EXHIBIT 121

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cancer

(KAN-ser)

A term for diseases in which abnormal cells divide without control and can invade nearby tissues. Cancer cells can also spread to other parts of the body through the blood and lymph systems. There are several main types of cancer. Carcinoma is a cancer that begins in the skin or in tissues that line or cover internal organs. Sarcoma is a cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue. Leukemia is a cancer that begins in blood-forming tissue, such as the bone marrow, and causes too many abnormal blood cells to be made. Lymphoma and multiple myeloma are cancers that begin in the cells of the immune system. Central nervous system cancers are cancers that begin in the tissues of the brain and spinal cord. Also called malignancy.

More Information

What Is Cancer?

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EXHIBIT 122

NIH NATIONAL CANCER INSTITUTE

Cancer in Children and Adolescents

How common is cancer in children?

Although cancer in children is rare, it is the leading cause of death by disease past infancy among children in the United States. In 2018, it is estimated that 15,590 children and adolescents ages 0 to 19 will be diagnosed with cancer and 1,780 will die of the disease in the United States (1). Among children ages 0 to 14 years, it is estimated that, in 2018, 10,590 will be diagnosed with cancer and 1,180 will die of the disease (1). Among adolescents ages 15 to 19 years, about 5000 will be diagnosed with cancer and about 600 will die of the disease.

Overall, among children and adolescents (ages 0 to 19) in the United States, the most common types of cancer are leukemias, brain and central nervous system tumors, and lymphomas. Among children (ages 0 to 14 years), the most common types of cancer are leukemias, followed by brain and other central nervous system tumors, lymphomas, soft tissue sarcomas (of which half are rhabdomyosarcoma), neuroblastoma, and kidney tumors (1). Among adolescents (ages 15 to 19 years), the most common types of cancer are brain and other central nervous system tumors and lymphomas, followed by leukemias, gonadal (testicular and ovarian) germ cell tumors, thyroid cancer, and melanoma (1).

As of January 1, 2015 (the most recent date for which data exist), approximately 429,000 survivors of childhood and adolescent cancer (diagnosed at ages 0 to 19 years) were alive in the United States (2). The number of survivors will continue to increase, given that the incidence of childhood cancer has been rising slightly in recent decades and that survival rates overall are improving.

What is the outlook for children and adolescents with cancer?

The overall outlook for children and adolescents with cancer has improved greatly over the last half-century. In the mid-1970s, 58% of children (ages 0 to 14 years) and 68% of adolescents (ages 15 to 19 years) diagnosed with cancer survived at least 5 years (1). In 2008–2014, 83.4% of children and 84.6% of adolescents diagnosed with cancer survived at least 5 years (2).

Although survival rates for most childhood cancers have improved in recent decades, the improvement has been especially dramatic for a few cancers, particularly acute lymphoblastic leukemia, which is the most common childhood cancer. Improved treatments introduced beginning in the 1960s and 1970s raised the 5-year survival rate for children diagnosed with acute lymphoblastic leukemia at ages 0 to 14 years from 57% in

1975 to 92% in 2012 (3). The 5-year survival rate for children diagnosed with non-Hodgkin lymphoma at ages 0 to 14 years has also increased dramatically, from 43% in 1975 to 91% in 2012 (3).

Because of these survival improvements, in more recent years brain cancer has replaced leukemia as the leading cause of cancer death among children (4).

By contrast, survival rates remain very low for some cancer types, for some age groups, and for some cancers within a site. For example, half of children with diffuse intrinsic pontine glioma (a type of brain tumor) survive less than 1 year from diagnosis (5). Among children with Wilms tumor (a type of kidney cancer), older children (those diagnosed between ages 10 and 16 years) have lower 5-year survival rates than younger children (6). For soft tissue sarcomas, 5-year survival rates in 2008–2014 among children and adolescents ages 0 to 19 years ranged from 65% (rhabdomyosarcoma) to 95% (chondrosarcoma) (7), but children with sarcomas who present with metastatic disease have much lower 5-year survival rates. And the 5-year survival rate for acute lymphoblastic leukemia in 2008-2014 was 91% for children younger than 15 years, compared with 74% for adolescents ages 15 to 19 years (7).

Some evidence suggests that adolescents and young adults with acute lymphoblastic leukemia may have better outcomes if they are treated with pediatric treatment regimens than if they receive adult treatment regimens (8). The improvement in 5-year survival rates for 15- to 19-year-olds with acute lymphoblastic leukemia may reflect greater use of these pediatric treatment regimens.

The cancer mortality rate—the number of deaths due to cancer per 100,000 people per year—among children and adolescents ages 0 to 19 years declined by more than 50% from 1975 to 2015 (2). Specifically, the mortality rate was 5.1 per 100,000 children and adolescents in 1975 and 2.3 per 100,000 children and adolescents in 2015. However, despite the overall decrease in mortality, approximately 1,800 children and adolescents still die of cancer each year in the United States, indicating that new advances and continued research to identify effective treatments are required to further reduce childhood cancer mortality.

Between 1999 and 2014, the cancer death rate dropped the most for 1- to-4-year-olds (a 26% drop), followed by that for 15- to 19-year-olds (a 22% drop), 10- to 14-year-olds (a 19% drop), and 5- to 9-year-olds (a 14% drop) (4).

What are the possible causes of cancer in children?

The causes of most childhood cancers are not known. Up to 10% of all cancers in children are caused by a heritable (germline) mutation (a mutation that can be passed from parents to their children). For example, about 45% of children with retinoblastoma, a cancer of the eye that develops mainly in children, inherited a mutation in a gene called RB1 from a parent (9). Inherited mutations associated with certain familial syndromes, such as Li-Fraumeni syndrome, Beckwith-Wiedemann syndrome, Fanconi anemia syndrome, Noonan syndrome, and von Hippel-Lindau syndrome, also increase the risk of childhood cancer.

Genetic mutations that initiate cancer can also arise during the development of a fetus in the womb. Evidence for this comes from studies of monozygotic (identical) twins in which both twins developed leukemia with an identical leukemia-initiating gene mutation (10).

Children who have Down syndrome, a genetic condition caused by the presence of an extra copy of chromosome 21, are 10 to 20 times more likely to develop leukemia than children without Down syndrome (11). However, only a very small proportion of childhood leukemia is linked to Down syndrome.

Most cancers in children, like those in adults, are thought to develop as a result of mutations in genes that lead to uncontrolled cell growth and eventually cancer. In adults, these gene mutations are often the result of exposure to cancer-causing environmental factors, such as cigarette smoke, asbestos, and ultraviolet radiation from the sun. One study found that melanoma in children and adolescents (ages 11–20 years) has many genomic similarities to melanoma that occurs in adults, including an enrichment of UV-induced mutations (12).

However, environmental causes of childhood cancer have been difficult to identify, partly because cancer in children is rare, and partly because it is difficult to determine what children might have been exposed to early in their development. In fact, most childhood cancers are not thought of as being caused by environmental exposures.

Nevertheless, several environmental exposures have been linked to childhood cancer. One is ionizing radiation, which can lead to the development of leukemia and other cancers in children and adolescents. For example, children and adolescents who were exposed to radiation from the atomic bombs dropped in Japan during the Second World War had an elevated risk of leukemia (13), and children who were exposed to radiation from the Chernobyl nuclear plant accident had an elevated risk for thyroid cancer (14). Children whose mothers had x-rays during pregnancy (that is, children who were exposed before birth) and children who were exposed after birth to diagnostic medical radiation from computed tomography (CT) scans have also been found to have an increased risk of leukemia and brain tumors, and possibly other cancers (15).

A number of other environmental exposures have also been reported to have possible associations with childhood cancer. However, because of challenges in studying these associations, such as recall bias and the difficulty of determining exposure at the relevant time period in a child's development, it is difficult to draw firm conclusions. Some types of childhood leukemia have been associated with father's tobacco smoking (16, 17); with exposure to certain pesticides used in and around the home (18) or by parents at their workplace (19, 20); with solvents, which are organic chemicals that are found in some household products; and with outdoor air pollution. Studies of childhood brain tumors have suggested possible associations with exposures to pesticides in and around the home (21) and maternal consumption of cured meats (22).

Researchers have also identified factors that may be associated with reduced risk of childhood cancer. For example, maternal consumption of folate has been associated with reduced risks of both leukemia and brain tumors in children (23). And being breastfed and having been exposed to routine childhood infections are

both associated with a lowered risk of developing childhood leukemia (24).

What does a child's cancer diagnosis mean for cancer risk in the rest of the family?

First- and second-degree relatives of a child diagnosed with cancer, particularly if diagnosed before age five, may be at increased risk for developing cancer if there is already a family history of cancer—that is, if the child's cancer is likely due to an inherited genetic syndrome (25). A clinician may advise as to whether a child could benefit from genetic testing or referral to a medical geneticist for evaluation (25–27).

How do cancers in adolescents and young adults differ from those in younger children?

Cancer occurs more frequently in adolescents and young adults ages 15 to 39 years than in younger children, although incidence in this group is still much lower than in older adults. According to the NCI Surveillance, Epidemiology, and End Results (SEER) program (7), each year in 2011–2015 there were:

- 16 cancer diagnoses per 100,000 children ages 0 to 14 years
- 72 cancer diagnoses per 100,000 adolescents and young adults ages 15 to 39 years
- 953 cancer diagnoses per 100,000 adults aged 40 years or older

The most frequent cancers diagnosed in adolescents and young adults (AYAs) are cancers that are more common among adults than younger children, such as breast cancer, melanoma, and thyroid cancer (28). But certain cancers, such as testicular cancer, are more typical of AYAs than of either younger children or adults (7). However, the incidence of specific cancer types varies widely across the adolescent and young adult age continuum.

Where do children with cancer get treated?

Children who have cancer are often treated at a children's cancer center, which is a hospital or a unit within a hospital that specializes in diagnosing and treating children and adolescents who have cancer. Most children's cancer centers treat patients through 20 years of age. The health professionals at these centers have specific training and expertise to provide comprehensive care for children, adolescents, and their families.

Recently, many Adolescent and Young Adult (AYA) cancer programs have been created to address the unique needs of teens and young adults. Areas of focus include long-term survivor care, access to clinical trial

enrollment, discussing and preserving future fertility, peer support, and psychosocial support that addresses their personal issues, including finances, education, occupational impacts, and transition to independence.

Children's cancer centers also participate in clinical trials. The improvements in survival for children with cancer that have occurred over the past half century have been achieved because of treatment advances that were studied and proven to be effective in clinical trials.

More than 90% of children and adolescents who are diagnosed with cancer each year in the United States are cared for at a children's cancer center that is affiliated with the NCI-supported Children's Oncology Group (COG). COG is the world's largest organization that performs clinical research to improve the care and treatment of children and adolescents with cancer. Each year, approximately 4,000 children who are diagnosed with cancer enroll in a COG-sponsored clinical trial. COG trials are sometimes open to individuals aged 29 years or even older when the type of cancer being studied is one that occurs in children, adolescents, and young adults.

Every children's cancer center that participates in COG has met strict standards of excellence for childhood cancer care. A directory of COG locations is available on their website. Families can ask their pediatrician or family doctor for a referral to a children's cancer center. Families and health professionals can call NCI's Cancer Information Service at 1–800–4–CANCER (1–800–422–6237) to learn more about children's cancer centers that belong to COG.

If my child is treated at a children's cancer center, will he or she automatically be part of a clinical trial?

No. Participation in a clinical trial is voluntary, and it is up to each family to decide if clinical trial participation is right for their child.

Can children who have cancer be treated at the National Institutes of Health (NIH) Clinical Center?

Children with cancer may be eligible to be treated in clinical trials at the NIH Clinical Center in Bethesda, Maryland. Because the NIH Clinical Center is a research hospital, only patients who have a specific type or stage of cancer that is under study can be accepted for treatment. In some cases, patients with conditions that are rare or difficult to diagnose may also be accepted for treatment at the Clinical Center. All patients who are treated at the Clinical Center must be referred by a physician.

NCI's Pediatric Oncology Branch conducts clinical trials for children, adolescents, and young adults with a wide variety of cancers. Patients with newly diagnosed cancer, as well as patients whose cancers have come

back after treatment, may be eligible to participate in a clinical trial. Physicians at the Pediatric Oncology Branch can also provide a second opinion on a patient's diagnosis or treatment plan. To refer a patient to the Pediatric Oncology Branch, the patient's health care provider should call 301–496–4256 (local) or 1–877–624–4878 (toll-free) weekdays between 8:30 a.m. and 5:00 p.m. ET. Parents can also call these numbers to learn if their child is eligible to participate in a clinical trial.

What should survivors of childhood cancer consider after they complete treatment?

Survivors of childhood cancer need follow-up care and enhanced medical surveillance for the rest of their lives because of the risk of complications related to the disease or its treatment that can last for, or arise, many years after they complete treatment for their cancer. Health problems that develop months or years after treatment has ended are known as late effects.

The specific late effects that a person who was treated for childhood cancer might experience depend on the type and location of his or her cancer, the type of treatment he or she received, and patient-related factors, such as age at diagnosis.

Children who were treated for bone cancer, brain tumors, and Hodgkin lymphoma, or who received radiation to their chest, abdomen, or pelvis, have the highest risk of serious late effects from their cancer treatment, including second cancers, joint replacement, hearing loss, and congestive heart failure (29, 30).

Long-term follow-up analysis of a cohort of survivors of childhood cancer treated between 1970 and 1986 has shown that cancer survivors remain at risk of complications and premature death as they age, with more than half of survivors having experienced a severe or disabling complication or even death by the time they reach age 50 years (31). Children treated in more recent decades may have lower risks of late effects due to modifications in treatment regimens to reduce exposure to radiotherapy and chemotherapy, increased efforts to detect late effects, and improvements in medical care for late effects (32).

It's important for childhood cancer survivors to have regular medical follow-up examinations so any health problems that occur can be identified and treated as soon as possible. The Children's Oncology Group (COG) has developed long-term follow-up guidelines for survivors of childhood, adolescent, and young adult cancers.

It is also important to keep a record of the cancer treatment that a child received. This record should include:

- The type and stage of cancer
- Date of diagnosis and dates of any relapses
- Types and dates of imaging tests

- Contact information for the hospitals and doctors who provided treatment
- Names and total doses of all chemotherapy drugs used in treatment
- The parts of the body that were treated with radiation and the total doses of radiation that were given
- Types and dates of all surgeries
- Any other cancer treatments received
- Any serious complications that occurred during treatment and how those complications were treated
- The date that cancer treatment was completed

The record should be kept in a safe place, and copies of the record should be given to all doctors or other health care providers who are involved with the child's follow-up care, even as the child grows into adulthood.

Many children's cancer centers have clinics where survivors of childhood cancer can go for follow-up until they reach their early 20s. Some cancer centers are now creating clinics dedicated to follow-up care for long-term survivors of pediatric and adolescent cancers.

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Related Resources

Adolescents and Young Adults with Cancer

Care for Childhood Cancer Survivors

Childhood Cancers

Childhood Cancers Research

Children with Cancer: A Guide for Parents

National Organizations That Offer Cancer-Related Support Services

NCI-COG Pediatric MATCH

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EXHIBIT 123

Key Statistics for Childhood Cancers

About 11,050 children in the United States under the age of 15 will be diagnosed with cancer in 2020. Overall, this accounts for less than 1% of all cancers. Childhood cancer rates have been rising slightly for the past few decades.

Because of major treatment advances in recent decades, 84% of children with cancer now survive 5 years or more. Overall, this is a huge increase since the mid-1970s, when the 5-year survival rate was about 58%. Still, survival rates can vary a great deal depending on the type of cancer and other factors. The survival rates for a specific type of childhood cancer can be found in our information for that cancer type.

After accidents, cancer is the second leading cause of death in children ages 1 to 14. About 1,190 children under the age of 15 are expected to die from cancer in 2020.

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EXHIBIT 124

OXFORD

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Article

ARTICLE

Colorectal Cancer Incidence Patterns in the United States, 1974–2013

Rebecca L. Siegel, Stacey A. Fedewa, William F. Anderson, Kimberly D. Miller, Jiemin Ma, Philip S. Rosenberg, Ahmedin Jemal

Affiliations of authors: Surveillance and Health Services Research, American Cancer Society, Atlanta, GA (RLS, SAF, KDM, JM, AJ); Division of Cancer Epidemiology and Genetics, National Cancer Institute, Rockville, MD (WFA, PSR).

Correspondence to: Rebecca L. Siegel, MPH, Surveillance and Health Services Research, American Cancer Society, 250 Williams Street, NW, Atlanta, GA (e-mail: rebecca.siegel@cancer.org).

Abstract

Background: Colorectal cancer (CRC) incidence in the United States is declining rapidly overall but, curiously, is increasing among young adults. Age-specific and birth cohort patterns can provide etiologic clues, but have not been recently examined.
Methods: CRC incidence trends in Surveillance, Epidemiology, and End Results areas from 1974 to 2013 (n = 490 305) were analyzed by five-year age group and birth cohort using incidence rate ratios (IRRs) and age-period-cohort modeling.
Results: After decreasing in the previous decade, colon cancer incidence rates increased by 1.0% to 2.4% annually since the mid-1980s in adults age 20 to 39 years and by 0.5% to 1.3% since the mid-1990s in adults age 40 to 54 years; rectal cancer incidence rates have been increasing longer and faster (eg, 3.2% annually from 1974–2013 in adults age 20–29 years). In adults age 55 years and older, incidence rates generally declined since the mid-1980s for colon cancer and since 1974 for rectal cancer. From 1989–1990 to 2012–2013, rectal cancer incidence rates in adults age 50 to 54 years went from half those in adults age 55 to 59 to equivalent (24.7 vs 24.5 per 100 000 persons: IRR = 1.01, 95% confidence interval [CI] = 0.92 to 1.10), and the proportion of rectal cancer diagnosed in adults younger than age 55 years doubled from 14.6% (95% CI = 14.0% to 15.2%) to 29.2% (95% CI = 28.5% to 29.9%). Age-specific relative risk by birth cohort declined from circa 1890 until 1950, but continuously increased through 1990. Consequently, compared with adults born circa 1950, those born circa 1990 have double the risk of colon cancer (IRR = 2.40, 95% CI = 1.11 to 5.19) and quadruple the risk of rectal cancer (IRR = 4.32, 95% CI = 2.19 to 8.51).
Conclusions: Age-specific CRC risk has escalated back to the level of those born circa 1890 for contemporary birth cohorts, underscoring the need for increased awareness among clinicians and the general public, as well as etiologic research to elucidate causes for the trend. Further, as nearly one-third of rectal cancer patients are younger than age 55 years, screening initiation before age 50 years should be considered.

Colorectal cancer (CRC) incidence rates have been declining in the United States for several decades, with the pace accelerating to 3% annually from 2003 to 2012 (1). The reduction in risk from 1975 to 2000 is attributed equally to changes in the prevalence of risk factors and the uptake of screening (2), while the recent steep decline is thought to be primarily driven by screening. A recent study speculated that underlying CRC risk also continues to decline (3), while others have reported increasing risk in adults younger than age 50 years, for whom screening is not recommended for those at average risk (4–8). However, none of these studies examined the temporal pattern simultaneously by age, calendar period, and year of birth for a comprehensive interpretation of the contemporary trend. To our knowledge, the last paper that examined trends in CRC by period and birth cohort was published in 1994 based on data through 1990 (9). Herein, we characterize trends in population-based CRC occurrence by tumor location, age at diagnosis, and year of birth using incidence data from 1974 to 2013 and age-period-cohort modeling (10). Age-period-cohort modeling is a quantitative tool used to enhance the understanding of disease trends by

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attempting to disentangle factors that influence all ages (period effects), such as changes in medical practice, from those that vary by generation (cohort effects), typically as a consequence of behavioral changes.

Methods

Study Design and Data Source

We conducted a retrospective cohort study of patients age 20 years and older diagnosed with invasive CRC from 1974 through 2013 in the nine oldest Surveillance, Epidemiology, and End Results (SEER) Program areas (Atlanta [from 1975], Connecticut, Detroit, Hawaii, Iowa, New Mexico, Seattle-Puget Sound, San Francisco-Oakland, and Utah) (11,12). The SEER program is the only source for historical population-based cancer incidence in the United States and is considered the gold standard for cancer registration worldwide because of the high quality of data. Diagnosis years 1974–2013 were selected to utilize the most recent available data while maintaining equivalent time period and age intervals, which are required for age-period-cohort modeling. Cases were stratified by tumor subsite (colon, ICD-O-3 codes C18.0, C18.2-C18.9, C26.0 [proximal colon, C18.0, C18.2-C18.4; distal colon, C18.5-C18.7]; rectum, C19.9, C20.9) and excluded appendiceal malignancies, which are considered distinct from those arising in the colorectum (13). Five percent of colon tumors were coded as overlapping or unspecified anatomic location and could not be included in subsite analysis. Because incidence trends during this time period are the same in men and women (14), data were not stratified by sex to improve statistical power.

Statistical Analysis

SEER*Stat (version 8.3.2; National Cancer Institute [NCI]) was used to access CRC cases and calculate delay-adjusted incidence rates, which correct for the lag in case capture affecting recent data years (15), and incidence rate ratios (IRRs) with accompanying 95% confidence intervals (CIs). All tests of statistical significance were two-sided, and a *P* value of less than .05 was considered statistically significant. Incidence rate ratios were considered statically significant when the 95% confidence interval did not include one. Incidence rates were calculated for eleven age groups (20–29, 30–39, 40–49, 50–54, 55–59...80–84, 85+), presented per 100 000 person-years, and age-adjusted to the 2000 US standard population for collective age groups (eg, age > 55 years). The magnitude and direction of temporal trends were quantified using the Joinpoint Regression Program (version 4.3.1.0; NCI), which uses permutation analysis to fit a series of joined straight lines on a logarithmic scale to observed rates to estimate the annual percent change (APC) and average annual percent change (16). We calculated the change in proportion of cases diagnosed in young adults (defined as age < 55 years based on like contemporary incidence trends) between 1989–1990 and 2012–2013 by adjusting to the 2012–2013 population in order to account for temporal shifts in underlying age distribution.

Birth cohort models were fitted using NCI's Age Period Cohort web tool (17). Age-period-cohort modeling provides estimates of parameters that describe relationships between observed incidence rates and age, calendar period, and birth cohort based on age groups and time periods of equal length (18). Input data were case and population counts for eight five-year time periods (1974–1978, 1979–1983...2009–2013) and 14 five-year age groups (20–24, 25–29...80–84, 85+) spanning 21 partially overlapping 10-year birth cohorts. Cohorts are referred to by mid-year of birth (1889, 1894...1989) corresponding to patients born beginning in 1887 through 1991. Cohort effects are presented graphically as IRRs adjusted for age and calendar period effects. To facilitate data interpretation, we chose reference values corresponding to the 1949 cohort, which had the lowest rates. (The choice of reference values is arbitrary and does not affect the interpretation of results.) We also present the local drift, which estimates the age-specific net annual percent change in incidence rates. Heat maps of residuals by age vs period were constructed to screen for systematic lack of fit. In addition, we examined how well observed rates agreed with confidence bands from the age-period-cohort model when the former were plotted together with the latter (Supplementary Figure 1, available online).

Results

There were 490 305 patients age 20 years and older diagnosed with CRC in the nine oldest SEER registries during 1974–2013. During the late 1970s and early 1980s, colon cancer incidence rates were declining in age groups younger than age 50 years and increasing in those age 50 years and older (Figure 1). Conversely, from the mid-1980s through 2013, rates declined in adults age 55 years and older, while increasing by 2.4% per year in adults age 20–29 years and by 1.0% per year in adults age 30–39 years. In the mid-1990s, rates also began increasing in adults age 40 to 49 years (1.3% per year) and 50 to 54 years (0.5% per year). Increasing trends in adults younger than age 50 years were confined to tumors in the distal colon, with the exception of adults age 40 to 49 years, among whom rates are also increasing for proximal tumors (Supplementary Table 1, available online). This is also the only age group for which tumors of unspecified or overlapping location are increasing. Notably, declines in adults age 55 years or older were also generally larger for distal than for proximal tumors.

Compared with colon cancer, incidence trends for rectal cancer are more prolonged for all age groups and the rise in young adults is steeper. Specifically, rectal cancer incidence rates increased by 3.2% per year from 1974 to 2013 in adults age 20 to 29 years and since 1980 in adults age 30 to 39 years, and by 2.3% per year since beginning in the 1990s in adults age 40 to 49 years and 50 to 54 years (Figure 2). In contrast, rates generally declined throughout the entire 40-year study period in adults age 55 years and older. The stronger, more sustained trends for rectal than for colon tumors are reflected in a notable crossover in the local drift, with rectal cancer incidence exhibiting a net increase of 3.9% to 4.0% annually in adults age 20 to 29 years coupled with a net decrease of 2.1% or more annually in adults age 75 and older (Figure 3A).

Oppositional trends by age are also causing a convergence in CRC incidence rates in adults age 50 to 54 years and 55 to 59 years (Supplementary Figure 1, available online). Whereas in the early 1990s both colon and rectal cancer incidence rates in adults age 50 to 54 years were half those in adults age 55 to 59 years, in 2012 to 2013 they were just 12.4% lower for colon (31.9 vs 36.4: IRR = 0.88, 95% CI = 0.81 to 0.94) and equivalent for rectum (24.7 vs 24.5: IRR = 1.01, 95% CI = 0.92 to 1.10). In addition, the age-adjusted proportion of incident cases in adults age 55 years and younger increased from 11.6% (95% CI = 11.1% to 12.2%) in 1989–1990 to 16.6% (95% CI = 16.0% to 17.1%) in 2012 to 2013 for

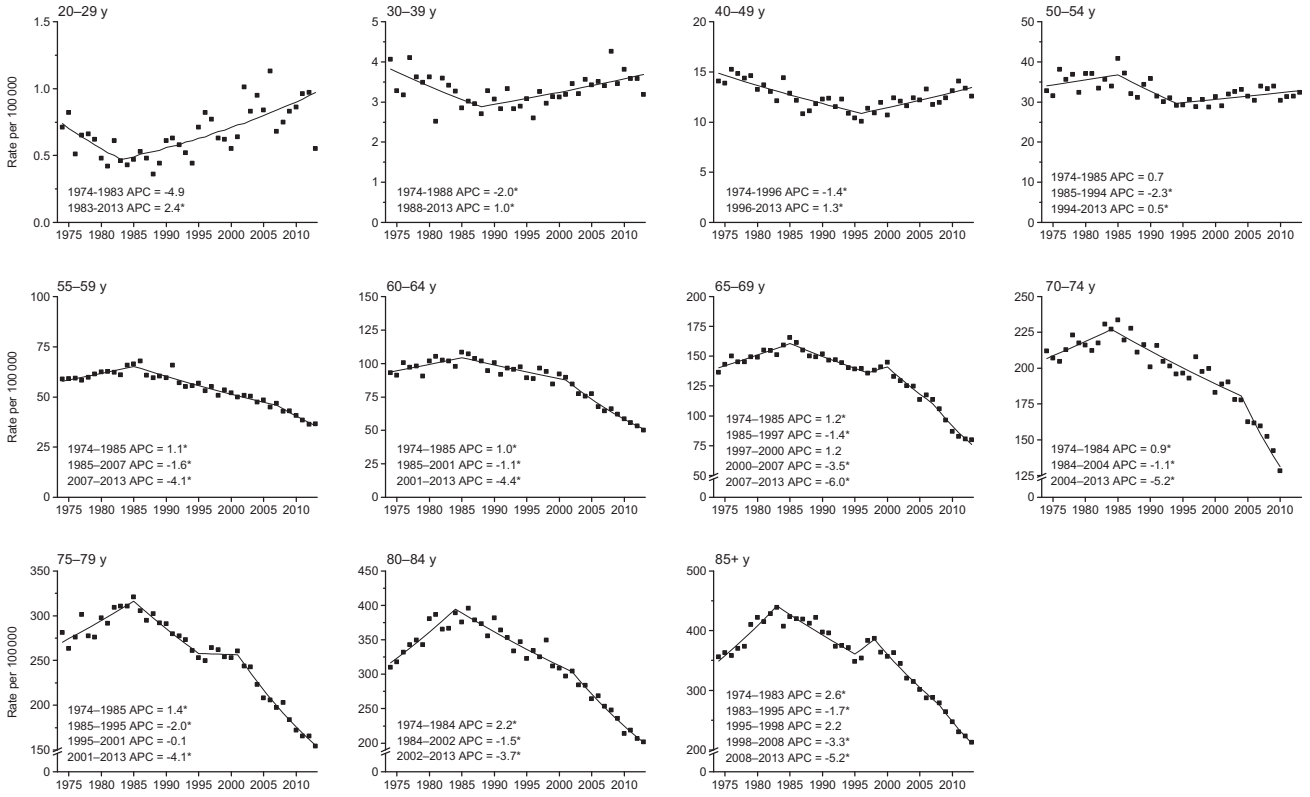

Figure 1. Annual percent change (APC) in age-specific colon cancer incidence rates in the United States, 1974–2013. An asterisk indicates that the APC is statistically significantly different from zero ($P < .05$) using a two-sided test based on the permutation method. In order to highlight trends, the scale of the y-axis varies.

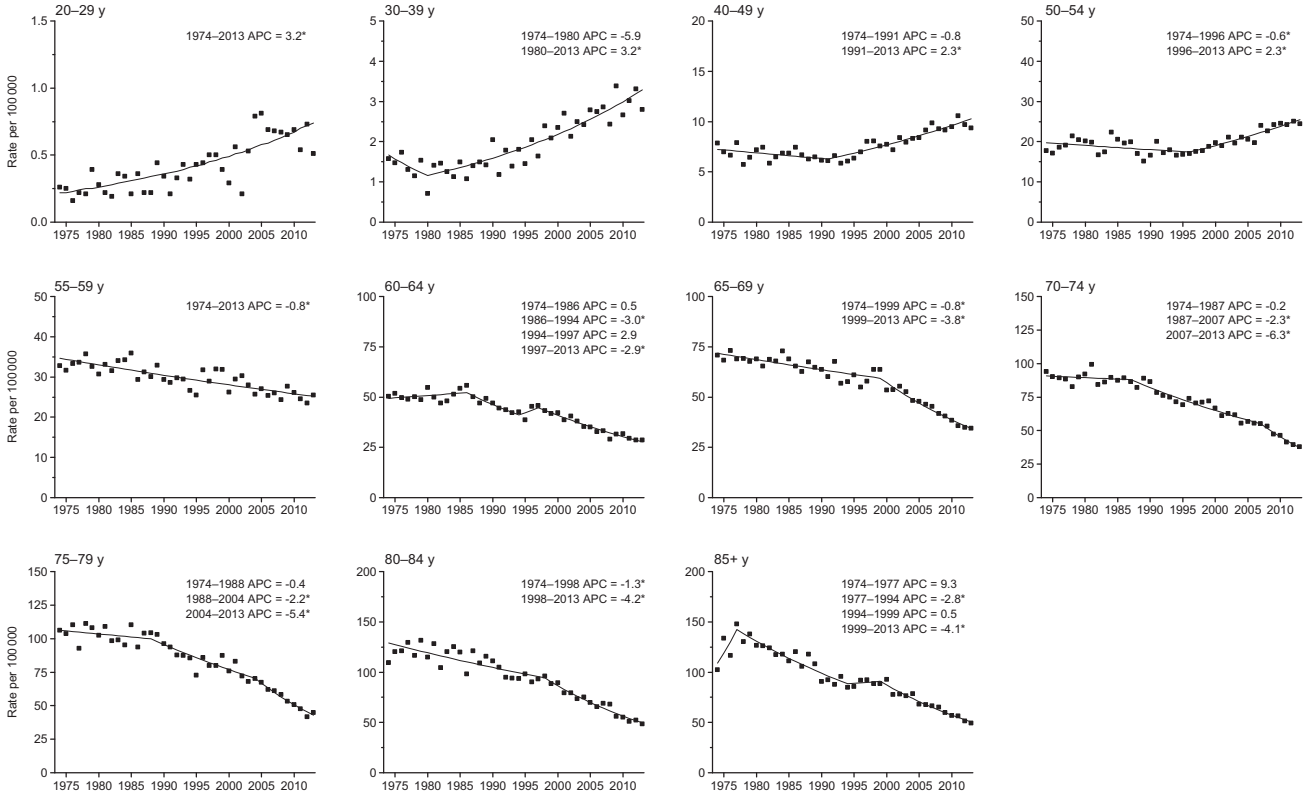

Figure 2. Annual percent change (APC) in age-specific rectal cancer incidence rates in the United States, 1974–2013. An asterisk indicates that the APC is statistically significantly different from zero ($P < .05$) using a two-sided test based on the permutation method. In order to highlight trends, the scale of the y-axis varies.

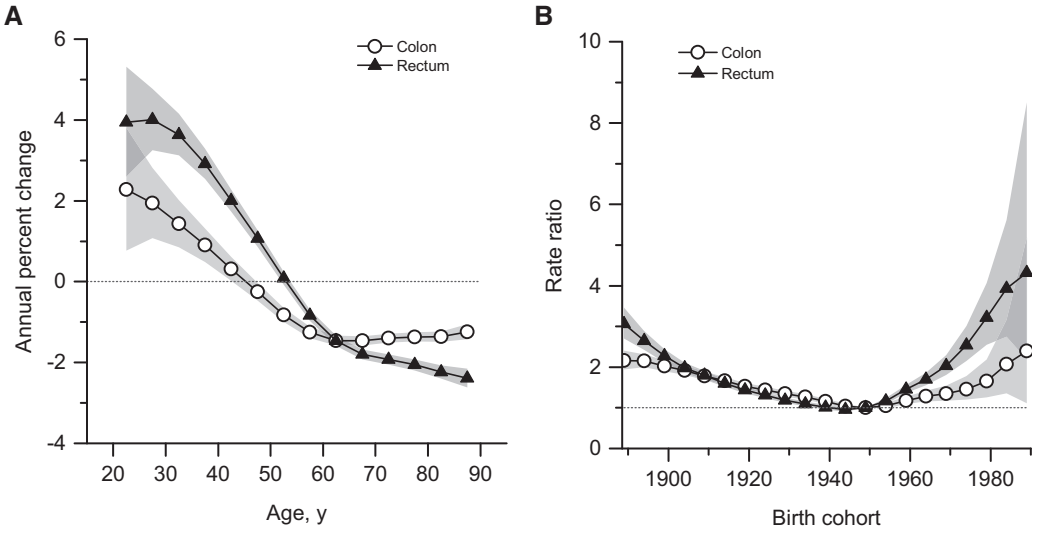

Figure 3. Summary age-specific annual percent change (i.e., local drift) and birth cohort rate ratios of colorectal cancer incidence rates in the United States. **A)** Local drift: summary age-specific annual percent change for colon and rectal cancer. **B)** Incidence rate ratios by birth cohort for colon and rectal cancer (referent cohort = 1949). **Shaded bands** indicate 95% confidence interval.

colon cancer and from 14.6% (95% CI = 14.0% to 15.2%) to 29.2% (95% CI = 28.5% to 29.9%) for rectal cancer.

Age-period-cohort modeling of CRC incidence data indicates both period and cohort effects, with deviations for each generally statistically significantly different from zero, particularly for rectal cancer (Supplementary Figure 2, available online). Quantitatively, however, period effects were dwarfed by cohort effects, with cohort deviations 10-fold higher than period deviations. Further, the local drift (net age-specific annual percent change) was statistically significant for all ages for colon cancer and, with the exception of adults age 50 to 54 years for rectal cancer (Figure 3A), consistent with the age-specific trend for that group shown in Figure 2.

Age-specific trends by birth cohort are presented as incidence rate ratios, for which the 1949 cohort is the referent group. Relative risks decreased for consecutive cohorts born from the late 1880s until the 1940s, then increased for subsequent cohorts (Figure 3B). Specifically, compared with people born circa 1950, those born circa 1890 had double the age-specific risk of colon cancer (IRR = 2.12, 95% CI = 1.91 to 2.36) and triple the risk of rectal cancer (IRR = 3.06, 95% CI = 2.71 to 3.47). These age-specific relative risks are comparable with those for the youngest birth cohort for both colon (IRR = 2.40, 95% CI = 1.11 to 5.19) and rectal cancer (IRR = 4.32, 95% CI = 2.19 to 8.51), despite wider confidence intervals because data are limited to young individuals, who have substantially lower disease rates. While the increase for colon cancer is primarily driven by distal tumors, risk for proximal tumors also appears to be increasing (Supplementary Figure 3, available online). Age-specific incidence trends by year of birth confirm the strong cohort effect (Supplementary Figure 4, available online). Residual analysis conducted to evaluate the goodness of fit of the age-period-cohort models revealed adequate agreement between the modeled and observed data (Supplementary Figure 1, available online).

Discussion

We found variations in CRC incidence patterns by age, tumor subsite, calendar period, and particularly birth cohort. The age-specific risk of CRC dropped for successive generations in the first half of the twentieth century, but has escalated back to the level of those born circa 1890 for current birth cohorts. The cohort effect was qualitatively similar for colon and rectal cancers, but quantitatively larger for rectal cancer, for which there was a net increase of 4% annually for people in their 20s coinciding with a net decrease of 2% annually for those age 75 years and older. As a consequence of these oppositional trends, the probability of a rectal cancer diagnosis for someone in their early 50s is now the same as it is for someone in their late 50s, whereas two decades ago it was just half.

In contrast to colon cancer, rectal cancer incidence has generally been declining in age groups older than 55 years since at least 1974, well before widespread screening, which was self-reported at less than 25% in 1987 (19). This may partly reflect detection and removal of precancerous lesions during clinical inspection of the rectum, which was common practice well before formal CRC screening (20). Inherent differences within the colorectum in the way environmental factors initiate and/or promote carcinogenesis (21), as well as the influence of unknown risk factors, may also have contributed.

While early-onset CRC has a familial component more often than late-onset disease, the majority of cases are sporadic (22). The strong birth cohort effects we observed signal relatively recent changes in exposures that influence risk. Established lifestyle factors associated with CRC include excess body weight, high consumption of processed meat and alcohol, low levels of physical activity and fiber consumption, and cigarette smoking (23,24). The rise in CRC in young adults has likely been attenuated by long-term declines in alcohol consumption and smoking (25), but fueled by increases in cumulative exposure to excess body fat, which have been demonstrated by studies of obesity trends by birth cohort (26). It is not surprising that the timing of the obesity epidemic parallels the rise in CRC because many behaviors thought to drive weight gain, such as unhealthy dietary patterns and sedentary lifestyles (27), independently increase CRC risk. Moreover, there are undoubtedly complex epigenetic interactions between obesity, sedentary behavior, and diet (28,29). Evolving research suggests that specific, unhealthy dietary elements, like high–glycemic load carbohydrates, may

trigger a cascade of detrimental health effects beyond caloric content (30). A recent study found that de novo introduction of a Western-style high-fat, low-fiber diet initiates inflammation and proliferation in the colonic mucosa within two weeks (31). These findings are consistent with the one-generation jump in CRC risk that has been observed among Japanese migrants to the United States that is attributed to diet (32).

Some of the increased CRC in recent birth cohorts may be due to the detection of prevalent subclinical disease because of rising colonoscopy utilization for diagnostic and screening purposes. According to the National Health Interview Survey, 13.6% of adults age 40 to 49 years reported having a colonoscopy in the past 10 years in 2013, compared with 6.4% in 2000 (Supplementary Table 2, available online) (33). During 2000 to 2011, approximately 17% of colonoscopies were performed in patients younger than age 50 years based on data from the National Endoscopic Database (34). Nevertheless, this is probably not a driving factor for the trends in early-onset disease because the most rapid gains are for individuals in their 20s and 30s, who are least likely to be screened. Moreover, rates have risen at a similar magnitude for early- and advanced-stage disease (7), which is inconsistent with a screening effect.

While primary prevention is the preferable course of action for cancer control, improving health behaviors and further identifying etiologic agents for CRC are long-term endeavors. In the meantime, a number of actions should be taken to ameliorate the rising burden of CRC in young adults. One is to educate the public and clinicians about the rising probability of disease in people younger than age 55 years. Young patients are 58% more likely than older patients to be diagnosed with distant- vs localized-stage CRC (35), largely due to delayed follow-up of symptoms, sometimes for years (36), because cancer is typically not on the radar of young adults or their providers (37). Another obstacle to timely diagnosis is less access to medical care; adults younger than age 55 years are three times more likely to be uninsured than those age 55 years and older—22% vs 7% in 2013 (38). The Affordable Care Act (ACA) may facilitate earlier detection for young CRC patients, as it has for other malignancies (39). The Commonwealth Fund ACA Tracking Survey reported that the proportion of adults age 19 to 34 years who were uninsured reduced from 28% in 2013, prior to the first open enrollment, to 18% in 2016, following the third open enrollment, with a similar decline (18% to 11%) among adults age 35 to 49 years (40).

Rapid declines in CRC incidence in the past decade in age groups older than 55 years are likely the result of increased uptake of screening, which rose from 38% in 2000 to 59% in 2013 in adults age 50 years or older (33). The larger decreases for distal than proximal tumors may reflect the longstanding effects of fecal occult blood tests and flexible sigmoidoscopy, which were the most common screening modalities among older adults until 2005, and possibly higher efficacy of colonoscopy for preventing distal cancers (41–43). However, our finding of rising CRC incidence rates for people in their early 50s, as well as younger age groups, highlights the need for increased adherence to recommended screening. Guidelines state that screening should commence at age 50 years for individuals at average risk of disease, but earlier for those at increased risk, which includes people with a family history of CRC or adenomatous polyps (44). Despite these recommendations, among people with an affected first-degree relative, those younger than age 50 years are half as likely to have had a colonoscopy as those age 50 years or older (45). Nationally, colonoscopy prevalence is lower in adults age 50 to 54 years than in adults age 55 to 59 years, although temporal trends are similar; reported receipt of a colonoscopy in the past 10 years increased from 14% in 2000 to 41% in 2013 in adults age 50 to 54 years and from 16% to 52% in adults age 55 to 59 years (Supplementary Table 2, available online). While national surveys do not collect information on age at screening initiation, one population-based study of non-Hispanic whites with higher-than-average educational attainment, one-quarter of whom were employed in health care, found a mean age at CRC screening initiation of 55 years (46).

Reversing increasing trends in adults age 50 to 54 years requires not only increased adherence to screening guidelines but also screening before age 50 years because the full benefit of polypectomy for preventing CRC requires about a decade to realize (47,48). Beginning screening at age 45 years is not supported by a recent review of the evidence for CRC screening (49,50) and would add approximately 20 million people to the screening-eligible population. Yet it is worth noting that in 2013 there were about 10 400 new CRCs diagnosed in adults age 40 to 49 years and 12 800 cases in adults age 50 to 54 years, similar to the total number of cervical cancers (12 300) (51), for which screening of 95 million women age 21 to 65 years is recommended (52). Moreover, Cancer Intervention and Surveillance Modeling Network (CISNET) researchers recently reported that beginning screening at age 45 years is "more effective and provided a more favorable balance between life-years gained and screening burden than starting at age 50 years" (49). Endoscopic screening could be particularly useful in stemming the tide of tumors in the distal colon and rectum (53), which are preponderant in young patients.

Our study is somewhat limited by its ecologic nature and the assumptions of the age-period-cohort model, specifically that interactions between age and period can be well described as a birth cohort phenomenon. Also, although the data fit our models well, existing models do not incorporate information on population-level screening or risk factors. Hence, parameters can help identify emerging trends and generate etiologic hypotheses, but the results do not provide any direct evidence about the role of specific exposures or interventions. Even so, as incidence trends in young adults often provide a bellwether of the future disease burden, our results are sobering. Additionally, long-term population-based cancer occurrence data in the United States are limited to nine SEER registries, and thus may not be generalizable to the broader population. However, a recent analysis of age-specific CRC incidence trends from 1998 to 2009 based on national data reported results similar to ours and those of other SEER-based studies (4,6).

In summary, the age-specific risk of a CRC diagnosis dropped for successive generations in the first half of the twentieth century, but escalated back to the level of those born in the late 1800s for current birth cohorts. As the proportion of rectal cancer diagnosed in adults younger than age 55 years years has doubled in just two decades, adherence to guideline-recommended screening initiation should be emphasized and initiation before age 50 years should be reconsidered. These results highlight the need for etiologic research to elucidate causes for the underlying increase in disease risk in young birth cohorts, as well as creative new strategies to curb the obesity epidemic and shift Americans toward healthier eating and more active lifestyles. Beyond awaiting scientific discovery and the widespread adoption of healthier living, meaningful action can be taken to mitigate premature morbidity and mortality from this disease through educational campaigns about the importance of timely follow-up of CRC symptoms, regardless of patient age, and age-appropriate screening.

ARTICLE

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EXHIBIT 125

Centers for Disease Control and Prevention
CDC 24/7: Saving Lives, Protecting People™

Reproductive Health

Infertility FAQs

Frequently Asked Questions

What is infertility?

In general, infertility is defined as not being able to get pregnant (conceive) after one year (or longer) of unprotected sex. Because fertility in women is known to decline steadily with age, some providers evaluate and treat women aged 35 years or older after 6 months of unprotected sex. Women with infertility should consider making an appointment with a reproductive endocrinologist—a doctor who specializes in managing infertility. Reproductive endocrinologists may also be able to help women with recurrent pregnancy loss, defined as having two or more spontaneous miscarriages.

Pregnancy is the result of a process that has many steps.

To get pregnant

- A woman's body must release an egg from one of her ovaries (ovulation).
- A man's sperm must join with the egg along the way (fertilize).
- The fertilized egg must go through a fallopian tube toward the uterus (womb).
- The fertilized egg must attach to the inside of the uterus (implantation).

Infertility may result from a problem with any or several of these steps.

Impaired fecundity is a condition related to infertility and refers to women who have difficulty getting pregnant or carrying a pregnancy to term.

Is infertility a common problem?

Yes. About 6% of married women aged 15 to 44 years in the United States are unable to get pregnant after one year of trying (infertility). Also, about 12% of women aged 15 to 44 years in the United States have difficulty getting pregnant or carrying a pregnancy to term, regardless of marital status (impaired fecundity).

Is infertility just a woman's problem? +

What causes infertility in men? +

What increases a man's risk of infertility? +

What causes infertility in women? +

What increases a woman's risk of infertility? +

How long should couples try to get pregnant before seeing a doctor? +

How will doctors find out if a woman and her partner have fertility problems? +

How do doctors treat infertility? +

What are some of the specific treatments for male infertility? +

What medicines are used to treat infertility in women? +

What is intrauterine insemination (IUI)? +

What is assisted reproductive technology (ART)? +

How often is assisted reproductive technology (ART) successful? +

What are the different types of assisted reproductive technology (ART)? +

Related links +

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EXHIBIT 126

al·ler·gy (al'er-jē),

1. Hypersensitivity caused by exposure to a particular antigen (allergen) resulting in a marked increase in reactivity to that antigen on subsequent exposure, sometimes resulting in harmful immunologic consequences.
See also: **allergic reaction**, **anaphylaxis**, **immune**. Synonym(s): **acquired sensitivity**, **induced sensitivity**

2. That branch of medicine concerned with the study, diagnosis, and treatment of allergic manifestations.

3. An acquired hypersensitivity to certain drugs and biologic materials.

[G. *allos*, other, + *ergon*, work]

EXHIBIT 127

allergic rhinitis

Also found in: **Dictionary**, **Thesaurus**, **Acronyms**, **Encyclopedia**, **Wikipedia**.
Related to allergic rhinitis: **asthma**, **sinusitis**

Allergic Rhinitis

Definition

Allergic **rhinitis**, more commonly referred to as hay fever, is an inflammation of the nasal passages caused by allergic reaction to airborne substances.

Description

Allergic rhinitis (AR) is the most common allergic condition and one of the most common of all minor afflictions. It affects between 10-20% of all people in the United States, and is responsible for 2.5% of all doctor visits. **Antihistamines** and other drugs used to treat allergic rhinitis make up a significant fraction of both prescription and over-the-counter drug sales each year.

There are two types of allergic rhinitis: seasonal and perennial. Seasonal AR occurs in the spring, summer, and early fall, when airborne plant pollens are at their highest levels. In fact, the term hay **fever** is really a misnomer, since allergy to grass pollen is only one cause of symptoms for most people. Perennial AR occurs all year and is usually caused by home or workplace airborne pollutants. A person can be affected by one or both types. Symptoms of seasonal AR are worst after being outdoors, while symptoms of perennial AR are worst after spending time indoors.

Both types of **allergies** can develop at any age, although onset in childhood through early adulthood is most common. Although allergy to a particular substance is not inherited, increased allergic sensitivity may "run in the family." While allergies can improve on their own over time, they can also become worse over time.

Causes and symptoms

Causes

Allergic rhinitis is a type of immune reaction. Normally, the immune system responds to foreign microorganisms, or particles, like pollen or dust, by producing specific proteins, called antibodies, that are capable of binding to identifying molecules, or antigens, on the foreign particle. This reaction between antibody and antigen sets off a series of reactions designed to protect the body from infection. Sometimes, this same series of reactions is triggered by harmless, everyday substances. This is the condition known as allergy, and the offending substance is called an allergen.

Like all allergic reactions, AR involves a special set of cells in the immune system known as mast cells. Mast cells, found in the lining of the nasal passages and eyelids, display a special type of antibody, called immunoglobulin type E (IgE), on their surface. Inside, mast cells store reactive chemicals in small packets, called granules. When the antibodies encounter allergens, they trigger release of the granules, which spill out their chemicals onto neighboring cells, including blood vessels and nerve cells. One of these chemicals, histamine, binds to the surfaces of these other cells, through special proteins called histamine receptors. Interaction of histamine with receptors on blood vessels causes neighboring cells to become leaky, leading to the fluid collection, swelling, and increased redness characteristic of a runny nose and red, irritated eyes. Histamine also stimulates **pain** receptors, causing the itchy, scratchy nose, eyes, and throat common in allergic rhinitis.

The number of possible airborne allergens is enormous. Seasonal AR is most commonly caused by grass and tree pollens, since their pollen is produced in large amounts and is dispersed by the wind. Showy flowers, like roses or lilacs, that attract

insects produce a sticky pollen that is less likely to become airborne. Different plants release their pollen at different times of the year, so seasonal AR sufferers may be most affected in spring, summer, or fall, depending on which plants provoke a response. The amount of pollen in the air is reflected in the pollen count, often broadcast on the daily news during allergy season. Pollen counts tend to be lower after a good rain that washes the pollen out of the air and higher on warm, dry, windy days.

Virtually any type of tree or grass may cause AR. A few types of weeds that tend to cause the most trouble for people include the following:

- ragweed
- sagebrush
- lamb's-quarters
- plantain
- pigweed
- dock/sorrel
- tumbleweed

Key terms

Allergen — A substance that provokes an allergic response.

Anaphylaxis — Increased sensitivity caused by previous exposure to an allergen1 that can result in blood vessel dilation (swelling) and smooth muscle contraction. Anaphylaxis can result in sharp blood pressure drops and difficulty breathing.

Antibody — A specific protein produced by the immune system in response to a specific foreign protein or particle called an antigen.

Antigen — A foreign protein to which the body reacts by making antibodies.

Granules — Small packets of reactive chemicals stored within cells.

Histamine — A chemical released by mast cells that activates pain receptors and causes cells to become leaky.

Mast cells — A type of immune system cell that is found in the lining of the nasal passages and eyelids, displays a type of antibody called immunoglobulin type E (IgE) on its cell surface, and participates in the allergic response by releasing histamine from intracellular granules.

Perennial AR is often triggered by house dust, a complicated mixture of airborne particles, many of which are potent allergens. House dust contains some or all of the following:

- house mite body parts. All houses contain large numbers of microscopic insects called house mites. These harmless insects feed on fibers, fur, and skin shed by the house's larger occupants. Their tiny body parts easily become airborne.
- animal dander. Animals constantly shed fur, skin flakes, and dried saliva. Carried in the air, or transferred from pet to owner by direct contact, dander can cause allergy in many sensitive people.
- mold spores. Molds live in damp spots throughout the house, including basements, bathrooms, air ducts, air conditioners, refrigerator drains, damp windowsills, mattresses, and stuffed furniture. Mildew and other molds release airborne spores that circulate throughout the house.

Other potential causes of perennial allergic rhinitis include the following:

- cigarette smoke
- perfume
- cosmetics
- cleansers
- copier chemicals
- industrial chemicals
- construction material gases

Symptoms

Inflammation of the nose, or rhinitis, is the major symptom of AR. Inflammation causes itching, sneezing, runny nose, redness, and tenderness. Sinus swelling can constrict the eustachian tube that connects the inner ear to the throat, causing a congested feeling and "ear popping." The drip of mucus from the sinuses down the back of the throat, combined with

increased sensitivity, can also lead to throat irritation and redness. AR usually also causes redness, **itching**, and watery eyes. **Fatigue** and headache are also common.

Diagnosis

Diagnosing seasonal AR is usually easy and can often be done without a medical specialist. When symptoms appear in spring or summer and disappear with the onset of cold weather, seasonal AR is almost certainly the culprit. Other causes of rhinitis, including infection, can usually be ruled out by a **physical examination** and a nasal smear, in which a sample of mucus is taken on a swab for examination.

Allergy tests, including skin testing and provocation testing, can help identify the precise culprit, but may not be done unless a single source is suspected and subsequent avoidance is possible. Skin testing involves placing a small amount of liquid containing a specific allergen on the skin and then either poking, scratching, or injecting it into the skin surface to observe whether redness and swellings occurs. Provocation testing involves challenging an individual with either a small amount of an inhalable or ingestable allergen to see if a response is elicited.

Perennial AR can also usually be diagnosed by careful questioning about the timing of exposure and the onset of symptoms. Specific allergens can be identified through allergy skin testing.

Treatment

Avoidance of the allergens is the best treatment, but this is often not possible. When it is not possible to avoid one or more allergens, there are two major forms of medical treatment, drugs and immunotherapy.

Drugs

ANTIHISTAMINES. Antihistamines block the histamine receptors on nasal tissue, decreasing the effect of histamine release by mast cells. They may be used after symptoms appear, though they may be even more effective when used preventively, before symptoms appear. A wide variety of antihistamines are available.

Older antihistamines often produce drowsiness as a major side effect. Such antihistamines include the following:

- diphenhydramine (Benadryl and generics)
- chlorpheniramine (Chlor-trimeton and generics)
- brompheniramine (Dimetane and generics)
- clemastine (Tavist and generics).

Newer antihistamines that do not cause drowsiness are available by prescription and include the following:

- astemizole (Hismanal)
- fexofenadine (Allegra)
- cetirizine (Zyrtec)
- azelastin HCl (Astelin).

Loratidine (Claritin) was available only by prescription but was released to over-the-counter status by the FDA.

Hismanal has the potential to cause serious heart **arrhythmias** when taken with the antibiotic erythromycin, the antifungal drugs ketoconazole and itraconazole, or the antimalarial drug quinine. Taking more than the recommended dose of Hismanal can also cause arrhythimas. Seldane (terfenadine), the original nondrowsy antihistamine, was voluntarily withdrawn from the market by its manufacturers in early 1998 because of this potential and because of the availability of an equally effective, safer alternative drug, fexofenadine.

LEUKOTRIENE RECEPTOR ANTAGONISTS. Leukotriene receptor antagonists (montelukast or Singulair and zafirlukast or Accolate) are a newer class of drugs used daily to help prevent **asthma**. They've also become approved in the United States to treat allergic rhinitis.

DECONGESTANTS. Decongestants constrict blood vessels to counteract the effects of histamine. This decreases the amount of blood in the nasopahryngeal and sinus mucosa and reduces swelling. Nasal sprays are available that can be applied directly to the nasal lining and oral systemic preparations are available. Decongestants are stimulants and may cause increased heart rate and blood pressure, headaches, isomnia, agitation and difficulty emptying the bladder. Use of topical decongestants for longer than several days can cause loss of effectiveness and rebound congestion, in which nasal passages become more severely swollen than before treatment.

TOPICAL CORTICOSTEROIDS. Topical **corticosteroids** reduce mucous membrane inflammation and are available by prescription. Allergies tend to become worse as the season progresses because the immune system becomes sensitized to particular antigens and can produce a faster, stronger response. Topical corticosteroids are especially effective at reducing this seasonal sensitization because they work more slowly and last longer than most other medication types. As a result, they are best started before allergy season begins. Side effects are usually mild, but may include headaches, nosebleeds, and unpleasant taste sensations.

MAST CELL STABILIZERS. Cromolyn sodium prevents the release of mast cell granules, thereby preventing release of histamine and the other chemicals contained in them. It acts as a preventive treatment if it is begun several weeks before the onset of the allergy season. It can be used for perennial AR as well.

Immunotherapy

Immunotherapy, also known as desensitization or allergy shots, alters the balance of antibody types in the body, thereby reducing the ability of IgE to cause allergic reactions. Immunotherapy is preceded by allergy testing to determine the precise allergens responsible. Injections involve very small but gradually increasing amounts of allergen, over several weeks or months, with periodic boosters. Full benefits may take up to several years to achieve and are not seen at all in about one in five patients. Individuals receiving all shots will be monitored closely following each shot because of the small risk of **anaphylaxis**, a condition that can result in difficulty breathing and a sharp drop in blood pressure.

Alternative treatment

Alternative treatments for AR often focus on modulation of the body's immune response, and frequently center around diet and lifestyle adjustments. Chinese herbal medicine can help rebalance a person's system, as can both acute and constitutional homeopathic treatment. Vitamin C in substantial amounts can help stabilize the mucous membrane response. For symptom relief, western herbal remedies including eyebright (*Euphrasia officinalis*) and nettle (*Urtica dioica*) may be helpful. Bee pollen may also be effective in alleviating or eliminating AR symptoms. A 2004 report said that **phototherapy** (treatment with a combination of ultraviolet and visible light) decreased the symptoms of allergic rhinitis in a majority of patients who did not respond well to traditional drug treatment.

Prognosis

Most people with AR can achieve adequate relief with a combination of preventive strategies and treatment. While allergies may improve over time, they may also get worse or expand to include new allergens. Early treatment can help prevent an increased sensitization to other allergens.

Prevention

Reducing exposure to pollen may improve symptoms of seasonal AR. Strategies include the following:

- stay indoors with windows closed during the morning hours, when pollen levels are highest
- keep car windows up while driving
- use a surgical face mask when outside
- avoid uncut fields
- learn which trees are producing pollen in which seasons, and avoid forests at the height of pollen season
- wash clothes and hair after being outside
- clean air conditioner filters in the home regularly
- use electrostatic filters for central air conditioning

Moving to a region with lower pollen levels is rarely effective, since new allergies often develop

Preventing perennial AR requires identification of the responsible allergens.

Mold spores:

- keep the house dry through ventilation and use of dehumidifiers
- use a disinfectant such as dilute bleach to clean surfaces such as bathroom floors and walls
- have ducts cleaned and disinfected
- clean and disinfect air conditioners and coolers
- throw out moldy or mildewed books, shoes, pillows, or furniture

House dust:

- vacuum frequently, and change the bag regularly. Use a bag with small pores to catch extra-fine particles
- clean floors and walls with a damp mop
- install electrostatic filters in heating and cooling ducts, and change all filters regularly

Animal dander:

- avoid contact if possible
- wash hands after contact
- vacuum frequently
- keep pets out of the bedroom, and off furniture, rugs, and other dander-catching surfaces
- have your pets bathed and groomed frequently

Resources

Periodicals

Finn, Robert. "Rhinoohototherapy Targets Allergic Rhinitis." *Skin & Allergy News* (July 2004): 62.

"What's New in: Asthma and Allergic Rhinitis." *Pulse* (September 20, 2004): 50.

rhinitis [ri-ni´tis]

inflammation of the mucous membrane of the nose; it may be either mild and chronic or acute. Viruses, bacteria, and allergens are responsible for its varied manifestations. Often a viral rhinitis is complicated by a bacterial infection caused by streptococci, staphylococci, and pneumococci or other bacteria. **HAY FEVER**, an acute type of allergic rhinitis, is also subject to bacterial complications. Many factors assist the invasion of the mucous membranes by bacteria, including allergens, excessive dryness, exposure to dampness and cold, excessive inhalation of dust, and injury to the nasal cilia due to viral infection.

It usually is not serious, but some forms may be contagious. The mucous membrane of the nose becomes swollen and there is a nasal discharge. Some types are accompanied by fever, muscle aches, and general discomfort with sneezing and running eyes. Breathing through the nose may become difficult or impossible. Often rhinitis is accompanied by inflammation of the throat and sinuses. If bacterial infection develops, the nasal discharge is thick and contains pus.

Acute rhinitis is the medical term for the **COMMON COLD**. *Chronic rhinitis* may result in permanent thickening of the nasal mucosa. Treatment of rhinitis is aimed at eliminating the primary cause and administration of decongestants to relieve nasal congestion.

acute rhinitis **common cold**.

allergic rhinitis any allergic reaction of the nasal mucosa, occurring perennially (**nonseasonal allergic RHINITIS**) or seasonally (**HAY FEVER**).

atrophic rhinitis a chronic form of nonallergic noninfectious rhinitis marked by wasting of the mucous membrane and the glands. It is sometimes the result of trauma, vascular damage by radiation therapy, and environmental irritants, and disease has also been implicated.

rhinitis caseo´sa that with a caseous, gelatinous, and fetid discharge.

fibrinous rhinitis **membranous rhinitis**.

hypertrophic rhinitis that with thickening and swelling of the mucous membrane.

membranous rhinitis chronic rhinitis with the formation of a false membrane, as in nasal **DIPHTHERIA**; called also **fibrinous rhinitis**.

nonseasonal allergic rhinitis allergic rhinitis occurring continuously or intermittently all year round, due to exposure to a more or less ever-present allergen, marked by sudden attacks of sneezing, swelling of the nasal mucosa with profuse watery discharge, itching of the eyes, and lacrimation. Called also **nonseasonal** or **perennial hay fever**.

seasonal allergic rhinitis **hay fever**.

vasomotor rhinitis

1. nonallergic rhinitis in which transient changes in vascular tone and permeability (with the same symptoms of allergic rhinitis) are brought on by such stimuli as mild chilling, fatigue, anger, and anxiety.

2. any condition of allergic or nonallergic rhinitis, as opposed to infectious rhinitis.

EXHIBIT 128

food allergy Sensitivity to one or more of the components of normal diets. Food allergy is much less common than unscientific claims might suggest and established methods of testing, including **DOUBLE-BLIND TRIALS** have shown that food allergy is not the basis of the many disorders commonly claimed to arise from it. **PEANUT ALLERGY** is becoming more common and may be dangerous. Monosodium glutamate can cause the '**CHINESE RESTAURANT SYNDROME**'. Tartrazine sensitivity is established. Other additives, such as sulphur dioxide, sulphites, azo dyes and benzoate preservatives also sometimes cause genuine allergic reactions, such as asthma. Allergy to basic foodstuffs seldom occurs.

EXHIBIT 129

Atopic dermatitis | definition of atopic dermatitis by Medical dictionary

https://medical-dictionary.thefreedictionary.com/atopic+dermatitis

atopic dermatitis

Also found in: **Dictionary**, **Thesaurus**, **Acronyms**, **Encyclopedia**, **Wikipedia**.

Related to atopic dermatitis: **eczema**

Atopic Dermatitis

Definition

Eczema is a general term used to describe a variety of conditions that cause an itchy, inflamed skin rash. Atopic **dermatitis**, a form of eczema, is a non-contagious disorder characterized by chronically inflamed skin and sometimes intolerable itching.

go bananas over (something)

go bananas over (something)To express great excitement about something in an exuberant manner. She totally went bananas over that… Go To Article

She totally went bananas over that... *Go To Article*

RELATED ITEMS

pack up
To put one's belongings into a box, suitcase, or other... *Watch Video*

hang on (someone's) lips
hang on (someone's) lipsTo listen very closely, intently, or... *Watch Video*

let (one) have it
Watch Video

Description

Atopic dermatitis refers to a wide range of diseases that are often associated with stress and allergic disorders that involve the respiratory system, like **asthma** and hay **fever**. Although atopic dermatitis can appear at any age, it is most common in children and young adults. Symptoms usually abate before the age of 25 and do not affect the patient's general health.

About one in ten babies develop a form of atopic dermatitis called infantile eczema. Characterized by skin that oozes and becomes encrusted, infantile eczema most often occurs on the face and scalp. The condition usually improves before the child's second birthday, and medical attention can keep symptoms in check until that time.

When atopic dermatitis develops after infancy, inflammation, blistering, oozing, and crusting are less pronounced. The patient's sores become dry, turn from red to brownish-gray, and skin may thicken and become scaly. In dark-skinned individuals, this condition can cause the complexion to lighten or darken. **Itching** associated with this condition is usually worst at night. It can be so intense that patients scratch until their sores bleed, sometimes causing scarring and infection.

Atopic dermatitis affects about 3% of the population of the United States, and about 80% of the people who have the condition have one or more relatives with the same condition or a similar one. Symptoms tend to be most severe in females. Atopic dermatitis can erupt on any part of the skin, and crusted, thickened patches on the fingers, palms, or the soles of the feet can last for years. In teenagers and young adults, atopic dermatitis often appears on one or more of the following areas:

- elbow creases
- backs of the knees
- ankles
- wrists
- face
- neck
- upper chest
- palms and between the fingers

Causes and symptoms

While allergic reactions often trigger atopic dermatitis, the condition is thought to be the result of an inherited over-active immune system or a genetic defect that causes the skin to lose abnormally large amounts of moisture. The condition can be aggravated by a cycle that develops in which the skin itches, the patient scratches, the condition worsens, the itching

worsens, the patient scratches, etc. This cycle must be broken by relieving the itching to allow the skin time to heal. If the skin becomes broken, there is also a risk of developing skin infections which, if not recognized and treated promptly, can become more serious.

Atopic dermatitis can erupt on any part of the skin. In infants, it often appears on the face, scalp, and knees, while it develops on the elbows, neck, back of the knees, and ankles in adults.

(Illustration by Electronic Illustrators Group.)

Key terms

Corticosteroid — A steroid hormone produced by the adrenal gland or as a synthetic compound that reduces inflammati redness, rashes, and irritation.

Dermatitis — Inflammation of the skin.

Symptoms of atopic dermatitis include the following:

- an itchy rash and dry, thickened skin on areas of the body where moisture can be trapped
- continual scratching
- chronic **fatigue**, caused when itching disrupts sleep

An individual is more at-risk for developing the condition if there is a personal or family history of atopic dermatitis, hay fever, asthma, or other allergies. Exposure to any of the following can cause a flare-up:

- hot or cold temperatures
- wool and synthetic fabrics
- detergents, fabric softeners, and chemicals
- use of drugs that suppress immune-system activity

Certain foods, such as peanuts, cow's milk, eggs, and fish, can trigger symptoms of atopic dermatitis. A small percentage of patients with atopic dermatitis find that their symptoms worsen after having been exposed to dust, feather pillows, rough-textured fabrics, or other materials to which dust adheres.

Diagnosis

Diagnosis of atopic dermatitis is usually based on the patient's symptoms and personal and family health history. Skin tests do not generally provide reliable information about this condition.

Treatment

Atopic dermatitis cannot be cured, but the severity and duration of symptoms can be controlled. A dermatologist should be consulted when symptoms first appear, and is likely to recommend warm baths to loosen encrusted skin, followed by applications of petroleum jelly or vegetable shortening to prevent the skin's natural moisture from escaping.

Externally applied (topical) steroids or preparations containing coal tar can relieve minor itching, but coal tar has an unpleasant odor, stains clothes, and may increase skin-cancer risk. Excessive use of steroid creams in young children can alter growth. Pregnant women should not use products that contain coal tar. Topical steroids can cause itching, burning, **acne**, permanent stretch marks, and thinning and spotting of the skin. Applying topical steroids to the area around the eyes can cause **glaucoma**.

Oral **antihistamines**, such as diphenhydramine (Benadryl), can relieve symptoms of allergy-related atopic dermatitis. More concentrated topical steroids are recommended for persistent symptoms. A mild tranquilizer may be prescribed to reduce **stress** and help the patient sleep, and **antibiotics** are used to treat secondary infections.

Cortisone ointments should be used sparingly, and strong preparations should never be applied to the face, groin, armpits, or rectal area. Regular medical monitoring is recommended for patients who use cortisone salves or lotions to control widespread symptoms. Oral cortisone may be prescribed if the patient does not respond to other treatments, but patients who take the medication for more than two weeks have a greater-than-average risk of developing severe symptoms when the treatment is discontinued.

Allergy shots rarely improve atopic dermatitis and sometimes aggravate the symptoms. Since **food allergies** may trigger atopic dermatitis, the doctor may suggest eliminating certain foods from the diet if other treatments prove ineffective.

If symptoms are extremely severe, ultraviolet light therapy may be prescribed, and a wet body wrap recommended to help the skin retain moisture. This technique, used most often with children, involves sleeping in a warm room while wearing wet pajamas under dry clothing, rain gear, or a nylon sweatsuit. The patient's face may be covered with wet gauze covered by elastic bandages, and his hands encased in wet socks covered by dry ones.

A physician should be notified if the condition is widespread or resists treatment, or the skin oozes, becomes encrusted, or smells, as this may indicate an infection.

Alternative treatment

Alternative therapies can sometimes bring relief or resolution of atopic dermatitis when conventional therapies are not helping. If the condition becomes increasingly widespread or infected, a physician should be consulted.

Helpful alternative treatments for atopic dermatitis may include:

- Taking regular brisk walks, followed by bathing in warm water sprinkled with essential oil of lavender (*Lavandula officinalis*); lavender oil acts as a nerve relaxant for the whole body including the skin
- Supplementing the diet daily with zinc, fish oils, vitamin A, vitamin E, and evening primrose oil (*Oenothera biennis*)-all good sources of nutrients for the skin
- Reducing or eliminating red meat from the diet
- Eliminating or rotating potentially allergic foods such as cow's milk, peanuts, wheat, eggs, and soy
- Implementing **stress reduction** techniques in daily life.

Herbal therapies also can be helpful in treating atopic dermatitis. Western herbal remedies used in the treatment of this

condition include burdock (*Arctium lappa*) and *Ruta (Ruta graveolens)*. Long-term herbal therapy requires monitoring and should be guided by an experienced practitioner.

Other alternative techniques that may be useful in the treatment of atopic dermatitis include:

- Acupressure (**acupuncture** without needles) to relieve tension that may trigger a flare
- Aromatherapy, using essential oils like lavender, thyme (*Thymus vulgaris*), jasmine (*Jasminum officinale*) and chamomile (*Matricaria recutita*) in hot water, to add a soothing fragrance to the air
- Shiatsu massage and **reflexology**, performed by licensed practitioners, to alleviate symptoms by restoring the body's natural balance
- Homeopathy, which may temporarily worsen symptoms before relieving them, and should be supervised by a trained alternative healthcare professional
- Hydrotherapy, which uses water, ice, liquid, and steam, to stimulate the immune system
- Juice therapy to purify the liver and relieve bowel congestion
- Yoga to induce a sense of serenity.

Prognosis

Atopic dermatitis is unpredictable. Although symptoms occur less often with age and sometimes disappear altogether, they can recur without warning. Atopic dermatitis lowers resistance to infection and increases the risk of developing **cataracts**. Sixty percent of patients with atopic dermatitis will experience flares and remissions throughout their lives.

Prevention

Research has shown that babies weaned from breast milk before they are four months old are almost three times more likely than other babies to develop recurrent eczema. Feeding eggs or fish to a baby less than one year old can activate symptoms, and babies should be shielded from such irritants as mites, molds, pet hair, and smoke.

Possible ways to prevent flare-ups include the following:

- eliminate activities that cause sweating
- lubricate the skin frequently
- avoid wool, perfumes, fabric softeners, soaps that dry the skin, and other irritants
- avoid sudden temperature changes

A doctor should be notified whenever any of the following occurs:

- fever or relentless itching develop during a flare
- an unexplained rash develops in someone who has a personal or family history of eczema or asthma
- inflammation does not decrease after seven days of treatment with an over-the-counter preparation containing coal tar or steroids
- a yellow, tan, or brown crust or pus-filled blisters appear on top of an existing rash
- a person with active atopic dermatitis comes into contact with someone who has cold sores, **genital herpes**, or another viral skin disease

Resources

Organizations

American Academy of Dermatology. 930 N. Meacham Road, P.O. Box 4014, Schaumburg, IL 60168-4014. (847) 330-0230. Fax: (847) 330-0050. **http://www.aad.org**.

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EXHIBIT 130

Anaphylaxis | definition of anaphylaxis by Medical dictionary

anaphylaxis

Also found in: **Dictionary**, **Thesaurus**, **Acronyms**, **Encyclopedia**, **Wikipedia**.
Related to anaphylaxis: **anaphylaxis management**, **idiopathic anaphylaxis**

Anaphylaxis

Definition

Anaphylaxis is a rapidly progressing, life-threatening allergic reaction.

Description

Anaphylaxis is a type of allergic reaction, in which the immune system responds to otherwise harmless substances from the environment. Unlike other allergic reactions, however, anaphylaxis can kill. Reaction may begin within minutes or even seconds of exposure, and rapidly progress to cause airway constriction, skin and intestinal irritation, and altered heart rhythms. In severe cases, it can result in complete airway obstruction, **shock**, and **death**.

Causes and symptoms

Causes

Like the majority of other allergic reactions, anaphylaxis is caused by the release of histamine and other chemicals from mast cells. Mast cells are a type of white blood cell and they are found in large numbers in the tissues that regulate exchange with the environment: the airways, digestive system, and skin.

On their surfaces, mast cells display antibodies called IgE (immunoglobulin type E). These antibodies are designed to detect environmental substances to which the immune system is sensitive. Substances from a genuinely threatening source, such as bacteria or viruses, are called antigens. A substance that most people tolerate well, but to which others have an allergic response, is called an allergen. When IgE antibodies bind with allergens, they cause the mast cell to release histamine and other chemicals, which spill out onto neighboring cells.

The interaction of these chemicals with receptors on the surface of blood vessels causes the vessels to leak fluid into surrounding tissues, causing fluid accumulation, redness, and swelling. On the smooth muscle cells of the airways and digestive system, they cause constriction. On nerve endings, they increase sensitivity and cause **itching**.

In anaphylaxis, the dramatic response is due both to extreme hypersensivity to the allergen and its usually systemic distribution. Allergens are more likely to cause anaphylaxis if they are introduced directly into the circulatory system by injection. However, exposure by ingestion, inhalation, or skin contact can also cause anaphylaxis. In some cases, anaphylaxis may develop over time from less severe **allergies**.

Anaphylaxis is most often due to allergens in foods, drugs, and insect venom. Specific causes include:

- Fish, shellfish, and mollusks
- Nuts and seeds
- Stings of bees, wasps, or hornets
- Papain from meat tenderizers
- Vaccines, including flu and **measles** vaccines
- Penicillin
- Cephalosporins
- Streptomycin
- Gamma globulin
- Insulin
- Hormones (ACTH, thyroid-stimulating hormone)
- **Aspirin** and other NSAIDs
- Latex, from exam gloves or condoms, for example.

Exposure to cold or **exercise** can trigger anaphylaxis in some individuals.

Key terms

ACTH — Adrenocorticotropic hormone, a hormone normally produced by the pituitary gland, sometimes taken as a treatment for arthritis and other disorders.

Antibody — An immune system protein which binds to a substance from the environment.

NSAIDs — Non-steroidal antiinflammatory drugs, including aspirin and ibuprofen.

Tracheostomy tube — A tube which is inserted into an incision in the trachea (tracheostomy) to relieve upper airway

obstruction.

Symptoms

Symptoms may include:

- Urticaria (**hives**)
- Swelling and irritation of the tongue or mouth
- Swelling of the sinuses
- Difficulty breathing
- Wheezing
- Cramping, vomiting, or **diarrhea**
- Anxiety or confusion
- Strong, very rapid heartbeat (**palpitations**)
- Loss of consciousness.

Not all symptoms may be present.

Diagnosis

Anaphylaxis is diagnosed based on the rapid development of symptoms in response to a suspect allergen. Identification of the culprit may be done with RAST testing, a blood test that identifies IgE reactions to specific allergens. Skin testing may be done for less severe anaphylactic reactions.

Treatment

Emergency treatment of anaphylaxis involves injection of adrenaline (epinephrine) which constricts blood vessels and counteracts the effects of histamine. Oxygen may be given, as well as intravenous replacement fluids. **Antihistamines** may be used for skin rash, and aminophylline for bronchial constriction. If the upper airway is obstructed, placement of a breathing tube or tracheostomy tube may be needed.

Prognosis

The rapidity of symptom development is an indication of the likely severity of reaction: the faster symptoms develop, the more severe the ultimate reaction. Prompt emergency medical attention and close monitoring reduces the likelihood of death. Nonetheless, death is possible from severe anaphylaxis. For most people who receive rapid treatment, recovery is complete.

Prevention

Avoidance of the allergic trigger is the only reliable method of preventing anaphylaxis. For insect allergies, this requires recognizing likely nest sites. Preventing **food allergies** requires knowledge of the prepared foods or dishes in which the allergen is likely to occur, and careful questioning about ingredients when dining out. Use of a Medic-Alert tag detailing dr allergies is vital to prevent inadvertent administration during a medical emergency.

People prone to anaphylaxis should carry an "Epi-pen" or "Ana-kit," which contain an adrenaline dose ready for injection.

Resources

Other

The Meck Page. February 20, 1998. **http://www.merck.com**.

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EXHIBIT 131

ALLERGY RESEARCH

Allergy Facts
Allergy Capitals
Anaphylaxis in America
Atopic Dermatitis in America
Access to Pseudoephedrine
Consensus Study of Food Allergies
Extreme Allergies and Climate Change
My Life With Food Allergy Report
Patient Focused Drug Development

Allergy Facts and Figures

An allergy is when your immune system reacts to a foreign substance, called an allergen. It could be something you eat, inhale into your lungs, inject into your body or touch. This reaction could cause coughing, sneezing, itchy eyes, a runny nose and a scratchy throat. In severe cases, it can cause rashes, hives, low blood pressure, breathing trouble, asthma attacks and even death.

There is no cure for allergies. You can manage allergies with prevention and treatment. More Americans than ever say they manage allergies. It is among the country's most common, but overlooked, diseases.

How Many People Do Allergies Affect?

- More than 50 million Americans have experienced various types of allergies each year.[13]
- Allergies are the 6th leading cause of chronic illness in the U.S.[1]

How Many People Get Sick from Allergies?

- Allergic conditions are the most common health issues affecting children in the U.S.[1] In 2015, 8.2 percent of adults and 8.4 percent of children were diagnosed with hay fever.[2]
- People visit the emergency room about 200,000 times each year because of food allergies.[1]

How Many People Die from Allergies?

- The most common triggers for anaphylaxis, a life-threatening reaction, are medicines, food and insect stings.[3] Medicines cause the most allergy related deaths.[3]
- African-Americans and the elderly have the deadliest reactions to medicines, food or unknown allergens.[5]

What Are the Costs of Allergies?

- Annual cost of allergies exceeds $18 billion.[1]
- Food allergies cost about $25 billion each year.[6]

What Are Indoor and Outdoor Allergies?

Types of indoor and outdoor allergies include sinus swelling, seasonal and returning allergies, hay fever and nasal allergies. Many people with allergies often have more than one type of allergy. The most common indoor/outdoor allergy triggers are: **tree, grass and weed pollen**, **mold spores**, **dust mites**, **cockroaches**, and **cat, dog** and rodent dander.

- Immunotherapy (allergy shots) helps reduce hay fever symptoms in about 85 percent of people with allergic rhinitis.[1]
- **Allergic rhinitis**, often called hay fever[1] affects 6.1 million of the children population and 20 million of the adult population.[2,1]
- In 2015, white children were more likely to have hay fever than African-American children.[2]

- The same triggers for indoor/outdoor allergies also often cause **eye allergies**.

How Common Are Skin Allergies?

Skin allergies include skin inflammation, eczema, hives, chronic hives and contact allergies. Plants like poison ivy, poison oak and poison sumac are the most common skin allergy triggers. But skin contact with cockroaches and dust mites, certain foods or latex may also cause skin allergy symptoms.

- In 2015, 8.8 million children had skin allergies.[2]
- Children age 0-4 are most likely to have skin allergies.[2]
- In 2015, African-American children in the U.S. were more likely to have skin allergies than white children.[2]

How Common Are Food Allergies?

Children have food allergies more often than adults. Eight foods cause most food allergy reactions. They are milk, soy, eggs, wheat, peanuts, tree nuts, fish and shellfish.

- Peanut is the most common allergen followed by milk and shelfish.[3]
- In 2015, 4.2 million children in the US have food allergies.[2]
- In 2014, 5.4 percent of US children under age 18 had food allergies.[1]

How Common Are Drug Allergies?

- Penicillin is the most common allergy trigger for those with drug allergies. Up to 10 percent of people report being allergic to this common antibiotic.[7]
- Bad drug reactions may affect 10 percent of the world's population. These reactions affect up to 20 percent of all hospital patients.[3]

How Common Is Latex Allergy?

- About 1 to 6 percent of people in the U.S. have a latex allergy.[8]
- Health care workers are becoming more concerned about latex allergies. About 8-12 percent of health care workers will get a latex allergy.[8]

How Common Is Insect Allergy?

People who have insect allergies are often allergic to bee and wasp stings and poisonous ant bites. Cockroaches and dust mites may also cause nasal or skin allergy symptoms.

- Insect sting allergies affect 5 percent of the population.[9]
- At least 90-100 deaths occur each year in the United States due to insect sting anaphylaxis.[10]

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HELPFUL LINKS

Asthma Capitals
Asthma Facts
Asthma Research
Asthma Triggers

Allergy Capitals
Allergy Facts
Allergy Research
Allergy Treatment

AAFA Chapters
Find a Local Support Group
Join Our Action Network
Kids With Food Allergies

Join Our Asthma Community
Health Professionals
Fundraise for AAFA
Make a Donation
Shop to Support

for life without limits™
Asthma and Allergy Foundation of America
1235 South Clark Street Suite 305, Arlington, VA 22202
Phone: 1-800-7-ASTHMA (1.800.727.8462)
Contact Us Online

EXHIBIT 132

Centers for Disease Control and Prevention
CDC 24/7: Saving Lives, Protecting People™

National Center for Health Statistics

Allergies and Hay Fever

Data are for the U.S.

Morbidity: Adults aged 18 and over

- Number with diagnosed hay fever in the past 12 months: 19.2 million
- Percent with diagnosed hay fever in the past 12 months: 7.7%

Source: Summary Health Statistics Tables for U.S. Adults: National Health Interview Survey, 2018, Tables A-2b, A-2c [PDF – 166 KB]

Morbidity: Children under age 18 years

- Number with reported hay fever in the past 12 months: 5.2 million
- Percent with reported hay fever in the past 12 months: 7.2%
- Number with reported respiratory allergies in the past 12 months: 7.1 million
- Percent with reported respiratory allergies in the past 12 months: 9.6%
- Number with reported food allergies in the past 12 months: 4.8 million
- Percent with reported food allergies in the past 12 months: 6.5%
- Number with reported skin allergies in the past 12 months: 9.2 million
- Percent with reported skin allergies in the past 12 months: 12.6%

Source: Summary Health Statistics Tables for U.S. Children: National Health Interview Survey, 2018, Tables C-2b, C-2c [PDF – 244 KB]

Physician office visits

- Number of physician office visits with allergic rhinitis as the primary diagnosis: 12.0 million
- Number of physician office visits with contact dermatitis or other eczema as the primary diagnosis: 3 million

Source: National Ambulatory Medical Care Survey: 2016 National Summary Tables, table 15 [PDF – 906 KB]

Emergency department visits

- Number of emergency room visits with contact dermatitis or other eczema as the primary diagnosis: 277,000

Source: National Hospital Ambulatory Medical Care Survey: 2017 Emergency Department Summary Tables, table 12 [PDF – 863 KB]

More data

- Trends in Allergy from Health, United States

Related Links

- Ambulatory Health Care Data
- National Health Interview Survey
- Centers for Disease Control and Prevention: Food Allergies in Schools
- Centers for Disease Control and Prevention: Asthma and Allergies in the Workplace
- National Institute of Allergy and Infectious Diseases
- American Academy of Allergy Asthma & Immunology

Page last reviewed: January 20, 2017
Content source: CDC/National Center for Health Statistics

EXHIBIT 133

FARE
Food Allergy Research & Education

Food Allergy Facts and Statistics for the U.S.

What Is a Food Allergy?

- A food allergy is an adverse health effect resulting from a specific immune response that occurs reproducibly on exposure to a given food.[1] The health effect, called an allergic reaction, occurs because the immune system attacks food proteins that are normally harmless.
- Food-induced anaphylaxis is a serious allergic reaction that is sudden in onset and may cause death.[1]
- A treatment for peanut allergy was approved in January 2020 by the U.S. Food and Drug Administration, but this treatment is not appropriate for every peanut allergy patient and is approved only for patients from age 4 through age 17.[69] There are no approved treatments for other food allergies. Strict avoidance of food allergens and early recognition and management of allergic reactions to food are important measures to prevent serious health consequences.[1]

To Which Foods Are People Allergic?

- More than 170 foods have been reported to cause reactions in the U.S.[1]
- Eight major food allergens – milk, egg, peanut, tree nuts, wheat, soy, fish and crustacean shellfish – are responsible for most of the serious food allergy reactions in the United States.[1]
- The most common food allergies in children are allergies to peanut, milk, shellfish and tree nut.[9]
- The most common food allergies in adults are allergies to shellfish, milk, peanut and tree nut.[7]
- Allergists consider sesame allergy to be an emerging concern. Sesame has caused severe reactions, including fatal anaphylaxis.[2,3,4,5,6]

How Many People Have Food Allergies?

- Approximately 32 million people in the United States have food allergies.[7,8,9]
- Nearly 11 percent of people age 18 or older – more than 26 million adults – have food allergies.[7,8]
- Results from a 2015-2016 survey of more than 38,000 children indicate that 5.6 million children, or nearly 8 percent, have food allergies.[8,9] That's one in 13 children, or roughly two in every classroom.
- Studies published in 2018 and 2019 estimate the U.S. population that reports convincing symptoms of allergic reactions to specific foods.[2,7,9]
    - shellfish: 8.2 million
    - milk: 6.1 million
    - peanut: 6.1 million
    - tree nuts: 3.9 million
    - egg: 2.6 million
    - fin fish: 2.6 million
    - wheat: 2.4 million
    - soy: 1.9 million
    - sesame: 0.7 million

- In a 2007 survey of 9,500 children conducted by the Centers for Disease Control and Prevention, 3.9 percent were reported to have a food or digestive allergy within the past year.[10]
- About 40 percent of children with food allergies have multiple food allergies (more than one food to which they're allergic).[9]

Food Allergies Are on the Rise

- The Centers for Disease Control & Prevention report that between 1997-1999 and 2009-2011, food allergy prevalence among children increased by 50 percent.[11]
- In the United States, the prevalence of childhood peanut or tree nut allergy appears to have more than tripled between 1997 and 2008.[12]
- The prevalence of childhood food allergy has increased at a rate of 2.1 percent per decade among blacks, 1.2 percent per decade among Hispanics and 1 percent per decade among whites, according to a study of self-reported allergy.[13]

Food Allergy Is a Serious Public Health and Economic Issue

- A food allergy is an impairment that limits a major life activity and may qualify an individual for protection under the Americans with Disabilities Act of 1990 (ADA) and Section 504 of the Rehabilitation Act of 1973.[14]
- Caring for children with food allergies costs U.S. families nearly $25 billion annually.[15]

Food Allergy Reactions Are Serious and Can Be Life-Threatening

- Every three minutes, a food allergy reaction sends someone to the emergency room.[16]
- Each year in the U.S., 200,000 people require emergency medical care for allergic reactions to food.[16]
- Pediatric hospitalizations for food allergy tripled between the late 1990s and the mid-2000s. Between 2004 and 2006, an average of 9,500 children received in-patient hospital care for food allergies each year.[10]
- More than 40 percent of children with food allergies have experienced a severe allergic reaction such as anaphylaxis.[9]
- Medical procedures to treat anaphylaxis resulting from food increased by 377 percent between 2007 and 2016.[68]

Serious Allergic Reactions (Anaphylaxis) Require Immediate Treatment

- Prompt injection of epinephrine (adrenaline) within minutes of the onset of anaphylaxis symptoms is crucial to successfully treating an anaphylactic reaction. A self-injectable epinephrine device is available by prescription.[17]
- Not recognizing the severity of an anaphylactic reaction and treating promptly (i.e., within minutes) with epinephrine is a risk factor for fatalities.[18,19,20]
- More than one dose of epinephrine may be required.[21]
- It is possible to have anaphylaxis without any skin symptoms, such as rash or hives.[22]
- Symptoms of anaphylaxis may recur after initially subsiding (known as a biphasic reaction). Experts recommend an observation period of 4 to 6 hours in the emergency room to monitor that the reaction has been resolved.[21,23]

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Food Allergy Impacts Quality of Life

- About one in three children with food allergy reports being bullied as a result. Among children with allergies to more than two foods, over half report being bullied due to food allergy.[24]
- Compared to children who do not have a medical condition, children with food allergy are twice as likely to be bullied.[25]
- More than one-quarter of parents surveyed during food allergy appointments report that their children do not participate in camp or sleepovers because of food allergy. More than 15 percent do not go to restaurants, and more than 10 percent avoid child care settings or playdates at friends' houses. Ten percent home-school their children to prevent food allergen exposure.[26]
- Among parents of young children in the first year after food allergy diagnosis, most avoid restaurants and about half restrict social activities or travel.[25]
- Mothers of food-allergic children under age five have significantly higher blood-pressure measurements and report significantly greater levels of psychosocial stress than mothers whose preschool-aged children do not have food allergies.[27]

Who is at Highest Risk for Developing Food Allergy?

- Compared to non-Hispanic white children, African American children are at significantly elevated risk of developing food allergy.[9]
- Children from rural communities are less likely to have food allergies than children from urban centers.[28]
- Children from households earning less than $50,000 per year are less likely to report food allergies than are children from households earning more than $50,000 per year.[9]
- Among inner-city children with a family history of hay fever, eczema or asthma, one preschool-aged child in 10 is allergic to eggs, milk, or peanuts.[29]
- Compared to children without food allergy, children with food allergy are more than twice as likely to have asthma and more than three times as likely to have respiratory allergy or eczema.[10]
- Food allergies may trigger or be linked to eosinophilic gastrointestinal diseases.[30]
- While most food allergies develop during childhood, medical records data suggest that at least 15 percent of patients with food allergies are first diagnosed in adulthood.[31] More than one in four adults with food allergies report that all of their food allergies developed during adulthood, and nearly half of adults with food allergy report developing at least one food allergy during adulthood.[7]

Who Is at Highest Risk for Fatal Anaphylaxis?

- Although a severe or fatal reaction can happen at any age, teenagers and young adults with food allergies are at the highest risk of fatal food-induced anaphylaxis.[18,19,20]
- Individuals with food allergies who also have asthma may be at increased risk for severe or fatal food allergy reactions.[18,20]

Under What Circumstances Do Reactions Occur?

- Food allergy reactions typically involve foods that are believed to be safe. Allergic reactions can result from mislabeling or cross-contact during food preparation.[19,32,33,34]
- Limited skin contact with peanut butter or inhaling peanut butter from a short distance is unlikely to elicit a significant allergic reaction. These results cannot be generalized to more

extensive contact or to other forms of peanut.[35,36] *Note: Limited contact with peanut butter presents a greater risk to young children, who frequently put their hands in their mouths.*

- Food proteins released into the air in vapor or steam from cooked foods can potentially cause allergic reactions. Reactions from vapor or steam can resemble reactions to inhaled allergens that cause hay fever or asthma symptoms, such as pollen or animal dander.[37,38]

Where Do Reactions Occur?

- Reports suggest that the majority of fatal food allergy reactions are triggered by food consumed outside the home.[18,19,20]
- One study looking at peanut and tree nut allergy reactions in restaurants and other food establishments found that reactions were frequently attributed to desserts, that Asian restaurants and take-out dessert stores (bakeries, ice cream shops) were common sources of foods that triggered reactions, and that the food establishment was often not properly notified of a food allergy by the customer with the allergy.[39]
- Research on self-reported reactions occurring on commercial airlines indicates that peanut and tree nut reactions on planes have resulted from ingestion, contact and inhalation. Ingestion of an allergen remains the main concern for severe reactions.[40,41,42]

Are Food Allergy Reactions Common at School?

- More than 15 percent of school-aged children with food allergies have had a reaction in school.[43,44]
- In a 2013-2014 survey of schools participating in a program to provide undesignated (stock) epinephrine for emergency use, over 600 schools – more than 10 percent – reported at least one case of anaphylaxis.[45]
- Approximately 20-25 percent of epinephrine administrations in schools involve individuals whose allergy was unknown at the time of the reaction.[46]
- In one large school district during the 2012-2013 school year, more than half of the 38 individuals who were treated with district-supplied emergency epinephrine were experiencing their first severe reaction.[47]
- Food allergy reactions can happen in multiple locations throughout the school, and are not limited to the cafeteria. Care must be exercised during bake sales, classroom parties and opportunities for snacking.[20,46]

Avoiding Allergens Requires Careful Reading of Labels and Stringent Cleaning Procedures

- Even trace amounts of a food allergen can cause a reaction.[48,49,50,51,52,53]
- Some studies have shown that most individuals with peanut and soy allergies can safely eat highly refined oils made from these ingredients. However, cold-pressed, expeller-pressed, or extruded oils should be avoided. Talk to your doctor about oils made from ingredients to which you are allergic.[54,55,56,57,58,48]
- According to the Food Allergen Labeling and Consumer Protect Act (FALCPA) the major eight allergens must be declared in simple terms, either in the ingredient list or via a separate allergen statement. However, FALCPA does not regulate the use of advisory/precautionary labeling (e.g., “may contain,” “made in a facility that also processes”).[59]
- Advisory/precautionary labeling is voluntary. The terms do not reflect specific risks, and random product testing has found allergen levels ranging from undetectable to amounts that can cause allergic reactions.[1,32]

- A study showed that peanut protein was detected in 7.3 percent of products bearing advisory/precautionary labeling for peanut.[33]
- A study showed that peanut can be cleaned from the hands of adults by using running water and soap or commercial wipes, but not by applying antibacterial gels. In addition, peanut was easily removed from surfaces by using common household cleaning sprays or sanitizing wipes but not by wiping with dishwashing liquid.[60]

Can Food Allergies Be Outgrown?

- Although allergies to milk, egg, wheat and soy often resolve in childhood, research suggests that children may outgrow at least some of these food sensitivities more slowly than was found in previous decades, with many children still allergic beyond age 5.[1]
- Allergies to peanuts, tree nuts and shellfish are generally lifelong.[1]

Food Allergy Prevention and Treatment

- The Learning Early About Peanut Allergy (LEAP) study provided evidence that the age at which a child first eats peanut and the frequency of peanut in the diet can influence whether the child develops an allergy to peanut. LEAP findings demonstrate that early, sustained consumption of peanut products is associated with a substantial and significant decrease in the likelihood of developing peanut allergy.[61]
- In 2017, findings from LEAP and related studies led to the release of new guidelines for introduction of peanut.[62]
- A follow-up to the LEAP trial, Persistence of Oral Tolerance to Peanut (LEAP-On), showed that decreased peanut allergy risk among children who consumed peanut throughout early childhood persists even after the children avoid peanut from ages 5 to 6.[63]
- Several immunotherapy approaches are being investigated. Immunotherapy involves intentional exposure to the food allergen, starting with very small amounts and increasing more or less gradually depending on the approach and the protocol. The goal of immunotherapy is to raise the threshold dose of food protein that results in a food allergy reaction. Successful immunotherapy can result in the ability to eat a significant/increased amount of the problem food without a reaction. This can be lost if the problem food is not consumed on an ongoing basis. Immunotherapy results in sustained unresponsiveness when a patient can discontinue exposure for a period of time and still safely eat the problem food. However this is typically only for weeks to several months. Some therapies under investigation include:
    - Oral immunotherapy (OIT)* – To raise the threshold dose at which food allergy reactions occur, progressively greater amounts of allergen are eaten (usually every 2 weeks and under medical supervision) until a maintenance dose is reached. Reported rates of desensitization – that is, increased food allergen tolerance, typically to a preset target amount – vary widely for OIT, ranging from 30 percent to more than 90 percent of trial participants.[64,65] Side effects can be severe, including anaphylaxis and eosinophilic esophagitis.[65] A treatment for peanut allergy was approved in January 2020 by the U.S. Food and Drug Administration, but this treatment is not appropriate for every peanut allergy patient and is approved only for patients from age 4 through age 17.[69]
    - Sublingual immunotherapy (SLIT)* – Food protein is dissolve in liquid and held under the tongue for a time before being spat out or swallowed. As with OIT, the dose of allergen is increased over time until a maintenance dose is reached, although the doses typically used in SLIT are smaller. The desensitization achieved with SLIT can

be equivalent to desensitization achieved with OIT, but SLIT is less likely to cause serious allergic reactions.[66]

- Epicutaneous immunotherapy (EPIT, or skin patch) – EPIT delivers food protein via patches applied to the skin. Clinical trials indicate that EPIT can result in desensitization, especially to peanut. Compared to OIT, EPIT has a better safety profile. [67]

*Oral immunotherapy and sublingual immunotherapy are being conducted both in clinical trials and in private practice.

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EXHIBIT 134

NCHS Data Brief ■ No. 121 ■ May 2013

Trends in Allergic Conditions Among Children: United States, 1997–2011

Kristen D. Jackson, M.P.H.; LaJeana D. Howie, M.P.H., C.H.E.S.; Lara J. Akinbami, M.D.

Key findings

Data from the National Health Interview Survey, 1997–2011

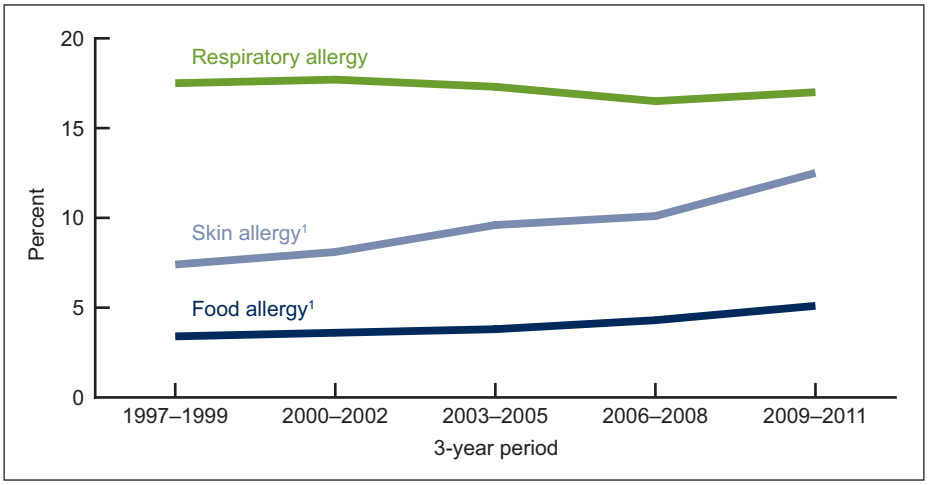
- The prevalence of food and skin allergies increased in children under age 18 years from 1997–2011.
- The prevalence of skin allergies decreased with age. In contrast, the prevalence of respiratory allergies increased with age.
- Hispanic children had a lower prevalence of food allergy, skin allergy, and respiratory allergy compared with children of other race or ethnicities. Non-Hispanic black children were more likely to have skin allergies and less likely to have respiratory allergies compared with non-Hispanic white children.
- Food and respiratory allergy prevalence increased with income level. Children with family income equal to or greater than 200% of the poverty level had the highest prevalence rates.

Allergic conditions are among the most common medical conditions affecting children in the United States (1–5). An allergic condition is a hypersensitivity disorder in which the immune system reacts to substances in the environment that are normally considered harmless (6,7). Food or digestive allergies, skin allergies (such as eczema), and respiratory allergies (such as hay fever) are the most common allergies among children. Allergies can affect a child's physical and emotional health and can interfere with daily activities, such as sleep, play, and attending school (8,9). A severe allergic reaction with rapid onset, anaphylaxis, can be life threatening. Foods represent the most common cause of anaphylaxis among children and adolescents (10,11). Early detection and appropriate interventions can help to decrease the negative impact of allergies on quality of life (6). This report presents recent trends in the prevalence of allergies and differences by selected sociodemographic characteristics for children under age 18 years.

Keywords: allergy • National Health Interview Survey

The prevalence of food and skin allergies increased in children aged 0–17 years from 1997–2011.

Figure 1. Percentage of children aged 0–17 years with a reported allergic condition in the past 12 months: United States, 1997–2011



[1]Significant increasing linear trend for food and skin allergy from 1997–1999 to 2009–2011.
SOURCE: CDC/NCHS, Health Data Interactive, National Health Interview Survey.

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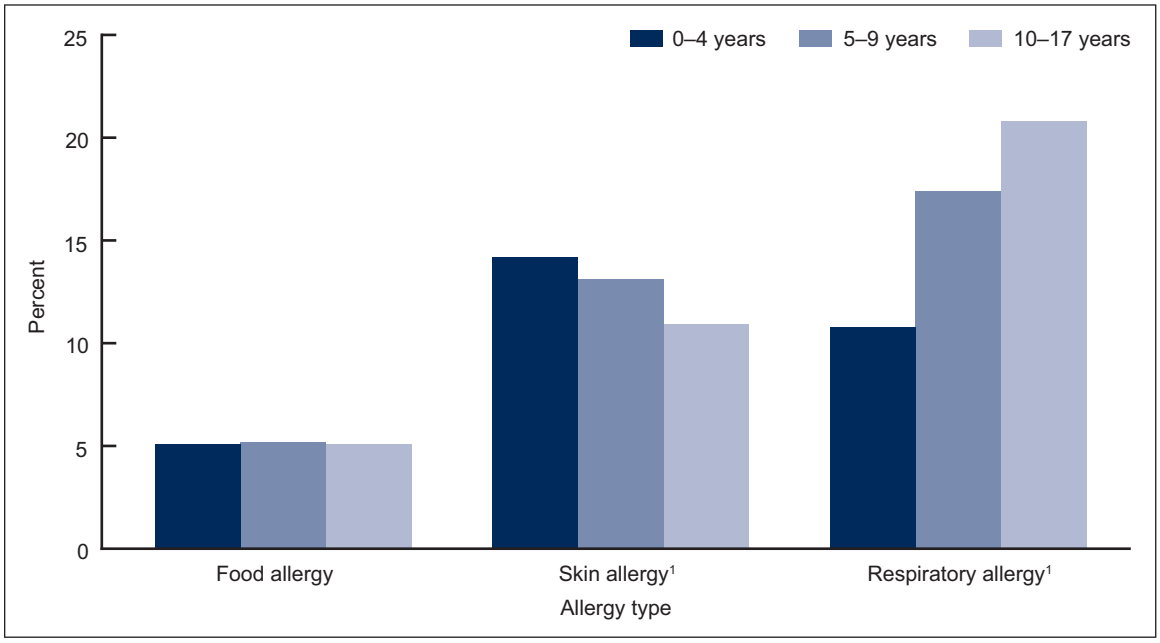
Among children aged 0–17 years, the prevalence of food allergies increased from 3.4% in 1997–1999 to 5.1% in 2009–2011. The prevalence of skin allergies increased from 7.4% in 1997–1999 to 12.5% in 2009–2011. There was no significant trend in respiratory allergies from 1997–1999 to 2009–2011, yet respiratory allergy remained the most common type of allergy among children throughout this period (17.0% in 2009–2011). Skin allergy prevalence was also higher than food allergy prevalence for each period from 1997–2011 (Figure 1).

Younger children were more likely to have skin allergies, while older children were more likely to have respiratory allergies.

Food allergy prevalence was similar among all age groups. Skin allergy prevalence decreased with the increase of age (14.2% among 0–4 years, 13.1% among 5–9 years, and 10.9% among 10–17 years); while respiratory allergy prevalence increased with the increase of age (10.8% among 0–4 years, 17.4% among 5–9 years, and 20.8% among 10–17 years) (Figure 2).

Figure 2. Percentage of children aged 0–17 years with a reported allergic condition in the past 12 months, by age group: United States, average annual 2009–2011

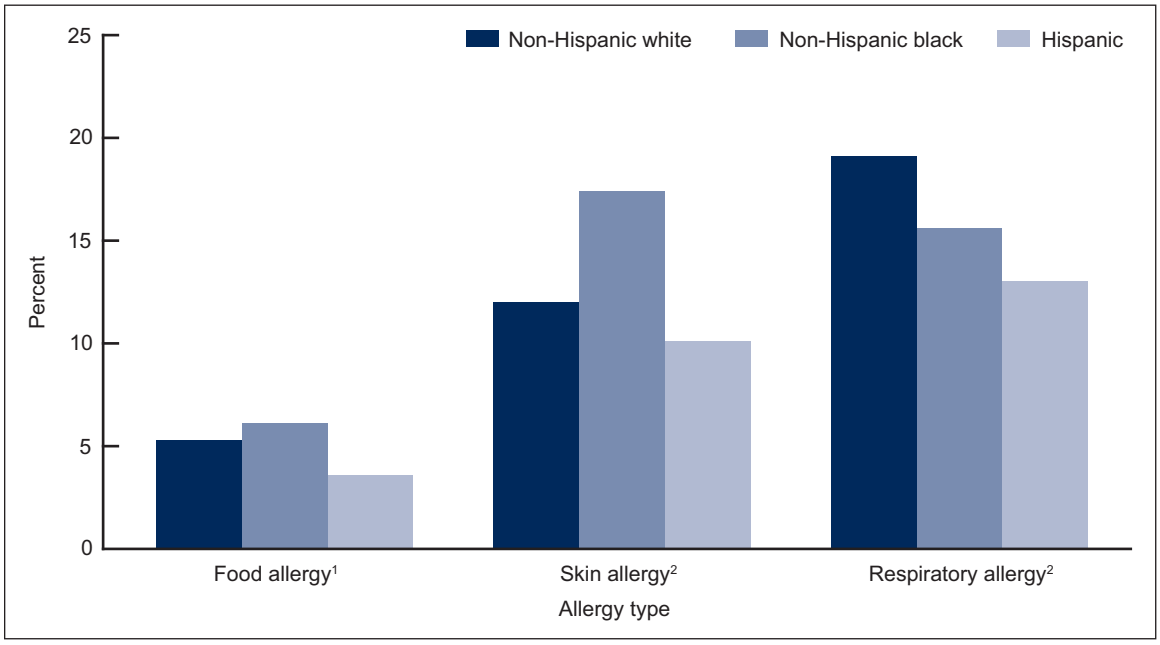


[1]Significant trend by age group.
SOURCE: CDC/NCHS, Health Data Interactive, National Health Interview Survey.

Hispanic children had lower rates of all three types of allergies compared with children of other race or ethnicities. Non-Hispanic black children were more likely to have skin allergies and less likely to have respiratory allergies compared with non-Hispanic white children.

Hispanic children had a lower prevalence of food allergy (3.6%), skin allergy (10.1%), and respiratory allergy (13.0%) compared with non-Hispanic white and non-Hispanic black children. Non-Hispanic black children had a higher percentage of reported skin allergy (17.4%) compared with non-Hispanic white children (12.0%) and a lower percentage of respiratory allergy (15.6%) compared with non-Hispanic white children (19.1%) (Figure 3).

Figure 3. Percentage of children aged 0–17 years with a reported allergic condition in the past 12 months, by race and ethnicity: United States, average annual 2009–2011

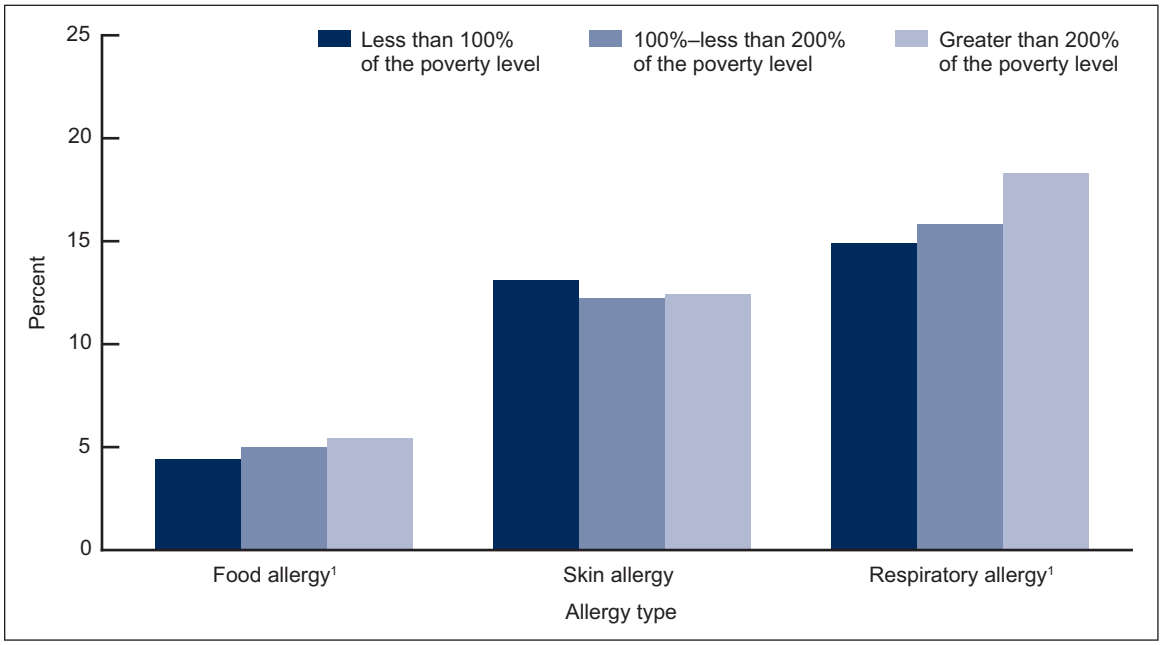


[1]Hispanic significantly different than all other race groups.
[2]The differences between all race groups are statistically significant.
SOURCE: CDC/NCHS, Health Data Interactive, National Health Interview Survey.

The prevalence of food and respiratory allergy, but not skin allergy, increased with higher income levels.

The prevalence of both food allergy and respiratory allergy increased with the increase of income level. Among children with family income less than 100% of the poverty level, 4.4% had a food allergy and 14.9% had a respiratory allergy. Food allergy prevalence among children with family income between 100% and 200% of the poverty level was 5.0%, and respiratory allergy prevalence was 15.8%. Among children with family income above 200% of the poverty level, food allergy prevalence was 5.4%, and respiratory allergy prevalence was 18.3%. There was no significant difference in the prevalence of skin allergy by poverty status (Figure 4).

Figure 4. Percentage of children aged 0–17 years with a reported allergic condition in the past 12 months, by poverty status: United States, average annual 2009–2011



[1]Significant trend by poverty status.
SOURCE: CDC/NCHS, Health Data Interactive, National Health Interview Survey.

Summary

Among children under age 18 years in the United States, the prevalence of food and skin allergies increased from 1997–1999 to 2009–2011. The prevalence of respiratory allergy, which is the most prevalent type of allergy among children, did not change during this period. There was no significant difference in food allergy prevalence between age groups. However, skin allergy decreased with the increase of age, and respiratory allergy increased with the increase of age. The prevalence of allergies varies by race and ethnicity, with Hispanic children having the lowest prevalence of food, skin, and respiratory allergies compared with non-Hispanic white and non-Hispanic black children. Non-Hispanic black children were more likely to have skin allergies and less likely to have respiratory allergies compared with non-Hispanic white children. The prevalence of allergies differed by poverty status. Food allergy and respiratory allergy increased with the increase of income level, but there was no difference in the prevalence of skin allergy by poverty status.

Definitions

Respiratory allergy prevalence: Estimated based on affirmative responses to either of the two National Health Interview Survey (NHIS) question(s): "During the past 12 months, has your child had hay fever?" and "During the past 12 months, has your child had any kind of respiratory allergy?"

Food allergy prevalence: Estimated based on an affirmative response to the NHIS question: "During the past 12 months, has your child had any kind of food or digestive allergy?"

Skin allergy prevalence: Estimated based on an affirmative response to the NHIS question: "During the past 12 months, has your child had eczema or any kind of skin allergy?"

Poverty status: Based on family income, family size, and the number of children in the family; and for families with two or fewer adults, on the age of the adults in the family. The poverty level is based on a set of income thresholds that vary by family size and composition. Families or individuals with income below their appropriate thresholds are classified as below the poverty level. These thresholds are updated annually by the U.S. Census Bureau to reflect changes in the Consumer Price Index for all urban consumers (12). Estimates by poverty status from NHIS are based on both reported and imputed family income (13).

Data source and methods

Prevalence estimates for allergic conditions were obtained from the Health Data Interactive (HDI) table, "Allergic conditions, ages 0–17: U.S., 1997–2011," available from the Health Data Interactive website: http://www.cdc.gov/nchs/hdi.htm. NHIS data were used to estimate the prevalence of allergic conditions for this HDI table.

NHIS data are collected continuously throughout the year for the Centers for Disease Control and Prevention's National Center for Health Statistics by interviewers from the U.S. Census Bureau. NHIS collects information about the health and the health care of the civilian noninstitutionalized U.S. population. Interviews are conducted in respondents' homes, but follow-ups to complete the interviews may be conducted over the telephone. The Sample Child component collects detailed data on health conditions for a randomly selected child in households with at least one child. All of the data in the Sample Child component are obtained from a proxy respondent and not from medical records. A responsible adult, usually a parent, responds to the survey questions as proxy for the sample child. For further information about NHIS and the questionnaire, visit the NHIS website at http://www.cdc.gov/nchs/nhis.htm.

NHIS is designed to yield a sample that is representative of the civilian noninstitutionalized population of the United States, and the survey uses weighting to produce national estimates. Data weighting procedures are described in more detail elsewhere (14). Point estimates and estimates of corresponding variances for the HDI estimates were calculated using SUDAAN software (15) to account for the complex sample design of NHIS. The Taylor series linearization method was chosen for variance estimation.

Differences between percentages were evaluated using two-sided significance tests at the 0.05 level. Terms such as "higher" and "lower" indicate statistically significant differences. Terms such as "no difference" indicate that the statistics being compared were not significantly different. Lack of comment regarding the difference between any two statistics does not necessarily suggest that the difference was tested and found to be not significant. All estimates shown in this report have a relative standard error less than or equal to 30%. The significance of trends was tested using weighted least squares regression models of the log of each outcome and Joinpoint software (16) to determine whether an apparent change over time was statistically significant, taking into account the standard error for each data point. Because there were limited data points over the period, linear regression (zero joinpoints) was specified for all models.

About the authors

Kristen D. Jackson, LaJeana D. Howie, and Lara J. Akinbami are with the Centers for Disease Control and Prevention's National Center for Health Statistics, Office of Analysis and Epidemiology.

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Centers for Disease Control and Prevention
National Center for Health Statistics
3311 Toledo Road
Hyattsville, MD 20782

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PEDIATRIC ALLERGY AND IMMUNOLOGY (JM PORTNOY AND CE CIACCIO, SECTION EDITORS)

Anaphylaxis in Children: Current Understanding and Key Issues in Diagnosis and Treatment

Chitra Dinakar

Published online: 20 July 2012

Abstract Anaphylaxis is a severe allergic reaction that is rapid in onset and may cause death. Since it is unpredictable and potentially fatal, prompt recognition and treatment are vital to maximize a positive outcome. The occurrence of anaphylaxis is increasing across all ages in the United States, with increased risk of worse outcome in teenagers/young adults and in those with comorbid conditions such as asthma. Gaps in the assessment of patient-specific risk factors, identification and prevention of triggers, recognition of signs/symptoms, and pharmacologic treatment of anaphylaxis have been identified at the physician and caregiver/patient level. A PubMed literature search (January 2000–December 2011) was conducted to identify publications on childhood anaphylaxis using the following terms: food allergy, food allergens, food hypersensitivity, epinephrine, epinephrine auto-injectors, anaphylactic triggers, and anaphylaxis. This review will critically appraise these key issues and highlight strategies that might result in improved management of anaphylaxis in children.

Keywords Epinephrine · Food allergy · Food hypersensitivity · Pediatric · Children · Anaphylaxis · Diagnosis · Treatment

Introduction

Anaphylaxis is a potentially fatal condition that can occur without warning [1]. Prompt diagnosis and treatment are crucial [2••, 3••]. Previously, the lack of a universal definition for anaphylaxis resulted in misdiagnosis, underreporting, and miscoding, impeding epidemiological research on this condition. To address this issue, a definition was standardized in 2005 by the Joint Task Force on Practice Parameters, representing the American Academy of Allergy, Asthma and Immunology (AAAAI), the American College of Allergy, Asthma and Immunology (ACAAI), and the Joint Council of Allergy, Asthma and Immunology (JCAAI) [4]. The Joint Task Force defined anaphylaxis as "a condition caused by an [immunoglobulin E] IgE-mediated reaction" that is "often life-threatening and almost always unanticipated." Anaphylactoid reactions were defined as non-IgE-mediated reactions with the same clinical picture as anaphylaxis. When both IgE-mediated and non-IgE-mediated mechanisms were a possible cause, the term anaphylactic was used to describe the reaction.

Due to the wide variability in defining anaphylaxis, incidence and prevalence data should be interpreted with caution [5]. In a population-based study in Rochester, Minnesota from 1990–2000, the annual age- and sex-adjusted incidence of anaphylaxis was estimated to be 49.8 per 100,000 person-years [6]. In this study, age-specific rates were highest for ages 0–9 years (75.1 per 100,000 person-years) and 10–19 years (65.2 per 100,000 person-years). In contrast, a previous study in Seattle, Washington from 1991–1997 estimated the rate of anaphylaxis in children and adolescents to be 10.5 per 100,000 person-years [7]. The reason for this difference is likely due to differences in the definitions of anaphylaxis used. In addition, although both studies also reviewed samples of records with less specific "allergy" codes, only the Rochester study included these cases in the estimated incidence.

Although overdiagnosis of anaphylaxis can occur (due to overlap of symptoms with panic attack, hyperventilation, vasovagal episode, etc.), underdiagnosis is more common as an episode may not be recognized due to the absence of

C. Dinakar (✉)
Section of Allergy, Asthma and Immunology, Children's Mercy Hospital and Clinics, University of Missouri-Kansas City,
2401 Gillham Road,
Kansas City, MO 64108, USA
e-mail: cdinakar@cmh.edu

cutaneous findings or misinterpretation of nonspecific signs (eg, confusion, nausea, dyspnea). Underreporting and miscoding can also lead to an underestimation of prevalence [5, 8]. Of the approximately 12.4 million allergy-related emergency department visits from 1993–2004, only 1 % received the diagnosis of anaphylaxis [9]. Several studies have shown that anaphylaxis is often miscoded or misclassified, with 21 %–57 % of food allergy or anaphylaxis cases coded with less specific allergy or anaphylaxis codes (eg, unspecified allergy) [6–8]. As the percentage of cases identified from review of nonspecific diagnoses was similar in the Rochester study (25 %), and the Seattle study (21 %), the frequency of miscoding appears to be similar in both adults and children.

Anaphylaxis Triggers

Across all age groups, the most common triggers for anaphylaxis are ingested foods (33 %), insect stings (19 %), and medications (14 %) [6]. Less common triggers include cats, latex, cleaning agents, environmental allergens, and exercise. For about a quarter of cases, the trigger is unknown. In children, food-induced anaphylaxis is the most common trigger and accounts for 37 %–85 % of cases, whereas insect bites/stings account for 5 %–13 % and medications account for 5 %–12 % [10–12]. Despite differences between studies, food allergy is clearly the most common cause of anaphylaxis in children.

In children, the most common food allergens are milk products (19 %–29 %), peanuts (9 %–36 %), tree nuts (9 %–19 %), eggs (5 %–22 %), shellfish (4 %–17 %), and fruits and vegetables (9 %) [10, 13, 14••, 15•]; regional differences most likely account for the differences between studies. In the United States, shellfish is the most common food allergen in persons aged ≥5 years, whereas eggs, fruits, peanuts, and tree nuts are more common in those aged <5 years [8]. The most common medication allergens are antibiotics (67 %) [10, 12].

Data suggest that the prevalence of food allergy is increasing. An analysis of multiple United States national surveys showed that food allergy in school-aged children increased from 3.3 % in 1997 to 3.9 % in 2007 [16]. In a recent United States survey of 38,480 children, the prevalence of food allergy was 8 %, with 38.7 % of those having a history of severe reactions [14••].

Information regarding the prevalence of fatal allergic reactions is limited. In an Australian study, the causes of anaphylaxis fatalities were drugs or probably drugs (58 %), insect bites/stings (18 %), undetermined (13 %), food (6 %), and other (5 %) [17]. Although most admissions for food-induced anaphylaxis occurred in children less than 5 years of age, all food-induced anaphylaxis fatalities were in patients between 8 and 35 years of age. Similarly, although insect sting-induced admissions peaked between 5 and 9 years of age, most insect sting-induced anaphylaxis deaths occurred between 35 and 84 years of age. Based on extrapolation of data from a United States-based population study, it is estimated that there are about 150 deaths annually due to food-induced allergic reactions [18]. In this study, fatalities occurred in patients aged 2 to 33 years, with 9 % in children less than 7 years of age and 53 % in teenagers.

In addition to these IgE-mediated triggers of anaphylaxis, other causes of anaphylaxis that should be considered include: galactose alpha-1,3-galactose, a carbohydrate contained in red meat that was recently described to cause anaphylaxis [19]; non-IgE-triggered mechanisms, such as through the complement and coagulation pathways initiated by oversulfated chondroitin components in heparin [20]; reactions in patients with mastocytosis and mast cell disorders; and idiopathic anaphylaxis [21••].

Diagnosis and Management

Recommendations for the management of anaphylaxis are predominantly based on expert opinion and consensus. The AAAAI/ACAAI/JCAAI practice parameter and the World Allergy Organization guidelines provide an evidence-based approach to diagnosis and management of anaphylaxis [3••, 21••]. In addition, guidelines published by the National Institute of Allergy and Infectious Diseases (NIAID) for the diagnosis and management of food allergy [2••] provide a paradigm for the acute management of food-induced anaphylaxis, which is similar to treatment of anaphylaxis as a result of other causes. A version of this guideline that focuses on the pediatric population is also available [22].

Guidelines for both adults and children stress rapid diagnosis as being key to optimal management [2••, 3••, 21••, 22]. Anaphylaxis affects multiple organs, including the skin, respiratory tract, gastrointestinal tract, cardiovascular system, and central nervous system [23]. Signs and symptoms of anaphylaxis for adults and children are summarized in Table 1 [3••, 11]. Although cutaneous symptoms predominate in adults, the primary presenting symptoms in children are respiratory in nature (e.g., wheezing, shortness of breath) [11]. In addition, cardiovascular symptoms tend to be less common in children (17 %) than in adults (30 %–35 %) [3••, 11]. This could be due to increasing age and comorbid disease in adults; however, it could also be due to differences in the prevalence of triggers between adults and children. Food related causes, which tend to cause respiratory tract involvement, are more common in children whereas medication and venom causes, which tend to cause cardiovascular reactions, are more common in adults [10].

Table 1 Signs and symptoms of anaphylaxis

Symptoms	All ages [3••]		Children [11]	
	Clinical features	Frequency	Clinical features	Frequency
Respiratory	Dyspnea, wheeze	45 %–50 %	Difficulty/noisy breathing	83 %
	Upper airway angioedema	50 %–60 %	Wheeze	59 %
	Rhinitis	15 %–20 %	Cough	33 %
			Swelling tongue	13 %
			Swelling/tightness in throat	11 %
			Difficulty talking/hoarse voice	13 %
Cutaneous	Urticaria, angioedema	85 %–90 %	Urticaria	72 %
	Flushing	45 %–55 %	Angioedema	55 %
	Pruritus without rash	2 %–5 %	Pruritus	11 %
Gastrointestinal	Nausea, vomiting, diarrhea, cramping pain	25 %–30 %	Vomiting, diarrhea, abdominal cramps	29 %
Cardiovascular	Dizziness, syncope, hypotension	30 %–35 %	Hypotension, pale and floppy, impaired/loss of consciousness, collapse	17 %

Typically, exposure to the triggering allergen is followed by the rapid development of symptoms over minutes to several hours. In both adults and children, the time course of the reaction may be uniphasic (occurring immediately after exposure and resolving with or without treatment in minutes to hours), biphasic (recurring after the apparent resolution of initial symptoms, usually about 8 h after the first reaction), or protracted (persisting for hours or days following the initial reaction) [2••, 22]. Early recognition of signs and symptoms, timing of the reaction, and existence of comorbid conditions and concomitant factors can aid in diagnosis [2••].

The development of diagnostic criteria represents an important advancement in anaphylaxis management, and it is estimated that these criteria enable health care providers to identify about 95 % of cases [1]. Nevertheless, accurate diagnosis in children presents challenges. This is partially due to the inability of children to accurately describe their symptoms [24], and the lack of cutaneous symptoms in about 18 % of cases [10].

Parents and caregivers of children with food allergies are often unable to recognize and manage anaphylaxis. In studies evaluating parents, only 48 % of parents could identify more than one symptom that would require use of epinephrine [25], and only 43.5 % reported receiving education regarding their child's food allergy and management of his/her reactions [26]. Venues where children are supervised or receive care, such as schools and child care centers, also need to be prepared to recognize and manage anaphylaxis. In a study of anaphylactic events in 48 Massachusetts public school districts, of 114 subjects who received epinephrine in the school setting (46 % were of elementary age), school personnel were unaware that the individual had a life-threatening allergy in 24 % of cases [27].

Physicians may also be unable to correctly diagnose food-induced anaphylaxis because of inadequate knowledge of food allergies. In a case-based survey of 419 pediatricians without specialized allergy training, only 56 % of respondents could appropriately recognize and treat food-induced anaphylaxis [28]. An analysis of referrals to a pediatric allergy clinic found that only 34.5 % of food allergy cases were accurately diagnosed [26]. These observations underscore a need to educate physicians and families on recognition of anaphylaxis and improve competence in recognizing this potentially fatal condition.

Treatment

First-Line Treatment

Evidence-based guidelines recommend the prompt administration of epinephrine as first-line treatment for an anaphylactic episode [2••, 3••, 21••]. Timely administration of epinephrine can be life-saving and help delay the progression of a life-threatening reaction so that medical attention can be provided [29]. Table 2 outlines the basic steps for management of anaphylaxis [21••].

In children, the recommended dose of epinephrine is 0.01 mg/kg of a 1:1000 (1 mg/mL) solution via intramuscular injection into the mid-anterolateral thigh [3••]. Auto-injector dosing for epinephrine is 0.15 mg for children who

Table 2 Basic management of anaphylaxis

1. Have a written emergency protocol for the recognition and treatment of anaphylaxis and rehearse it regularly.
2. Remove exposure to the trigger if possible (eg, discontinue an intravenous diagnostic or therapeutic agent that seems to be triggering symptoms).
3. Assess the patient's circulation, airway, breathing, mental status, skin, and body weight (mass).

Promptly and simultaneously, perform steps 4–6

4. Call for help: resuscitation team (hospital) or emergency medical services (community) if available.
5. Inject epinephrine (adrenaline) intramuscularly in the mid-anterolateral aspect of the thigh (0.01 mg/kg of a 1:1000 (1 mg/mL) solution), maximum of 0.3 mg for children (0.5 mg for adults); record the time of the dose and repeat it in 5–15 min, if needed. Most patients respond to 1 or 2 doses.
6. Place the patient in a position of comfort and elevate the lower extremities. (Note: in adults, fatality can occur within seconds if the patient stands or sits suddenly. It is not known if this also applies to children.)
7. When indicated, give high-flow supplemental oxygen (6–8 L/min) by face mask or oropharyngeal airway.
8. Establish intravenous access using needles or catheters with wide-bore cannulae (14–16 gauge). When indicated, give 1–2 L of 0.9 % (isotonic) saline rapidly (e.g., 10 mL/kg in the first 5–10 min to a child).
9. When indicated at any time, perform cardiopulmonary resuscitation with continuous chest compressions.[a]

In addition,

10. At frequent, regular intervals monitor patient's blood pressure, cardiac rate and function, respiratory status, and oxygenation (monitor continuously, if possible).

[a] Resuscitation guidelines recommend initiating cardiopulmonary resuscitation with chest compressions only (hands-only), before giving rescue breaths. In children, the rate should be at least 100 compressions/min at a depth of 5 cm (4 cm in infants)

(Adapted from Simons et al. [21••])

weigh 10–25 kg and 0.3 mg for those who weigh >25 kg [22]. Repeated dosing of epinephrine is recommended for suboptimal response or symptom progression [2••, 3••]. Intravenous infusion of epinephrine or intravenous bolus should be considered if shock has developed or cardiac arrest is imminent [22].

According to a consensus statement from the AAAAI regarding the management of anaphylaxis in patients with a previous anaphylactic reaction in the child care setting, epinephrine should be given at the start of any reaction occurring subsequent to contact with a known or suspected allergen [30]. Calling for medical help and concurrent elimination of additional allergen exposure is also recommended [2••]. Although it is generally recommended to place the patient in a recumbent position with lower extremities elevated, individuals who are experiencing respiratory distress, which is common in children, and/or vomiting should instead be placed in a comfortable position with lower extremities elevated [21••]. If possible, supplemental oxygen and fluid resuscitation should be provided [31].

Epinephrine has alpha- and beta-adrenergic properties through which it increases blood pressure, prevents and relieves hypotension and shock, decreases upper airway obstruction (e.g., in the larynx), decreases urticaria and angioedema, and decreases wheezing [21••]. Patients may experience self-limiting effects after epinephrine administration, such as pallor, tremor, anxiety, palpitations, dizziness, and headache [21••]. In both adults and children, significant adverse effects, such as ventricular arrhythmias, hypertensive crisis and pulmonary edema, can occur after an overdose of epinephrine by any route of administration, although typically they are reported after intravenous dosing (e.g., rapid infusion, bolus administration, dosing error due to administration of concentrated solution appropriate for intramuscular injection). Misunderstanding about the correct dose and route of epinephrine administration in hospital and emergency department settings can lead to serious cardiovascular complications from overdose [32•]. In infants especially, it is important to use caution when calculating and drawing up an epinephrine dose, to stay vigilant for changes in vital signs, and to ensure use of age-appropriate blood pressure norms [21••].

Epinephrine Auto-Injectors

Epinephrine auto-injectors (EAIs) are the cornerstone of treatment in the first-aid management of anaphylaxis in the community setting. For allergic reactions occurring in the community setting, it is recommended to administer the patient's EAI without delay [31]. A second dose can be administered after 5–10 min based on patient status.

EAIs are often prescribed because of their ease of use and ability to rapidly produce peak epinephrine concentrations following intramuscular injection [33, 34]. Children at risk for anaphylaxis may need to carry 2 doses of epinephrine for several reasons: the first dose may not be administered effectively; symptoms may persist despite a successful first injection; or the patient may experience biphasic anaphylaxis. In a study of children with multiple food allergies, 19 % of food-induced anaphylactic reactions required ≥2 doses of

epinephrine and 6 % of reactions required ≥3 doses [35]. Existing EAIs, EpiPen (Dey Pharma, L.P., Basking Ridge, NJ), TwinJect (Shionogi Inc, Florham Park, NJ), Adrenaclick (Shionogi Inc.), Anapen (Lincoln Medical Ltd, Salisbury, Wiltshire, UK), and Jext (ALK Abelló Ltd, Reading, Berkshire, UK) are available in 2 pre-set doses of epinephrine (0.15 and 0.3 mg). It is important to note that at the time this review was written, Twinject was no longer manufactured in the United States and Adrenaclick was not marketed anymore.

While EAIs have significantly improved emergency care of anaphylactic reactions, there are several limitations with the devices currently available. One of the primary limitations is their symmetrical, pen-like appearance, which can result in accidental needle sticks [35]. From 1994–2007, prior to the redesign of the EpiPen, a total of 15,190 cases of unintentional injections from EAIs were reported with the number of reports increasing significantly ($P<0.001$) annually across all age groups [24]. In addition to adverse effects such as local ischemia of the digit [36•], accidental finger injections may also result in partial or complete loss of the epinephrine dose for the person having an anaphylactic episode, known as the "lost dose hazard". The symptoms and signs of local ischemia reported include pain or numbness, pallor, cyanosis, hypothermia, absence of sensation or hyperesthesia, and weak or absent pulse, as well as skin peeling, sensory loss, neuropraxia, and protracted ischemia reperfusion pain. It is to be noted that none developed tissue necrosis and that overall epinephrine is associated with a good safety profile and most adverse events related to unintentional injection of epinephrine resolve without additional complications within 2–24 h with or without treatment [36•]. Studies indicate that only 25 %–55 % of patients carry their EAI with them at all times, as recommended [29, 37•]. This may be due, at least in part, to the bulky size and shape of currently available EAIs. It is important to note that the second-dose feature available in some types of EAIs (i.e., TwinJect; Shionogi Inc) requires handling an exposed, used needle [38••]. In the school setting, such injectors should be disposed of after the first dose has been used to reduce the risk of needle-stick injury. Therefore, a separate unit should be used if a second dose is required [38••]. The pre-set, fixed dose ranges (0.15 and 0.3 mg) of currently available auto-injectors can be a limitation in the pediatric setting as the 0.15-mg dose may be too strong for infants and toddlers weighing <15 kg, and the 0.3-mg dose may be subtherapeutic for children weighing >30 kg, particularly those who are overweight or obese [39]. However, data to indicate what dose is correct, inadequate, or adequate in children are currently lacking and further studies are needed.

Current EAIs have a needle length of 1.27 cm for the 0.15 mg dose EAI and 1.58 cm for the 0.3 mg dose EAI, which may be too short to penetrate the subcutaneous tissue to achieve intramuscular injection in children who are overweight or obese [40•]. Therefore, with currently available EAIs, it is important to recognize that children who are overweight or obese may be inadvertently receiving a subcutaneous injection, which will result in delayed epinephrine absorption and a lower plasma concentration of epinephrine [33]. To address this problem, additional research on needle length is necessary. The United States Food and Drug Administration has issued guidance to the medical device industry regarding the incorporation of human-factor engineering principles into improving the design and safety of medical devices [41]. Much of this guidance involves identifying and preventing user-related hazards. As this guidance becomes operational, it is hoped that further research will be undertaken to address the discussed unmet needs.

In order for EAIs to be effective, they must be used correctly and in a timely manner. However, there is a lack of patient/caregiver education and ongoing skill-retention regarding symptom recognition and proper epinephrine administration in several settings [25, 42]. In a survey that assessed patient/parent knowledge and usage of EAIs, 86 % of families indicated they kept the epinephrine device with them at all times, yet only 71 % of participants had their device with them at their office visit, and only 32 % of participants could correctly demonstrate how to use the EAI [29]. Furthermore, 10 % of participants possessed devices that were expired, leaving just 55 % of families with unexpired epinephrine on hand at the time of the survey [29]. School-aged children (aged ≥5 years) were less likely than younger children to have their EAI with them when eating lunch (25 % vs 42 %) or a snack (28 % vs 37 %) [37•]. In a recent study of 14,677 patients who filled an initial prescription for an EAI only 46 % ever refilled the prescription (63 % for children), and only 11 % refilled it at all the expected refill times [43]. It has been shown that parental empowerment and training on the use of an EAI significantly ($P\leq0.05$) correlates with greater parental comfort with administration [44], and EAI-training improves the odds of having an EAI readily available [37•]. In a quality-improvement project at the Children's Mercy Hospital (Kansas City, MO) in which data from 277 patients at-risk for anaphylaxis was collected, less than half (44 %) of the patients had their EAI devices with them. The most common reason cited (47%–56%) for not having the EAI device during their visit was not realizing they had to carry it at all times (other reasons included forgot, expired, lost, etc.). At the initial visit, only about 3 out of 5 caregivers were able to correctly perform all the steps to use the device. Our study reveals that a systematic and periodic process of screening, education, and re-education on carriage, and knowledge of use of this device is needed. This process is now routine and mandatory in our clinics for all patients at risk for anaphylaxis.

Preparedness for appropriately treating anaphylactic reactions is suboptimal in child care centers. In a survey of 42

child care centers tending children aged 6 months to 6 years in the suburbs of Chicago, only 24 % of center directors initially stated that they would administer EpiPen for a severe allergic reaction and only 55 % had trained staff for this emergency [13]. An assessment of the long-term effectiveness of an allergy seminar identified a need for recurring anaphylaxis education among child care providers [42]. Although 77 % of child care center directors could demonstrate EAI technique at 4 weeks post-training, knowledge decreased to 48 % at 6 months, and 31 % at 1 year.

Gaps in anaphylaxis management extend to clinicians as well, including incomplete understanding of how and when to use EAIs, and inadequate provision of, or arrangement for patient education. An assessment of 29 attending pediatricians found that only 24 % provided families with written indications for use of the EAI, only 21 % could correctly demonstrate technique, and 14 % of pediatricians familiar with EpiPen/EpiPen Jr incorrectly thought the device should be refrigerated [29]. In a separate survey of food-allergic individuals or caregivers (n=1887), only 58.7 % of respondents reported receiving training from the prescriber on the use of auto-injectors [45]. Further, 86.6 % of participants did not recall receiving oral counseling during dispensation of EAI at the pharmacy [45]. These findings highlight the need for all health care professionals to become comfortable with EAI usage and to educate patients and their families appropriately. Health care professionals should also remind patients that EAIs expire 1 year after dispensing [2••], and need to be stored at room temperature [38••].

Physicians can help strengthen parental education by appropriate referral to an allergist for children with anaphylaxis [3••]. Just 1 visit to an allergy clinic has been reported to improve parental knowledge of allergen avoidance, management of allergic reactions, and use of an EAI [26]. Physicians should also develop emergency action plans (e.g., http://www.foodallergy.org/files/FAAP.pdf) for children with food allergies [3••]. An e-mail survey of 1885 individuals who had survived anaphylaxis or had been responsible for someone who had survived anaphylaxis, reported that 62 %–64 % of participants did not have a plan of action readily available [46]. Food-allergy emergency action plans are essential in the school setting also. A recent survey of elementary and middle school nurses from 43 schools in South Carolina found food-allergy emergency action plans in place in only 44 % of schools [47]. As an added consideration, Bansal et al. reported that most child care centers (98 %; n=41) do not have medications on hand to treat an allergic reaction unless provided by the caregiver [13]. Thus, in addition to establishing a standard anaphylaxis-management protocol, state/federal policy must permit schools to have a supply of epinephrine on hand for general use [27, 38••].

In summary, in the community, prompt administration of epinephrine via an EAI is crucial. Ongoing education is required to ensure patients and child care providers always have an unexpired EAI with them, know how to correctly use their EAI, and know what to do in case of a severe allergic reaction. In addition, health care professionals need to become comfortable with EAI usage and provide appropriate education for patients/caregivers.

Second-Line Treatment

Second-line treatment options in the outpatient setting include β_2-agonists, antihistamines, and glucocorticoids. These agents may be administered in a hospital-based setting, along with vasopressors, glucagon, and activated charcoal [2••, 3••, 48]. Although commonly used, data supporting the role or effectiveness of second-line treatment options in the management of anaphylaxis are limited [49, 50]. Hence, these second-line treatments should be considered only as adjunct therapy to epinephrine.

Preventive Measures

Preventive measures may reduce the risk of anaphylaxis in susceptible individuals. Anaphylaxis education should begin prior to discharge from the health care facility and include a prescription for an EAI, education on technique, recommendation of a medical identification bracelet or wallet card, and referral to an allergy/immunology specialist for assessment of triggers. Anaphylaxis triggers should be identified by taking a detailed history, and be confirmed when possible by using allergen skin tests or serum allergen specific IgE levels [21••].

Avoidance of suspected triggers or co-triggers is key to the management of anaphylaxis; however, this approach is often unsuccessful due to inadequate patient education and understanding of allergen avoidance, and a lack of awareness of triggers, particularly during a first episode [51, 52]. Physicians should provide families of allergic children with detailed information regarding relevant triggers and how to avoid them. In addition, the Food Allergy and Anaphylaxis Network website contains information on food allergen avoidance, among other issues, and may be a useful resource for families and physicians (http://www.foodallergy.org).

In addition to avoidance, the guidelines recommend patients should receive a prescription for an EAI and referral to an allergy/immunology specialist [3••]. However, retrospective studies report low compliance with these guidelines in both adults and children, although concordance with recommended care was somewhat better among patients who were admitted to the hospital (59 % prescribed EAI; 35 % referred to allergist) [53]. In children considered to have food-induced anaphylaxis, 51 %–63 % received an EAI prescription and 24 %–33 % were referred for follow-up [14••, 15•]. Among those admitted to hospital, 94 % were

prescribed an EAI and 69 % were referred to an allergist [15•]. These data indicate that many patients are not receiving basic tools to prevent or manage subsequent anaphylaxis events.

Several other preventive measures are available for allergen triggers, such as immunotherapy for insect bites/sting reactions, avoidance of the drug or therapeutic substitution with a non–cross-reacting medication, and desensitization protocols for drug allergy. Patients with frequent episodes of idiopathic anaphylaxis may benefit from prophylactic treatment with a systemic glucocorticoid and an H1-antihistamine [3••] or prophylactic omalizumab injections [21••].

Experimental approaches to prevent food-allergy reactions include immunotherapy [54, 55], anti-IgE therapy [56], and possibly early introduction of solids and allergenic food contrary to past infant-feeding guidelines [57]. Preclinical data hint at the potential efficacy of Chinese herbal medicine [58] and probiotic supplementation [59] in food allergy prevention. However, studies are needed to investigate the effectiveness and safety of the above approaches for preventing anaphylaxis in children with food allergy.

Conclusions

Numerous gaps in the diagnosis and management of anaphylaxis in children exist at both the physician and caregiver/patient level. The key to successful management involves recognition of populations at risk (e.g., children with food allergies) and rapid diagnosis with early initiation of effective evidence-based therapy. Ongoing education is required to improve the ability of physicians and families to recognize anaphylaxis in children. Epinephrine is the recommended first-line treatment for anaphylaxis in both inpatient and outpatient settings, and should be administered promptly upon recognition of signs and symptoms. Although second-line treatments are available, they should be considered only as adjunct therapy to epinephrine. Ongoing education is required to ensure patients and child care providers understand the importance of always having an unexpired EAI with them, knowing how to correctly use their EAI, and knowing what to do in case of a severe allergic reaction.

Epinephrine auto-injectors allow for the prompt administration of epinephrine in the community setting and must be prescribed to all patients at risk. Advances in the design of these devices to improve safety and convenience of carriage and use, may further aid in successful management of children with anaphylaxis.

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- • Of importance
- •• Of major importance

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EXHIBIT 136

Original Investigation

The Economic Impact of Childhood Food Allergy in the United States

Ruchi Gupta, MD, MPH; David Holdford, RPh, PhD; Lucy Bilaver, PhD; Ashley Dyer, MPH; Jane L. Holl, MD, MPH; David Meltzer, MD, PhD

IMPORTANCE Describing the economic impact of childhood food allergy in the United States is important to guide public health policies.

OBJECTIVE To determine the economic impact of childhood food allergy in the United States and caregivers' willingness to pay for food allergy treatment.

DESIGN, SETTING, AND PARTICIPANTS A cross-sectional survey was conducted from November 28, 2011, through January 26, 2012. A representative sample of 1643 US caregivers of a child with a current food allergy were recruited for participation.

MAIN OUTCOMES AND MEASURES Caregivers of children with food allergies were asked to quantify the direct medical, out-of-pocket, lost labor productivity, and related opportunity costs. As an alternative valuation approach, caregivers were asked their willingness to pay for an effective food allergy treatment.

RESULTS The overall economic cost of food allergy was estimated at $24.8 (95% CI, $20.6-$29.4) billion annually ($4184 per year per child). Direct medical costs were $4.3 (95% CI, $2.8-$6.3) billion annually, including clinician visits, emergency department visits, and hospitalizations. Costs borne by the family totaled $20.5 billion annually, including lost labor productivity, out-of-pocket, and opportunity costs. Lost labor productivity costs totaled $0.77 (95% CI, $0.53-$1.0) billion annually, accounting for caregiver time off work for medical visits. Out-of-pocket costs were $5.5 (95% CI, $4.7-$6.4) billion annually, with 31% stemming from the cost of special foods. Opportunity costs totaled $14.2 (95% CI, $10.5-$18.4) billion annually, relating to a caregiver needing to leave or change jobs. Caregivers reported a willingness to pay of $20.8 billion annually ($3504 per year per child) for food allergy treatment.

CONCLUSIONS AND RELEVANCE Childhood food allergy results in significant direct medical costs for the US health care system and even larger costs for families with a food-allergic child.

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Author Affiliations: Smith Child Health Research Program, Ann & Robert H. Lurie Children's Hospital of Chicago, Chicago, Illinois (Gupta); Center for Healthcare Studies, Northwestern University Feinberg School of Medicine, Chicago, Illinois (Gupta, Bilaver, Dyer, Holl); Department of Pediatrics, Northwestern University Feinberg School of Medicine, Chicago, Illinois (Gupta, Holl); Department of Pharmacotherapy and Outcomes Science, Virginia Commonwealth University School of Pharmacy, Richmond (Holdford); School of Nursing and Health Studies, Northern Illinois University, DeKalb (Bilaver); Departments of Medicine and Economics and Harris School of Public Policy, University of Chicago, Chicago, Illinois (Meltzer).

Corresponding Author: Ruchi Gupta, MD, MPH, Center for Healthcare Studies, 750 N Lake Shore Dr, Chicago, IL 60611 (r-gupta@northwestern.edu).

Food allergy is a growing public health concern in the United States that affects 8% of children.[1] Childhood food allergy results in significant direct medical costs to the health care system and imposes substantial costs on families. Direct medical costs to the health care system stem from office visits, rescue medications, emergency department (ED) visits, and hospitalizations. Costs borne by families include medical and nonmedical expenses, specifically out-of-pocket, lost productivity, and opportunity costs.

To our knowledge, no research has offered a comprehensive assessment of the economic burden of food allergies in the United States. A previous study, which examined direct and indirect costs associated with food-induced allergic events in children and adults, relied primarily on data from federal data sources to identify significant health care costs.[2] The authors acknowledge that costs were underestimated because not all food allergy cases could be identified using *International Classification of Diseases, Ninth Revision* codes. Moreover, many critical costs to families were not captured from these data.

Indeed, not much data exist about the costs borne by families such as buying special foods or forgoing full-time employment to care for a child with a food allergy. Previous research has articulated how having a child with a food allergy impairs families' overall quality of life through limiting social interac-

tions and adversely affecting family finances.[3-7] However, the total effect of direct medical and nonmedical costs on families has yet to be comprehensively described.

To better allocate resources dedicated to preventing and treating food allergy, policy makers must first understand its economic effect on the health care system and families. To quantify the overall economic impact of food allergy, we conducted a national survey of caregivers of food-allergic children. We hypothesized that the economic impact of food allergy in the United States is significant and has been underestimated.

Methods

Study Design

Caregivers of a child with a current food allergy were surveyed between November 28, 2011, and January 26, 2012, and asked to assess direct medical, out-of-pocket, lost labor productivity, and opportunity costs due to their child's food allergy. Caregivers were also asked about their willingness to pay (WTP) for an effective food allergy treatment as an alternative valuation method. The sample (N = 1643) was weighted to represent the population of US children with current food allergies (**Table 1**). The institutional review board of Lurie Children's Hospital of Chicago approved the study protocol.

Survey Development

The survey was developed by physicians, health services researchers, and health economists. Cognitive interviews (n = 5) were conducted to ensure general understandability and consistency of response. Quality control testing was carried out to ensure that skip logic and randomization were met. A pretest of 52 surveys was electronically administered to verify the reliability of question responses and to assess respondent burden, which is the effort that is required for caregivers to provide adequate responses to the survey.

The final survey, which incorporated feedback from the cognitive interviews, quality control testing, and pretest, consisted of items assessing child and household demographics, food allergy severity and reaction history, estimates of resource use, lost productivity, health insurance coverage, and WTP. Household information comprised zip code, income, and other demographic descriptors. The survey is available upon request.

Study Participants

Eligible participants included caregivers able to complete the survey in English who resided in US households with at least 1 child with a current food allergy. Caregivers were recruited using a dual-sample approach. The first sample of 629 caregivers was selected by resampling a previous sample of US families with a food-allergic child (N = 3339). The earlier study, which recruited families to estimate the prevalence of childhood food allergy in the United States, is described in depth by Gupta et al.[1] The second sample consisted of 1014 caregivers who were recruited electronically through a food allergy support and advocacy organization. In households with multiple food-allergic children, 1 child was randomly selected and caregivers were instructed to complete the survey for the selected child. Caregiver recruitment and survey administration were conducted by Knowledge Networks, a survey research firm based in Menlo Park, California.

Table 1. Characteristics of Study Sample Among 1643 Children Surveyed With Childhood Food Allergy

Characteristic	% (95% CI)
Sex	
Male	50.6 (46.4-54.8)
Female	49.4 (45.2-53.6)
Age, y	
≤2	13.4 (10.7-16.5)
3-5	18.2 (15.5-21.3)
6-10	27.0 (23.5-30.7)
11-13	17.0 (14.0-20.6)
14-17	24.5 (20.8-28.5)
Race/ethnicity	
White	75.9 (71.6-79.6)
Black	10.7 (8.0-14.3)
Asian	4.1 (2.7-6.2)
Hispanic	7.6 (5.4-10.6)
Other	1.6 (0.8-3.2)
Annual household income, US$	
<25 000	14.5 (11.3-18.4)
25 000-49 999	27.1 (23.2-31.4)
50 000-99 999	39.2 (35.2-43.3)
100 000-149 999	12.8 (10.7-15.2)
≥150 000	6.4 (5.3-7.8)
Geographic region	
Midwest	24.4 (21.1-28.1)
Northeast	20.5 (17.4-24.0)
South	33.4 (29.5-37.6)
West	21.7 (18.3-25.4)
Health insurance status	
Insured	88.9 (85.4-91.7)
Not insured	11.1 (8.3-14.6)
Type of food allergy	
Milk	22.3 (19.3-25.6)
Egg	11.1 (9.4-13.1)
Peanut	28.7 (25.7-31.9)
Soy	5.3 (4.1-6.9)
Wheat	5.8 (4.4-7.5)
Tree nut	15.0 (13.1-17.1)
Fin fish	6.2 (4.4-8.7)
Shellfish	18.6 (15.3-22.4)
Pea	3.7 (2.6-5.3)
Sesame	4.6 (3.4-6.0)
Other	37.6 (33.3-42.2)
Severity of food allergy	
Mild reaction in lifetime	91.1 (88.4-93.2)
Severe reaction in lifetime	41.2 (37.3-45.2)
An anaphylactic reaction occurred	10.0 (8.3-12.1)

Table 2. Direct Medical Costs of Childhood Food Allergy[a]

Characteristic	Children With Visit, % (SE)	Visits per Child, Mean (SE)	Cost, US$		
			Visit	Child	Overall Annual (in Millions)
Visits					
Pediatrician	42 (2)	.82 (.05)	112[b]	92	543
Allergist	41 (2)	.79 (.05)	175[b]	138	819
Pulmonologist	14 (1)	.07 (.01)	175[b]	12	71
Nutritionist	17 (1)	.16 (.04)	100[b]	16	96
Alternative provider	17 (1)	.23 (.05)	100[b]	23	136
Emergency department	13 (1)	.18 (.02)	711[c]	129	764
Inpatient hospitalization stays	4 (1)	.05 (.01)	6269[c]	314	1863
Total direct medical costs				724	4292

[a] Direct medical costs are medical costs borne by the health care system associated with the prevention, diagnosis, and treatment of food allergies.

[b] Source: Hospital Outpatient Prospective Payment System.[8]

[c] Source: Patel et al.[2]

Measuring Direct Medical Costs

Direct medical costs include the costs to the health care system for the diagnosis, treatment, and prevention of childhood food allergy. Events resulting in resource use were identified by asking caregivers of allergic children about the number of times a child had outpatient visits, ED visits, and inpatient hospitalizations related to a food allergy in the past year. Costs associated for each event were estimated from several sources. Costs for outpatient visits were taken from Medicare cost data.[8] The mean cost associated with an ED visit was based on prior calculations using the Healthcare Cost and Utilization Project Nationwide Emergency Department Sample for visits due to food allergy and food-induced anaphylaxis.[2] The mean cost for an inpatient admission was also taken from prior estimates based on the Healthcare Cost and Utilization Project Nationwide Inpatient Sample[2] and inflated using the inpatient care component of the consumer price index for 2011.[8] The direct medical costs per child were estimated by multiplying the mean number of each type of visit by the mean cost per visit. This number was then multiplied by the total number of US children with food allergies (5.9 million).[1] The sum of costs for all visits equals the total direct medical costs (**Table 2**).

Measuring Total Costs Borne by the Family

Costs borne by families comprised lost labor productivity, out-of-pocket, and opportunity costs.

Total lost labor productivity costs were estimated by multiplying the mean number of hours reported by caregivers accompanying the child to health visits for food allergy by the mean number of each type of visit and the total number of US children with food allergies. The time spent for each of these visits was then valued using the mean national hourly labor wage from September 2011 ($30.11/hr).[8] The sum of caregiver hours spent for all health care visits equals the total lost labor productivity (eTable in the Supplement).

Total direct out-of-pocket costs were estimated by multiplying the percentage of caregivers reporting each type of cost by the mean caregiver-reported cost and the number of US children with food allergies (**Table 3**).

Total opportunity costs were estimated by multiplying the percentage of caregivers reporting lost opportunity in the labor market by the mean caregiver-reported cost and the number of US children with food allergies (**Table 4**).

Measuring WTP

The WTP questions were based on established elicitation methods to assess individuals' valuation of health care treatments and outcomes.[9] Caregivers were asked 2 questions to determine the maximum amount they would be willing to pay per month (out of pocket or through insurance) for a safe and effective treatment that allowed the child to eat all foods. Monthly costs were used since consumers typically pay for prescription drugs and insurance on a monthly basis. For each response, caregivers had to select an expenditure range (eg, $1000-$5000) and then report the maximum amount they would be willing to spend within the range. To assess for potential bias due to the predetermined ranges, we randomized caregivers to a second version of the survey with expenditure ranges that were twice as large. Answers from these 2 versions were pooled, and differences in WTP were compared to assess any bias. To reduce the sensitivity of mean estimates of WTP to outliers, we excluded responses of more than $10 000 per month from the analysis. The total WTP was calculated by multiplying the mean annual amount by the number of food-allergic children in the United States.

Statistical Analysis

Data were weighted using base and poststratification weights to adjust for potential biases from sampling design and survey response. Base and panel demographic poststratification weights were adjusted for known selection deviations in the sampling design used in the study by Gupta et al.[1] Additional poststratification weights were constructed when the study sample was finalized using age, sex, income, type of food allergy, and reaction severity. The study-specific poststratification weights were devised to match benchmark distributions from Gupta et al. Means, proportions, and medians used to calculate costs were computed using the sample weights. All analyses were conducted using Stata 12.0 (StataCorp).

Monte Carlo probabilistic sensitivity analyses were performed to develop 95% CIs around mean cost estimates. A total of 10 000 simulations were run for mean estimates of direct medical costs, direct out-of-pocket costs, lost labor productivity costs, opportunity costs, and all costs combined. Distributions were fit for each input using the means and SEs where appropriate. The method of moments approach was used to estimate α and β parameters for the γ and β distributions. Vari-

Table 3. Out-of-Pocket Costs of Childhood Food Allergy[a]

Variable	% Reporting Cost (SE)	Mean Direct Out-of-pocket Costs, US$ (SE)	Cost per Child, US$	Overall Annual Cost (in Millions), US$
Visits to the physician's office or health clinic (including copays)	52.5 (2.2)	160 (14)	84	499
Visits to the emergency room (including copays)	16.1 (1.6)	247 (42)	40	235
Overnight stays at the hospital	10 (1.4)	411 (182)	41	244
Travel to and from health care visits (including ambulance use; parking expenses)	27.7 (1.8)	91 (14)	25	149
Epinephrine injectors (Epipen, Epipen Jr)	35.9 (1.9)	87 (4)	31	184
Antihistamines (Allegra, Benadryl, Claritin, Zyrtec)	50.8 (2.2)	62 (4)	32	188
Other prescription/nonprescription medication	29.3 (1.9)	122 (13)	36	211
Non-traditional medicine (such as herbal products)	15 (1.6)	123 (30)	19	110
Costs associated with special diets and allergen-free foods	37.7 (2.0)	756 (59)	285	1689
Additional/change in child care	6.7 (0.8)	2158 (323)	145	857
Legal guidance	2.3 (0.6)	402 (122)	9	55
Counseling or mental health services	4.5 (0.7)	571 (123)	26	152
Special summer camp	3 (0.7)	702 (183)	21	125
A change in schools was needed due to this child's food allergy	4.2 (0.7)	2611 (497)	110	650
Other out-of-pocket expenses (eg, cleaning supplies, skin care products, transportation)	9.2 (1.1)	396 (86)	36	216
Any out-of-pocket costs	**74.3 (2.1)**	**1252 (90)**	**931**	**5516**

[a] Out-of-pocket costs: medical costs borne by patient associated with the prevention, diagnosis, and treatment of food allergies. Includes all costs associated with protecting the child from exposure to allergens, including special child care arrangements. The out-of-pocket costs exclude the top 1% of reported costs in each category.

Table 4. Opportunity Cost of Childhood Food Allergy[a]

Characteristic	Cost			
		US$		
	Reporting, % (SE)	Opportunity, Mean (SE)	Per Child	Overall Annual (in Billions)
Choice of career has been restricted	5.7 (0.9)	15 655 (2471)	892	5.3
A job had to be given up	4.9 (0.7)	29 657 (4151)	1453	8.6
A job was lost through dismissal	1.9 (0.6)	14 849 (7479)	282	1.7
A job change was required	2.5 (0.6)	10 605 (3161)	265	1.6
Any job-related opportunity cost (total amount)[b]	9.1 (1.0)	32 719 (4166)	2977	17.6
Any job-related opportunity cost (maximum amount)[c]	9.1 (1.0)	26 363 (2545)	2399	14.2

[a] Opportunity cost is the additional cost associated with activities forgone as a result of a child's allergy. The opportunity costs exclude the top 1% of reported costs in each category.

[b] All possible responses were used to calculate job-related opportunity cost.

[c] Only the maximum of 4 possible responses was used to calculate job-related opportunity cost.

ance for ED costs and inpatient costs was calculated with the SDs and sample sizes from a previous study.[2] Outpatient physician costs and the hourly wage rate were varied between ±25% of the base estimate. Nonparametric 95% CIs were constructed using the lower and upper 2.5% of all simulations.

Results

After weighting, the sample of 1643 children with a current food allergy matched the distribution of US children with a food allergy on key characteristics, including race (75.9% white non-Hispanic), sex (50.6% male), and age (68.5% aged 6 years and older) (Table 1). Most families reported an annual household income above $50 000 (58.4%), and 88.9% reported that their food-allergic child had health insurance. The sample was evenly distributed across 4 US geographic regions, with a slightly higher concentration in the South (33.4%). The most common food allergies were peanut (28.7%), milk (22.3%), and shellfish (18.6%). Consistent with previous findings, 91.1% reported ever having a mild reaction, and 41.2% reported ever having a severe reaction.

Total annual direct medical costs due to food allergy were estimated at $4.3 billion or $724 per child (Table 2). Hospitalizations accounted for the largest proportion of direct medical cost ($1.9 billion), followed by outpatient visits to allergists ($819 million), ED visits ($764 million), and pediatrician visits ($543 million).

Annual lost labor productivity costs associated with caregivers accompanying their child to medical visits totaled $773 million or $130 per child (eTable in the Supplement). Lost labor productivity due to caregiver time attending allergist vis-

Table 5. Comparison of WTP and Total Reported Costs

Characteristic	Annual Costs, US$			
			95% CI	
	Total (in Billions)	Per Child	Total (in Billions)	Per Child
WTP[a]	20.8	3504	15.7-25.7	2652-4344
Total costs borne by families	20.5	3457	16.7-24.9	2816-4208
Out-of-pocket costs for treatment[b]	5.5	931	4.7-6.4	793-1080
Lost labor productivity	0.77	130	0.53-1.00	89-175
Opportunity costs[b,c]	14.2	2399	10.5-18.4	1771-3104
Total direct medical costs	4.3	724	2.8-6.3	472-1063
Total reported costs	24.8	4184	20.6-29.4	3475-4960

Abbreviation: WTP, willingness to pay.

[a] The 95% CIs for WTP estimates were computed using linearized SEs while all other 95% CIs were computed with the probabilistic method. Monthly WTP responses greater than $10 000 have been excluded.

[b] Top 1% of responses from each question in these categories has been excluded.

[c] Only the maximum of 4 possible responses was used to calculate any job-related opportunity cost.

its accounted for the largest proportion of costs ($318 million), followed by pediatrician visits ($165 million) and ED visits ($148 million).

Annual out-of-pocket costs borne by the caregivers of children with food allergy totaled $5.5 billion or $931 per child (Table 3). The highest proportion of direct out-of-pocket costs stemmed from caregiver estimates of the cost of special diets and allergen-free foods ($1.7 billion). The second largest driver of costs was due to additional or changes in child care due to food allergy ($857 million), followed by changes in schools ($650 million).

Annual opportunity costs due to forgone labor market activities were estimated at $14 billion or $2399 per child (Table 4). A job-related opportunity cost was reported by 9.1% of caregivers. The largest proportion of caregivers reported restriction of career choice (5.7%), followed by giving up a job (4.9%), needing to change jobs (2.5%), and losing a job (1.9%).

Annual medical, out-of-pocket, lost labor productivity, and lost opportunity costs in the United States totaled $24.8 billion or $4184 per child (**Table 5**). Caregivers' WTP for an effective treatment totaled $20.8 billion or $3504 per child. After excluding direct medical costs, which are typically covered by insurance, the total $20.8 billion WTP for treatment almost equaled the $20.5 billion for reported costs borne by caregivers.

A probabilistic sensitivity analysis was performed to account for the uncertainty relating to all costs. Simulations of each type of cost yielded 95% CIs presented in Table 5. Total reported costs spanned a 95% CI from $20.6 billion to $29.4 billion annually.

The results of the probabilistic sensitivity analysis reflect uncertainty surrounding the model parameters, including each event associated with resource use, the number of adults accompanying the child for prevention and care, the time involved in accessing health care services, and the costs assigned to allergic events.

The 95% CI around WTP spanned $15.7 billion to $25.7 billion (Table 5). By comparing responses across randomly assigned expenditure ranges, we assessed the sensitivity of the WTP estimates. We found insignificant differences in mean WTP by expenditure range.

Discussion

To our knowledge, this is the first study to comprehensively quantify the economic impact of childhood food allergy in the United States. Overall, food allergy costs $24.8 billion annually or $4184 per child. The total cost comprises significant direct medical costs for food allergy ($4.3 billion) and even larger costs borne by families ($20.5 billion). The cost borne by families is remarkably similar to caregivers' WTP for a food allergy treatment ($20.8 billion).

Few studies describe the economic impact of food allergy on children and their families. One study by Patel et al[2] estimated that the annual cost of food-induced allergic reactions in a combined child and adult population was $500 million. The authors acknowledge they underestimated the prevalence of childhood food allergy and excluded many other costs, including those borne by families. In addition, a recent European study found that food-allergic children had 4 more annual visits to health care professionals than children without food allergies, which resulted in an additional annual direct medical cost of $1334 per child.[10] Similar to the Patel et al study, the European study did not report other costs, especially those borne by families.

To put food allergy costs in context, we compare them with asthma costs as asthma affects a similar number of US children.[10] Direct medical costs for asthma have been estimated at $3259 per person (child or adult), which is about 5 times the cost per food-allergic child ($724 per child).[11] Prescription medications account for more than 50% of direct medical costs associated with asthma.[10] In contrast, few prescription medication options exist for treating food allergies. Overall costs for outpatient visits for persons with asthma are also considerably higher.[10] In contrast, costs for ED and inpatient visits are similar for asthma and food allergy.[10]

This study found that direct medical costs were a small portion of the total economic burden of childhood food allergy. For example, out-of-pocket costs due to copayments, medications, special diets, and special child care arrangements amounted to $5.5 billion ($931 per child). Food allergy places unique out-of-pocket cost burdens on families such as pur-

chasing allergen-free foods ($1.7 billion). Other unique out-of-pocket costs are incurred to avoid unintentional exposure to food allergens, including special child care arrangements ($857 million), changing schools ($650 million), and attending special summer camps ($125 million).

Lost opportunities, including a change or loss of a job, had the highest associated cost at $14.2 billion. This cost was higher than all other food allergy–related costs combined. Although assessing lost opportunities is complex, evidence now suggests that childhood food allergy has a profound effect on families' finances by altering caregivers' careers. Previous studies have clearly articulated how food allergy affects daily life for the food-allergic child and his or her caregiver(s).[3-7] The constant threat of an accidental exposure of the allergen throughout a child's environment, including school, extracurricular, and social activities, may limit social interactions for the child or require a caregiver to be present more frequently.[7] Moreover, caregivers often need to be at school, social events, or camp to educate and affirm the seriousness of their child's condition.[6,7,12,13] In case of an emergency, caregivers may not be able or willing to take a job that requires travel or many hours away from their child.

Caregivers reported they would pay $3504 per year per child for food allergy treatment. These WTP estimates are remarkably similar to estimates of total food allergy costs minus direct medical costs ($3457 per family). The human capital method used alongside WTP has estimated costs of other disease states.[14] The fact that parents were willing to pay a similar amount for treatment that they currently incur due to food allergy seems to confirm the consistency and validity of the 2 analytical approaches.

This study has several limitations common to survey research since it relies on self-reporting by caregivers of events and costs during 1 year. Poor recall and misunderstanding of the question may influence the results. Another potential limitation is that the sample of families used in this study may not be representative of the population of families with food-allergic children in the United States. For example, the second sample of caregivers was recruited from a food allergy support and advocacy organization. To address this issue, we weighted and adjusted the data for known selection deviations in the sampling design and finalized them using age, sex, income, type of food, and reaction severity. Another limitation is that the cost estimates did not include childhood mortality or missed school days due to food allergy.

In summary, childhood food allergy in the United States places a considerable economic burden on families and society. Results from this study reveal significant direct medical costs to the US health care system and even larger costs to families with a food-allergic child. Furthermore, we found that caregivers' WTP for food allergy treatment is similar to the total costs borne by families associated with out-of-pocket expenses, lost labor productivity, and lost opportunity. Given these findings, research to develop an effective food allergy treatment and cure is critically needed. Moreover, unlike other common childhood diseases in which most costs are borne by the health care system, childhood food allergy disproportionately burdens family finances. Ultimately, to reduce the economic effect on families due to lost opportunity, additional policies to ensure safe environments and to provide health insurance coverage of special needs for food-allergic children are essential.

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Analysis and interpretation of data: Gupta, Holdford, Bilaver, Holl, Meltzer.
Drafting of the manuscript: All authors.
Critical revision of the manuscript for important intellectual content: Holdford, Bilaver, Holl, Meltzer.
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Correction: This article was corrected on October 9, 2013, to fix Tables 3 and 5.

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EXHIBIT 137

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Role of aluminum adjuvant in producing an allergic rhinitis animal model

L. Xi[1,2], E. Fan[2], Y. Zhao[1,2], Y. Li[2], Y. Zhang[1,2] and L. Zhang[1,2]

[1]Department of Otolaryngology, Head and Neck Surgery,
Beijing Tongren Hospital, Capital Medical University, Beijing, China
[2]Key Laboratory of Otolaryngology,
Head and Neck Surgery, Ministry of Education of China
Beijing Institute of Otolaryngology, Beijing, China

Corresponding author: L. Zhang
E-mail: luozhangcn@163.com

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ABSTRACT. This study evaluated different dosage forms of aluminum adjuvant in generating allergic rhinitis animal models. Forty female BALB/c mice were assigned to four groups, including three dosage forms of aluminum adjuvant [powder, gel, and hydrosolvent of aluminum hydroxide, $Al(OH)_3$] mixed with ovalbumin to simulate the symptoms of allergic rhinitis and one control group. Although the aluminum adjuvants were in different dosage forms, the content was 5 mg after conversion in all groups. The fourth group was given normal saline instead as a control. Mice of the powder group displayed typical symptoms of allergic rhinitis. We also found discrete eosinophils in the nasal mucosa of mice from the hydrosolvent group; however, no eosinophils were found in the gel group. These two groups both displayed cytotoxic symptoms and foreign body granuloma. Aluminum adjuvant used in producing animal models can induce foreign body granuloma and other untoward reactions, which are associated with the

dosage level and form.

Key words: Aluminum adjuvant; Animal model; Allergic rhinitis; Foreign body granuloma; Colloid chemistry

INTRODUCTION

Immunoadjuvants are commonly used to reinforce the immunogenicity of an antigen in inducing an allergic rhinitis (AR) animal model. The ideal immunoadjuvant must promote the antigen to generate classical symptoms and pathological changes without inducing untoward reactions. Because of their good adsorbability, aluminum adjuvants can be used to induce humoral immunity reactions and stimulate T-helper2 (Th2) cells to generate a high level of antibodies, and are thus the first choice as immunoadjuvants. However, different dosage forms and methods applying aluminum adjuvants have yielded different results in different experiments. Inevitably, unexpected damage on organs will often disturb such studies. This study focused on the issue of using an aluminum adjuvant to establish an AR animal model and the comparison of three different dosage forms of aluminum adjuvant.

MATERIAL AND METHODS

Animals

The BALB/c mice were provided by the animal center of Capital Medicine University, and were maintained in a specific pathogen free environment. All forty mice were females in good health, 6 to 8 weeks of age, and ranged in weight from 17 to 24 g. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Capital Medicine University.

Grouping and establishment of AR animal model

We used 50 µg ovalbumin (OVA) blended with 5 mg different dosage forms of aluminum hydroxide [$Al(OH)_3$] as a sensitogen to produce the animal models of AR (de Serres, 1988). The forty mice were randomly assigned to four groups by the drawing lots method. The first group was treated with 50 µg OVA and 5 mg $Al(OH)_3$ powder. The second group was treated with 50 µg OVA and 0.385 mL aluminum hydroxide gel. The third group was treated with 50 µg OVA and 0.125 mL injected aluminum. The fourth group was treated with 0.9% sodium chloride instead, and acted as the control group. The animal model was produced in two stages: sensitization and provocation. In the first stage, mice in the case groups (groups 1 to 3) were injected with 50 µg OVA and 5 mg/mL $Al(OH)_3$ through an enterocoelic injection every two days for a total of seven times while mice in the control group were treated with 0.9% sodium chloride instead. The provocation stage started at the end of the sensitization stage. Mice in the case groups were nasally administrated 20 µL OVA in 10 µL 0.9% sodium chloride in each nasal cavity and the control group was administrated 0.9% sodium chloride

only. At the same time, we observed and recorded behaviors and symptoms. Twenty-four hours after the last provocation treatment, mice were scarified by cervical dislocation and the necessary tests were conducted.

Electron microscopy observations of pathology and transmission

The nasal cavities of mice were removed and were fixed in neutral methanol for 24 h. After using 10% EDTA for decalcification for 3 weeks, 4 μm nasal cavity coronal slices was embedded in paraffin and stained with hematoxylin and eosin (HE) for microscope observations. The abdominal cavities of all mice were observed for histopathological assessments.

The granulation tissue on the surfaces of the liver and spleen was observed through transmission electron microscopy in the second group of mice. Several 1 mm^3 sections living tissue were immediately fixed in 2.5% glutaraldehyde, rinsed with phosphate-buffered saline (PBS), fixed in osmic acid, dehydrated in acetone step by step, embedded in EP-812, and then were made into ultrathin sections, stained with lead acetate and sodium citrate, and then features were examined under a JME-1010 transmission electron microscope. At the same time, the chemical constituent of granuloma was examined by JEM-2100F industry transmission electron microscopy and energy spectrum analysis.

RESULTS

Physical examination and behavioral observations

The appearance of mice in the first group was normal, and after basic sensitization and challenging, the most frequently observed symptoms included nose scratching, rhinorrhoea, sneezing, and swelling of the mucosa around the nasal cavity. The mice in the second group showed enlargement of the abdominal region, slowed movement, dull fur, and these mice became thin and emaciated. Administration of celiocentesis resulted in hemorrhagic ascites (Figure 1A). However, this group did not show classic symptoms after sensitization and challenging. Interestingly, 5 of the 10 mice of the third group showed ascites (Figure 1B) and their general conditions were better than those of the second group. In the sensitization stage, nose scratching and rhinorrhea were evident. The fourth group of mice (controls) showed no changes in behavior.

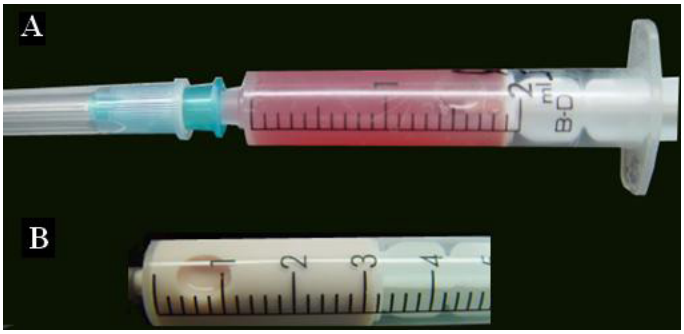

Figure 1. A. Second group [Al $(OH)_3$ gel] = Bloody ascites from the abdominocentesis. **B.** Third group [Al $(OH)_3$ hydrosolvent] = the chylia ascites.

Observation of nasal mucosa under HE staining

In the first group [$Al(OH)_3$ powder], there were abundant eosinophils aggregating at the lateral nasal walls and in the mucosa of the inferior turbinate (Figure 2A). Mucosa and goblet cells showed obvious proliferation and active secretion. Staining with Alcian blue-periodic acid Schiff (AB-PAS) revealed a large quantity of acid mucopolysaccharides in goblet cells. Lymphocyte and neutrophil infiltration and the quantity of plasmocytes increased in the lamina propria. In the second group [$Al(OH)_3$ gel], there were no eosinophils found in the nasal mucosa of the mice. Cilia were arranged in a disorderly manner and there were some coloboma in the nasal mucosa. In the third group [$Al(OH)_3$ hydrosolvent], eosinophils were occasionally found in the nasal mucosa. In the fourth group (control), the nasal mucosa was intact and the ciliated epithelium lined up. There were discrete goblet cells and a small amount of eosinophils. The basement membrane was intact (Figure 2B).

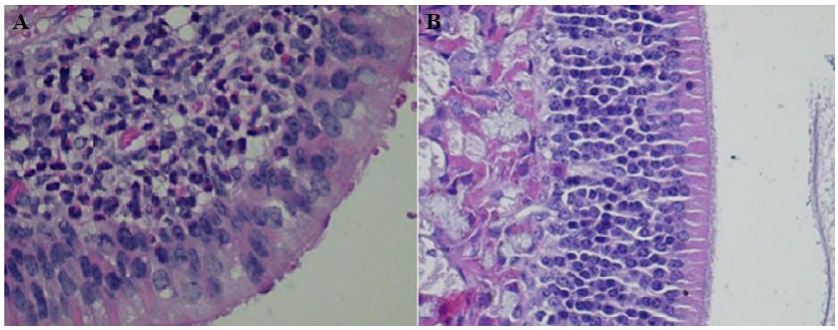

Figure 2. A. First group [Al $(OH)_3$ powder] = there were eosnophiles in the nasal inferior Turbinate (40X). **B.** Control group = the normal nasal cavity and nasal septum (40X).

Coelom pathological examination

In the first group, the mice showed normal activity and flesh fur. There were no abnormalities found in the examination of the viscera. In the second group, the structure of the hepatic lobule was clear, whereas foreign body granuloma emerged inside the tunication of the liver (Figure 3A). A granular gray substance was found in the granuloma cells and it showed a strong refraction (Figure 3B). Moreover, halos could also be seen around some of this substance. Similarly, the splenci cortex nodule atrophied, granuloma was found in the tunication of the spleen, and there were many neutrophils in the areas combined of the granuloma and spleen. Discrete polymorphonuclear macrophagocytes emerged in the splenic sinus. The structure of all levels of bronchia, alveola, and alveolar ducts were clear, but hyperemia of the alveolar septum capillary was obvious. A small amount of neutrophils and eosinophils were found in the bronchial mucosa. The structures of the glomerulus and renal tubule were clear. Early stage granuloma emerged in the retroperitoneum of the kidney. Except for some granuloma emerging in the lobule, the structure of the thymus was normal. In the third group, the focal granuloma was the same as that of the second group, and emerged in the liver, spleen, and kidney, and some lymphocytes and eosinophils were also found in the mesenchyme. In the fourth group, the pathological examinations of the liver, spleen, and kidney were all normal.

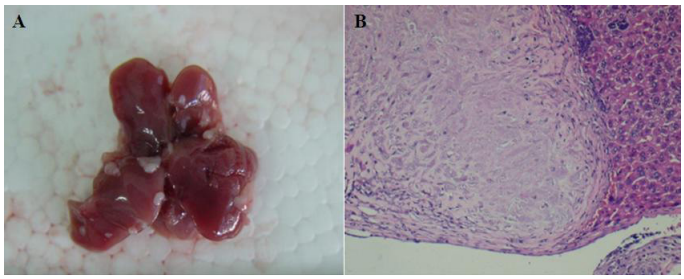

Figure 3. A. Second group = many white nodules emerged in the liver. **B.** Second group = there were foreign body granulomas found inside of the tunication of liver.

JEM-1010 transmission microscope observations

Many phagocytes were found collected in the granuloma of the spleen and liver. In the cytoplasm, a large number of phagosomes emerged, whose single layer was full of phagocytosed substance (Figure 4A). There were two types of phagocytosed substances. One was observed under high magnification and was arranged in bundles, and the other was observed under low magnification and appeared as tiny particles. Around the phagocytes, the mitochondria were swelling and some of the mitochondrion ridges disappeared. Moreover, the double layer of some mitochondria disappeared and formed vacuole structures. Fibroblasts and several lymphocytes and phagocytes could be seen around the capillary in the granuloma tissue. There was a small amount of collagen fibers in the mesenchyme. The structure of the granuloma of the third group was similar to that of the second group except for a smaller amount of phagocytes.

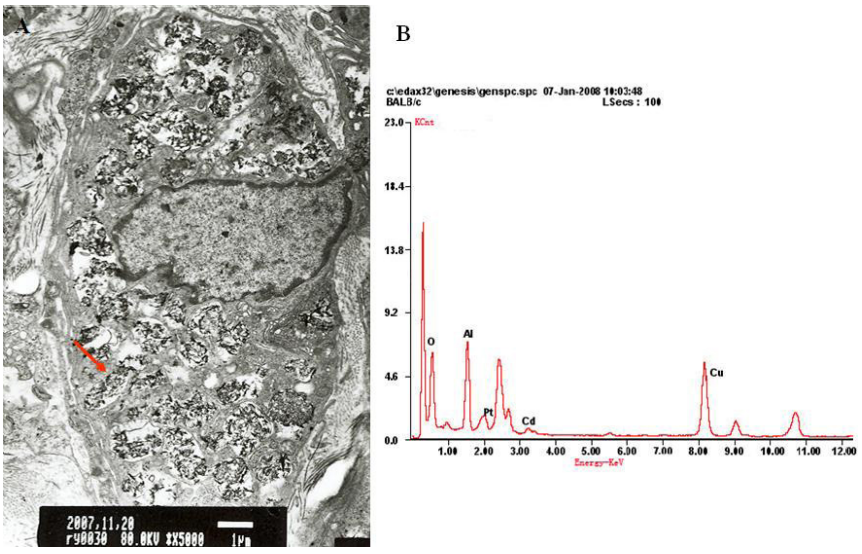

Figure 4. A. JEM-1010 observation of the second group, the arrows pointed out the needle-like structure in the cytoplasm of phagocyte (5000X). **B.** Second group [Al $(OH)_3$ gel] = JEM-2100F energy spectrum analysis of spleen, the foreign body in the sample showed peak value at aluminum.

JEM-2100F energy spectrum analysis

The electron microscope analysis showed a peak value at aluminum. This demonstrated that high magnification fine needles and oval substances and low magnification tiny particles both contained aluminum (Figure 4B).

DISCUSSION

Establishment of an animal model for AR is crucial for studying the mechanism of AR. The main principle of producing the animal model is that the induced symptoms and pathology can be easily duplicated. As the basic step for AR functional studies, a successful animal model is determined by observations of the behavior of animals and histological changes.

OVA is a protein with good antigenicity and carrier activity. Although OVA also has immunogenicity, the addition of some adjuvants are still necessary to reinforce the immunogenicity in establishing an animal model. $Al(OH)_3$ is widely used in animal experiments owing to its nontoxic properties and good adsorbability. It can induce humoral immunity reactions and simulate Th2 cells to generate antibodies at high levels, and also satisfies safety requirements. At present, OVA+ $Al(OH)_3$ is widely accepted as fundamental material for producing animal models around the word (Sehmi et al., 1997).

However, there are some problems associated with the use of $Al(OH)_3$ as an adjuvant. The adjuvant should be able to effectively generate the accurate animal model while not resulting in unacceptable pathological changes. Therefore, it is better to minimize the amount of adjuvant-allergen complex used in order to induce the expected immune response and the least adverse reaction (Wiedermann-Schmidt and Maurer, 2005). Two traditional dosage forms of aluminum adjuvant are widely used by researchers. The first involves placing the aluminum suspension into the solution containing the antigen to form a protein-aluminum salt complex, and the other is putting the solution containing the antigen into $Al(OH)_3$ solution or aluminum phosphate to form aluminum-containing vaccines. Recent studies found that making the aluminum adjuvant into nanometer form could induce the specific antibody in mice quickly and at a high level, and that compared with other forms of aluminum adjuvant, it could induce humoral immunity earlier (Frey et al., 1999). After consulting the relevant literature, we found the amount of OVA used in animal models generally ranges from 10 to 100 μg (Yamaki et al., 2005; Tumes et al., 2007; Liu et al., 2010; Oh et al., 2011), whereas the amount of $Al(OH)_3$ ranges from 1 and 5 mg. Moreover, there are several forms of $Al(OH)_3$ used in these experiments including powder, gel, solution, and other dosage forms (Yamaki et al., 2005; Zhao et al., 2005; Su et al., 2006; Tumes et al., 2007; Mo et al., 2011). Our study focused on these three dosage forms (powder, gel, and solution) in the production of an AR animal model.

In the first group of mice (powder), there were no abdominal bulges and no white precipitated substance appeared. A large amount of eosinophils aggregated in the mucosa of the nasal lateral wall and inferior turbinate. Goblet cells proliferated, lymphocytes infiltrated, and the amount of plasmocytes increased. These observations demonstrated that the animal model was successfully produced. Because of the difficulty for $Al(OH)_3$ powder to dissolve, we observed some powder precipitating on the bottom of the bottle, and the adjuvant-allergen complex existed in the form of suspension. Although we tried to blend the solution sufficiently, the liquid extracted in the syringe still contained relatively less $Al(OH)_3$. Therefore,

we estimated that the effective concentration was below that specifically designed [50 µg OVA + 5 mg $Al(OH)_3$ powder]. We could then extrapolate that the concentration of the suspension was appropriate for establishing successful animal models.

Mice of the $Al(OH)_3$ gel group showed toxic symptoms and foreign body granuloma was found in all ten mice. The aluminum was detected by energy spectrum analysis of the needle-like foreign body. Early evidence about the safety problem of aluminum adjuvants were discussed in a workshop focused on the use of aluminum adjuvants in vaccine acquisition that was held in Puerto Rico in May of 2000. During the meeting, the scientists discussed macrophages myofascial inflammation (MMF), which was first found in France, and then pointed out that there were many macrophages around the muscle fibers containing PAS-positive crystal structures, which were ultimately identified as aluminum. This supported that damage of the MMF was caused by the aluminum adjuvant. Thus, although aluminum adjuvants have been widely used in the production of animal models, this method still has safety problems that require attention.

In the third group of mice (solution), we used the same amount $Al(OH)_3$ solvents as used in the $Al(OH)_3$ gel; however, no AR symptoms were apparent, although foreign body granuloma was observed in the viscera. Compared with the $Al(OH)_3$ gel, the particles of $Al(OH)_3$ solvents were smaller, ranging between 1 nm and 100 nm. Based on colloid chemistry, $Al(OH)_3$ solvents easily form a colloidal dispersion system, in which the particles range from 1 to 100 nm. Although there were still physical boundary surfaces because of the lower sinker rate, we could nonetheless obtain an approximate concentration compared with previous designs. Thus, we speculated that the overdose of aluminum in the third group caused the foreign body granuloma and immunosuppression instead of AR symptoms and pathological changes. Aluminum metabolism in the animals showed that the aluminum adjuvants were absorbed by citric acid in the tissue fluid and were then quickly discharged from the body.

We administrated 5 mg $Al(OH)_3$ in three different dosage forms in 6-8 week-old female mice, and only the powder group successfully produced the allergic rhinitis animal model. We can speculate various reasons to explain these results. Because of the slight solubility of $Al(OH)_3$, it will form a colloidal dispersion system. The sedimentation rate of the colloidal solid is much slower than that of the solute particles in the suspension. Although we blended the liquid well when we extracted the liquid from test tubes, we nonetheless obtained different concentrations. The gel group was set to 5 mg/mL, and the concentration of the powder group was lower than that of the hydrosolvent group, and they were both lower than that of the gel group. Therefore, after the intraperitoneal injection, the extra colloidal solid could not be well absorbed and instead aggregated in the viscera, stimulating phagocytocis around the cell, and ultimately forming the foreign body granuloma. Accordingly, we speculated that excessive aluminum could cause foreign body granuloma and other adverse reactions. A previous study demonstrated that excessive aluminum could result in different kinds of inflammation caused by complement activation (Clements and Griffiths, 2002).

There are few reports available that focus on the concentration and toxicological effects of aluminum. Our animal model experiment showed that when the dosage form of aluminum adjuvant was changed, we could not simply convert the concentration because of consequent changes in the isoelectric point and physical and chemical properties, which can play important roles in influencing the results. Understanding how to use the different dosage forms of aluminum adjuvant in animal experiments could help to guarantee the safety of experimental animals and generate accurate AR animal models.

Another interesting finding was that there was not an increase in the number of eosinophils in the nasal mucosa of mice from the gel and hydrosolvent groups, which demonstrated that extra aluminum adjuvant could induce immunosuppression and other adverse actions. To generate successful animal models, researchers need to understand the extrinsic factors (type of allergen, concentration, dosage form, and correct method of immunoadjuvant use) and animal factors (genetic background, gender, age), and so on. Ignoring any of the factors mentioned above could result in a bad outcome. In particular, scientists should pay particular attention to these factors when aluminum adjuvants are used in the establishment of animal models, which will benefit the animal experiment.

CONCLUSION

The immunoadjuvant $Al(OH)_3$ was routinely administered during the establishment of AR animal models for the enhancement of the immunogenicity of antigenic substances. The dosage form of immunoadjuvants directly determined whether or not a model was successfully established, and moreover, an overdose of $Al(OH)_3$ could result in immunosuppressive action, leading to foreign body granuloma and ascites of internal organs. This study is expected to provide an experimental basis for the safe use of $Al(OH)_3$ in animal models and serves as a reminder of the importance and considerations in using $Al(OH)_3$ in AR studies.

Conflicts of interest

The authors declare no conflict of interest.

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Development of an Animal Model to Evaluate the Allergenicity of Food Allergens

Birgit Ahrens[a] David Quarcoo[a, c] Sabine Buhner[b, d] Gerald Reese[f]
Stefan Vieths[f] Eckard Hamelmann[a, e]

Departments of [a]Pediatric Pneumology and Immunology, [b]Gastroenterology and [c]Institute of Occupational Medicine, Charité Universitätsmedizin, Berlin, [d]Human Biology Technische Universität, Munich, [e]University Children's Hospital, Ruhr University, Bochum, and [f]Paul-Ehrlich-Institut, Division of Allergology, Langen, Germany

Key Words
Food allergy · Immunological responses · Permeability index

Abstract
Scope: Considering the increasing numbers of patients suffering from food allergy (FA) as well as the great variety of novel foods and food compositions, an unmet need exists for the development of preclinical approaches to characterize the allergenic potential of proteins. The aim of our study was to evaluate the allergenicity of different food allergens in a rat model. ***Methods:*** Brown Norway rats were sensitized to protein extracts (RuBisCO, apple, soy, peanut, garden pea) or ovalbumin (OVA) combined with *Bordetella pertussis* and aluminium hydroxide, followed by oral allergen challenges. ***Results:*** Allergen-specific serum immunoglobulin production and the proliferation of mononuclear cells from spleen confirmed sensitization. To assess functional alterations in the gut, intestinal permeability was measured, which increased in sensitized and challenged animals compared to non-sensitized controls. Allergens with high allergenic potential (peanut, OVA, soy) caused a stronger immunological response than allergens with low allergenic potential, such as RuBisCO and apple. Moreover, the immunological responses were reduced when using boiled instead of raw soy and pea proteins. ***Conclusion:*** This model mimics key features of FA and facilitates investigating the allergenicity of allergens in novel food or food compositions in vivo.

Introduction

Food allergy (FA) is an important health issue in westernized countries, with around 8% of children and 5% of adults affected [1]. Although any food protein can possibly act as an allergen, relatively few protein families cause the majority of allergic reactions [2]. Foods with the highest number of published reports are cow's milk, hen's egg, peanut, fish and shellfish [3]. The severity of reactions elicited by these food allergens may vary substantially, ranging from fatal reactions mostly observed in regards to peanut allergy [4] to milder responses detected

KARGER

E-Mail karger@karger.com
www.karger.com/iaa

1018–2438/14/1642–0089$39.50/0

Correspondence to: Prof. Eckard Hamelmann
University Children's Hospital, Ruhr University
Alexandrinenstrasse 5
DE–44791 Bochum (Germany)
E-Mail eckard.hamelmann@rub.de

Exhibit 138

in, for example, soybean- or apple-allergic patients [5–7]. The particular allergenicity of a food allergen is not only determined by its source, but also relies on the condition and the state of processing (cooked, boiled, roasted, etc.) of the food products [8]. Interestingly, although the epitopes of many known allergens have yet to be identified, there seems to be no clear structural or other property common to all food allergens allowing the reliable prediction of their allergenicity [9].

Considering the overall prevalence of FA [1] and the rising accessibility of novel foods and or food compositions, for example from transgenic crop plants, there is a growing need for the development of preclinical approaches that may help to characterize the allergenic potentials of proteins and to predict their risk of initiating severe anaphylactic reactions [9]. The example of the introduction of lupine flour *(Lupinus albus)*, a member of the legume family, to a variety of foods nearly 2 decades ago showed how important a careful selection in advance would have been. Lupine was supplied for food manufacturing for its textural properties in bakery products [10]. Unfortunately, lupine allergy develops by cross-reactivity in patients who are already sensitized to other members of the legume family, in particular peanut, soy or pea [10]. As allergic reactions to lupine became more and more frequent, lupine was added to the list of commonly allergenic foods in the EU 10 years after its introduction (Annex IIIa; directive 2000/13/EC).

Currently, the strategy for assessing the potential allergenicity of (genetically engineered) food is based on guidelines from the European Food Safety Authority (EFSA) and the Food and Agriculture Organization (FAO)/World Health Organization (WHO) [11–13]. A weight of evidence approach includes comparing the introduced protein with known allergens based on gene source, sequence homology and, if necessary, reactivity with serum from allergic patients, as well as evaluating the protein's stability against digestive enzymes [14].

Animal models have played a valuable role in increasing our understanding of the immunology and pathology involved in allergic responses to food proteins [15, 16]. Oral sensitization with food allergens in genetically apt rodents has resulted in the production of allergen-specific IgE and various phenotypical changes that mimicked the disease in human FA patients [17]. However, most of these studies did not assess responses in the gastrointestinal tract upon local (oral) allergen provocation and, therefore, missed a cardinal feature of the human disease. In the current study, we utilized our established rat model of FA [18] to associate immune responses and phenotypical changes after sensitization with oral allergen challenges in order to better delineate the allergic potential of different common food allergens.

Material and Methods

Animals

All experiments were approved by the animal research ethical institution, LAGetSi (Berlin, Germany). Male Brown Norway (BN) rats, aged 6–8 weeks, weighing 180–230 g (BfR, Berlin, Germany) and maintained on a peanut-, ovalbumin (OVA)-, apple-, pea- and soybean-free diet, arrived pathogen-free and were kept under controlled conditions.

Sensitization and Treatment

Animals were sensitized by intraperitoneal (i.p.) injections on days 1, 5 and 10 with the following food allergens adsorbed on aluminium hydroxide: 10 μg of OVA (main egg white protein, as the reference allergen), 2.5 μg of RuBisCO (ribulose-1,5-biphosphate carboxylase/oxygenase, a protein in green plants and the main abundant protein on earth), 2.5 μg of apple (extracted protein), 2.5 μg of green garden pea (protein extract from raw and/or boiled pea), 2.5 μg of soy (protein extract from raw and/or boiled soy) and 2.5 μg of peanut (protein extract from roasted peanut). The protein extract preparation has been described in detail previously [19]. In addition to the first allergen injection, animals were given a booster with a single i.p. injection of *Bordetella pertussis* whole-body vaccine (2×10^6).

Sensitized animals were challenged by gavage feeding with the following allergens on days 20 and 21: 100 μg of OVA (in 1 ml of PBS), 1 mg of RuBisCO, 1 mg of apple extract protein, 1 mg of soybean extract protein, 1 mg of pea extract protein and 1 mg of peanut extract protein. Animals of the negative control groups were sham-sensitized and challenged with PBS according to the same protocols. All animals were sacrificed and analyzed 24 h after the last allergen challenge, on day 22 (fig. 1).

Assessment of Immunoglobulin Levels

Serum antibody levels of total IgE and allergen-specific IgE and IgG were measured by enzyme-linked immunosorbent assay (ELISA) as previously described [20]. Briefly, mircrotiter plates coated with monoclonal anti-rat IgE antibody were incubated with diluted serum samples and biotinylated allergen, and streptavidin-peroxidase was used as a substrate. For biotinylation of RuBisCO, apple extract, pea extract, soybean extract, peanut extract and OVA, the biotinylation kit of Sigma® (Sigma, Deisenhofen, Germany) was used according to the manufacture's instructions. For quantification of specific IgE-serum levels, in-house controls were utilized, thus allowing no direct comparison between specific IgE levels against different allergens.

Cell Culture

Spleen and mesenteric lymph node mononuclear cells were purified by density gradient centrifugation (Lympholyte Rat; Cedarlane Laboratories, Hornby, Ont., Canada; 1,000 g, 20 min at room temperature) and suspended in RPMI 1640 containing 10% FCS (Biochrome, Berlin, Germany) for proliferation assays and cytokine production.

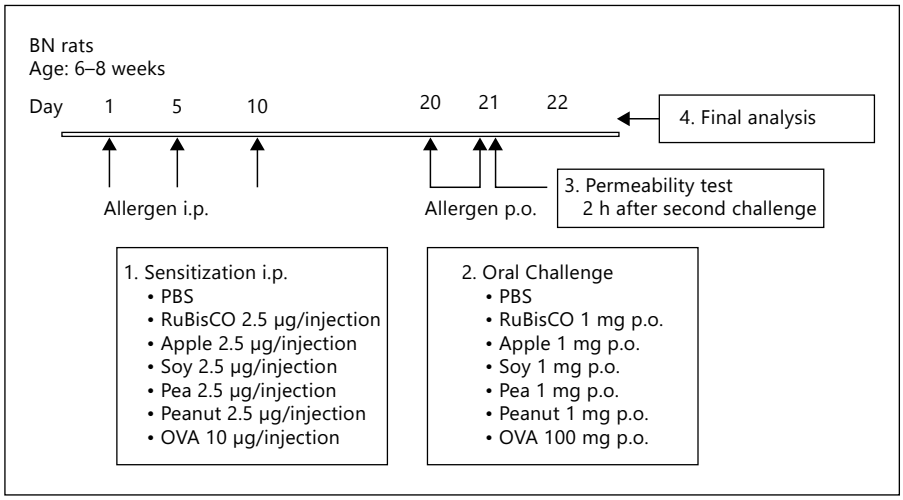

Fig. 1. Treatment protocol: BN rats were sensitized to different allergens i.p. on days 1, 5 and 10. The negative control group was sham-sensitized with PBS. The animals were challenged by gavage feeding of specific allergen extracts on days 20 and 21. Control animals received only PBS p.o. The BN rats were analyzed 24 h after the last challenge, on day 22.

Proliferative Responses

Mononuclear cells (3×10^5/well) were incubated in 96-well U-bottomed tissue-culture plates (Costar, Cambridge, Mass., USA) at 37°C and 5% CO_2, and stimulated for 96 h with mitogen (Concanavalin A, 2.5 µg/ml, Sigma) or allergen (100 µg/ml of OVA, Sigma; 100 µg/ml of RuBisCO, and 100 µg/ml of protein extracts from, apple, pea, soy, peanut). 3[H]-thymidine (Amershan Buchler, Braunschweig, Germany) was added for the last 18 h of the cell culture (1 µCi/well), and thymidine uptake was measured in a liquid scintillation counter (Wallac, Väsby, Sweden). The proliferation rate was calculated as multiples of 3[H]-thymidine incorporation by stimulated cells compared to that of non-stimulated cells.

Intestinal Permeability

Intestinal permeability was assessed using a sugar-recovery test as previously described and reported in detail [18]. The test is based on the measurement of the urinary excretion of orally (p.o.) administered non-metabolized sugar probe molecules. Lactulose and mannitol have been demonstrated to meet the criteria for usage as tracers [21]. Two hours after the second allergen challenge, the animals received 2 ml of PBS by oral route, containing lactulose (10 mg) and mannitol (5 mg), followed 2 h later by 2 ml of drinking water. The tracers recovered in the first 8 h in the urine were detected and quantified by high-performance liquid chromatography (HPLC), with pulsed electrochemical detection (Dionex, Idstein, Germany), chromatography module 250 × 40 mm Carbopac PA-1 column (Dionex), eluent 150 mmol NaOH and flow 1 ml/min [21]. Results were expressed as the percentage recovery of the ingested dose of the sugars. The ratio of recovered lactulose and mannitol (permeability index) served as a marker for intestinal permeability.

Statistical Analysis

Calculations were performed with the statistical software SPSS (SPSS Inc., Chicago, Ill., USA) and R (R Foundation for Statistical Computing, Vienna, Austria). Graphs were generated in GraphPad Prism (version 4; GraphPad Software Inc., San Diego, Calif., USA) and R. We performed the non-parametric two-sample Wilcoxon test (Mann-Whitney test) and p values <0.05 were regarded as significant.

Results

Immunoglobulin Production after Allergen Sensitization

In order to analyze the sensitization status of BN rats, total and specific IgE serum levels were measured before ('naive'; fig. 2) and after allergen sensitization and oral challenges (day 17; fig. 2a). In naive BN rats, total IgE averaged 534 ± 273 ng/ml. After sensitization with the different allergens, a significant increase of total IgE in each group was detected, showing the expected allergic immune response. The strongest increase was measured after sensitization with members of the legume family: raw pea (7,932 ± 4,978 ng/ml), raw soy (11,206 ± 8,727 ng/ml) and peanut (12,471 ± 7,936 ng/ml).

Allergen-specific IgE was detected by ELISA. A significant increase of specific IgE was detected after soybean, OVA and peanut sensitization. There was no significant increase of allergen-specific IgE after sensitization with pea; administration of extract of raw and boiled pea did not induce significant increases of pea-specific IgE (fig. 2b).

Proliferative Response of Mononuclear Cells

Spleen mononuclear cells from allergen-sensitized and challenged BN rats were stimulated in vitro with the respective allergen to measure allergen-specific proliferative responses. We detected significantly enhanced responses compared to cells from non-sensitized controls after sensitization with raw pea, raw soybean, peanut and OVA (fig. 3). No significant increase was detected after sensitization with RuBisCO, apple, and extracts of boiled pea and soybeans.

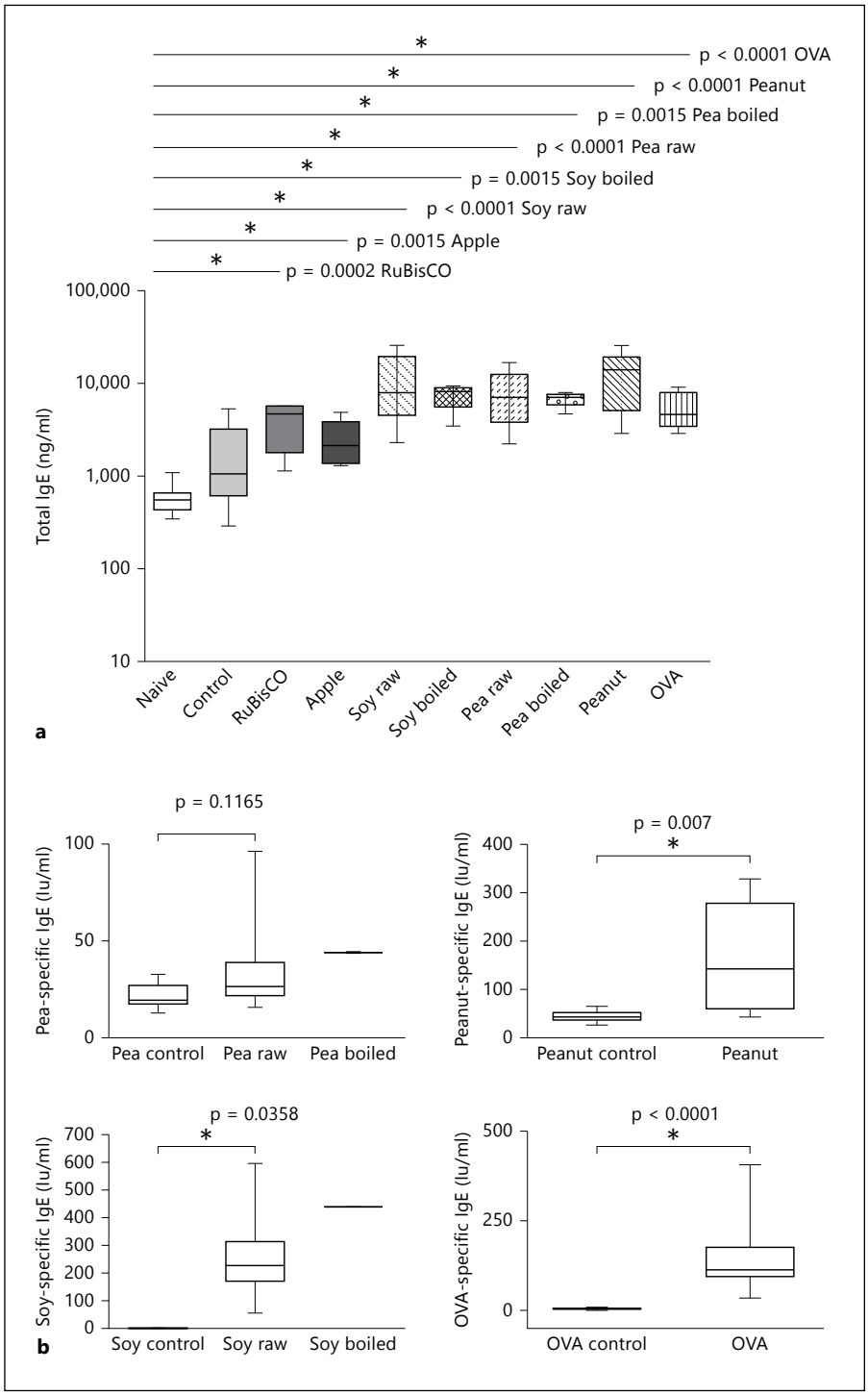

Fig. 2. Immunoglobulin levels in allergen-sensitized and challenged BN rats: total IgE (**a**), specific IgE (**b**). BN rats were treated as described in figure 1. On day 1 (before sensitization, 'naive') and on day 22, immunoglobulin levels in the serum of the animals were measured. Animals of the control groups were sham-sensitized and challenged with PBS according to the same protocols.

Intestinal Permeability upon Local Allergen Challenge

In order to evaluate local functional alterations following oral allergen challenges of sensitized animals, we analyzed changes in intestinal permeability. Urinary excretion of lactulose and mannitol was evaluated after oral uptake of the tracers. The lactulose-mannitol ratio (permeability index) served as a marker for intestinal permeability. Intestinal absorption of lactulose, a disaccharide containing galactose and fructose, occurs predominantly through a paracellular pathway via tight junctions, where-

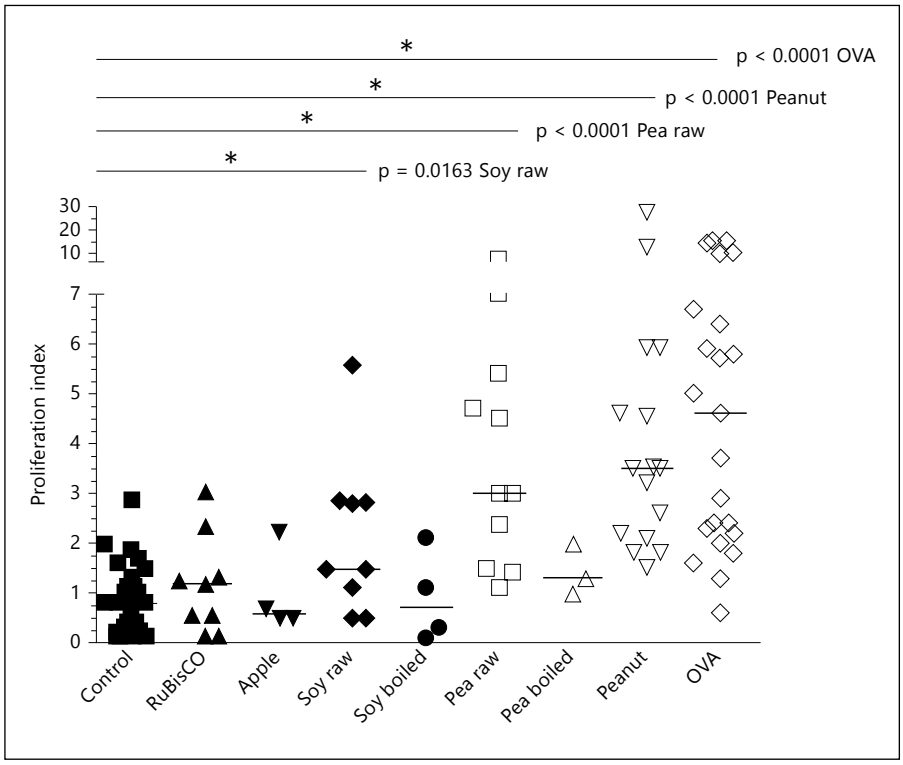

Fig. 3. Influence of allergen sensitization and challenge on allergen-specific proliferation. On day 22, spleen mononuclear cells were stimulated for 96 h with 100 µg/ml of OVA or 100 µg/ml of protein extracts from RuBisCO, apple, pea, soybean and peanut, and 3[H]-thymidine was added for the last 18 h.

as mannitol is absorbed mainly through an intracellular pathway. Allergen sensitization and repeated oral allergen challenges led to a significant increase in permeability after sensitization with RuBisCO, raw soybean, raw pea, roasted peanut and OVA. Sensitization solely with apple did not lead to a significant change in intestinal permeability (fig. 4). These data confirmed the loss of gut mucosal integrity and elevated intestinal permeability after local allergen challenges in animals sensitized with specific allergens.

Discussion

We have presented data from a rat model of FA [18] established to quantify and compare the allergenicity of different food allergens. This model was set up in analogy to the well-recognized model of allergic asthma, where a systemic i.p. sensitization with a model allergen is followed by an airway allergen challenge. Accordingly, in the model of FA presented here, the allergen-specific systemic sensitization is followed by an oral allergen challenge. The optimal sensitization protocol, concerning doses of allergen and choice and doses of adjuvant were determined in advance to induce a high and long-lasting IgE response. We used the purified protein OVA as a reference allergen for comparison with a range of food allergen extracts, since OVA is the most widely applied allergen in animal models. In this way we hoped to take account of the need 'to compare the allergenicity of food extracts versus the purified allergens' [22].

Our final resulting model comprised two hallmark features of FA in patients: allergen-specific immune responses and sensitization and alterations in the intestinal tract upon local allergen challenges. This distinguishes this model from most other existing animal models of FA and allowed testing for allergenicity of different food allergens in a somewhat more 'clinical situation'. The disruption of the intestinal barrier [23, 24] may very well be taken as a direct sign of the local allergic response. It indicates a pivotal event in the course of allergic gut inflammation since it allows peptides (epitopes) to cross the intestinal barrier and to stimulate the submucosal immune system, thus enhancing the local production of inflammatory cytokines, mediators and most probably specific IgE antibodies [25, 26]. The importance of barrier disruption has been highlighted in recent years, especially by publications regarding the loss-of-function variants of the epidermal barrier protein, filaggrin, which display a predisposing factor for atopic eczema [26]. The magni-

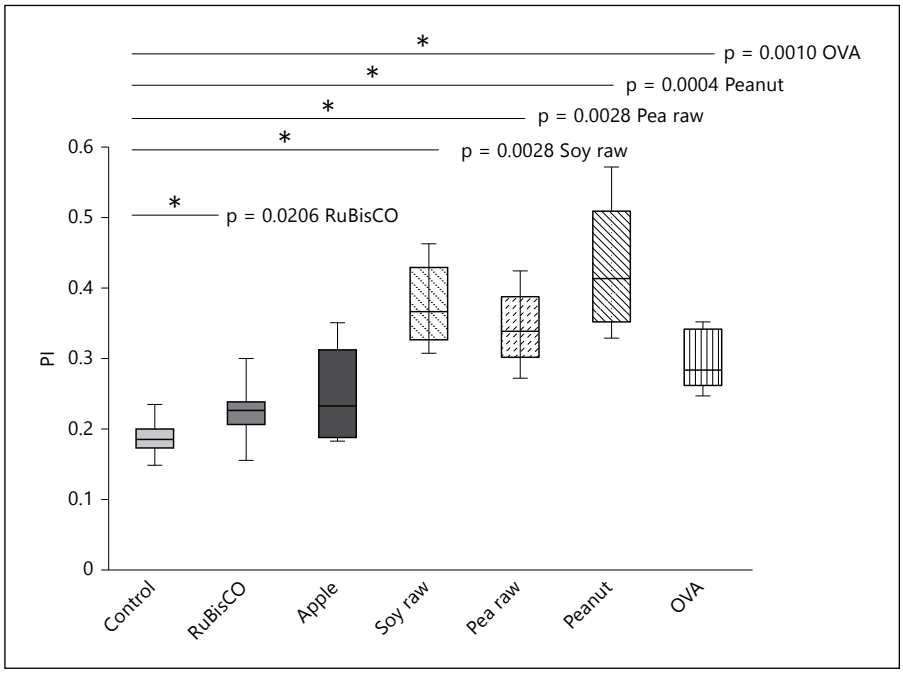

Fig. 4. Influence of allergen sensitization and challenge on intestinal permeability. BN rats were treated as described in figure 1. Two hours after the 2nd allergen challenge, animals received lactulose/mannitol in PBS p.o. The tracers recovered in the first 8 h in urine were detected by HPLC. The ratio of recovered lactulose/mannitol (permeability index, PI) served as a marker for intestinal permeability.

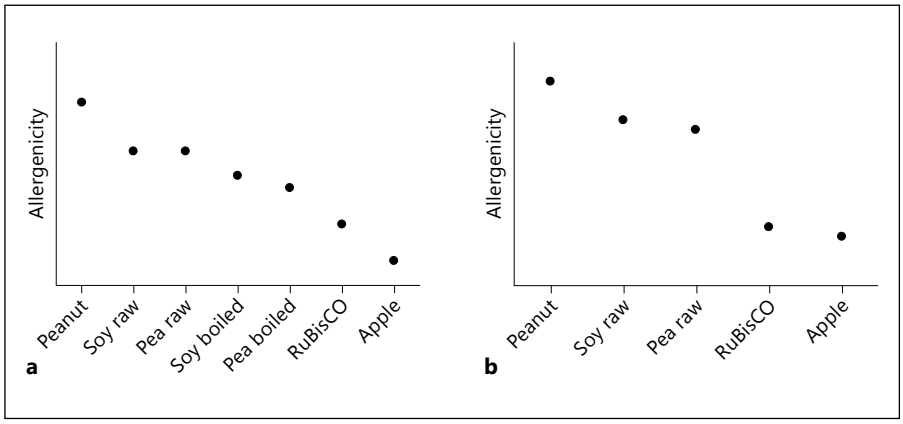

Fig. 5. Ranking of the allergenicity. Based upon the systemic and local responses after allergic sensitization and challenges, a scoring system was built. After calculation of the rank sum of the median total IgE levels and proliferative responses (**a**), as well as of the median total IgE levels, proliferative responses and intestinal permeability (**b**), a quantification of the allergenicity of the specific allergens by correlation with the specific score was performed and a ranking of the allergenicity of the employed food allergens was depicted. Specific IgE levels were not calculated since levels are allergen specific and therefore not comparable.

tude of the changes in the intestinal permeability caused by a given allergen is thus a suitable parameter for the (local) allergenicity.

On the basis of the assessment of these two aspects of FA, we compared different common food protein (plant) extracts, all of which are considered to have a different allergenicity, starting with RuBisCO, the most abundant protein on earth and major spinach protein, which is generally accepted as being non-allergic [27]. We further compared the effect of extracts from apple, raw or boiled green garden pea, raw or boiled soybean, and finally roasted peanut, which is considered to be the most aggressive allergenic food.

The systemic and local immune responses induced by allergic sensitization and challenges with apple, raw or boiled green garden pea, raw or boiled soybean, roasted peanut and RuBisCO differed significantly. Based upon the responses, we built a scoring system depicting the allergenic potential of the different allergens. We calculated the rank sum of the median total IgE levels and proliferative responses (fig. 5a) as well as of the median total IgE levels, proliferative responses and intestinal permeability (fig. 5b). We excluded specific IgE levels for this calculation, since the ELISA system is based on comparison of serum levels with allergen-specific controls for each food allergen and utilizes biotinylation of the

allergen extracts that may differ gradually for the different foods.

The scoring allowed us to build a ranking of the allergenicity of the employed food allergens in our model, with highest values for members of the legume family – roasted peanut, raw soy and raw pea – and lowest values for apple and RuBisCO. In regard to clinical symptoms, the rank of intestinal permeability may be more important than the rank of median total IgE levels and proliferative responses. When we overestimated the 'clinical symptom' intestinal permeability by multiplying it by 2, 5 or 10, the ranking order did not change between peanut, soy and pea.

In humans peanut allergy is accepted as the 'most serious of the hypersensitivity reactions to foods due to its persistence and high risk of severe anaphylaxis' [28]. When taking anaphylaxis as a parameter for severe allergic reactions, epidemiological data on elicitors of anaphylaxis often suggest legumes, especially peanut, to be a food group with all too frequent registered anaphylactic reactions. Other often-reported foods to elicit anaphylaxis are animal-derived food (including hen's egg, cow's milk, fish), tree nuts and fruits [29–31].

Epidemiological data have to be evaluated with care since they are influenced by many factors, such as allergy definitions, study populations, methodologies, ages and eating habits in different countries, etc. This represents one important reason for the development of the FA model presented here.

Pea, soy and peanut comprise up to 70% of their protein content in the form of 7S globulin storage proteins [32]. These belong (next to 11S globulins) to the cupin superfamily. 7S globulins (vicilin) share extensive immunological cross-reactivity in vitro and show a high sequence homology [32, 33]. However, clinically significant cross-reactivity is very rare [32, 33]. Importantly, the allergenicity in our model was scored much lower after boiling processes for pea and soy, compared with raw protein extracts. These differences were confirmed in immunoblot analysis of the sera of animals sensitized and challenged with either raw or boiled protein extracts (data not shown). Our observation confirms reports by Beyer et al. [8] who showed that methods of frying or boiling peanuts reduced their allergenicity compared with the method of dry roasting. Likewise, it is known that the allergenicity of soybeans is altered after manipulation [34]. Little is known about pea, which in westernized diet is mostly consumed blanched or cooked [32]. Raw pea is ranked rather high in our scoring, prompting one to consider its potential allergenicity in different forms of processed foods, especially in regard to the increasingly popular pea flour in bakery products.

In line with our method, Selgrade et al. [22] have also undertaken a ranking approach. They visualized a 'spectrum of allergenic potency of food extract based on perceived allergenicity in humans' by sketching 12 allergens in an allergic order, including peanut, egg and spinach, among others. Although this assortment lacks the basis of direct assessment, it is reflected to a great extent by our ranking order obtained from a straightforward comparison of different food allergens.

In conclusion, we utilized a rat model of FA to assess the allergenic potential of different employed food proteins and their potency to induce immunological changes on T and B cell responses as well as functional alterations. Of course, due to ethical reasons such an analysis is not possible in humans. Even more, the comparability is obscured by different genetic and/or environmental predispositions and backgrounds. We are fully aware that animal models per se have limitations in transferring observed reactions to clinical problems or symptoms in patients. Nevertheless, we believe, that this model provides an interesting tool to assess the potential allergenicity of novel food or food compositions. It may serve as a suitable test system for the allergenicity of unknown or modified allergens and may thus be used as a tool in future safety assessments.

Acknowledgements

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EXHIBIT 139

Asthma | definition of asthma by Medical dictionary

asthma

Also found in: **Dictionary**, **Thesaurus**, **Acronyms**, **Encyclopedia**, **Wikipedia**.
Related to asthma: **asthma attack**

Asthma

Definition

Asthma is a chronic (long-lasting) inflammatory disease of the airways. In those susceptible to asthma, this inflammation causes the airways to spasm and swell periodically so that the airways narrow. The individual then must wheeze or gasp for air. Obstruction to air flow either resolves spontaneously or responds to a wide range of treatments, but continuing inflammation makes the airways hyper-responsive to stimuli such as cold air, exercise, dust mites, pollutants in the air, and even stress and anxiety.

Description

According to the American Lung Association, as of 2007, about 34.1 million Americans, including 9 million children, had been diagnosed with asthma during their lifetime. This number appears to be both increasing, especially among children under age 6, while at the same time the disease is becoming more severe. Asthma is estimated to cause between 3,500 and 5,000 deaths annually in the United States. In 2007, it was responsible for 217,000 emergency room visits and 10.4 million office visits. Its estimated cost to the United States economy is $19.7 billion. Worldwide, asthma is estimated to affect 300 million people. Asthma is closely linked to allergies; about 75% of people with asthma also have allergies.
The changes that take place in the lungs of people with asthma makes the airways (the "breathing tubes," or bronchi and the smaller bronchioles) hyper-reactive to many different types of stimuli that do not affect healthy lungs. In an asthma attack, the muscle tissue in the walls of bronchi go into spasm, and the cells lining the airways swell and secrete mucus into the airways. Both these actions cause the bronchi to become narrowed (bronchoconstriction). As a result, an asthmatic person has to make a much greater effort to breathe in air and to expel it.
Cells in the bronchial walls, called mast cells, release certain substances that cause the bronchial muscle to contract and stimulate mucus formation. These substances, which include histamine and a group of chemicals called leukotrienes, also bring white blood cells into the area, which is a key part of the inflammatory response. Many individuals with asthma are prone to react to such "foreign" substances as pollen, house dust mites, or animal dander; these substances are called allergens. On the other hand, asthma affects many individuals who are not allergic in this way.
About two-thirds of all cases of asthma are diagnosed in people under age 18, but asthma also may first appear during adult years. While the symptoms may be similar, certain important aspects of asthma differ in children and adults.

Child-onset asthma

About 9 million American children have been diagnosed with asthma. Approximately 20% of cases begin in the first year of life. When asthma begins in childhood, it often does so in a child who is likely, for genetic reasons, to become sensitized to common allergens in the environment (atopic person). When these children are exposed to dust mites, animal proteins (i.e.,

animal hair, dander), fungi, or other potential allergens, they produce a type of antibody that is intended to engulf and destroy the foreign materials. This has the effect of making the airway cells sensitive to particular materials. Further exposure can lead rapidly to an asthmatic response. This condition of atopy is present in at least one-third and as many as one-half of the general population.

Adult-onset asthma

Allergies also may play a role when adults become asthmatic. Adults who develop asthma may be exposed to allergens in the workplace, such as certain forms of plastic, solvents, and wood dust. Other adults may be sensitive to aspirin, nonsteroidal anti-inflammatory drugs (NSAIDs such as ibuprofen), or other drugs. More women than men are diagnosed with adult-onset asthma. Compared to childhood-onset asthma, adult-onset asthma tends to be more continuous, while childhood asthma often is marked by asthmatic episodes followed by asthma-free periods.

Exercise-induced asthma

People who may not have allergies can still develop a form of asthma that is brought on by aerobic exercise. These episodes can last for several minutes and leave the individual gasping for breath. Some estimates suggest that 12-15% of Americans are susceptible to exercise-induced asthma. Breathing in cold air, aerobic exercise lasting more than 10 minutes, or shorter periods of very heavy aerobic exercise tend to bring on an exercise-induced asthma attack in susceptible individuals. Polluted air and certain chemicals (e.g., chlorine in pools, herbicides on a playing field) appear to increase the likelihood of an asthma episodes in sensitive individuals.

Causes and symptoms

In most cases, asthma is caused by inhaling an allergen that sets off the chain of biochemical and tissue changes leading to airway inflammation, bronchoconstriction, and wheezing. Avoiding or at least minimizing exposure to asthma triggers is the most effective way of treating asthma, so it is helpful to identify which specific allergen or irritant is causing symptoms in a particular individual. Once asthma is present, symptoms may be triggered or aggravated if the individual also has rhinitis (inflammation of the lining of the nose) or sinusitis (sinus inflammation). When stomach acid passes back up the esophagus (acid reflux), this also may worsen asthma symptoms. A viral infection of the respiratory tract (e.g., a cold) also may trigger or worsen an asthmatic reaction. Aspirin, NSAIDs, and beta-blocker drugs also may worsen the symptoms of asthma.
The most common inhaled allergens that trigger asthma attacks are:

- animal dander
- mites in house dust
- fungi (molds) that grow indoors
- cockroach allergens
- pollen
- chemicals, fumes, or airborne industrial pollutants
- smoke

Inhaling tobacco smoke, either by smoking or being around people who are smoking, can irritate the airways and trigger an asthmatic attack. Air pollutants such as wood smoke can have a similar effect. In addition, three factors that regularly produce attacks in certain asthmatic individuals, and may sometimes be the sole cause of symptoms are:

- inhaling cold air (cold-induced asthma)
- exercise-induced asthma
- stress or a high level of anxiety

Wheezing is often obvious, but mild asthma attacks may be confirmed only when the physician listens to the individual's chest with a stethoscope. Besides wheezing and being short of breath, the individual may cough and/or may report a feeling of "tightness" in the chest. Wheezing is often loudest when the individual breathes out (exhales) in an attempt to expel air through the narrowed airways. Some people with asthma are free of symptoms most of the time but occasionally may have episodes of shortness of breath. Others spend much of their time wheezing or have frequent bouts of shortness of breath

until properly treated. Crying or laughing may bring on an attack. Severe episodes often develop when the individual has a viral respiratory tract infection or is exposed to a heavy load of an allergen or irritant (e.g., breathing in smoke from a campfire). Asthma attacks may last only a few minutes or can continue for hours or even days (a condition called status asthmaticus).

Being short of breath may cause an individual to become visibly anxious, sit upright, lean forward, and use the muscles of the neck and chest wall to help move air in and out of the lungs. The individual may be able to say only a few words at a time before stopping to take a breath. Confusion and a bluish tint to the skin are clues that the oxygen supply is seriously low and that emergency treatment is needed. In a severe attack that lasts for an extended period, some of the air sacs in the lung may rupture so that air collects within the chest. This makes it even harder for the lungs to exchange enough air.

Diagnosis

Apart from listening to the individual's chest, the examiner should look for maximum chest expansion while taking in air. Hunched shoulders and contracted neck muscles are other signs of narrowed airways. Nasal polyps or increased amounts of nasal secretions often are noted in asthmatic individuals. Skin changes, such as atopic dermatitis or eczema, are indications that the individual is likely to allergies.

The physician will ask about a family history of asthma or allergies. A diagnosis of asthma may be strongly suggested when typical signs and symptoms are present. A test called spirometry measures how rapidly air is exhaled and how much air is retained in the lungs. Repeating the test after the individual inhales a bronchodilator drug that widens the airways will show whether the airway narrowing is reversible, which is a very typical finding in asthma. Often individuals use a related instrument, called a peak flow meter, to keep track of asthma severity when at home.

It often is difficult to determine what is triggering asthma attacks. Allergy skin testing may be used, although an allergic skin response does not always mean that the allergen being tested is causing the asthma. The body's immune system produces specific antibody to fight off each allergen. Measuring the amount of a specific antibody in the blood may indicate how sensitive the individual is to a particular allergen. If the diagnosis is still in doubt, the individual can inhale a suspect allergen while using a spirometer to detect airway narrowing. Spirometry also can be repeated after a bout of exercise when exercise-induced asthma is suspected. A chest x ray may be done to help rule out other lung disorders.

Treatment

The goals of asthma treatment are to prevent troublesome symptoms, maintain lung function as close to normal as possible, and allow individuals to pursue their normal activities including those requiring exertion. Individuals should periodically be examined and have their lung function measured by spirometry to make sure that treatment goals are being met. The best drug therapy is that which controls asthmatic symptoms while causing few or no side effects. Many people with asthma are treated with a combination of long-acting drugs taken on a regular basis to help prevent asthma attacks and short-acting (quick relief) drugs given by inhaler to reduce the immediate symptoms of an attack.

Drugs

The choice of initial drug treatment often depends on whether the asthma is classified as intermittent, mildly persistent, moderately persistent, or severely persistent, the age of the individual, other medical conditions that may be present, and other drugs the patient may be taking. It make take several attempts to find the best combination of drugs to control the asthma.

Beta-receptor agonists (bronchodilators)

These drugs, which relax the airways, often are the best choice for relieving sudden attacks of asthma and for preventing attacks of exercise-induced asthma. Some bronchodilators, such as albuterol (Ventolin, Proventil) and levalbuterol

(Xopenex), act mainly in lung cells and have little effect on other organs. Bronchodilators occasionally may be taken orally (i.e., pills or liquid), but normally they are administered through inhalers. The inhaled drugs go directly into the lungs and cause fewer side effects. These drugs generally start acting within minutes, but their effects last only four to six hours. Long-acting beta agonists LABAs) have been developed that can last up to 12 hours. These include salmeterol (Severent Diskus), fluticasone/salmeterol (Advair Diskus), arformoterol (Brovana), formoterol (Perforomist, Foradil), and budesonide/formoterol (Symbacort). In January 2008, the United States Food and Drug Administration (FDA) issued a warning that LABAs may increase the chance of severe asthma episodes and asthma-caused death. LABAs are not recommended as a first-line treatment for asthma. Additional information on these drugs was being gathered at the time this entry was written. The FDA suggests that people taking LABAs discuss the risks and benefits with their physician.

Leukotriene receptor antagonists

The leukotriene receptor antagonists such as montelukast (Singulair), zafirlukast (Accolate), and Zyflo (zileuton) control inflammation of the airways by blocking the action of leukotrienes, which are chemicals involved in producing inflammation. These drugs are tablets taken by mouth on a regular basis to treat or prevent symptoms of asthma and exercise-induced asthma. In March 2008, the FDA released a preliminary warning that Singulair might cause behavior and mood changes, suicidal thinking and behavior, and suicide. The warning was preliminary, meaning a cause and effect relationship between these adverse reactions and the drug had not been definitely established, and that more information was needed. The FDA recommended that individuals taking Singulair or any other leukotriene receptor antagonist drug should be alert to these behavioral side effects but not stop taking these drugs until they had discussed their condition with a physician.

Corticosteroids

These drugs, which resemble natural body hormones, block inflammation and are often effective in relieving symptoms of chronic asthma and preventing asthma episodes, but they generally are not used to treat asthma attacks once they have begun. Examples include fluticasone (Flovent), triamcinolone (Azmacort), and beclomethasone (Vanceril, Beclovent, QVAR) all of which are taken by inhalation. When corticosteroids are taken by inhalation over a long time, asthma attacks become less frequent as the airways become less sensitive to allergens. Prendisone (Deltasone, Orasone, Meticorten) is given by mouth (i.e., pills) to speed recovery after treatment of initial symptoms of an asthma attack and sometimes to treat chronic asthma.

Corticosteroids are strong drugs and usually can control even severe cases of asthma over the long term and maintain good lung function. Corticosteroids may cause numerous side effects, however, including bleeding from the stomach, loss of calcium from bones, cataracts in the eye, and a diabetes-like state. Individuals using corticosteroids for lengthy periods also may have problems with wound healing, may gain weight, and may experience psychological problems. In children, growth may be slowed.

Other drugs

Cromolyn (Intal) and nedocromil (Tilade) are anti-inflammatory drugs that affect mast cells. They may be used as initial treatment to prevent asthmatic attacks. They may also prevent attacks when given before exercise or when exposure to an allergen cannot be avoided. To be effective, these drugs must be taken regularly even if there are no asthma symptoms. Anticholinergic drugs, such as atropine, may be useful in controlling severe attacks when added to an inhaled beta-receptor agonist. They help widen the airways and suppress mucus production.

Managing asthmatic attacks

A severe asthma attack should be treated as quickly as possible; professional emergency medical assistance may be needed, as an individual experiencing an acute attack may need to be given extra oxygen. Rarely is it necessary to use a

mechanical ventilator to help the individual breathe. An inhaler, usually containing a beta-receptor agonist, is inhaled repeatedly or continuously. If the individual does not respond promptly and completely, a corticosteroid may be given. A course of corticosteroid therapy, given after the attack is over, may make a recurrence less likely.
Many asthma experts recommend a device called a "spacer" to be used along with metered-dose inhalers. The spacer is a tube or bellows-like device held in or around the mouth into which the metered-dose inhaler is puffed. This device enables more medication from a metered-dose inhaler to reach the lungs.

Maintaining control

Long-term asthma treatment is based on inhaling appropriate drugs using a special inhaler that meters the dose. Individuals must be instructed in proper use of an inhaler to be sure that it will deliver the right amount of drug. Once asthma has been controlled for several weeks or months, a physician may recommend that the patient gradually cut down on drug treatment. The last drug added usually is the first to be reduced. Individuals should be seen by their physician every one to six months, or as needed, depending on the frequency of asthma episodes.
School-age and older children may also be prescribed peak flow meters, simple devices which measure how easy or difficult it is for a person to exhale. With home peak-flow monitoring, it is possible for many children with asthma to discern at an early stage that a flare-up is just beginning and adjust their medications appropriately.
Individuals with asthma do best when they have a written action plan to follow if symptoms suddenly become worse. This plan should address how to adjust their medication and when to seek medical help. A 2004 report found that individuals with self-management written action plans had fewer hospitalizations, fewer emergency department visits, and improved lung function. They also had a 70% lower mortality rate.
Referral to an asthma specialist should be considered if:

- a life-threatening asthma attack has occurred or if asthma is severe and persistent
- treatment for three to six months has not met its goals
- some other condition, such as nasal polyps or chronic lung disease, is complicating asthma treatment
- special tests, such as allergy skin testing or an allergen challenge, are needed
- intensive long-term corticosteroid therapy has been needed to control asthma.

Special populations

Infants and young children

It is especially important to closely watch the course of asthma in young individuals. Treatment is cut down when possible, and if there is no clear improvement, treatment should be modified. Asthmatic children often need medication at school to control acute symptoms or to prevent exercise-induced attacks. Parents or guardians of these children should consult the school district on their drug policy in order to assure that a procedure is in place to permit their child to carry an inhaler. The health care provider should write an asthma treatment plan for the child's school. Proper management will usually allow a child to take part in play activities. Only as a last resort should activities be limited.

The elderly

Older persons often have other types of lung disease, such as chronic bronchitis or emphysema. These must be taken into account when treating asthma symptoms. Side effects from beta-receptor agonist drugs (including a speeding heart and tremor) may be more common in older individuals.

Prognosis

More than half of all asthma cases in children resolve by young adulthood, but chronic infection, pollution, cigarette smoke, and chronic allergen exposure are factors which make resolution less likely. Infants and toddlers who have persistent wheezing even without viral infections and those who have a family history of allergies are most likely to continue to have asthma into the school-age years.
Most individuals with asthma respond well once the proper drug or combination of drugs is found, and most asthmatics are able to lead relatively normal, active lives. A few individuals will have progressively more trouble breathing and run a risk of going into respiratory failure, for which they must receive intensive treatment.

Prevention

Minimizing allergy episodes

Exposure to the common allergens and irritants that provoke asthmatic attacks often can be reduced or avoided by implementing the following:

- If the individual is sensitive to a family pet, remove the animal from the home. If this is not acceptable, keep the pet out of the bedroom (with the bedroom door closed), remove carpeting, and keep the animal away upholstered furniture.
- To reduce exposure to dust mites, remove wall-to-wall carpeting, keep humidity low, and use special covers for pillows and mattresses. Reduce the number of stuffed toys and wash them weekly in hot water.
- If cockroach allergen is causing asthma attacks, killing the roaches using poison, traps, or boric acid is preferable to using sprayed pesticides. Avoid leaving food or garbage exposed to discourage re-infestation.
- Keep indoor air clean by vacuuming carpets once or twice a week (with the asthmatic individual absent). Avoid using humidifiers and use air conditioning during warm weather so that windows can be kept closed. Change heating and air conditioning filters regularly. High-efficiency particulate air (HEPA) filters are available that are very effective in removing allergens from household air.
- Avoid exposure to tobacco or wood smoke.
- Do not exercise outdoors when air pollution levels are high or when air is extremely cold.
- When asthma is related to exposure at work, take all precautions, including wearing a mask and, if necessary, arranging to work in a safer area. Occupational safety and health (OSHA) regulations limit exposure to certain pollutants and potential allergens in the workplace.

Key Terms

Allergen
A foreign substance, such as mites in house dust or animal dander which, when inhaled, causes the airways to narrow and produces symptoms of asthma.

Atopy
A state that makes persons more likely to develop allergic reactions of any type, including the inflammation and airway narrowing typical of asthma.

Beta blockers

Drugs used to treat high blood pressure (hypertension) that limit the activity of epinephrine, a hormone that increases blood pressure.

Hypersensitivity

The state where even a tiny amount of allergen can cause the airways to constrict and bring on an asthmatic attack.

Spirometry

A test using an instrument called a spirometer that shows how difficult it is for an asthmatic individual to breathe. It is used to determine the severity of asthma and to see how well it is responding to treatment.

For Your Information

Resources

Books

- Allen, Julian Lewis et al. eds.*The Children's Hospital of Philadelphia Guide to Asthma: How to Help You Child Live a Healthier Life.* Hoboken, NJ: J. Wiley, 2004.

Websites

- "Asthma." *United States Centers for Disease Control and Prevention.* [cited January 20, 2009]. *http://www.cdc.gov/asthma*.
- "Asthma." *MedlinePlus.* January 16, 2009 [cited January 20, 2009]. *http://www.nlm.nih.gov/medlineplus/asthma.html* .
- Morris, Michael. "Asthma." *eMedicine.com.* July 10, 2008 [cited January 20, 2009]. *http://emedicine.medscape.com/article/296301-overview*.

Organizations

- Allergy and Asthma Network: Mothers of Asthmatics (AANMA). 2751 Prosperity Ave., Suite 150, Fairfax, VA 22031. Telephone: (800) 878-4403. Fax: (703) 573-7794. *http://www.aanma.org*.
- American Academy of Allergy, Asthma, and Immunology (AAAAI) 555 East Wells Street, Suite 1100, Milwaukee, WI 53202-3823. Telephone: (414) 272-6071. *http://www.aaaai.org*.
- American College of Allergy, Asthma, and Immunology 85 West Algonquin Road, Suite 550, Arlington Heights, IL 60005. Telephone: (847) 427-1200). Email: mail@acaai.org *http://www.acaai.org*.

Antiasthmatic agents
Allergens
Allergy
Asthma

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asthma [az´mah]

a condition marked by recurrent attacks of DYSPNEA, with airway inflammation and wheezing due to spasmodic constriction of the bronchi; it is also known as *bronchial asthma.* Attacks vary greatly from occasional periods of wheezing and slight dyspn severe attacks that almost cause suffocation. An acute attack that lasts for several days is called ***status asthmaticus;*** this is medical emergency that can be fatal. adj., *adj* asthmat´ic.

CAUSES. Asthma can be classified into three types according to causative factors. *Allergic* or *atopic asthma* (sometimes called *extrinsic asthma*) is due to an ALLERGY to ANTIGENS; usually the offending allergens are suspended in the air in the form of pollen, dust, smoke, automobile exhaust, or animal dander. More than half of the cases of asthma in children and young adults are of this type. *Intrinsic asthma* is usually secondary to chronic or recurrent infections of the bronchi, sinuses, or tonsils and adenoids. There is evidence that this type develops from a HYPERSENSITIVITY to the bacteria or, more commonly, viruses causing the infection. Attacks can be precipitated by infections, emotional factors, and exposure to nonspecific irritants. The third type of asthma, *mixed,* is due to a combination of extrinsic and intrinsic factors.

There is an inherited tendency toward the development of extrinsic asthma. It is related to a HYPERSENSITIVITY REACTION of the IMMUNE RESPONSE. The patient often gives a family medical history that includes allergies of one kind or another and a personal history of allergic disorders. Secondary factors affecting the severity of an attack or triggering its onset include events that produce emotional stress, environmental changes in humidity and temperature, and exposure to noxious fumes or other airborne allergens.

SYMPTOMS. Typically, an attack of asthma is characterized by dyspnea and a wheezing type of respiration. The patient usually assumes a classic sitting position, leaning forward so as to use all the accessory muscles of respiration. The skin is usually pale and moist with perspiration, but in a severe attack there may be cyanosis of the lips and nailbeds. In the early stages of the attack coughing may be dry; but as the attack progresses the cough becomes more productive of a thick, tenacious, mucoid sputum.

TREATMENT. The treatment of extrinsic asthma begins with attempts to determine the allergens causing the attacks. The cooperation of the patient is needed to relate onset of attacks with specific environmental substances and emotional factors that trigger or intensify symptoms. The patient with nonallergic asthma should avoid infections, nonspecific irritants, such as cigarette smoke, and other factors that provoke attacks.

Drugs given for the treatment of asthma are primarily used for the relief of symptoms. There is no cure for asthma but the disease can be controlled with an individualized regimen of drug therapy coupled with rest, relaxation, and avoidance of causative factors. Bronchodilators such as epinephrine and aminophylline may be used to enlarge the bronchioles, thus relieving respiratory embarrassment. Other drugs that thin the secretions and help in their ejection (expectorants) may also be prescribed.

The patient with status asthmaticus is very seriously ill and must receive special attention and medication to avoid excessive strain on the heart and severe respiratory difficulties that can be fatal.

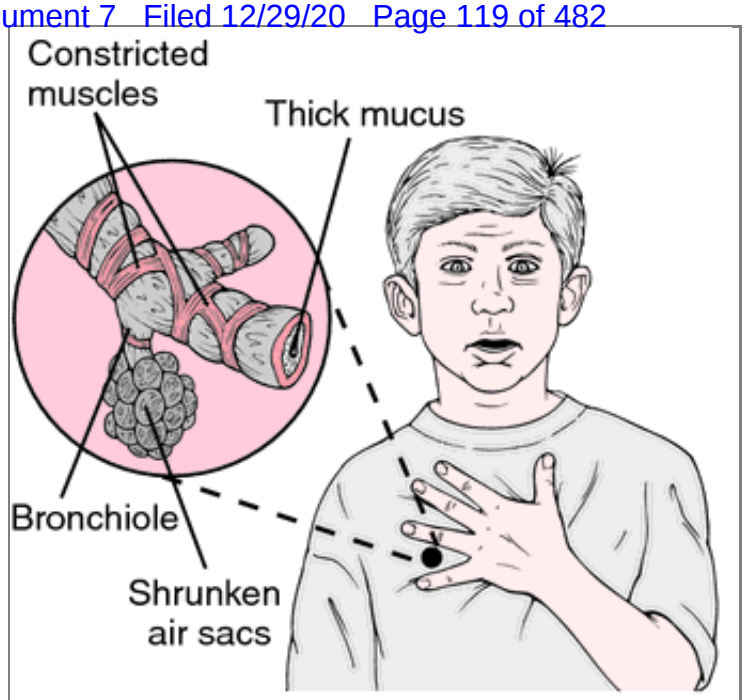
PATIENT CARE. Because asthma is a chronic condition with an irregular pattern of remissions and exacerbations, education of the patient is essential to successful treatment. The plan of care must be highly individualized to meet the needs of the patient and must be designed to encourage active participation in the prescribed program and in self care. Most patients welcome the opportunity to learn more about their disorder and ways in which they can exert some control over the environmental and emotional events that are likely to precipitate an attack.

Exercises that improve posture are helpful in maintaining good air exchange. Special deep breathing exercises can be taught to the patient so that elasticity and full expansion of lung and bronchial tissues are maintained. (See also **LUNG** and **CHRONIC OBSTRUCTIVE PULMONARY DISEASE**.) Some asthmatic patients have developed a protective breathing pattern that is shallow and ineffective because of a fear that deep breathing will bring on an attack of coughing and wheezing. They will need help in breaking this pattern and learning to breathe deeply and fully expand the bronchi and lungs.

The patient should be encouraged to drink large quantities of fluids unless otherwise contraindicated. The extra fluids are needed to replace those lost during respiratory distress. The increased intake of fluids also can help thin the bronchial secretions so that they are more easily removed by coughing and deep breathing.

The patient should be warned of the hazards of extremes in eating, exercise, and emotional events such as prolonged laughing or crying. The key words are modification and moderation to avoid overtaxing and overstimulating the body systems. Relaxation techniques can be very helpful, especially if the patient can find a method that effectively reduces tension.

Asthmatic patients fare better if they feel that they



Symptoms	Physical findings
• Shortness of breath	• Rapid, shallow respirations
•Wheezing	• Rapid pulse
• Difficult breathing	• Pallor or cyanosis
• Cough	• Diminished breath sounds
• Anxiety	• Generalized retractions
	• Frequent pausing to catch the breath when talking
	• Hyperexpansion of the chest

An asthma attack with respiratory distress. From Frazier et al., 2000.

do have some control over their disease and are not necessarily helpless victims of a debilitating incurable illness. There is no cure for asthma but there are ways in which one can adjust to the illness and minimize its effects.

allergic asthma (**atopic asthma**) that due to an atopic ALLERGY; see **ASTHMA**.

bronchial asthma **asthma**.

cardiac asthma a term applied to breathing difficulties due to pulmonary edema in heart disease, such as left ventricular failure.

extrinsic asthma

asthma caused by some factor in the environment, usually atopic in nature.

atopic asthma.

intrinsic asthma that due to a chronic or recurrent infection; see **ASTHMA**.

occupational asthma extrinsic asthma due to an allergen present in the workplace.

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asth·ma (az'mă), [MIM*600807]

An inflammatory disease of the lungs characterized by (in most cases) reversible airway obstruction. Originally, a term used to mean "difficult breathing"; now used to denote bronchial asthma.
[G.]

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asthma (ăz′mə, ăs′-)

n.

A chronic inflammatory disease of the lungs characterized by a narrowing of the airways and attacks of wheezing, coughing, and shortness of breath that are induced by triggers such as allergens, exercise, infections, and stress.

asth·mat′ic (-măt′ĭk) *adj. & n.*

asth·mat′i·cal·ly *adv.*

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EXHIBIT 140

Centers for Disease Control and Prevention
CDC 24/7: Saving Lives, Protecting People™

Asthma

Most Recent National Asthma Data

These tables feature the latest national and state statistics on the burden of asthma among children and adults. The data are from national and state surveillance systems administered by the Centers for Disease Control and Prevention (CDC). Links to sources are provided with each table to assist with finding additional information on the data and relevant tables and reports.

See also: State or Territory Data, Archived Most Recent Data

National Data

Prevalence | Asthma Attacks | Health Care Use | Mortality

National
Current Asthma[1] Prevalence (2018)

Characteristic[2]	Weighted Number with Current Asthma[1]	Percent (SE)
Total	**24,753,379**	**7.7 (0.20)**
Child (Age <18 years)	5,530,131	7.5 (0.37)
Adult (Age 18+ years)	19,223,248	7.7 (0.22)
All Age Groups		
0–4 years	744,172	3.8 (0.49)
5–14 years	3,552,191	8.6 (0.56)
15–19 years	2,204,217	11.0 (0.93)
20–24 years	1,741,490	8.1 (0.93)
25–34 years	2,895,111	6.5 (0.49)
35–64 years	9,587,682	7.7 (0.30)

65+ years	4,028,516	7.8 (0.40)
Child Age Group		
0–4 years	744,172	3.8 (0.49)
5–11 years	2,349,889	8.1 (0.60)
12–17 years	2,436,070	9.9 (0.73)
Young Teens (12–14 years)	1,202,302	9.8 (1.15)
Teenagers (15–17 years)	1,233,768	10.0 (0.91)
Adolescents (11–21 years)	4,601,301	10.3 (0.65)
Young Adults (22–39 years)	5,102,853	6.5 (0.36)
Sex		
Males	9,786,413	6.2 (0.25)
Boys (Age <18 years)	3,121,842	8.3 (0.54)
Men (Age 18+ years)	6,664,571	5.5 (0.27)
Females	14,966,966	9.1 (0.29)
Girls (Age <18 years)	2,408,289	6.7 (0.52)
Women (Age 18+ years)	12,558,677	9.8 (0.33)
Poverty Level[3]		
Below 100% of the poverty threshold	4,432,695	10.8 (0.60)
100% to less than 250% of the poverty threshold	7,069,790	8.1 (0.40)
250% to less than 450% of the poverty threshold	6,028,021	7.3 (0.40)
450% of poverty threshold or higher	7,222,873	6.5 (0.32)

Note: NH = Non-Hispanic, SE = Standard Error

[1]Includes persons who answered "yes" to the questions: "Have you EVER been told by a doctor or other health professional that you had asthma?" and "Do you still have asthma?"

[2]Numbers within selected characteristics may not sum to total due to rounding
[3]Poverty level is based on family income and family size using the U.S. Census Bureau's poverty thresholds.
Source: 2018 National Health Interview Survey (NHIS) Data, Table 3-1 and Table 4-1 (Note: Some Child Age Group data analyzed separately)

Current Asthma[1] Prevalence by Race and Ethnicity (2016-2018)

	Total		Child		Adult	
Race/Ethnicity	Weighted Number with Current Asthma[1]	Percent (SE)	Weighted Number with Current Asthma[1]	Percent (SE)	Weighted Number with Current Asthma[1]	Percent (SE)
White NH	15,496,008	8.0 (0.13)	2,560,627	6.8 (0.27)	12,935,381	8.2 (0.14)
Black NH	4,159,143	10.7 (0.35)	1,391,780	14.2 (0.75)	2,767,363	9.6 (0.39)
AIAN NH	252,177	10.4 (1.42)	64,276	10.2 (2.96)	187,901	10.5 (1.53)
Asian NH	829,238	4.5 (0.36)	142,508	3.8 (0.50)	686,730	4.7 (0.42)
Multiple NH	952,212	13.1 (0.96)	398,771	13.0 (1.17)	553,441	13.3 (1.33)
Hispanic	3,746,988	6.5 (0.30)	1,380,782	7.5 (0.46)	2,366,206	6.0 (0.35)
Puerto Rican[2]	780,533	14.0 (1.17)	228,118	13.6 (1.89)	552,414	14.2 (1.40)
Mexican[2]	1,916,450	5.4 (0.31)	782,776	6.6 (0.49)	1,133,674	4.8 (0.39)
Other Hispanic[2]	1,050,005	6.3 (0.52)	369,888	7.6 (0.87)	680,117	5.7 (0.57)

Note: NH = Non-Hispanic, AIAN = American Indian/ Alaska Native, SE = Standard Error
[1]Includes persons who answered "yes" to the questions: "Have you EVER been told by a doctor or other health professional that you had asthma?" and "Do you still have asthma?"

[2]As a subset of Hispanic

Source: 2016–2018 National Health Interview Survey (NHIS)

Page last reviewed: March 24, 2020

Content source: National Center for Environmental Health

EXHIBIT 141

Centers for Disease Control and Prevention
CDC 24/7: Saving Lives, Protecting People™

Vital Signs

Asthma in the US

Growing every year

May 2011
CDC Vitalsigns™

1 in 12

About 1 in 12 people (about 25 million) have asthma, and the numbers are increasing every year.

12M

About 1 in 2 people (about 12 million) with asthma had an asthma attack in 2008, but many asthma attacks could have been prevented.

56 Billion

Asthma cost the US about $56 billion in medical costs, lost school and work days, and early deaths in 2007.

Asthma is a lifelong disease that causes wheezing, breathlessness, chest tightness, and coughing. It can limit a person's quality of life. While we don't know why asthma rates are rising, we do know that most people with asthma can control their symptoms and prevent asthma attacks by avoiding asthma triggers and correctly using prescribed medicines, such as inhaled corticosteroids.

The number of people diagnosed with asthma grew by 4.3 million from 2001 to 2009. From 2001 through 2009 asthma rates rose the most among black children, almost a 50% increase. Asthma was linked to 3,447 deaths (about 9 per day) in 2007. Asthma costs in the US grew from about $53 billion in 2002 to about $56 billion in 2007, about a 6% increase. Greater access to medical care is needed for the growing number of people with asthma.

Latest Findings

Asthma is increasing every year in the US.

Too many people have asthma.

- The number of people with asthma continues to grow. One in 12 people (about 25 million, or 8% of the population) had asthma in 2009, compared with 1 in 14 (about 20 million, or 7%) in 2001.
- More than half (53%) of people with asthma had an asthma attack in 2008. More children (57%) than adults (51%) had an attack.
- 185 children and 3,262 adults died from asthma in 2007.
- About 1 in 10 children (10%) had asthma and 1 in 12 adults (8%) had asthma in 2009. Women were more likely than men and boys more likely than girls to have asthma.
- About 1 in 9 (11%) non-Hispanic blacks of all ages and about 1 in 6 (17%) of non-Hispanic black children had asthma in 2009, the highest rate among racial/ethnic groups.
- The greatest rise in asthma rates was among black children (almost a 50% increase) from 2001 through 2009.

Asthma Action Plan Stages

Green Zone: Doing Well

No cough, wheeze, chest tightness, or shortness of breath; can do all usual activities. Take prescribed longterm control medicine such as inhaled corticosteroids.

Yellow Zone: Getting Worse

Cough, wheeze, chest tightness, or shortness of breath; waking at night; can do some, but not all, usual activities. Add quick-relief medicine.

Red Zone: Medical Alert!

Very short of breath; quick-relief medicines don't help; cannot do usual activities; symptoms no better after 24 hours in Yellow Zone. Get medical help NOW.

Full Action Plan: http://www.cdc.gov/asthma/actionplan.html

Asthma has a high cost for individuals and the nation.

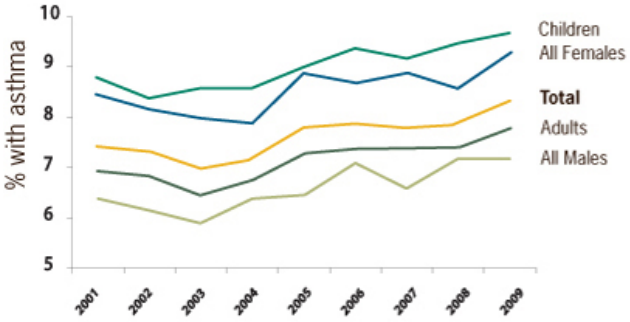
- Asthma cost the US about $3,300 per person with asthma each year from 2002 to 2007 in medical expenses.
- Medical expenses associated with asthma increased from $48.6 billion in 2002 to $50.1 billion in 2007. About 2 in 5 (40%) uninsured people with asthma could not afford their prescription medicines and about 1 in 9 (11%) insured people with asthma could not afford
their prescription medicines.
- More than half (59%) of children and one-third (33%) of adults who had an asthma attack missed school or work because of asthma in 2008. On average, in 2008 children missed 4 days of school and adults missed 5 days of work because of asthma.

Better asthma education is needed.

- People with asthma can prevent asthma attacks if they are taught to use inhaled corticosteroids and other prescribed daily long-term control medicines correctly and to avoid asthma triggers. Triggers can include tobacco smoke, mold, outdoor air pollution, and colds and flu.
- In 2008 less than half of people with asthma reported being taught how to avoid triggers. Almost half (48%) of adults who were taught how to avoid triggers did not follow most of this advice.
- Doctors and patients can better manage asthma by creating a personal asthma action plan that the patient follows.

Who's At Risk?

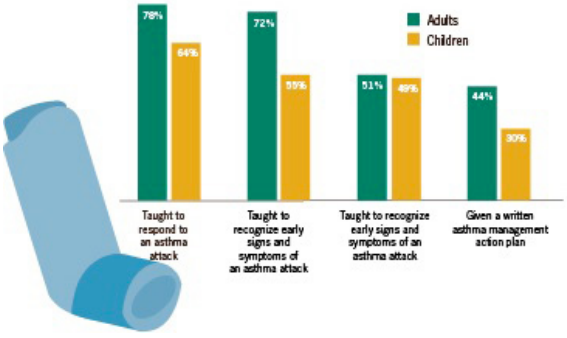
Asthma by age and sex US, 2001-2009



Percentages are age-adjusted

SOURCE: National Center for Health Statistics; 2010.

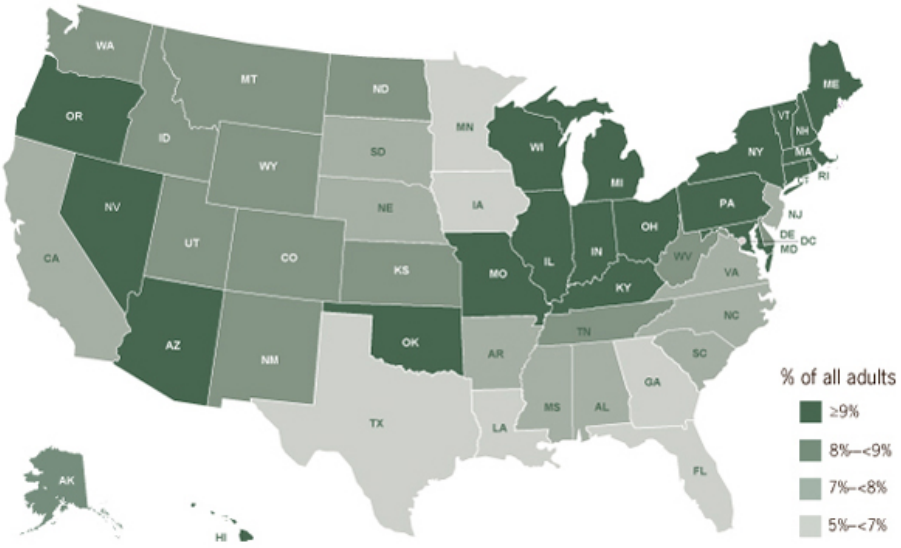
Asthma self-management education by age, US, 2008



SOURCE: National Health Interview Survey, 2008, asthma supplement.

U.S. State Info

Adults with asthma in the US, 2009



SOURCE: Behavioral Risk Factor Surveillance System, 2009

What Can Be Done

Federal, state, and local health officials can

- Track asthma rates and the effectiveness of control measures so continuous improvements can be made in prevention efforts.
- Promote influenza and pneumonia vaccination for people with asthma.
- Promote improvements in indoor air quality for people with asthma through measures such as smoke-free air laws and policies, healthy schools and workplaces, and improvements in outdoor air quality.

Health care providers can

- Determine the severity of asthma and monitor how much control the patient has over it.
- Make an asthma action plan for patients. Use this to teach them how to use inhaled corticosteroids and other prescribed medicines correctly and how to avoid asthma triggers such as tobacco smoke, mold, pet dander, and outdoor air pollution.
- Prescribe inhaled corticosteroids for all patients with persistent asthma.

People with asthma and parents of children with asthma can

- Receive ongoing appropriate medical care.
- Be empowered through education to manage their asthma and asthma attacks.
- Avoid asthma triggers at school, work, home, and outdoors. Parents of children with asthma should not smoke, or if they do, smoke only outdoors and not in their cars.
- Use inhaled corticosteroids and other prescribed medicines correctly.

Schools and school nurses can

- Use student asthma action plans to guide use of inhaled corticosteroids and other prescribed asthma medicines correctly and to avoid asthma triggers.
- Make students' quick-relief inhalers readily available for them to use at school as needed.
- Take steps to fix indoor air quality problems like mold and outdoor air quality problems such as idling school buses.

Employers and insurers can

- Promote healthy workplaces by reducing or eliminating known asthma triggers.
- Promote measures that prevent asthma attacks such as eliminating co-payments for inhaled corticosteroids and other prescribed medicines.
- Provide reimbursement for educational sessions conducted by clinicians, health educators, and other health professionals both within and outside of the clinical setting.
- Provide reimbursement for long-term control medicines, education, and services to reduce asthma triggers that are often not covered by health insurers.

Science Behind the Issue

- MMWR
- Science Clips

Related Pages

- Vital Signs Issue details: Morbidity and Mortality Weekly Report (MMWR)
- Asthma – What You Need to Know [PODCAST – 01:19 minutes]
- Asthma – What You Need to Know [PSA – 0:60 seconds]

On Other Web Sites

- The Community Guide – Asthma Control
- MedlinePlus – Asthma
- MedlinePlus – Asthma in Children
- Living With Asthma: CDC Vital Signs [VIDEO – 02:08 minutes]
- CDC Medscape Commentary: Asthma Control During Travel [VIDEO – 3:46 minutes]

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EXHIBIT 142

Serebrisky D and Wiznia A. Pediatric Asthma: A Global Epidemic. *Annals of Global Health*, 2019; 85(1): 6, 1–6. DOI: https://doi.org/10.5334/aogh.2418

REVIEW

Pediatric Asthma: A Global Epidemic

Denise Serebrisky and Andrew Wiznia

The global prevalence, morbidity and mortality related to childhood asthma among children has increased significantly over the last 40 years. Although asthma is recognized as the most common chronic disease in children, issues of underdiagnosis and undertreatment persist. There are substantial global variations in the prevalence of asthma symptoms in children, with up to 13-fold differences between countries. The rising number of hospital admissions for asthma may reflect an increase in asthma severity, poor disease management and/or the effect of poverty. The financial burden of asthma is relatively high within developed countries (those for which data is available) spending 1 to 2% of their healthcare budget on this condition. Established in 1989, the Global Initiative for Asthma (GINA) attempts to raise awareness about the increasing prevalence of asthma, improve management and reduce the burden of asthma worldwide. Despite global efforts, GINA has not achieved its goal, even among developed nations. There are multiple barriers to reducing the global burden of asthma, including limited access to care and/or medications, and lack of prioritization as a public healthcare priority. In addition, the diversity of healthcare systems worldwide and large differences in access to care require that asthma management guidelines be tailored to local needs.

Introduction

Pediatric asthma is a serious public health problem around the world. The World Health Organization estimated that approximately 300 million people currently have asthma worldwide, and with current trends rising, it is expected to reach 400 million by 2025 [1]. Nearly 250,000 people die prematurely each year from asthma, and most of all these deaths are preventable. Globally, death rates from asthma in children range from 0 to 0.7 per 100,000 people [2]. Among children, asthma is the most common chronic disease, ranking among the top 20 conditions worldwide for disability-adjusted life years in children [3].

Increasing Prevalence

The most accurate information regarding the prevalence of asthma in children around the world is available from the International Study of Asthma and Allergies in Childhood (ISAAC). Phase I of this study was completed in 1994–1995 and involved over 700,000 schoolchildren aged 6–7 and 13–14 years from 56 countries. The study revealed marked geographic variations in the prevalence of asthma. Countries with low prevalence of asthma (2–4%) were mostly in Asia, Northern Africa, Eastern Europe, and Eastern Mediterranean regions, whereas countries with high prevalence (29–32%) were located in South East Asia, North America and Latin America [4, 5] Phase III of ISAAC was conducted during 2000–2003 and involved over 1,100,000 school children from 98 countries [5–7]. Phase III of the study also showed significant geographic variations in asthma prevalence. While English-speaking countries and countries in Latin America had the highest rates of asthma per capita, the disease appeared to be less often recognized yet more severe in Africa, the Indian subcontinent and the Eastern Mediterranean. Several factors may explain these observations. In low-income countries and amongst ethnic minorities in developed countries, there may be less awareness that wheezing may be a symptom of asthma, even among those with frequent episodes [8]. This hypothesis is supported by findings showing that undiagnosed asthma among current wheezers was more common in children from lower income countries. Second, asthma care may be poorer in developing countries, leading to underdiagnosis. However, recent data showed that, unfortunately, suboptimal asthma management is a global phenomenon [9, 10]. Differences in environmental exposures to air pollutants as well as infective agents may also contribute to the greater severity observed in developing countries [6].

Morbidity and Mortality

Current statistics show substantial levels of morbidity and mortality among children with asthma. For example, worldwide trends indicate an increasing number of hospitalizations for asthma among young children, which can be attributed to increased severity, poor disease management, and poverty [1, 11, 12].

A survey of households in 29 countries in North America, Europe and Asia identified individuals with asthma who were symptomatic in the last year or were taking asthma medications [9]. Over 10,000 children and adults were interviewed. A substantial negative impact of asthma

Department of Pediatrics, Jacobi Medical Center, Bronx, NY, US
Corresponding author: Denise Serebrisky, MD (Denise.Serebrisky@nychhc.org)

on patients' lives was documented with a high number of school and work days lost, restrictions on lifestyle and requirements for urgent care. Another survey conducted in Norway showed that less than half of children admitted to a hospital for asthma had taken an inhaled corticosteroid on a regular basis, and in Turkey, a similar survey showed that only one fifth of children diagnosed with asthma were on daily anti-inflammatory therapy before the admission [13]. Interestingly, in all the studies, there was a clear overdependence on short-acting bronchodilators to manage acute asthma exacerbations. There are many reasons for these high rates of undertreatment with controller medications, including failure of medical providers to correctly classify the severity of their patient's asthma, poor access to medications, and low rates of long-term adherence.

Childhood asthma accounts for many lost school days and may deprive children of both academic achievement and social interaction, particularly in underserved populations [14] and minorities [15]. The Asthma Insights and Reality (AIR) surveys, conducted worldwide from 1998 to 2001, were aimed at assessing variations in asthma severity and control and the state of asthma management with respect to the Global Initiative for Asthma (GINA) recommendations [9]. These surveys provided direct evidence of suboptimal asthma control in many patients worldwide, despite the availability of effective therapies, with long-term management commonly falling short of the goals of the GINA guidelines. For example, among patients with severe persistent asthma, use of anti-inflammatory therapy ranged from 26% in Western Europe to 9% in Japan; the percentage of asthmatic patients with at least one unscheduled emergency visit in the past year ranged from 47% in Japan to 29% in the United States [9].

Hospitalizations for asthma are an important measure of disease severity, but data from low and middle-income countries is mostly unavailable [16]. Countries that implemented asthma management plans have observed decreases in hospitalization rates [17, 18] although they remain elevated among low socioeconomic status and minority populations [19]. Similarly, a recent European study showed that a large proportion of asthmatics had uncontrolled asthma; 57% of treated asthmatics were not well-controlled and 17% had an asthma-related hospitalization over the last year [20].

Disability-adjusted life years (DALYs), a metric that incorporates years of life lost as well as years lived with disability, is an accepted measure of disease burden [21]. The global ranking of asthma DALYs in children compared with other causes of DALYs is shown in **Table 1**. Unfortunately, asthma is among the top 20 causes of DALYs for children of all ages and among the top 10 causes in the mid-childhood (ages 5–14 years). In the older age group (10–19 years), asthma has become more common cause of DAYLs over the last decade.

It is estimated that asthma death rates have fallen worldwide by about one-third between 1990 and 2010; from 250 per million to 170 per million among males and from 130 per million to 90 per million among females [22]. However, there are large differences between countries. Data from the United States, Canada, New Zealand, Australia, Western Europe, Hong Kong and Japan show a mortality rate peak of 0.62/100,000 people in the mid-1980s among children and young adults with a progressive decline in the mid-2000s to mortality rates as low as 0.23/100,000 people [23, 24]. These findings coincide with the introduction of national and international asthma management guidelines, suggesting the potential positive impact of policy measures to curtail asthma mortality [24].

Table 1: Global Ranking of Disability-adjusted Life Years in Children Due to Asthma Compared to Other Conditions.

Age Group	Year	
	1990	2010
1–4 years	18	18
5–9 years	6	8
10–14 years	6	3
15–19 years	16	12

Pediatric Asthma and Air Pollution

Air pollution is particularly hazardous to the health of susceptible populations like children and the elderly. Children are at the highest risk because they inhale a higher volume of air per body weight than adults [25]. Numerous studies have shown that children living in environments near traffic have increased risk of asthma symptoms, asthma exacerbations, school absences, asthma hospitalizations as well as new-onset asthma [26–29]. These effects are larger in children living in metropolitan than those living in rural areas [30]. Recent data from the Aphekom project, a study focused on Improving Knowledge and Communication for Decision Making on Air Pollution and Health in Europe, indicated that near-road traffic-related pollution accounted for 15% of all pediatric asthma cases [31]. Rapid urbanization and industrialization throughout the world have increased air pollution and therefore population exposures. Worldwide, the main sources of outdoor pollutants are fuel combustion from vehicles, construction and agricultural operations, power plants and industries. Further complicating this problem, it is now recognized that global warming will increase the effect of outdoor air pollution on health [32, 33].

Indoor levels of air pollutants (excluding environmental tobacco smoke) have also been related to asthma prevalence and/or symptoms [34]. Indoor environments depend on the quality of air that penetrates from outdoors and on the presence of indoor air pollution. Approximately half of the world's population burns biomass for cooking and heating purposes, mostly in poorly ventilated areas [35]. The combustion process produces pollutants such as carbon monoxide, nitrogen oxide, sulfur dioxide and particular matters known to be risk factors for respiratory diseases such as asthma, obstructive lung disease and cancer [35, 36]. The concentration of these pollutants is particularly hazardous since most children spend about 90% of their time in confined environments. The World

Health Organization classifies indoor air pollution as the eighth most important risk factor for disease, responsible for approximately 3% of the global burden of asthma (up to 5% in low income countries). According to the Global Burden of Disease Study 2015, household air pollution from solid fuels accounted for about 5.5 million deaths worldwide in 2013 [37]. Many of these studies have demonstrated associations between exposure to indoor pollutants and the risk for several respiratory allergic conditions, including asthma.

Epidemiological studies have also shown an association between indoor dampness and mold with increased asthma incidence and prevalence regardless of the presence of atopy [38]. The respiratory health effects of smoke exposure have also been well documented. Studies have consistently shown that exposure to environmental tobacco smoke is an important risk factor for childhood asthma and as well as greater asthma morbidity in children of all ages [39–42].

The Economic Burden of Pediatric Asthma

The monetary costs of asthma are substantial and include both direct medical costs (e.g., hospitalizations, emergency room visits, medical practitioner visits and medication), and indirect nonmedical costs (e.g., time lost from work or school, decreased productivity at work or school, premature death) [1, 43]. Globally, the economic costs of asthma exceed those of tuberculosis and human immunodeficiency virus/acquire immune deficiency syndrome (HIV/AIDS) combined [44]. Developed economies spend 1 to 2% of their healthcare budget on asthma [1, 45]. In the United States, asthma cost about $3,300 per patient each year in medical expenses. Medical costs associated with asthma increased from $48.6 billion in 2002 to $50.1 billion in 2007, and will likely keep growing. Unfortunately, about 40% of uninsured people and 11% of those with insurance cannot afford their asthma prescription medicines, leading to poorer outcomes [46]. Indirect costs are also substantial, in England, 69% of parents or partners of parents of asthmatic children reported having to take time off work because of their child's asthma, and 13% had lost their jobs [47].

The economic burden of asthma disproportionately affects those with the most severe disease. In both Western and developing countries, patients with severe asthma are responsible for approximately 50% of all direct and indirect costs, even though they represent just 10 to 20% of all asthma sufferers [11, 45]. By contrast, the 70% of asthma patients with "mild" disease account for only 20% of total asthma costs.

Despite the high cost of asthma care, several studies suggest that cost containing programs can be successfully implemented. A model of disease management conducted in Finland from 1994 2004 had a massive effect in reducing asthma mortality, morbidity and costs. The program focused on early diagnosis, active anti-inflammatory treatment, and networking between primary care providers and pharmacists. Health care costs were reduced from €500 million to €230 million [18]. Similarly, an education program implemented in the United States resulted in a 35% decrease in overall hospitalization rates, a 27% decrease in asthma-related visits to the emergency department, and a 19% decrease in outpatient visits; thus, suggesting a positive impact on care costs [48]. This type of program should be implemented in other countries while also adapting to local socio-economic conditions and cultural practices.

Reducing the Global Burden of Pediatric Asthma

Unfortunately, there are many barriers to reducing the worldwide burden of asthma (**Table 2**). For the governments of much of the world population, asthma is not a healthcare priority. In developing countries, many patients have very limited access to care and essential medications. For example, in Brazil, the proportion of asthmatics using inhaled corticosteroids ranges only from 6–9% largely due to cost-related barriers [49]. In addition, asthma management must compete with other prevalent chronic illnesses for a share of available medical care resources. For example, in Africa, the most urgent healthcare priorities are poor nutrition, poor housing, and infectious diseases (especially HIV/AIDS). However, even in developed countries, access to care and ongoing management may be suboptimal, particularly for minority populations. Studies from the United States and Canada have found that minority children are less likely to be prescribed inhaled corticosteroids, even those with full prescription coverage [50, 51]. Moreover, the increasing prevalence of asthma implies that as the number of asthma patients increases, asthma-related expenditures will become an even more important challenge.

GINA guidelines stress that until there is a greater understanding of the factors that cause pediatric asthma and measures become available to reduce its prevalence, the focus should be on cost-effective management approaches that are available to most patients [24]. In addition to more research on the fundamental causes and pathogenesis of asthma, there are also urgent needs for: 1) effective patient management systems, particularly in primary care; 2) better and prompter diagnoses; 3) implementation of guidelines that are tailored to the local needs; 4) better referral and treatment patterns, including use of controller medications; and 5) cooperation

Table 2: Barriers to Reducing the Burden of Asthma.

Economic Poverty, inadequate resources, poor education, illiteracy, lack of sanitation and poor infrastructure.
Health Care System Low public health priority, poor health-care infrastructure, difficulty implementing guidelines developed in wealthier countries, limited availability of and access to medication, lack of patient education and resources.
Cultural Multiplicity of languages, religious and cultural beliefs, concerns about medications.
Environmental Tobacco, pollution, occupational exposures.

between healthcare officials and primary care providers to develop, implement and sustain management programs that will work at a local level.

In summary, there has been a significant increase on the global prevalence, morbidity and mortality related to asthma among children over the last 40 years. Governments should commit to research, intervention and monitoring to reduce the burden of asthma in the world, develop cost-effective innovative strategies to prevent the disease and more integrated approach to treatment, thus avoiding premature and unwanted deaths and improving the quality of life of asthmatic children and their families. It is also important to continue the efforts of monitoring asthma prevalence and severity globally and to implement new actions to reduce the worldwide burden of asthma.

Competing Interests

The authors have no competing interests to declare.

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EXHIBIT 143

Centers for Disease Control and Prevention

National Center for Environmental Health

You Can Control Your Asthma

You can control your asthma! When you control your asthma, you will breathe easier, be as active as you would like, sleep well, stay out of the hospital, and be free from coughing and wheezing. Learn about controlling your asthma at CDC's asthma site.

Asthma is one of the most common lifelong chronic diseases. One in 13 Americans (nearly 25 million) lives with asthma, a disease affecting the lungs and causing repeated episodes of wheezing, breathlessness, chest tightness, and coughing.

Although asthma cannot be cured, you can control your asthma successfully to reduce and to prevent asthma attacks, also called episodes. Successful asthma management includes knowing the warning signs of an attack, avoiding things that may trigger an attack, and working with your doctor to develop your personal Asthma Action Plan. CDC's National Asthma Control Program has worked to help millions of people with asthma in the United States gain control over their disease since 1999. CCARE, Controlling Childhood Asthma and Reducing Emergencies, is the program's new objective of preventing 500,000 Emergency Department (ED) visits and hospitalizations due to asthma by August 31, 2024.

Asthma deaths have decreased over time and varied by demographic characteristics. The rate of asthma deaths decreased from 15 per million in 2001 to 10 per million in 2018. Deaths due to asthma are rare and are thought to be largely preventable, particularly among children and young adults.

Asthma deaths have decreased over time.

In most cases, we don't know what causes asthma, and we don't know how to cure it. Some things may make it more likely for one person to have asthma than another person. If someone in your family has asthma, you are more likely to have it. Regular physical exams that include checking your lungs and checking for allergies can help your healthcare provider make the right diagnosis. Then you and your healthcare provider can make your own asthma management plan so that you know what to do based on your own symptoms.

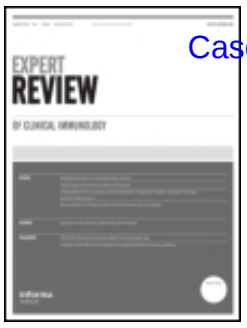
Using your asthma medicine as prescribed and avoiding common triggers that bring on asthma symptoms, such as smoke (including second-hand and third-hand tobacco smoke), household pets, dust mites, and pollen will help you control your asthma.

Make sure you are up to date on vaccinations that help protect your health. Respiratory infections like influenza (flu), can be very serious for you, even if your asthma is mild or your symptoms are well-controlled by medication. Respiratory infections can trigger asthma attacks and make your asthma symptoms worse and is more likely to lead to other infections like pneumonia. Get the recommended vaccines to help you stay healthier. Learn how to manage your asthma during an emergency.

Remember—*you can control your asthma!*

Page last reviewed: May 4, 2020

EXHIBIT 144



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Prevention of asthma: where are we in the 21st century?

Phaedra Propp & Allan Becker

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EXPERT REVIEWS

Prevention of asthma: where are we in the 21st century?

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Phaedra Propp[1,2] and Allan Becker*[1–3]

[1]*The Manitoba Institute of Child Health, Winnipeg, MB R3E 3P4, Canada*
[2]*Allergy Genes and the Environment Network (AllerGen nce) of the Networks of Centres of Excellence, McMaster University, 1280 Main Street West, Hamilton, ON L8S 4K1, Canada*
[3]*Department of Pediatrics and Child Health, Section of Allergy and Clinical Immunology, University of Manitoba, Winnipeg, MB R3T 2N2, Canada*
**Author for correspondence:*
Tel.: +1 204 789 3755
Fax: +1 204 789 3986
becker@cc.umanitoba.ca

Asthma is the most common chronic disease of childhood and, in the latter part of the 20th century, reached epidemic proportions. Asthma is generally believed to result from gene-environment interactions. There is consensus that a 'window of opportunity' exists during pregnancy and early in life when environmental factors may influence its development. We review multiple environmental, biologic and sociologic factors that may be important in the development of asthma. Meta-analyses of studies have demonstrated that multifaceted interventions are required in order to develop asthma prevention. Multifaceted allergen reduction studies have shown clinical benefits. Asthma represents a dysfunctional interaction with our genes and the environment to which they are exposed, especially in fetal and early infant life. The increasing prevalence of asthma also may be an indication of increased population risk for the development of other chronic non-communicable autoimmune diseases. This review will focus on the factors which may be important in the primary prevention of asthma. Better understanding of the complex gene–environment interactions involved in the development of asthma will provide insight into personalized interventions for asthma prevention.

Keywords: allergens • asthma • children • environment • genes • microbiota • nutrition • prevention • stress

Asthma is generally believed to be a disease whose inception and persistence is driven by gene-environment interactions. The most important of those interactions may occur in early life and even *in utero*. Recent Genome-Wide Association Studies (GWAS) for asthma have highlighted the importance of genes involving epithelial danger signaling and the innate immune response, as well as the more classic allergy associated pathways [1]. Data supporting the role of environmental risk factors for the development of asthma include a focus on the following factors: nutrition, allergens (both inhalant and ingestant), pollutants (particularly environmental tobacco smoke [ETS]), microbes and psychosocial factors.

There are several forms of prevention to be considered: primary, secondary and tertiary. Tertiary prevention is what we undertake in our clinical practises. We attempt to modify factors which influence established disease, for example, avoiding those allergens to which a child is sensitive or introduction of pharmacotherapy. Secondary prevention focuses on interventions to prevent progression of a disease process that may have downstream consequences. For example, knowing that 40% of the children with atopic dermatitis will subsequently develop asthma, the Early Treatment of Atopic Child (ETAC) study was a randomized controlled trial of pharmacologic therapy to prevent asthma in toddlers with atopic dermatitis [2]. Primary prevention is the attempt at intervention in advance of any apparent disease and will be the focus of this article.

The Canadian asthma primary prevention study

Based on the best available evidence at the time, in 1994 we initiated a multifaceted primary prevention study of asthma during the last trimester of pregnancy where, based on immediate family history, there was a high risk of the child developing asthma [3]. We focused on avoidance of active smoking and exposure to ETS in home, modifying the maternal diet to decrease exposure to major food allergens (especially peanut) *in utero* and during the first year and environmental control of house dust mites (HDMs) and pets. We encouraged breastfeeding during the first year of life or, if breastfeeding was not possible, use of a modified formula and delay of introduction of solid foods until after 6 months of age. We also

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encouraged families to avoid childcare settings with large numbers of children.

Preventions were introduced without concern that there may be any risk of adverse effects. However, within months of beginning the study we began to worry whether we may be creating an environment that may be deleterious for these children. Fortunately, by the age of 7 years old, the multifaceted intervention program was associated with a highly significant decrease in asthma [4].

Breastfeeding

Most early studies have shown a 20–25% decrease in asthma is associated with breastfeeding. Oddy found that exclusive breastfeeding (or more specifically, delay in introduction of cow's milk) during the first 4 months of life was associated with a lower risk of childhood asthma by 6 years of age [5]. Other studies have shown a protective effect of prolonged breastfeeding on asthma during the first two years of life [6] and an effect of breastfeeding on asthma risk reduction at ages 4, 5 and 8, particularly for children with a family history of asthma [7]. Breastfeeding for 4 months or more seems to be protective against later asthma compared to a shorter period of breastfeeding [8,9]. In one study [9], exclusive breastfeeding per month, for up to 15 months reduced the risks of current asthma in children up to the age of 6 years, however, the protective effects weakened over time.

Despite these findings, the benefits of breastfeeding for the development of asthma are currently somewhat controversial. Two prospective birth cohorts reported an increased risk for asthma associated with breastfeeding. In the Tucson Children's Respiratory Study, Wright and colleagues [10] observed a significantly increased risk for asthma upon breastfeeding by an asthmatic mother if, by the age of 6 year, the child was atopic. In the Dunedin birth cohort, Sears and colleagues have shown that breastfeeding for >4 weeks was associated with an increase in development of asthma at 9 years of age [11]. As well, in another cohort, [12] total breastfeeding for <10 months was associated with an increased risk of asthma, specifically non-atopic asthma.

However, there are multiple variables that may modify the beneficial impact of breastfeeding. Our Study of Asthma Genes and the Environment (SAGE), a 1995 birth cohort of children, in the province of Manitoba, Canada has demonstrated an interaction between exclusive breastfeeding, overweight and asthma [13]. We showed that breastfeeding for <12 weeks was associated with overweight by the age of 9 years and breastfeeding for <12 weeks plus the presence of overweight was associated with an increased risk for asthma (adjusted odds ratio [OR]: 1.81; 95% CI: 1.11–2.95) [13].

It is well recognized that breastfeeding decreases wheezing episodes in early life; however, the critical issue is whether breastfeeding prevents development of persistent asthma. Oddy *et al.*, [14] found the fatty acid composition in breast milk affected the development of allergy and asthma, possibly because of anti-inflammatory and immune-modulating effects associated with omega-3 (n-3) fatty acids. In addition, breastfeeding may reduce the impact of air pollution in children, especially among younger children [15].

It has been assumed that breastfeeding is preventive for early childhood wheezing and the subsequent development of asthma. However, given that recent studies have questioned this assumption, we ought not to advise families that breastfeeding will prevent development of asthma. Regardless of its effect on development of asthma, breastfeeding should be encouraged for all of its other positive benefits, such as its high nutrient content [16], contribution to the transfer of maternal antibodies and its role in protection against infectious diseases [17].

Dietary factors

Nutrients

Various nutrients have been investigated in terms of their involvement in the onset of asthma. The increase in asthma prevalence in the last quarter of the 20th century was paralleled by the increased use of folic acid in pregnancy and increased fortification of foods with folate. Studies have found that maternal folate intake during pregnancy was associated with an increased risk of asthma in the offspring [18,19]. This association was stronger for synthetic folic acid supplements possibly because they contain more potent methyl donors than dietary folate [20]. One could hypothesize that methyl donors may be responsible for epigenetic effects on disease expression by increasing DNA methylation, particularly for expression of some immune modulating genes allowing for more likely development of asthma. Still, studies are needed to better define this issue.

A number of factors associated with pro- or anti-inflammatory or antioxidant effects have been studied. For example, higher levels of n-6 polyunsaturated fatty acids (PUFAs), which are more pro-inflammatory, in food were shown to relate to elevated levels of exhaled nitric oxide and asthma symptoms [21]. However, total fat intake, saturated fatty acids, monounsaturated fatty acids and cholesterol were found to have no effect on wheeze [21,22]. Hodge *et al.* [23] have shown an association between decreased asthma and regular ingestion of cold water fish high in n-3 fatty acids.

Vitamin D intake may be through diet, sunlight or supplementation and may be beneficial in the prevention of childhood asthma. Currently, clinical trials of vitamin D supplementation on subsequent asthma are being conducted. These stem from findings by Weiss and Litonjua [24], suggesting that there may be a role for vitamin D in asthma intake and lung function development. Likewise, Erkkola, Kaila and Nwaru [25] found that maternal vitamin D levels during pregnancy were associated with a decreased risk of asthma and allergic rhinitis in children at the age of 5 years. Although studies seem to point to a beneficial effect of vitamin D on asthma prevention, the issue remains controversial. For example, Gale *et al.* [26] found that maternal exposure to higher concentrations of vitamin D during pregnancy were associated with

an increased risk of asthma in children at the age of 9 years. A recent systematic review by Nurmatov, Devereux and Sheikh [27] concluded that maternal intake of vitamins D and E was protective for the development of wheezing illnesses in the offspring. Although research regarding this topic is still in its infancy stage, studies have suggested that vitamin D may protect infants from asthma through the regulation of chromatin modification enzymes [28,29].

Childhood asthma had also been associated with reduced dietary vitamin E intake by the mother [30,31] but a meta-analysis by Allen, Britton and Leonardi-Bee [32] concluded that vitamin E intake had no effect on asthma.

High levels of cord blood selenium and iron in pregnant mothers have been associated with a lower prevalence of persistent and late onset wheeze in their children [33]. Selenium deficiencies in pregnancy have therefore been associated with an increased risk of persistent asthma in the child [34].

Introduction of 'allergenic' foods

Beginning in the 1990s, many national pediatric agencies and societies recommended delay of solid food introduction, especially for children at a high risk for development of allergy. These recommendations were widely adopted based on a belief that this would reduce the risk of developing food allergy. The theory that the immune system is not yet matured and that the gastrointestinal tract is more permeable during the first year of life appeared to be the rationale for these recommendations. Data from our SAGE 1995 provincial birth cohort showed no relationship of prematurity or low birth weight with development of food allergy [35] and there are data showing a lack of protective effect of food restriction on the incidence of childhood asthma [36–38]. In fact, there are new data comparing early versus delayed introduction of peanut that show a much higher prevalence of peanut allergy in children where introduction of peanut was delayed [39]. On the other hand, intake of certain foods may play a preventative role. Nagel *et al.* [40] found that eating green vegetables, fruit and fish was protective against allergic asthma. Mediterranean diets have a high vegetable and fruit content and provide a potential protective factor against asthma [41,42].

Probiotics

An extensively hydrolyzed formula with a mixture of synbiotics (prebiotics plus probiotics) has been shown to lower the risk of asthma-like symptoms in infants with atopic dermatitis compared to infants receiving a placebo [43]. Even so, researchers are uncertain and even doubtful about the role played by probiotics in development of asthma. In fact, a meta-analysis by Osborn and Sinn [44] showed insufficient evidence to recommend probiotics for prevention of any allergic disease (asthma, rhinitis, eczema or food allergy).

Medication

Antibiotic use has been shown to associate with development of asthma in the later stages of life [45–47]. Kozyrskyj and colleagues found an association between broad spectrum antibiotic use in the first year of life and asthma at the age of 7 years in a cohort of 13,116 Manitoba children [45]. Children receiving four or more doses of antibiotics had an even higher risk of developing asthma at the age of 7 years. The risk of asthma was doubled when antibiotics were used in the treatment of non-respiratory tract infections. Results were evident even after adjusting for reverse causation, health-care utilization bias and many well-known risk factors for asthma. Marra *et al.* found similar results when they looked at the association between amoxicillin, penicillin, cephalosporin, sulfonamide, macrolide and other antibiotics used in first year and development of asthma in early childhood [46]. All of the tested antibiotics, with the exception of sulfonamide, were associated with an increased risk of asthma where increasing courses of antibiotics were related to increasing risk. These results have been replicated by Stensballe *et al.*, [47] whereas Celedón *et al.* could not find any associations [48].

The intake of paracetamol, a mild analgesic, may be associated with asthma in both children and adults [49]. Frequent use of paracetamol by pregnant women was associated with asthma in their offspring.

Inhalant allergens

Sensitization to indoor inhalant aeroallergens is generally more important than sensitization to outdoor allergens for the presence or development of asthma. Data suggest a direct relationship between exposure to increasing concentration of HDMs, sensitization and the development of asthma [50,51]. Sensitization of an infant to inhalant allergens may actually begin during pregnancy. However, studies have not been able to show a relationship between exposure during pregnancy and evidence of umbilical cord blood response to a common allergen such as HDM [52,53].

While there appears to be a linear relationship between exposure and sensitization to HDM, exposure to animal allergen does not appear to be as simple as perceived. There are studies that demonstrated a decreased risk of developing allergy, especially to cat allergen, with increased exposure [54,55]. In a recent review of over 22,000 school age children from 11 birth cohorts in Europe, no increase or decrease in asthma relating to pets in the home, in early life, was observed [56]. That being said, the results of exposure to pets are conflicting as there have been studies that found increased risk of sensitization to pet allergens [57,58], asthma and wheezing [59–61].

At present, we have insufficient data to provide support for recommendations to discourage or encourage attempts to reduce or increase exposure during pregnancy or early life towards common allergens to which sensitization is associated with subsequent development of asthma.

Pollutants

ETS has been the focus of many studies which have shown that exposure to tobacco smoke both prenatally [62] and after birth is associated with measurable harmful effects, including a greater risk of developing asthma symptoms in early childhood [63]. As well, there are demostrable effects on fetal airway development and subsequent decrements in lung function in

Table 1. Primary prevention of asthma studies outcomes.

Study	Intervention type	Intervention	Outcome	Ref.
Childhood Asthma Prevention Study (CAPS)	Mono-allergen	Avoidance of HDM or dietary restrictions; Avoidance of ETS; first 5 years of life	3 years: Reduced cough (in atopic children only), reduced sensitization to HDM; no effect on wheeze	[130]
			5 years: For HDM avoidance group: No effect on asthma, wheeze, atopy; increase in eczema For dietary restrictions group: no effect on asthma, wheezing, eczema and atopy	[131]
Canadian Asthma Primary Prevention Study (CAPPS)	Multi-allergen	Avoidance of HDM, pets, ETS, day care and cow's milk, seafood or peanuts; first year of life	2 years: Reduced asthma, reduced recurrent wheeze; no effect on atopy or recurrent cough	[125]
			7 years: Reduced asthma and wheeze; no effect on allergic rhinitis, atopic dermatitis or bronchial hyper-responsiveness	[4]
Isle of Wight Study	Multi-allergen	Avoidance of HDM; dairy, nuts, wheat, eggs or soy introduced after 9 months; first 9 months of life	1 year: Reduced physician diagnosed asthma, reduced sensitization to food allergens, reduced eczema	[132]
			2 years: Reduced sensitization to cats, reduced atopy; non-significant trends to physician diagnosed asthma, eczema and sensitization to food allergens	[133]
			4 years: Reduced sensitization to mite, reduced sensitization to cats, reduced atopy, reduced eczema; non-significant trends to sensitization to food allergens, wheeze and cough	[134]
			8 years: Reduced sensitization to mite, reduced atopy, reduced nocturnal cough, and reduced bronchial hyperresponsivenes;s nonsignificant trends to wheeze, sensitization to cats, sensitization to food allergens, and physician diagnosed asthma	[124]
			18 years: Reduced asthma; no effect on atopy	[127]
Manchester Asthma and Allergy Study (MAAS)	Mono-allergen	Avoidance of HDM; implemented from prenatal period	1 year: Reduced asthma; no effect on atopy or eczema	[58]
			3 years: No effect on wheeze, cough, rhinitis or eczema	[135]

ETS: Environmental tobacco smoke; HDM: House dust mite.

Table 1. Primary prevention of asthma studies outcomes (cont.).

Study	Intervention type	Intervention	Outcome	Ref.
Prevention and Incidence of Asthma and Mite Allergy Study (PIAMA)	Mono-allergen	Avoidance of HDM; implemented from prenatal period	1 year: Reduced sensitization to HDM; no effect on atopy	[136]
			2 years: Reduced nighttime cough without a cold; no effect on respiratory symptoms, atopic dermatitis or atopic sensitization	[137]
Prevention of Asthma in Children Study (PREVASC)	Multi-allergen	Avoidance of HDM, pets and ETS; implemented from prenatal period	2 years: No effect on wheeze, cough, rhinitis or eczema	[126]
Study on the Prevention of Allergy in Children in Europe (SPACE)	Multi-allergen	Avoidance HDM and pets; introduction of solid foods after 6 months; Introduction of milk, eggs and fish after 12 months; implemented from birth	1 year: Reduced sensitization to HDM and reduced atopy; no effect on wheeze	[138]
			2 years: No effect on sensitization to HDM, wheeze, eczema, asthma or rhinitis	[139]

ETS: Environmental tobacco smoke; HDM: House dust mite.

later childhood, respiratory infections and increased risk of chance of wheezing, exercise induced wheezing, nocturnal cough, physician-diagnosed asthma and allergic sensitization [64–67]. Significant differences have been shown for lung function between newborn and 4-week-old infants who were born to smoking mothers compared with non-smoking mothers. Those born to smoking mothers had significantly higher risk of wheezing illnesses in the first year of life [68].

Maternal smoking during pregnancy is the most direct avenue of prenatal ETS exposure [69] and a systematic review and meta-analysis [63] concluded that prenatal smoking had the strongest effect at the lower ages and postnatal maternal smoking seemed to be only relevant to older children. The relationship between prenatal smoking and wheeze was positive across all ages but the relationship between prenatal maternal smoking and asthma weakened with increasing age [63]. The relationship of passive smoking with asthma was weaker than with wheeze. Gilliland *et al.* [70] found that ETS exposure was modified by the child's glutathione-S-trasnferase (GSTM1) genotype, however, it may only modify the effects of infant lung function but not later childhood asthma [71].

Maternal, paternal and grandmaternal smoking, all have been deemed harmful. The risk of ETS exposure on asthma development may be transmitted across two generations, specifically *via* grandmaternal smoking [72]. Grandmaternal smoking during the mother's pregnancy alone was a risk factor for grandchild asthma independent of maternal smoking, but a child had the highest risk of asthma development if both the grandmother and the mother smoked during pregnancy [72].

Traffic-related air pollutants have been the focus of a great deal of research as well. Children, residing nearby busy roadways, had increased asthma hospitalizations, decreased lung function and increased prevalence and severity of wheezing and allergic rhinitis [73]. In several studies there was a positive relationship between traffic-related pollution and physician-diagnosed asthma apparent in school aged children [74–77]. A recent study suggests that exposure to out of door pollutants may only be important for those children who were exposed to ETS *in utero* and in infancy [78].

Three air pollutants of particular interest regarding asthma prevention are particulate matter, ozone and nitrogen oxides [74–76,79]. Diesel particulates may have a specific impact, providing adjuvant effect for the development of asthma [80]. Socioeconomic status (SES) and amount of exposure are confounders which may also influence the impact of traffic-related pollutants. Low-income minority children who, more often, live near pollution sites with heavy-traffic seem to be at a high risk for asthma [81]. Roadway density and proximity to highway at the prenatal residence also may influence asthma [82]. Patel *et al.* [82] witnessed a stronger association with the individual's concurrent proximity to highway than with their proximity only prenatally or in early childhood exposure. This demonstrates the importance of cumulative pollutant exposure on asthma.

Microbial effects

Viral infections

Respiratory viral illness during early life has been associated with an increased likelihood for asthma. In particular,

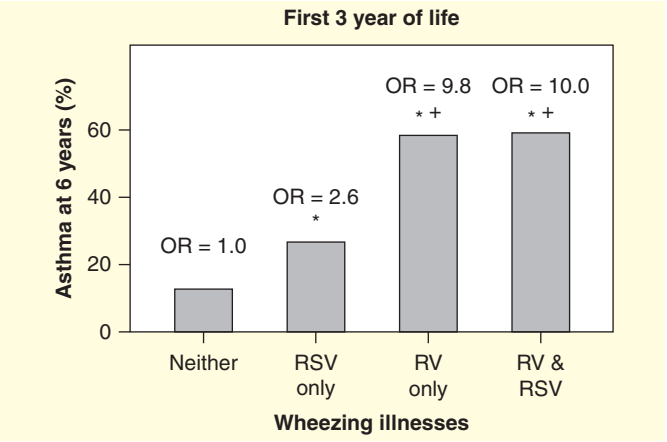

Figure 1. Risk of asthma at age 6 years in children who wheezed during the first 3 years of life with rhinovirus, respiratory syncytial virus, or both.
*p < 0.05 versus Neither; **p < 0.05 versus RSV only.
OR: Odds ratio; RSV: Respiratory syncytial virus; RV: Rhinovirus.
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respiratory syncytial virus (RSV) and human rhinovirus virus (RV) have been implicated as potential important factors in the development of asthma [83–85]. The Tucson Children's Respiratory Study identified several potential risk factors associated with wheezing in childhood. Family size, birth order and day care attendance may all influence exposure to viral infections and increase the risk of wheezing in early life but may decrease potential for persistent asthma by school age [86,87].

Recently, wheezing episodes associated with human rhinovirus (HRV) have been found to be a stronger predictor of school-age asthma than RSV induced wheezing [88,89]. In the Childhood Origins of Asthma Study (COAST), study of high-risk children, HRV associated wheezing illnesses in infancy were the most significant predictors of wheezing throughout the participants first 3 years of life [90] and for asthma at the age of 6 years (Figure 1) [89,91]. In addition, Caliskan and colleagues were able to identify a specific gene-environment interaction relating to HRV wheezing episodes [92]. In both the COAST and Copenhagen Prospective Study on Asthma in Childhood (COPSAC) birth cohorts, they found associations between asthma and five asthma-associated 17q21 SNPs that were restricted to children with HRV wheezing illness in early childhood [92]. No interaction was discovered between *17q21* genotype and RSV wheezing illness.

Microbiota

The 'hygiene hypothesis' and the more recently coined 'microflora hypothesis' posit that the interaction with microbiota may, in fact, be beneficial as regarding the prevention of asthma. Studies have shown asthma and allergy risk to be lower in children raised on farms where they are exposed to stables and consume raw farm milk [93] and where bedrooms have high levels of bacterial derived lipopolysaccharide endotoxin [94,95]. A lower incidence of asthma was related to higher amount of dust, extracellular polysaccharides and endotoxin in the mother's mattress [95]. Farmers may be exposed to asthma-protective microbiota *via* other routes, such as through farm animals' raw milk consumption, more common in the farming community [96,97]. Similarly, homes with animals, particularly dogs, have higher levels of endotoxins and children in those home have lesser allergy [54]. We have recently shown a significant difference in the gut microbiota of infants from homes with pets and older siblings [98].

An inverse association has been observed [99–101] between *Helicobacter pylori* infections in infancy and incidence of asthma and allergy. *H. pylori* infections neonatally, but not in adulthood, prevent allergic asthma in mouse models through the induction of Tregs [102]. Dendritic cells exposed to *H. pylori* can become tolerogenic, driving Treg differentiation through IL-18 production and causing asthma protection. Intriguingly, as the prevalence of asthma has increased [103], the presence of *H. pylori* also appears to have decreased.

Exposure to bacteria prenatally and during delivery may be particularly impactful on the child's development of asthma, either in a positive or detrimental way. Some studies [104,105] have found that exposure to gram-negative bacteria (*Acinetobacter lwoffii*) *in utero* provided potential protection against development of atopy and asthma. Research has shown that infants born *via* Cesarean section are at an increased risk for the development of asthma and atopy [106,107]. Exposure of the infant to the mother's vaginal microflora through vaginal delivery may be beneficial, modifying development of their immune responses and prevention of allergic diseases. We have also shown a significant effect of the gut microbiota in infants born *via* Cesarean section compared with those born vaginally [108].

Lange *et al.* [109] found that higher counts of maternal gut aerobic bacteria and enterococci in the mothers' third trimester stool samples were associated with an increased risk of infant wheeze up to the age of 6 months. This relationship was evident even when controlling for breastfeeding, day care attendance and maternal atopy. Benn *et al.* [110] reported a higher risk of hospitalizations for wheezing when mothers were vaginally colonized with *Ureaplasma urealyticum* and a higher risk of asthma when colonized with *Staphylococci*.

Psychosocial factors

The social environment, in which a child is exposed to, may also contribute to the development and severity of asthma. Lower SES has been shown to associate with higher asthma prevalence rates and hospitalizations [111]. Still to be answered is whether early life lower SES increases vulnerability for asthma later in life. Most studies on the topic are cross-sectional, and find associations between current SES and numerous child health outcomes [111]. Higher levels of parental stress, related to the lower SES, may also relate to increases in the risk of infant wheezing [112]. Prenatal maternal stress has also been found to associate with elevated cord blood IgE [113] as well as childhood wheeze [114–116], asthma, eczema and allergic

Table 2. What recommendations can we currently offer our patients?

Encourage	Discourage
Breastfeeding	Exposure to environmental tobacco smoke
Vaginal delivery	Use of broad spectrum antibiotics during the first year of life
A positive supportive environment: decrease stress and encourage families to make choices with which they are comfortable	

rhinitis [116]. Additionally, maternal distress that persisted from birth to early school age was associated with an increased risk for asthma in the child [117]. These relationships are not explained by an increased level of stress in the child [118] leading Wright *et al.*, [119] to propose an impact of the hypothalamic-pituitary-adrenal axis on fetal immune developmental processes. Consistent with this, we have shown a lower cortisol response to a stressor among school aged children with asthma who had been exposed to persistent maternal stress in early life [120].

The connection of asthma with psychosocial aspects seems logical because data showing a connection between stress and immune-related Th cells. One murine model looked at the impact of perceived stress and learned helplessness on airway inflammation [121]. Mice that experienced uncontrollable stress also experienced more narrowing of airways and goblet cell hyperplasia, features of asthma pathogenesis, than those mice in the control group. After an ovalbumin (OVA) challenge, Deshmukh *et al.*, reported a lymphocyte population highly saturated with CD4+ T cells, indicative of increases in IL-4, IL-13 and lack of IFN-γ production. Mice that experienced control over the stressful situation were able to dampen inflammatory disease by limiting CD4+ T cells and production of cytokines related to Th2 cells. Helpless mice, however, were unable to do this and, in fact, had increased levels of B cells and eosinophils.

Research supporting this model has found an association between lower levels of family support and higher levels of nighttime asthma symptoms, lower pulmonary functioning in the morning and higher eosinophil and IL-4. Likewise, in a sample of 15,357 adults, increasing numbers of adverse childhood experiences related to a 70% increase in risk for hospitalization with Th1, 80% increase in risk for hospitalization with Th2 and 100% increased risk for rheumatic disease [122].

Is primary prevention of asthma possible?

Birth cohort studies provide some evidence for consideration (Table 1). A meta-analysis by Van Schayck *et al.*, [123] demonstrated that studies of intervention focused on a single exposure (mono-allergen), did not significantly affect asthma development, whereas multifaceted interventions had evidence for asthma prevention. When considering multifaceted allergen reduction studies at <5 years, including the Isle of Wight study [124], The Canadian asthma primary prevention study (CAPPS) [125] and the Prevention of Asthma in Children (PREVASC) study [126] from the Netherlands, the impact on asthma prevention was significant (OR: 0.73; 95% CI: 0.55–0.97). Two multifaceted studies had published data beyond the age of 5 years (CAPPS [4] and the Isle of Wight [124] and demonstrated a significant effect for children younger than 5 years of age and for children older than 5 years of age (OR: 0.52; 95% CI: 0.32–0.84). The Isle of Wight study [127] has shown continuing positive benefits of the early life intervention through to 18 years of age. However, exactly which components of the intervention were important, and what specific mechanistic changes were induced, remain elusive.

Large population based studies such as the Canadian healthy infant longitudinal development (CHILD) study, which assess multiple aspects of genetics and the environment, will be critical to better inform future prevention trials. One particularly effective approach has been demonstrated by the Global Allergy and Asthma European Network (GA^2LEN) initiative which brings together a number of European birth cohorts and compares study designs, exposures and outcomes in order to increase the power of individual birth cohorts [128].

Expert commentary

What recommendations can we currently offer our patients?

When we began the multifaceted CAPPS birth cohort in 1994, we were sure about what was bad (environmental tobacco smoke) and what was good that we could encourage (breastfeeding, delaying introduction of 'allergenic' foods, avoidance of pets, elimination of HDMs and a 'clean environment' including avoiding exposure to lots of other children). The intervening years have turned much of what we 'knew' on its head.

What do we know now? Do we know enough to begin a new prevention of asthma cohort? Do we know enough to advise our patients and their families? Well, ETS is still bad and breastfeeding (for reasons other than prevention of allergy and asthma) is still good. Where possible, vaginal delivery should be encouraged and use of broad spectrum antibiotics during the first year of life should be discouraged. Additionally, for premature infants, recent data have shown that preventative treatment with palivizumab can help in preventing development of asthma in those high risk children [129]. Possibly the most important factor for us to consider is the need to provide a positive supportive environment to decrease stress and encourage families to make choices with which they are comfortable. It is time to stop making mothers feel guilty about choices they make (Table 2).

Five-year view

It is increasingly clear that there are multiple genes and environmental factors that interact in the development of asthma. It is critical to have studies, such as CHILD, that are of sufficient size and depth to truly understand these gene-environment interactions. That level of understanding will be critical for us to provide families with truly effective personalized approaches toward prevention of asthma, allergies and other non-communicable diseases. Over the next decade we will have data on scientific gene-environment interactions that will allow for the approach to become common practice: at least for high risk families.

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Key issues

- Asthma has become the most common chronic disease of childhood and has reached epidemic proportions in the past few decades.
- The 'hygiene hypothesis' suggests that early exposure to microbiota may be beneficial against asthma development.
- However, certain respiratory viral illnesses during early life have been associated with an increased likelihood for asthma. Most recently, wheezing episodes with human rhinovirus, rather than respiratory syncytial virus, have been shown to be highly associated with persistent asthma.
- Pollutants, especially tobacco smoke, are associated with an increased risk for asthma.
- The beneficial effect of breastfeeding on asthma prevention is controversial and caution should be taken into account when advising families that breastfeeding will prevent asthma. However, breastfeeding should be encouraged for all of its other benefits.
- There is no reason to delay solid foods beyond 4–6 months of life and early introduction of some solids may prevent allergy to that food.
- Early life exposure to pets has not been associated with prevalence of asthma. In fact, dogs in the home may be associated with less asthma.
- Antibiotic use and paracetamol intake in early life may be associated with an increased risk of asthma.
- Stress in early life has been associated with more asthma.
- Multifaceted interventions have been shown to be effective in the prevention of asthma, whereas single interventions (e.g., eliminating dust mite exposure) have not worked.

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EXHIBIT 145

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Animal models of asthma

Jason H. T. Bates, Mercedes Rincon, and Charles G. Irvin

Vermont Lung Center and Center for Immunology and Infectious Disease, University of Vermont College of Medicine, Burlington, Vermont

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Bates JH, Rincon M, Irvin CG. Animal models of asthma. *Am J Physiol Lung Cell Mol Physiol* 297: L401–L410, 2009. First published June 26, 2009; doi:10.1152/ajplung.00027.2009.—Studies in animal models form the basis for much of our current understanding of the pathophysiology of asthma, and are central to the preclinical development of drug therapies. No animal model completely recapitulates all features of the human disease, however. Research has focused primarily on ways to generate allergic inflammation by sensitizing and challenging animals with a variety of foreign proteins, leading to an increased understanding of the immunological factors that mediate the inflammatory response and its physiological expression in the form of airways hyperresponsiveness. Animal models of exaggerated airway narrowing are also lending support to the notion that asthma may represent an abnormality of the airway smooth muscle. The mouse is now the species of choice for asthma research involving animals. This presents practical challenges for physiological study because the mouse is so small, but modern imaging methodologies, coupled with the forced oscillation technique for measuring lung mechanics, have allowed the asthma phenotype in mice to be precisely characterized.

allergic inflammation; mouse; airway smooth muscle; lung impedance

ASTHMA IS A PRIME EXAMPLE of a "complex disease," a term that has become popular for characterizing those pathologies that appear to have multifaceted etiologies and an entanglement of underlying mechanisms. The moniker of complex disease, however, often tends to belie a general lack of understanding about what is going on, and asthma is no exception. Indeed, asthma is more properly regarded as a syndrome than a disease because it is defined on the basis of clinical characteristics rather than underlying mechanisms, and there remains a great deal of controversy about which of the possible mechanisms are the most important (20). The principal characteristics of asthma are reversible airflow obstruction, hyperresponsiveness of the lungs to challenge with smooth muscle agonists, and airway inflammation. Not surprisingly, allergy heads the list of causative suspects, but it is by no means alone. Exercise, cold air, and emotional stress are also known triggers of asthma (1), leading to the notion that asthma itself represents merely the common clinical endpoint of a number of distinct disease processes (42, 53, 91).

As with most human diseases, studies in laboratory animals have produced much of what we currently think we know about the mechanisms responsible for asthma. Obviously, the relevance and validity of these studies are tied to how well we can produce accurate animal equivalents of human asthma. The development of such "animal models" is still very much a work in progress; although many of the various features of asthma have been convincingly recapitulated in animals, invariably every animal model misses some important aspect of the human syndrome (97). Also, very few animals spontaneously develop a condition with any similarity to asthma, the most reminiscent being an allergic syndrome in cats (72) and the condition known as heaves in horses (24). If asthma is a condition peculiar to humans for some reason, then having it manifest in its entirety in a laboratory animal may simply be impossible. On the other hand, given that we still do not fully understand what asthma in humans actually is, it remains difficult to know whether an animal really has it or not. Accordingly, much of the challenge in studying animal models of asthma lies in phenotyping them properly, particularly as asthma is defined in humans in terms of phenotype rather than underlying pathology. In this review, we therefore focus on the issue of how to assess the relevant function and structure in the lungs of laboratory animals, and what clues this has given us about the possible mechanistic underpinnings of asthma in humans.

Development of Animal Models

Early animal models of asthma were developed in a variety of species (90) and focused on the phenomenon of airways hyperresponsiveness (AHR), defined as excessive bronchoconstriction in response to a standardized challenge. The challenging agent was usually a smooth muscle agonist such as methacholine or histamine. These agonists were intended to mimic the actions of mediators released as part of an overexuberant immune response thought to be behind an attack of allergic asthma. In a similar vein, some animal models employed hyperpnea to mimic the asthma brought on by exercise (35). In any case, the motivation behind these models arose from the idea that asthma is primarily a matter of excessive shortening of the airway smooth muscle (98). This notion was also

Address for reprint requests and other correspondence: J. H. T. Bates, Univ. of Vermont College of Medicine, HSRF 228, 149 Beaumont Ave., Burlington, VT 05405-0075 (e-mail: jason.h.bates@uvm.edu).

reflected in the predominant use of β-agonists to relax smooth muscle as frontline therapy for most asthma cases.

In the early 1980s, a greater awareness of the role of inflammation in asthma developed, driven by an increased understanding of allergic immunology together with observations that human asthmatics frequently exhibited marked symptoms when challenged with antigens of various kinds. The first inflammatory cell to be linked firmly to asthma pathogenesis was the eosinophil (27, 63), followed soon after by the T cell (66). This led to increasing use of corticosteroids in the routine treatment of asthma. Current asthma guidelines (1) call for combining corticosteroids with long-acting β-agonists in treating persistent asthma, which seems appropriate given its current definition comprising both obstructive and inflammatory components. In fact, it now seems that treating asthma with a β-agonist alone may be inadvisable. Indeed, monotherapy with some long-acting forms has recently been designated as unacceptable by the Food and Drug Administration.

As a result of studies with animal models, we now know that allergic asthma involves a complex interplay between the innate and adaptive immune systems. The involvement of adaptive immunity begins with naïve CD4 T cells that differentiate into T helper (Th) cells with the potential to regulate the fate, function, and location of a variety of other immune cell components. Different subsets of Th cells have been defined on the basis of the cytokines they secrete, with Th1 and Th2 cells being the best known examples. The production of Th1 cells is promoted primarily by IL-12, whereas differentiation into Th2 cells occurs in the presence of IL-4. In turn, Th1 cells produce IFNγ, whereas Th2 cells produce IL-4, IL-5, and IL-13. Allergic asthma is associated with a Th2 type of immune response, since the Th2 cytokines are known to cause many of the features of the disease (36, 52). Th2 cytokines, particularly IL-5 and to certain extent IL-4, are also responsible for linking adaptive to innate immunity by promoting eosinophil proliferation in bone marrow and subsequent migration to the lung. Eosinophilia in the airways has long been linked to allergic asthma, although exactly why has been controversial. Nevertheless, their pivotal role in altering lung function in allergic mice has been demonstrated in experiments with eosinophil knockout animals (58).

The Th2 cytokine IL-4 is also implicated in the dominance of antigen-specific IgE over other antibody isotypes in allergic asthma. Although a number of cytokines such as IL-21 are involved in promoting B cells to produce antibody and generation of plasma cells, only IL-4 and IL-13 are known to promote isotype switching to IgE (71). Binding of antigen-specific IgE to its Fcε receptor (FcεR) on mast cells then promotes degranulation, leading to secretion of a number of bronchoactive mediators including histamine. Increased IgE levels would seem to contribute to the pathogenesis of asthma, although clinical trials with anti-IgE therapy (Xolair, omalizumab) show only modest therapeutic efficacy (19).

Th2 cells are not the only T cell variants associated with allergic airway inflammation, however. Recently, a role has been demonstrated for natural killer T (NKT) cells, which are characterized (55) by the presence of an invariant T cell receptor (Vα14 Jα18 associated with Vβ8, Vβ7, and Vβ2) and are restricted by the CD1d MHC I-like molecule and recognize glycolipids instead of protein antigen (16). Despite their relatively low numbers, NKT cells have the capacity to rapidly produce high levels of various cytokines upon antigen recognition (16) and are found in the lung. Transfer of NKT cells from mice with allergic airway inflammation to naïve mice is sufficient to induce airway hyperreactivity (3).

Another CD4 subset that has emerged within the last two years is the Th17 cell. Both TGFβ and IL-6 are necessary, although not sufficient, for the differentiation of Th17 cells (17, 62, 86). These cells produce mainly IL-17, some IL-22 and IL-21, small amounts of IFNγ, and no IL-4 (46, 69). Although IL-17 is clearly detectable in allergically inflamed lungs, its role in the pathogenesis of the allergic response remains unclear. It seems that IL-17 may be needed for the initial development of airway inflammation in mice, but administration of IL-17 decreases eosinophilia. Also, adoptive transfer of Th17 cells induces neutrophilia and imparts resistance to steroid therapy, suggesting that IL-17 may function to promote neutrophil recruitment (55) and could therefore be particularly important in severe asthma (64). IL-17 has also been shown to interfere with epithelial cell production of eotaxin (77), which plays a major role in the recruitment and activation of eosinophils (70). IL-17 may thus regulate the eosinophil-neutrophil balance in the lung.

Despite the wealth of knowledge we now have about the role of immunity in allergic asthma, the complex pathophysiology of the disease caused researchers to have a difficult time agreeing over whether asthma is due primarily to altered immune status or to an abnormality of airway smooth muscle, or even both simultaneously (5). Work has thus proceeded along both fronts, driven by the respective convictions of the scientists involved. The development of animal models of asthma has been correspondingly schizophrenic. Nevertheless, the asthma world has come together on one thing regarding animal models: the species. As with most animal-based biomedical research, mice now dominate the scene because of the immunological and molecular tools available to study them, together with their obvious practical advantages related to cost and gestation period (23, 50). This is not to say, of course, that the mouse is the only species used to model asthma. Rat models have also been widely employed (21, 84), notably the Brown-Norway strain because of its propensity to exhibit a later allergic response following antigen challenge (84). Monkeys have also attracted attention as a useful model that bridges the gap to human relevance more than rodent species (28, 39, 61). Nevertheless, most asthma-related research continues to be pursued in the mouse. The development of transgenic mice that exhibit various lung pathologies is now a huge research enterprise, and mouse models of lung disease have been the subject of a number of recent reviews that cover their various pathophysiological features in detail (11, 23, 49, 50, 54, 67).

Mouse Models of Allergic Asthma

Allergic mouse models of asthma are generated by first sensitizing an animal to a foreign protein, most commonly ovalbumin. This is typically done by injecting the protein intraperitoneally along with an adjuvant, typically aluminum hydroxide, that serves to enhance the protein's immunogenicity for reasons that are complex and not entirely understood (56). After the immune system has had a chance to mount a reaction against the antigenic protein, which takes several days,

the animal receives a further antigen exposure either directly to the lungs in the form of an aerosol or via postnasal drip following nasal instillation. This elicits an inflammatory reaction in the lungs characterized by an influx of eosinophils, epithelial thickening, and AHR. There is wide variation, however, in the precise recipe used by different investigators to perform these various steps. A typical scenario is to sensitize an animal with two intraperitoneal injections spaced a week or two apart, wait another week, and then challenge the animal by exposure to a 1% ovalbumin aerosol for 30 min each day for 3 days (87). The features of allergic inflammation typically reach their peak a day or two after the final exposure, although the time course of this process has not been fully characterized.

Many variations on all aspects of the above theme have been tried. For example, inhalational exposure to NO_2 has been used as a means of bolstering the immune response in place of an intraperitoneal adjuvant (18), invoking the notion that airborne pollutants may play a role in the development of asthma in industrialized societies. Also, questions about the relevance of ovalbumin to human asthma have led to the use of antigens such as extracts of house dust mite (30) and aspergillus (40), which are more representative of those that occur naturally. Of particular recent interest has been the issue of chronicity of antigen exposure. The sensitization and challenge procedure described above is very acute, yet asthma in humans is typically a chronic disease that exhibits clear features of airway remodeling in its later stages (51). Researchers have thus been motivated to explore the effects of longer-term antigen exposures in mice. Somewhat disappointingly, extended exposure to ovalbumin aerosol in at least some mouse strains such as the BALB/c leads to tolerization, characterized by the waning of the inflammatory and hyperresponsive phenotypes (9, 94). There have been some reports that other antigens such as house dust mites produce a more extended asthma-like picture (30). Furthermore, simultaneous exposure to multiple antigens produces inflammation in the absence of adjuvant, and may even break through the tolerization barrier to produce a chronic phenotype (38). Nevertheless, the chronic model has yet to be optimized, particularly in view of the rather modest degree of AHR that has so far been demonstrated. Indeed, the generation of allergic mouse models of asthma remains something of a black art, no doubt due to the immensely complex and often poorly understood biological processes involved.

Fully elucidating the biological complexities of allergic mouse models of asthma is going to take some time, but it has moved ahead in recent years thanks to significant technical advances in phenotyping using both lung function assessment and imaging. The measurement of lung mechanical function in mice poses a particular challenge, of course, due to the small size of the animal (50). Measurement techniques developed in larger animals or humans frequently do not do well when scaled down to the mouse. The frustration this causes has led to a temptation to cut corners in the assessment of the mouse phenotype, the most problematic result of which has been the use of the parameter known as enhanced pause (Penh) to assess AHR (43). Due to its attractions of noninvasiveness and simplicity, as well as its availability in a commercial device (31, 32), Penh has become widely used in the study of AHR. Unfortunately, however, this use has invariably been inappropriate. Penh is merely a nonspecific feature of the breathing pattern and cannot be taken as a measure of lung mechanics, as has been discussed extensively elsewhere (7, 65). Of course, because the mechanical properties of the lung have an important bearing on the pattern of breathing (43), it is conceivable that a change in the breathing pattern, such as might be reflected in Penh, could signal the presence of some event worthy of more detailed investigation, such as activation of irritant receptors in the lung (2). On the other hand, one can just as easily screen for a change in breathing pattern using the obvious parameters of breathing frequency and/or tidal volume that seem to be just as efficacious as Penh (2) but do not suffer from the latter's obscurity of meaning. Indeed, we would go so far as to suggest that the use of Penh be avoided completely (7). In any case, the pattern of breathing can never serve as a substitute for a measure of mechanical function.

Recently, two approaches have been used to extend unrestrained plethysmography by combining it with an independent noninvasive measure of changes in lung volume to estimate specific airway resistance (14, 76). These approaches are theoretically sound and may eventually prove useful for rapid screening, but the information they provide is noisy and subject to the vagaries of the animal's spontaneous breathing pattern. The problem underlying these approaches has been ascribed to the "phenotyping uncertainty principle" (11), which states that there is a fundamental trade-off to be made between the maintenance of natural measurement conditions and precision. Penh sits at one extreme of this trade-off; a conscious animal that is free to choose its own pattern of activity and breathing exists in its most natural state, but the measurements of lung mechanics one can obtain under these conditions are noisy and very nonspecific.

At the other extreme of the phenotyping uncertainty principle lies the measurement of lung mechanics using the forced oscillation technique in anesthetized, tracheostomized, paralyzed mice (11). Here the animal's breathing pattern is entirely under the control of the experimenter, and the upper airways are bypassed so that their mechanical properties do not interfere with those of interest (i.e., the lung). On the other hand, one is left with the conundrum that the extensive interventions employed to achieve this level of experimental control may have caused the mechanical properties of the lung to depart significantly from their natural state. Nevertheless, our understanding of AHR in mice has advanced considerably in recent years using this approach. Intermediate between the above two extremes of the phenotyping uncertainty principle lie methods, such as the measurement of transfer impedance in restrained animals (47), that are based soundly in physical theory, but which are subject to greater variation as a result of fewer constraints on the behavior of the animal.

Assessing Lung Mechanics in Mice

The assessment of lung mechanics in mice, or any experimental animal for that matter, boils down, in essence, to determining how difficult (or easy) it is to drive a given volume of gas into the lungs over a given period of time. At its most basic level, this process can amount simply to examining the peak airway pressures achieved during regular mechanical ventilation (11, 29, 50). Clearly, an increased peak pressure indicates an increased impediment to lung inflation, although in an entirely nonspecific manner. Much greater specificity is

achieved with the forced oscillation technique, in which an appropriate oscillatory flow signal is applied to the airway opening while the airway pressure is measured. In fact, flow signals containing a single dominant frequency determined by the number of breaths per minute have been used for decades to obtain estimates of lung resistance and elastance (6, 74). Numerical techniques based on the Fourier transform (13) have been used more recently to determine resistance and elastance at a number of different frequencies simultaneously when the oscillatory flow signal contains components at those frequencies. This multifrequency information about the respiratory system is encapsulated in a complex function of frequency known as the input impedance (*Zin*) (74). Being a complex function, *Zin* actually consists of two independent components known as the real part and the imaginary part, themselves both functions of frequency. The real part is known as the resistance because, at any particular frequency, it is equal in value to that of the conventional resistance that would be measured by oscillating the system at only that frequency. The imaginary part of *Zin* is known as the reactance, and is equal to conventional elastance scaled by the inverse of frequency. Obtaining *Zin* in a mouse is technically challenging because of the animal's small size, but is now routinely achieved using either a commercially available device known as the Flexivent that is based around a computer-controlled piston oscillator (78) or a wavetube coupled to a loudspeaker (44).

Interpreting *Zin* is performed on the basis of a mathematical model of the lung with a structure that is interpretable in physiological terms. The appropriate model depends on the frequency range over which *Zin* is measured. When frequency is kept below ~20 Hz in mice, the model that has come to dominate the advanced investigation of lung mechanics is the so-called constant phase model introduced by Hantos and colleagues (45). This model has a particularly simple structure consisting of a single viscoelastic lung compartment served by a single airway. The airway has a resistance *Raw* that reflects its caliber and length. The mass of the air in the airway also provides the model with an inertance, although this is so small in mice that it can be neglected below the 20-Hz maximum frequency typically used to determine *Zin* (44, 75, 87). The viscoelastic compartment is characterized by two parameters, *G* and *H*, that account for the resistance and elastic properties, respectively, of the lung tissues. It has been found empirically that the impedance of the viscoelastic compartment has a special form in which the ratio of its real part to its imaginary part does not change with frequency. It is this feature that gives rise to the name "constant phase."

The equation for the impedance of the constant phase model of the lung is

$$Zin(f) = R_N + i2\pi fI + \frac{G - iH}{(2\pi f)^{\alpha}} \quad (1)$$

where f is frequency in Hz and

$$\alpha = \frac{2}{\pi}\tan^{-1}\left(\frac{H}{G}\right) \quad (2)$$

R_N in *Eq. 1* is referred to as a Newtonian resistance because it has been found experimentally to behave like a classic resistor, but experiments with alveolar capsules in open-chest animals have confirmed that it provides an accurate measure of the overall resistance of the airway tree (83). *G* and *H* together encapsulate the mechanical properties of the lung periphery. When the lung behaves in a mechanically homogeneous fashion, *G* and *H* reflect the intrinsic dissipative and elastic properties, respectively, of the tissues. Experimental studies have shown, however, that *G* invariably increases proportionately more than *H* when the lung becomes mechanically heterogeneous in such as way that ventilation is not apportioned to different regions of the lung according to the volume of each region, but rather becomes differentially affected due to inequities in regional airway resistance and tissue stiffness. This finding is supported by theoretical analysis (8) and computational modeling (60, 82). An increase in the ratio *G*/*H*, known as hysteresivity (34), thus serves as a convenient marker for the presence of regional heterogeneities, which typically accompany most lung diseases and are also brought on transiently by induced bronchoconstriction (87). By contrast, if a fraction of the lung becomes isolated from the airway opening either by closure of the subtending airways or by atelectasis, collectively referred to as derecruitment, estimates of *G* and *H* will increase in the same proportion, leaving hysteresivity unchanged (4). The parameters R_N, *G*, and *H* of the constant phase model together thus provide a means for distinguishing changes taking place in the conducting airways vs. changes in the lung periphery, and of inferring the presence of ventilation heterogeneity vs. derecruitment.

Figure 1 shows an example of the use of R_N, *G*, and *H* to infer details about the nature of AHR in mice. Allergic inflammation was induced in BALB/c mice using the standard 3-day challenge protocol described above. Groups of inflamed and control animals were then exposed for 40 s to an aerosol of methacholine and then switched immediately to a 5-min protocol during which *Zin* was measured approximately every 15 s. All three impedance parameters increased following cessation of the challenge, with the responses from the inflamed animals being substantially greater than those in the controls. The biggest difference between inflamed and control animals, however, occurred in *H*, the parameter that measures lung elastance, indicating that the major effect of inflammation was manifest in the lung periphery. What is more, the ratio *G*/*H* remained largely unchanged throughout, implying that the peripheral effect that occurred was due to derecruitment of lung units. Closure of small airways was subsequently confirmed directly using microcomputed tomography (59) on the basis of images such as those shown in Fig. 2, which also serve to illustrate that the airway tree of the mouse is structurally rather different to that of the human.

Linking Structure to Function

Despite the technical advance represented by the forced oscillation technique over earlier methods that yielded only single values for lung resistance and elastance, the parameters of the constant phase model still constitute an extremely simplistic view of a very complicated organ. Using only measurements of pressure and flow at the airway opening, it is not currently possible to reconstruct a more detailed picture of the lung than that contained in R_N, *G*, and *H*. However, one can often use computer simulation to address questions about what might be causing experimentally observed changes in these parameters. Here one tries to construct as detailed a model of

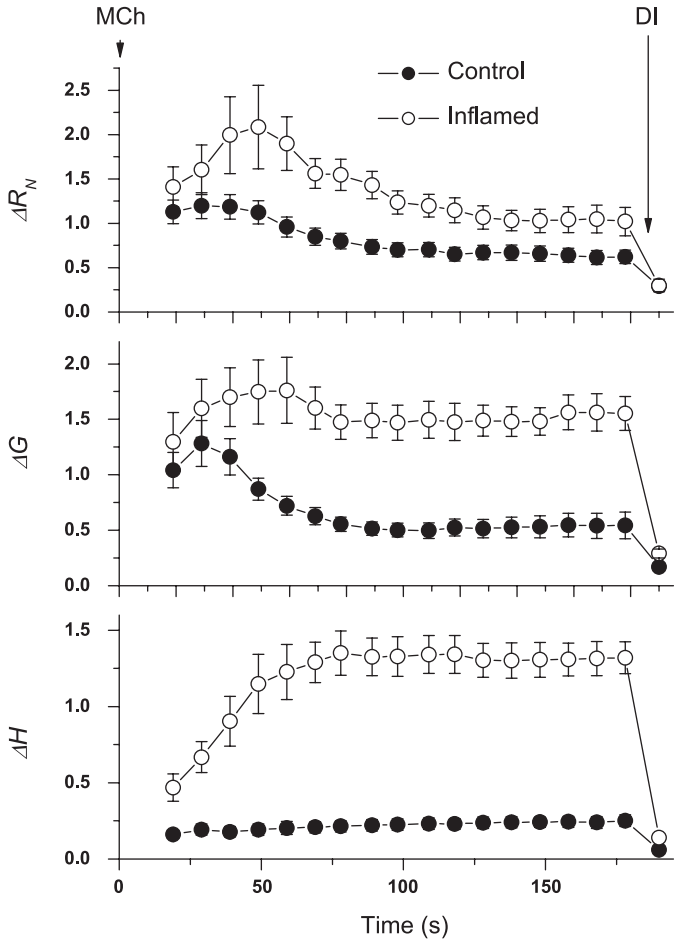

Fig. 1. The parameters of respiratory input impedance (R_N, G, and H) expressed as fractional changes above baseline (ΔR_N, ΔG, and ΔH, respectively) in control and allergically inflamed mice following a 40-s challenge with an aerosol of methacholine. MCh, the time of completion of delivery of methacholine. DI, the time of delivery of 2 deep lung inflations to 25 cmH_2O. [From Wagers et al. (87).]

the lung as anatomical and physiological knowledge will allow. The model is then used to simulate measurements of airway pressure and flow under conditions that match those used experimentally. The model thus serves as a virtual laboratory in which the feasibility of specific hypotheses can be addressed, providing in silico experimentation to complement conventional in vivo and in vitro experiments.

An example of this approach has been employed to investigate what might be responsible for the AHR observed in acutely inflamed BALB/c mice (87). A computational model of the mouse lung was constructed in which every airway in the entire airway tree was individually accounted for, with tissue units appended to each terminal bronchiole. The model was calibrated by adjusting its parameters until it exhibited changes in R_N, G, and H similar to those seen in normal mice exposed to an aerosol of methacholine. To achieve this, however, it was not enough simply to simulate the effects of smooth muscle shortening by narrowing the model airways. Derecruitment of some fraction of the model lung was also required to mimic the increases in lung elastance observed experimentally. This was achieved by completely closing any airway in the model that narrowed to a specified critical radius, thereby mimicking the effects of formation of a liquid bridge across the airway lumen. In silico experimentation was then performed with the model in an attempt to make it hyperresponsive to methacholine in a way that matched experimental observations in allergically inflamed mice. Simply increasing the extent of smooth muscle shortening reproduced the observed changes in R_N but failed to match the changes in G and H. However, if the degree of shortening was kept the same as in control mice, but the epithelial layer in the model was thickened to match histological observations in the inflamed animals and the critical airway closure radius was also increased somewhat, the model was able to accurately mimic the behavior of all three impedance parameters (15). This suggests that the mechanism giving rise to AHR in the acutely inflamed BALB/c mouse does not involve an abnormality of the airway smooth muscle, but instead is linked to physical changes taking place in the periphery of the lung itself. Of course, results such as these are dependent on the validity of the model used to make the simulations, and no model is a perfect representation of reality. However, the model used in this case was based reasonably closely on the anatomy of the mouse lung, so the results of such simulations can lend strong support to one hypothesis over another.

The use of computational modeling can also raise important questions for future investigation. For example, geometric amplification of airway luminal narrowing can potentially occur either by epithelial thickening or by increased mucus secretion. Certainly, epithelial hypertrophy is a characteristic feature of allergic inflammation (87), but so is mucus hypersecretion. Mucus is produced by goblet cells in the airway epithelium, and the appearance of excessive mucus in the airways can lead to significantly impaired lung function. Furthermore, IL-13, one of the Th2 cytokines, is the major inducer of epithelial cell mucus (93), signaling through the IL-13/4 receptor-α complex (92) and epidermal growth factor receptor (96). We do not know which of these two factors, epithelial thickening or excessive mucus, is dominant in allergically inflamed mice. Experimenting with mice in which these two effects are individually manipulated would thus be an important area for future research.

Mouse Models of Excessive Airway Smooth Muscle Shortening

Although allergy and inflammation are widely recognized as central to the pathogenesis of asthma, there is a school of thought that places the principal blame for asthma squarely at the feet of the airway smooth muscle. Although the jury is still out on this question, considerable effort has been devoted to identifying animal models in which AHR results from excessive smooth muscle shortening. Related to this, some degree of airway smooth muscle hypertrophy has been identified in several chronic models of allergic inflammation (57, 79). The epithelium probably also plays an important role in ASM hyperplasia and subsequent AHR; selectively activating the nuclear transcription factor NF-κB in the airway epithelium results in airway smooth muscle hyperplasia and associated AHR (73). There is also evidence that the alterations in smooth muscle associated with inflammatory remodeling result in an increase in the speed of muscle contraction (80). It is not entirely clear why this should be associated with AHR and

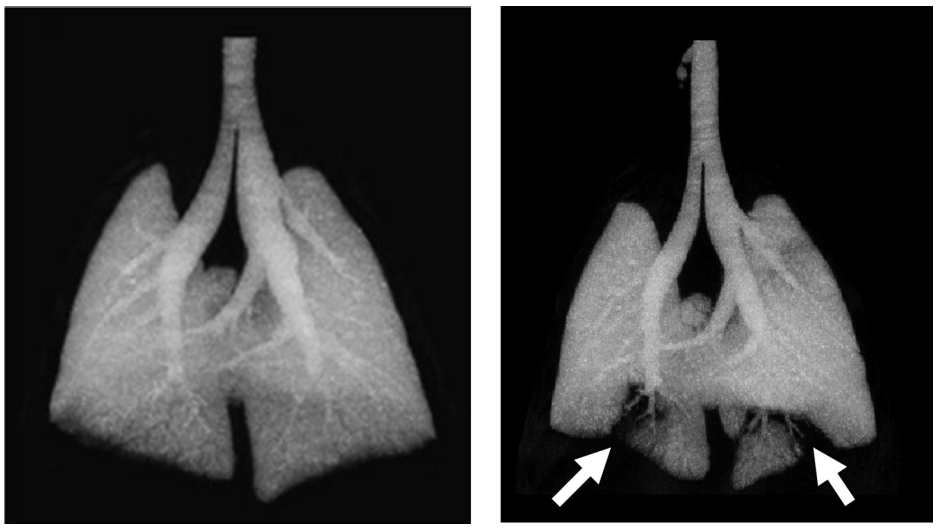

Fig. 2. Minimum intensity projection images of a normal mouse lung (*left*) and an allergically inflamed mouse lung (*right*) following ventilation with pure oxygen obtained using microcomputed tomography. The missing basal air spaces in the inflamed lung (arrows) represent atelectatic regions that became consolidated after absorption of the oxygen by the capillary blood. Note the large bulbous appearance of the central airways and the asymmetrical branching pattern of the airway tree, which is typical of the mouse but very different in the human. [Adapted with permission from Lundblad et al. (59).]

asthma, although there is speculation that it may allow the smooth muscle to more easily move into a latch state from which escape is difficult (33).

Enhanced shortening of airway smooth muscle has been induced by treating animals with cationic proteins (Fig. 3). Mice receiving an intratracheal instillation of poly-L-lysine were found to be hypersensitive to methacholine when it was delivered as an aerosol but not as an intravenous injection (48), implicating degradation of the normal barrier function of the epithelium as the causative factor. In other words, by making the underlying smooth muscle more accessible to agonists introduced into the airways, bronchoconstriction was enhanced both in terms of magnitude and speed of onset. Poly-L-lysine is an analog of the highly charged cationic proteins found in all inflammatory cells, particularly the eosinophil, suggesting a way in which the events accompanying allergic inflammation might lead to hypersensitivity and AHR (85).

Differences in the intrinsic contractility of airway smooth muscle also arise spontaneously between different strains of animal. The A/J mouse, for example, has been shown to be hyperresponsive, and, in particular, to exhibit bronchoconstriction that develops particularly rapidly following challenge (26, 88). There are also clear differences in natural airways responsiveness between different strains of rat, the Fischer and Brown-Norway strains being key examples (37, 89). Accordingly, we now recognize that genetic factors play a major role in determining AHR.

The Role of Lung Volume

The degree of airway smooth muscle shortening elicited by challenge with a standard dose of agonist is highly dependent on transpulmonary pressure. Most of the pulmonary airways are embedded in the lung parenchyma, which transmits transpulmonary pressure from the airway lumen to the pleural surface. The parenchymal attachments to the airway wall exert an outward tethering force that opposes shortening of the airway smooth muscle. The mitigating effect of these parenchymal forces is dramatically demonstrated by the way in which airways responsiveness is decreased by an increase in transpulmonary pressure of only a few cmH_2O (10, 22). Thus, a normoresponsive animal can be rendered very asthma-like simply by reducing its lung volume, just as has been demonstrated to be the case in human subjects (25). There are also some animal studies showing that chronic alterations in lung volume can lead to persistent changes in airways responsiveness, suggesting the possibility that airway smooth muscle somehow eventually adapts to a new lung volume (81). Of course, the forces of parenchymal tethering are not the only loads opposing airway smooth muscle shortening; the stiffness of the airway wall itself has also been shown to play a significant role in this regard (12, 68). In addition, the airway remodeling known to accompany some inflammatory conditions presumably has the potential to alter wall stiffness, but whether this tends to work for or against AHR remains controversial. It has recently been shown, for example, that there is no evidence of an effect on AHR due to mechanical changes in the airway wall of the acutely inflamed mouse (22). On the other hand, more chronic allergen exposures have shown evidence of an airway remodeling effect on AHR (79).

It must be mentioned that a great deal of animal work related to the hyperresponsiveness or otherwise of airway smooth muscle takes place in vitro, usually with tracheal smooth muscle, which is easily isolated and subjected to controlled force-length studies. The precision of the measurements that can be made with this preparation has led to the discovery of many intriguing phenomena such as the ability of smooth muscle to adapt its force generating capacity to baseline length (95). The biochemistry associated with the different phosphorylation states of the smooth muscle crossbridge is also rather complicated (41). It remains to be seen how significant some of these phenomena really are for asthma at the level of the entire animal or human subject. Nevertheless, computational modeling of the dynamic behavior of activated airway smooth muscle and its mechanical integration into the airway wall has shown promise in accounting for bronchoconstriction in vivo (10, 12, 22).

Mouse Models of Severe Asthma

In the animal laboratory, AHR is defined in statistical terms; a hyperresponsive animal exhibits significantly worse lung function than a control animal when subjected to a standardized challenge. Whether or not AHR is biologically significant,

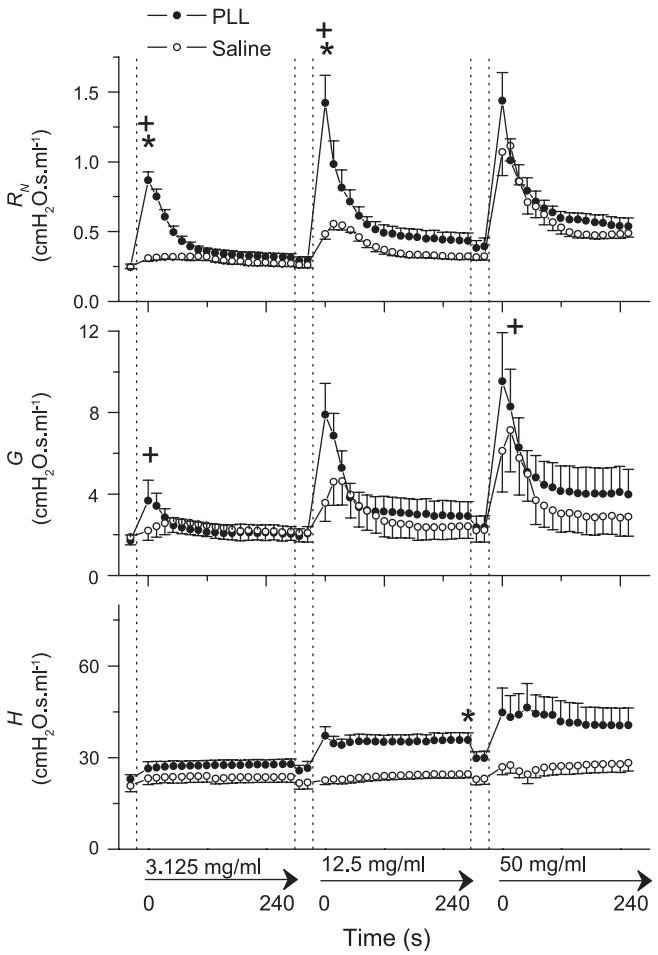

Fig. 3. The time course of bronchoconstriction in BALB/c mice following aerosolization of 3.125, 12.5, and 50 mg/ml methacholine. The open circles are saline-treated animals; the closed circles are animals treated with poly-L-lysine. The vertical dotted lines bracket parameter values obtained following deep lung inflations given to reestablish baseline conditions. Note the exaggerated response in R_N compared with G and H in the animals treated with poly-L-lysine compared with controls. This contrasts with the effects of allergic inflammation shown in Fig. 1. *Significant difference in magnitude of response; +significant difference in timing of peak. $P < 0.05$. [From Bates et al. (15).]

however, is often not considered. In fact, the degree of AHR is generally rather modest in most animal models considered to exhibit the phenomenon; a response in airway resistance of only two- to threefold above normal is not unusual (87, 88). In one sense, this may be apropos, given that much of human asthma also rumbles along chronically in a fashion that is perhaps more irritating than outright dangerous. On the other hand, in the subset of individuals who are afflicted with the severe form of the syndrome, the decrement in lung function accompanying an asthma attack can be extreme and even fatal.

What distinguishes severe asthma from its more common milder form remains an area of active research that has so far yielded few answers. It is therefore perhaps not surprising that animal models of correspondingly severe AHR are also few in number. One reason for this may be the tendency for researchers to focus on single causes, in pursuit of the most important mechanism responsible for the AHR of asthma. It has recently been suggested, however, that the key to severe asthma may be the simultaneous presence of multiple mechanisms; allergically inflamed mice treated with poly-L-lysine were shown to exhibit an extreme level of AHR that was easily fatal and that could be attributed to the synergistic interaction between an enhanced ability of smooth muscle to narrow the conducting airways and an increased propensity for liquid bridges to occlude peripheral airways (9). The mechanism behind this synergy is illustrated in Fig. 4. A severe asthma-like phenotype can also be achieved by coexpressing IL-5 and eotaxin 2 in the same animal; IL-5 leads to systemic eosinophilia, whereas selective eotaxin 2 upregulation in the airway epithelium directs chemotaxis and markedly activates the diapedesing eosinophils (70). The physiological manifestations are nearly identical to the antigen/cationic protein model (9) supporting the multiple mechanism hypothesis for the genesis of extreme AHR.

Summary and Conclusions

Asthma still appears to be a uniquely human disease despite decades of attempts to recapitulate its features in animals. Nevertheless, animal models of AHR have provided many of the key insights we currently have about the possible causes of asthma and have served as invaluable test beds for pharmacotherapy. Mouse models of asthma now represent the bulk of the scientific industry in this field because they can be explored with the most complete range of biological reagents and genomic knowledge. Recent developments in physiological phenotyping and three-dimensional imaging have added greatly to the investigative armamentarium. Combined with computational models of lung function, we are now able to make some rather precise links between structure and function in the mouse lung that enable AHR to be tied to underlying mechanisms. Of course, at the end of the day we are still going to face the issue that a mouse is not a human, so there will always be a gap in our understanding that biological relevance and experimental ethics prevent us from bridging completely.

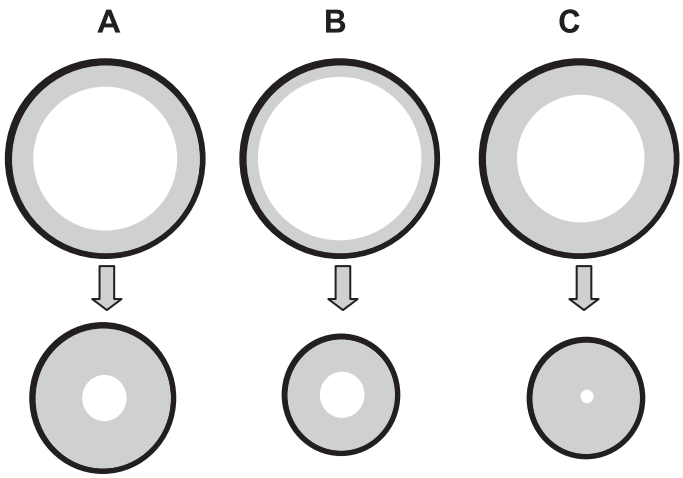

Fig. 4. Mechanical and geometric mechanisms for airways hyperresponsiveness. *A*: a modest degree of shortening of the airway smooth muscle (black ring) impinging on an airway wall that thickened is due to epithelial hypertrophy and/or mucus hypersecretion (gray annulus) and leads to a substantial degree in luminal area (white). *B*: the same degree of luminal narrowing can be caused by accentuated smooth muscle shortening in the presence of a normal airway lining. *C*: both mechanisms together lead to a dramatic reduction in luminal area and may even lead to complete airway closure.

Nevertheless, we expect the development and investigation of animal models of asthma to continue for some time into the future, where a great deal of new knowledge awaits.

GRANTS

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SHORT COMMUNICATION

Kinetics of asthma- and allergy-associated immune response gene expression in peripheral blood mononuclear cells from vaccinated infants after *in vitro* re-stimulation with vaccine antigen

Anne I. Lahdenperä[a], Lennart J. Nilsson[a], Karin Regnström[b,c,*]

[a] *Division of Paediatrics, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, 58185 Linköping, Sweden*
[b] *Department of Pharmacy, Uppsala University, Box 580, 75123 Uppsala, Sweden*
[c] *Department of Pharmaceutical Sciences, School of Pharmacy, University of Connecticut, 69 North Eagleville Road, Storrs, CT 06269, United States*

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Summary The global expression of immune response genes in infants after vaccination and their role in asthma and allergy is not clearly understood. Pharmacogenomics is ideally suited to study the involved cellular responses, since the expression of thousands of genes can be assessed simultaneously. Here, array technology was used to assess the expression kinetics of immune response genes with association to asthma and allergy in peripheral blood mononuclear cells (PBMC) of five healthy infants after vaccination with Infanrix-Polio + Hib. At 12 h after *in vitro* re-stimulation of the PBMC with pertussis toxin (PT) antigen, 14 immune response pathways, 33 allergy-related and 66 asthma-related genes were found activated.

Introduction

Little is known about the gene expression changes involved in immune responses in adults and infants after vaccination, even less is known about asthma- and allergy-associated genes expressed during or after immune responses. An often-cited theory is that an imbalance between the Th1 and Th2 response is responsible for the development of asthma [1]. A pronounced T-reg and Th1 immune response has been found after specific immunotherapy of allergic rhinitis patients [2]. Immunisation with pertussis vaccines has been reported to induce both a Th1 and Th2 immune response [3].

Recent studies using microarray technology have lead to the discovery of several important markers for asthma and allergy [4–10], but it is unknown if they are expressed during or after immune responses. Pharmacogenomics is ide-

* Corresponding author at: Department of Pharmaceutical Sciences, School of Pharmacy, University of Connecticut, 69 North Eagleville Road, Storrs, CT 06269, United States.
E-mail address: karin.regnstrom@uconn.edu (K. Regnström).

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ally suited to study cellular responses related to immune response and it was shown earlier that cytokine markers measured on the gene expression level by array technology are in good correlation with the corresponding protein levels measured by conventional technology [11]. Furthermore, additional gene markers for the adaptive as well as the innate immune responses can be evaluated [12,13], as well as genes involved in toxicity, inflammation, apoptosis, stress and oncogenesis [11—15]. Array technology holds the promise to find hitherto unknown immune response genes with associations to asthma and allergy-related inflammatory processes [16].

Earlier it was shown, that the ideal time-points for the evaluation of immune response gene expression in vaccinated mice are 4 and 24 h after *in vitro* re-stimulation of lymphocytes with the vaccine antigen [11]. But, the ideal time-point for the expression of immune response genes with an association to asthma and allergy in peripheral blood mononuclear cells (PBMC) of humans has not yet been determined.

Here, array technology was used in a small-scale study to determine the ideal time-point for the expression of immune response genes with association to asthma and allergy in PBMCs, which were isolated from five infants vaccinated with Infanrix-Polio + Hib and *in vitro* re-stimulated with the vaccine antigen, pertussis toxin (PT), for various time-points.

Materials and methods

Clinical subjects

Venous blood samples were collected from five infants at the age of 6 months (born November—December 2000). The infants were vaccinated with Infanrix-Polio + Hib vaccine (GlaxoSmithKline Beecham, Rixensart, Belgium) at the age of 3 and 5 months, respectively. The study was approved by the Regional Ethics Committee for Human Research at the University Hospital of Linköping. The identities of the clinical subjects were blinded.

Isolation and *in vitro* re-stimulation of PBMC

The experiments were performed as described previously in detail [15]. Briefly, venous blood samples were drawn into heparin-treated tubes (Vacuette, Greiner Labortechnik, Kremsmünster, Austria). PBMCs were separated on Ficoll Paque Density gradient (Amersham, Piscataway, NJ) according to standardised methodology and cryopreserved in freezing medium (50% fetal calf serum, 40% RPMI 1640 and 10% DMSO) (Sigma—Aldrich, St. Louis MO) until analysis. After thawing, the cell viability was checked with Trypan blue (Sigma—Aldrich) exclusion test. The cells were resuspended in AIM-V serum free medium (Life Technologies, Täby, Sweden) with 20 μM β-mercaptoethanol (Sigma—Aldrich, St. Louis MO) to a concentration of 1×10^6 cells/ml. Cell aliquots were *in vitro* cultivated for 0, 4, 12, 24 and 48 h at 37 °C in a humidified atmosphere with 5% CO_2, with serum free AIM-V medium alone (negative control, at 0 h) or with 1 μg/ml pertussis toxin at 4—48 h (GlaxoSmithKline Biologicals, Rixensart, Belgium). The PT was heat-inactivated for 20 min in an 80 °C water bath to avoid the antigen's high mitogenic potential. Because the blood volume that can be taken from infants is limited, the number of cells per infant was too low to allow for the isolation of a RNA quantity, which was sufficient for array experiments of four time-points and one control per infant. The cell aliquots per infant were therefore pooled after *in vitro* re-stimulation at each time-point, respectively. The cells were collected, washed in cold PBS, snap frozen in liquid N_2 and stored at −70 °C until RNA isolation.

RNA isolation and array analysis

Total RNA was isolated from the lymphocytes, quality checked and used in cDNA array experiments as described previously [11—15]. Briefly, total RNA was isolated from pooled samples of PBMCs using the RNeasy® Mini Kit (Qiagen, Hilden, Germany). DNase treatment was performed with DNase I (Promega, Madison, WI) and RNA quantification was performed with RiboGreen®RNA Quantitation kit (Molecular Probes, Eugene, OR). RNA (4 μg) was converted to cDNA (Clontech, Mountain View CA), radioactively labeled with α-^{32}P-dATP (PB10204) (Amersham Biosciences, Piscataway, NJ) and hybridized to Atlas Human cDNA Arrays (Clontech, Mountain View, CA) with 1176 genes, according to the manufacturers guidelines. The arrays were exposed to phosphor imaging screens for 2, 3 or 5 days (at room temperature). Gene expression levels were quantified by the CYCLONE®Storage Phosphor System (PerkinElmer, Waltham MA) and the image data were processed using the AtlasImage software version 2.0 (Clontech, Mountain View, CA).

Bioinformatics analysis

The data was treated statistically as described previously [11—15]. To identify genes with significant gene expression changes of at least two times above control and to group them according to their expression levels across all samples, the data was clustered using the software GeneCluster [17]. Data-mining tools from the World Wide Web, the software PubGene (version 2.6, Oslo, Norway) [18] and Ingenuity Pathways Analysis (version 5.0, Redwood City, CA) were used to assess the biological functions of the genes and their associations to asthma and allergy.

Results and discussion

Expression kinetics of genes reported in the literature

As a first step of evaluation the literature was searched for immune response genes that were reported to be up- or down-regulated in patients with asthma and allergy (see Table 1). The time-points of their gene expression levels (high, low or not detected) according to our array results were noted.

As shown in Table 1 the expression of the majority of these genes (34 genes out of a total of 52 genes) can be assessed at 12 h after *in vitro* re-stimulation with the PT antigen. This is not surprising, since these genes are involved

Table 1 Kinetics of immune response gene expression in human PBMC after *in vitro* re-stimulation with PT antigen for 4, 12, 24 and 48 h compared with negative control

Gene name	Genbank accession no.	4 h	12 h	24 h	48 h	Asthma association
CCR1	NM001925	—	++	—	—	Yes
CCR2	NM000647	++	+	—	—	Yes
CD4	M35160	—	+	—	+	Yes
CD8	NM001768	—	—	—	++	Yes
CD27 (T-cell antigen)	M63928	—	+	—	—	No
CD30	M83554	++	—	—	++	Yes
CD33	M23197	—	+	—	—	Yes
CD40L	X67878	++	+	+	—	Yes
CD70	L08096	—	+	—	—	No
CD72	M54992	—	+	—	—	Yes
CD102	NM000873	—	—	++	+	No
CD103	L25851	—	—	+	—	Yes
CD104	NM001005619	—	—	++	+	No
CD106	M60335	—	+	—	—	Yes
ICAM-1 (CD54)	NM000201	—	+	—	—	Yes
IL-1Rbeta	X59770	++	—	—	++	No
IL-2	A14844	—	++	+	—	Yes
IL-2Rbeta (CD122)	M26062	+	+	—	—	No
IL-2R gamma	D11086	—	++	+	+	Yes
IL-3	M14743	—	++	—	—	Yes
IL-4	M13982	—	++	—	—	Yes
IL-4Ralpha (CD124)	X52425	—	+	—	—	Yes
IL-5	X04688	—	—	++	+	Yes
IL-5 R alpha	M75914	—	+	—	—	Yes
IL-6R	M20566	+	—	—	—	Yes
IL-6	X04602	+	++	+	—	Yes
IL-7	J04156	—	++	+	—	Yes
IL-8	Y00787	—	++	—	—	Yes
IL-10	M57627	++	++	—	—	Yes
IL-11	M57765	—	—	—	++	Yes
IL-10R	U00672	—	—	++	—	No
IL-12 R	U03187	++	—	—	++	Yes
IL-13	L06801	—	++	+	—	Yes
IL-15	U14407	—	—	++	+	Yes
IL-16	M90391	—	++	+	—	Yes
IFN beta	M28622	—	++	—	—	No
IFN gamma	X01992	++	++	—	—	No
IFN gamma R	J03143	++	—	—	—	No
IRF-1	X14454	—	++	—	—	Yes
ITGB8	M73780	+	+	—	—	No
Jak3	U31631	—	—	++	—	Yes
CCL13	NM005408	+	+	—	—	Yes
PAX-5	NM016734	—	++	+	—	No
STAT1	NM007315	—	+	++	—	Yes
STAT2	NM005419	—	—	++	—	No
TGF beta R1	X11695	—	++	+	+	No
TGF beta II	P61862	—	+	++	—	Yes
TGF beta III	NM003239	++	—	—	++	No
TGF beta R III	L07594	++	+	—	—	No
TNF beta	P01374	—	—	—	++	No
TNF R	M67454	—	—	++	+	No
YES	M15990	—	++	++	—	Yes

Expression levels of the genes are shown as high (++), low (+) or no expression (—). The genes were chosen for their association with asthma and allergies according to Refs. [4—9,20—23]. Only genes with sequences represented on the Human 1.2 array are shown. Association of the genes with asthma according to the literature was assessed using the data-mining tool PubGene (see also Fig. 2).

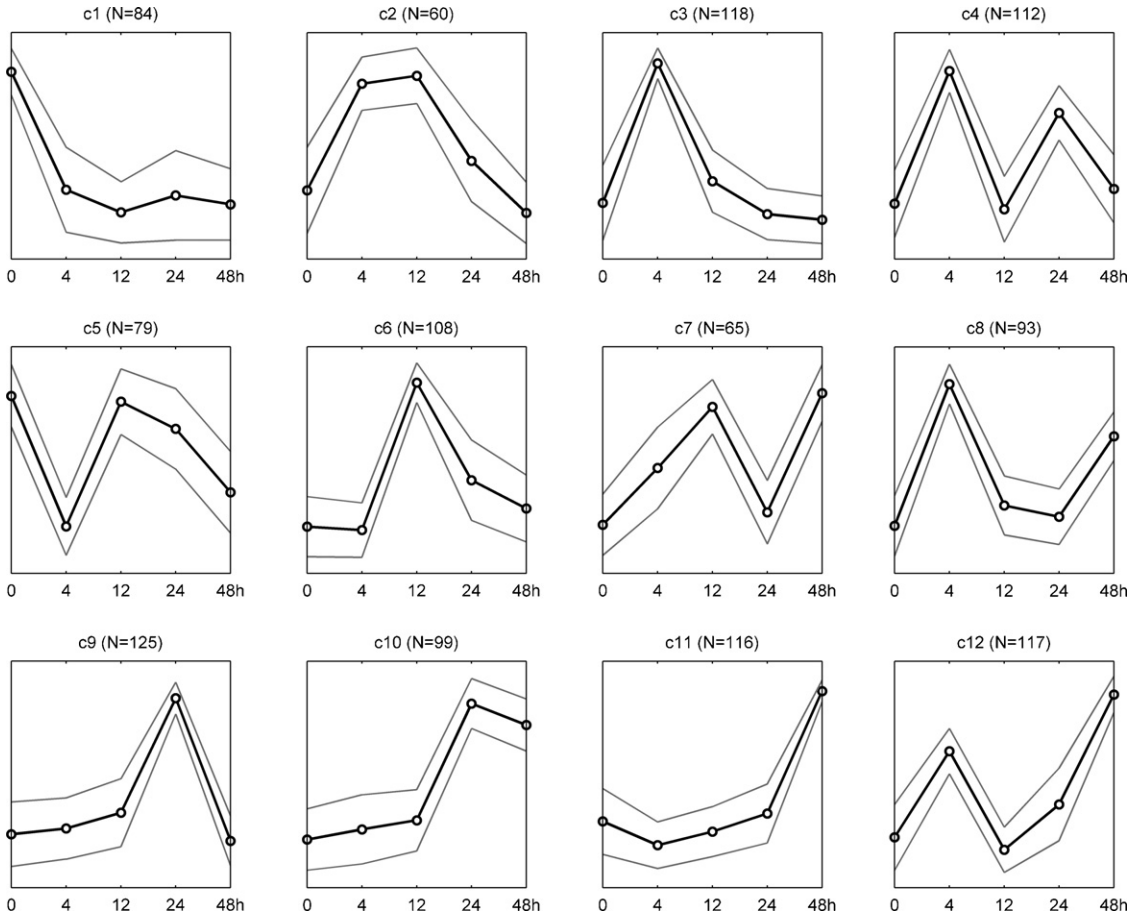

Figure 1 Gene expression differences in lymphocytes from infants at 4, 12, 24 and 48 h after *in vitro* re-stimulation with PT antigen and non-treated lymphocytes. Self-organizing map clusters according to Tamayo's algorithm [17] using the criteria for significant gene expression described in ''Materials and methods''. Bold black lines indicate the mean expression profiles; grey lines indicate the SD. *N* represents the number of genes in each cluster.

in immune response and it is known from earlier array experiments in mice that immune response genes are highly activated at 4 h and 24 h after *in vitro* re-stimulation with tetanus toxoid or diphtheria toxoid, respectively [11,12]. In addition, it has been shown earlier in mice, that the expression level of genes involved in Th1 responses is high between 2 and 18 h after stimulation with ovalbumin peptide [19]. There were only 18 genes without measurable gene expression at 12 h after *in vitro* re-stimulation of PBMCs with PT antigen, of which 9 genes had no association with asthma in the literature (see Table 1).

Other non-immune response genes with association to allergies, which were reported in the literature (see references of Table 1), were also detected at 12 h after *in vitro* re-stimulation with PT antigen. These genes comprised thymosin-β4, HMG-1 (high mobility group box 1), DPP4 (dipeptyl peptidase 4), MMP7 (matrix metalloproteinase 7) and SOD1 (superoxide dismutase 1) (data not shown). MMP7 and SOD1 have also an additional association with asthma according to the literature (see Table 1).

In the next step, the analysis was focused on finding additional genes with significant expression at 12 h after *in vitro* re-stimulation with PT-antigen and possible involvement in asthma and allergy. Clustering of the gene expression data using the self-organizing map software [17], which groups genes according to their expression behavior, resulted in 12 clusters (c1—c12) containing between 60 and 125 genes (Fig. 1). No clusters were found with a significant down-regulation in the 12 h-sample versus the control (0 h) and all the other time-point samples. The clusters containing genes with up-regulation at 12 h after *in vitro* re-stimulation with PT antigen were c2, c6 and c7 with a total number of genes of 233. These three clusters contained the 33 genes listed in Table 1, resulting in 200 additional genes with up-regulation at 12 h.

Additional genes with significant expression levels at 12 h and their functions

To determine the function of these additional genes found in clusters c2, c6 and c7, the expression data of these genes were submitted to the bioinformatics tool ingenuity, which searches submitted gene lists against a pathway database. The software's output shows the number of submitted genes per pathway and the corresponding statistical error. According to this software, the main functions associated with the 233 genes obtained by GeneCluster (some genes were listed

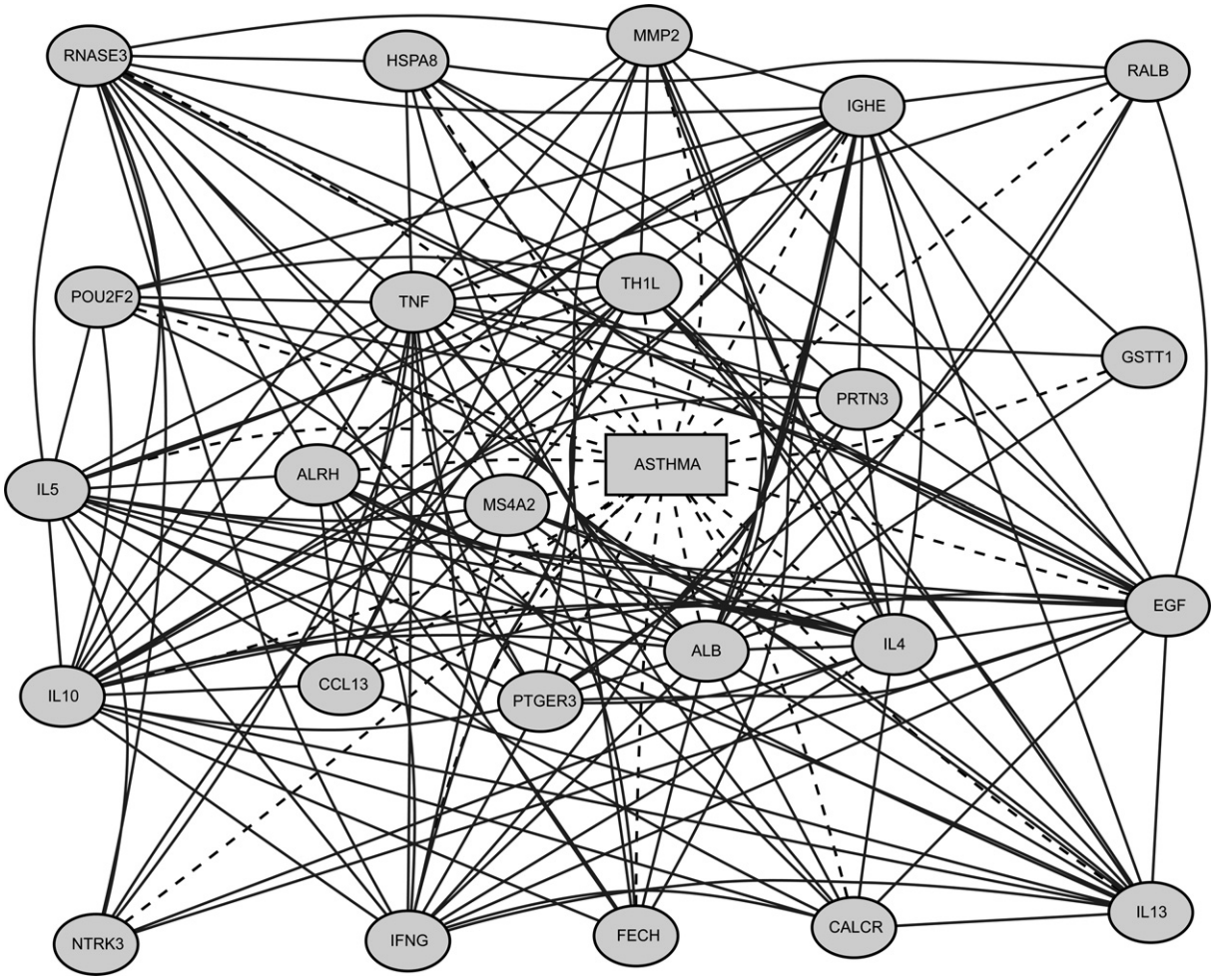

Figure 2 Literature network of cluster c2 genes. A list of 60 genes was submitted to PubGene to identify the association of co-expressed genes with asthma in the literature. A dark gray node represents one gene. Black lines indicate co-citation in the literature (the number of articles varied between 5 and 2320). The association with the keyword asthma is shown as a black-dotted line.

with more than one function) were immune response (33 genes; 9.42 E^{-17}), cellular growth and proliferation of lymphocytes (37 genes; 3.34 E^{-24}), T lymphocytes (29 genes; 1.12E^{-19}), B lymphocytes (17 genes; 1.15E^{-14}), leukocytes (37 genes; 1.75 E^{-24}), activation of lymphocytes (23 genes; 4.29 E^{-17}), T lymphocytes (21 genes; 1.62 E^{-17}); B lymphocytes (8 genes; 2.57E^{-8}), activation of phagocytes (11 genes; 5.88 E^{-9}), activation of mononuclear cells (5 genes; (2.54E^{-8})), inflammatory disease (32 genes; 2.84E^{-17}), cytotoxic reaction (8 genes; 4.65E^{-10}), antibody response (7 genes; 4.78E^{-9}), phagocytosis (11 genes, 5.29E^{-9}). Furthermore, cancer genes were up-regulated (67 genes; 7.07 E^{-18}), apoptosis genes involved in hematological disease (35 genes; 1.5 E^{-12}) and immunological disease (25 genes; 1.5 E^{-12}).

Taken together, this analysis elucidates the suitability of this time-point for the evaluation of immune response reactions as well as allergy-related processes.

The data-mining tool PubGene [18] was used to search the clusters c2, c6 and c7 with up-regulation at 12 h for genes, which are associated with asthma in the literature (the keyword allergy was not recognized by the software). The gene names were translated into the primary gene symbols used by the software and submitted together with the keyword asthma. In clusters c2: 15 genes out of 60; c6: 31 genes out of 108 and c7: 20 genes out of 65 were found to be associated with asthma. Of the total of 233 submitted genes were 66 genes (28%) reported to be involved in asthma.

The results for cluster c2 are shown in Fig. 2. Beside the 15 on the array represented and up-regulated genes, the software found 9 additional genes, which are associated by co-citation in the literature with the keyword asthma and with the other genes of the literature network. These 9 genes were: ALB (serum albumin); IL-4 and IL-13, which were represented on the array and measurable at 12 h; IL-5 which was represented on the array, but was not detectable at 12 h; ALRH, IGHE (heavy immunoglobulin component of IgE), MS4A2 (Fc fragment of IgE, high affinity I, receptor for), RNASE3 (eosinophil cationic protein) and TNF, which were not represented on the array, but are well-known to be involved in asthma. In cluster c6 and c7, these 9 genes were listed again, respectively; but two additional genes for each cluster were found: AKT1 (v-akt murine thymoma viral oncogene homolog 1); FLT1 (vascular endothelial growth factor receptor); MAPK1 (mitogen-activated protein kinase 1)

and TBP (TATA box-binding protein) were found by the software (data not shown). Taken together, the software found 13 additional genes with involvement in asthma.

In summary, these results point towards the suitability of the 12 h time-point after *in vitro* re-stimulation of PBMCs with PT antigen for the detection of significantly expressed immune genes with an association to asthma and allergy. These comprise the 33 allergy- and asthma-related genes shown in Table 1, the 14 pathways (containing between 5 and 37 genes) involved in immune response, related cellular reactions and allergic reactions, as well the 66 genes associated with asthma in the literature.

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EXHIBIT 147

Erythema multiforme | definition of erythema multiforme by Medical dictionary

https://medical-dictionary.thefreedictionary.com/erythema+multiforme

erythema multiforme

Also found in: **Dictionary**, **Thesaurus**, **Financial**, **Encyclopedia**, **Wikipedia**.

Related to erythema multiforme: **Erythema migrans**, **erythema marginatum**, **erythema multiforme major**

Erythema Multiforme

Definition

Erythema multiforme is a skin disease that causes lesions and redness around the lesions.

cause a scene

To create a loud, typically angry disturbance or display in public, such that it draws attention to those involved. *Go To Article*

Exhibit 147

RELATED ITEMS

hew down
hew downTo cut something down, typically a... *Watch Video*

have a pink fit
have a pink fitTo become angry. Primarily heard in UK. Don't... *Watch Video*

have a cadenza
have a cadenzaTo become angry, agitated, or irritated.... *Watch Video*

Description

Erythema multiforme appears on the skin and the mucous membranes (the lining of the mouth, digestive tract, vagina, and other organs). Large, symmetrical red blotches appear all over the skin in a circular pattern. On mucous membranes, it begins as blisters and progresses to ulcers. A more advanced form, called Stevens-Johnson syndrome, can be severe and even fatal.

Causes and symptoms

Erythema multiforme has many causes, most commonly are drugs. Penicillin, **sulfonamides**, certain epilepsy drugs, aspirin, and **acetaminophen** are the most likely medication-induced causes. Erythema multiforme can also be caused by certain diseases. Herpes virus and mycoplasma pneumonia are likely infectious causes.

Diagnosis

The appearance of the rash is sufficiently unique to identify it on sight. Having identified it, the physician will determine the underlying cause.

Treatment

Erythema multiforme is inadvertently treated when the causative agent, whether it be a drug or a disease, is treated. In severe cases, cortisone-like medication is often used along with general supportive measures and prevention of infection.

Prognosis

As a rule, the rash abates by itself without damaging the skin. Only in the case of infection, severe blistering, or continued use of an offending drug does complications occur.

Resources

Books

Fauci, Anthony S., et al., editors. *Harrison's Principles of Internal Medicine*. New York: McGraw-Hill, 1997.

Key terms

Herpes virus — Viruses that can infect the skin, mucous membranes, and brain, and they are responsible for such disea herpes simplex, chicken pox, and shingles.

Mycoplasma pneumonia — An incomplete bacterium that infects the lung.

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erythema [er″ĭ-the´mah]

redness of the skin caused by congestion of the capillaries in the lower layers of the skin. It occurs with any skin injury, infection, or inflammation.

erythema chro´nicum mi´grans a ring-shaped erythema due to the bite of a tick of the genus *Ixodes;* it begins as an erythematous plaque several weeks after the bite and spreads peripherally with central clearing. Often there are also sys symptoms, including chills, fever, headache, vomiting, backache, and stiff neck. See also LYME DISEASE.

gyrate erythema (**erythema gyra´tum**) erythema multiforme characterized by the development of lesions that tend to migrate and spread peripherally with central clearing.

erythema ab ig´ne permanent erythema produced by prolonged exposure to excessive nonburning heat. It is seen most often on the legs of women, but under appropriate environmental circumstances, it can occur anywhere on the body in ei sex.

erythema indura´tum a chronic necrotizing vasculitis, usually occurring on the calves of young women; see also **BAZIN'S DISEASE**.

erythema infectio´sum a mild, self-limiting disease of childhood characterized by a lacelike skin rash symmetrically distributed on the hands, arms, and legs, with few or no other symptoms; occasionally there is a low grade fever, and the condition often clears up without specific treatment. The incubation period is six days to two weeks. This disease is conta and originally was believed to be a form of rubella; because the rash can resemble that of scarlet fever and German mea is important to differentiate this mild condition from those more serious ones. Called also **fifth disease**.

erythema margina´tum a type of erythema multiforme in which the reddened areas are disk-shaped, with elevated edge

erythema margina´tum rheuma´ticum a superficial, often asymptomatic, form of gyrate erythema associated with some cases of rheumatic fever, which is characterized by the presence on the trunk and extensor surfaces of the extremities of transient eruption of flat to slightly indurated, nonscaling, and usually multiple lesions.

erythema mi´grans **geographic tongue**.

erythema multifor´me a symptom complex representing a reaction of the skin and mucous membranes secondary to various known, suspected, and unknown factors, including infections, ingestants, physical agents, malignancy, and pregn The conditions in the complex are characterized by the sudden onset of a reddened macular, bullous, papular, or vesicula eruption, the characteristic lesion being the iris, bull's eye, or target lesion, which consists of a central papule with two or concentric rings. The complex includes a mild self-limited mucocutaneous form (erythema multiforme minor) and a severe sometimes fatal, multisystem form (**STEVENS-JOHNSON SYNDROME**).

erythema nodo´sum a type of **PANNICULITIS** occurring usually as a **HYPERSENSITIVITY REACTION** to multiple provoking agents, including various infections, drugs, sarcoidosis, and certain enteropathies. It may also be of idiopathic origin. It most ofter affects young women and is characterized by the development of crops of transient, inflammatory, nonulcerating nodules are usually tender, multiple, and bilateral, and most commonly located on the shins; the lesions involute slowly, leaving br patches without scarring. The acute disease is often associated with fever, malaise, and arthralgias. A chronic variant sor

EXHIBIT 148

Toxic epidermal necrolysis | definition of toxic epidermal necrolysis by Medical dictionary

https://medical-dictionary.thefreedictionary.com/toxic+epidermal+necrolysis

toxic epidermal necrolysis

Also found in: **Dictionary**, **Thesaurus**, **Acronyms**, **Encyclopedia**, **Wikipedia**.
Related to toxic epidermal necrolysis: **Acute Generalized Exanthematous Pustulosis**

Toxic Epidermal Necrolysis

Definition

Toxic epidermal necrolysis is a rare condition that causes large portions of the epidermis, the skin's outermost layer, to detach from the layers of skin below. A reaction to a medication is the primary cause.

Description

Toxic epidermal necrolysis (TEN) begins with **fever**, cough, and other nonspecific symptoms, and is soon followed by purplish, bloody-looking lesions on the skin and mucous membranes. These early lesions, typically found on the head, neck, and upper chest, soon merge and blister. Sheets of epidermis then begin to detach from the skin layers below. In time, the entire surface of the skin may be involved, with detachment of 100% of the epidermis.

Causes and symptoms

The main cause of TEN is a severe drug reaction. Some investigators believe there may be additional infectious causes. A severe reaction in transplant patients, called **graft-vs.-host disease**, can also produce TEN. One study reported more than 100 different drugs as causes of TEN. The drugs most commonly implicated, however, include antibacterial **sulfonamides** such as sulfadiazine, **antibiotics** such as aminopenicillins and **cephalosporins**, and anticonvulsants like phenytoin. TEN is extremely rare. Researchers estimate that there are 0.2 cases per million users of aminopenicillins and 4.5 cases per million users of sulfonamides.

Exactly what leads to detachment of the epidermis remains unclear. People with TEN seem to have difficulty metabolizing the offending drug. Some researchers suggest that certain substances that should be cleared from the body instead get deposited on the outer shell of the epidermis, causing an immune response that leads the body to "reject" the skin.

Diagnosis

Diagnosis is made primarily on the appearance and spread of the **skin lesions**, and on a history that includes introduction of a new medication within the previous one to three weeks. A biopsy of the early lesions will confirm the diagnosis. Physicians will consider other potential diseases that cause similar symptoms before reaching a diagnosis of TEN. One is **erythema multiforme**, a recurrent skin disorder that produces lesions similar in appearance to TEN. However, this disorder is not caused by a drug reaction and does not lead to sheet-like shedding of the skin. Another disease, Stevens-Johnson syndrome, is a drug-induced skin disease that some experts believe is really a milder form of TEN. **Staphylococcal scalded skin syndrome** (SSSS) also looks like TEN, but it is caused by a staphylococcal infectio Unlike TEN, which occurs rarely in children, SSSS primarily affects infants, young children, and adults with weakened imı systems.

Treatment

There is no specific treatment for TEN. Patients are typically treated in an intensive care unit or in a burn unit and receive treatment similar to that given to patients with major **burns**. With the loss of skin, severe **dehydration** is a major risk, so health care workers will attempt to replace fluids intravenously. Nutritional supplementation from a tube routed thr the nose to the stomach may also be contemplated to promote the healing of the skin. Infection is a major risk, so some physicians "paint" the open lesions with topical **antiseptics**. Others use skin grafts taken from cadavers or cultured skin substitutes to cover large open areas until healing can occur. Some investigators believe system **corticosteroids** are useful in the treatment of TEN. But since these medications have also been implicated as a cause in some cases of TEN are known to supress the immune system, their use should be considered carefully.

Key terms

Epidermis — The outermost layer of the skin.

Erythema multiforme — A recurrent skin disorder that produces lesions similar in appearance to TEN, but is not caused a drug reaction and does not lead to sheet-like shedding of the skin.

Staphylococcal scalded skin syndrome — A disease caused by *Staphylococcus aureus*, in which large sheets of skin may peel away from the body. It most often affects infants, young children, and people with weakened immune systems.

Stevens-Johnson syndrome — A drug-induced skin disease that some experts believe is really a milder form of TEN.

Prognosis

About 25-30% of patients with TEN die. Elderly patients, those with extensive skin lesions, and those with **AIDS** have the worst prognosis. Widespread systemic infection (**sepsis**) is the primary cause of **death**. Survivors, however, will be completely healed in three to four weeks.

Prevention

There is no prevention for TEN. No reliable test can indicate that a specific drug may cause TEN in a specific patient. Some researchers believe skin tests of potentially offending drugs may prove useful in the future.

Resources

Organizations

American Academy of Dermatology. 930 N. Meacham Road, P.O. Box 4014, Schaumburg, IL 60168-4014. (847) 330-0230.Fax: (847) 330-0050. **http://www.aad.org**.

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necrolysis [nĕ-krol´ĭ-sis]

separation or exfoliation of necrotic tissue.

toxic epidermal necrolysis an exfoliative skin disease in which erythema spreads rapidly over the body, followed by blis much like those seen in a second degree burn. It may be caused by drug reactions, infections (viral, bacterial, or fungal), neoplastic disease, graft-versus-host reaction, and chemical exposure.

EXHIBIT 149

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Erythema multiforme, Stevens Johnson syndrome, and toxic epidermal necrolysis reported after vaccination, 1999–2017

John R. Su [a,*], Penina Haber [a], Carmen S. Ng [a,1], Paige L. Marquez [a], Graça M. Dores [b], Silvia Perez-Vilar [b,2], Maria V. Cano [a]

[a] Immunization Safety Office, Division of Healthcare Quality Promotion, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA, United States
[b] Office of Biostatistics and Epidemiology, Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, United States

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ABSTRACT

Background: Since the last review of vaccine safety surveillance data for erythema multiforme (EM), Stevens Johnson syndrome (SJS), SJS/TEN, and toxic epidermal necrolysis (TEN) (EM/SJS/TEN), over 37 new vaccines have been introduced in the United States. We sought to describe reported EM/SJS/TEN after vaccines during 1999–2017.

Methods: We identified U.S. reports of EM/SJS/TEN received by the Vaccine Adverse Event Reporting System (VAERS) during 1999–2017. We stratified analysis by condition (EM, SJS, or TEN), and analyzed reports by serious or non-serious status, sex, age group, time from vaccination to symptom onset, exposure to known causes of EM/SJS/TEN, and vaccines administered. We used Empirical Bayesian data mining to detect vaccine-AE pairs reported more frequently than expected.

Results: Of 466,027 reports to VAERS during 1999–2017, we identified 984 reports of EM, 89 reports of SJS, 6 reports of SJS/TEN, and 7 reports of TEN. Few reports of EM (9%), and most reports of SJS (52%), SJS/TEN (100%), and TEN (100%) were serious. Overall, 55% of reports described males, 48% described children aged < 4 years; 58% of EM/SJS/TEN occurred ≤ 7 days after vaccination. Few reports (≤5%) described exposure to known causes of EM/SJS/TEN. Overall, childhood vaccines (e.g., combined measles, mumps, and rubella vaccine) were most commonly reported. We identified 6 deaths; 4 were exposed to medications associated with EM/SJS/TEN. EM after smallpox vaccine was reported disproportionately among people aged 19–49 years.

Conclusions: EM/SJS/TEN were rarely reported after vaccination; data mining identified a known association between EM and smallpox vaccine.

1. Introduction

Dermatologic adverse events (AEs) are among the most frequently reported AEs after vaccination. The most common dermatologic AEs are redness, swelling, and tenderness at the injection site [1], which can occur in up to 90% of persons receiving vaccinations [2]. At the other extreme are AEs, such as Henoch-Schönlein purpura after the combined measles, mumps, and rubella (MMR) vaccine, for which only isolated case reports exist [3]. While some dermatologic AEs can be common to many vaccines (such as tenderness at the injection site), other dermatologic AEs can be specific to a particular vaccine, such as vesicular lesions after varicella vaccine.

Some dermatologic AEs that have been reported to occur after vaccination involve hypersensitivity reactions, such as an allergy to the aluminum in some adjuvanted vaccines [4], or lichen planus

Abbreviations: AE, adverse event; BSA, body surface area; CDC, Centers for Disease Control and Prevention; DTaP, combined diphtheria, tetanus, and acellular pertussis vaccine; EM, erythema multiforme; FDA, Food and Drug Administration; IIV, trivalent inactivated influenza vaccine; MGPS, Multi-Item Gamma Poisson Shrinker; MedDRA, Medical Dictionary for Regulatory Activities; MMR, combined measles, mumps and rubella vaccine; NDMA, N-methyl-D-aspartate; NSAID, non-steroidal anti-inflammatory drug; PNC7, 7-valent conjugated pneumococcal vaccine; PT, Preferred Term; SJS, Stevens Johnson Syndrome; TEN, toxic epidermal necrolysis; VAERS, Vaccine Adverse Event Reporting System.

* Corresponding author at: Immunization Safety Office, Centers for Disease Control and Prevention, 1600 Clifton Rd, MS V18-4, Atlanta, GA 30329, United States.
E-mail address: ezu2@cdc.gov (J.R. Su).
[1] Currently at Division of Health Economics, Policy and Management, Hong Kong University School of Public Health, Hong Kong.
[2] Currently at Office of Surveillance and Epidemiology, Center for Drug Evaluation and Research, U.S. Food and Drug Administration, United States.

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after hepatitis B vaccine [5]. Erythema multiforme (EM) is a hypersensitivity reaction characterized by papules, classically with a ringed, target-like appearance often involving the palms of the hands and soles of the feet [1]. More severe hypersensitivity reactions include Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN), both of which involve blistering and sloughing of skin. [6] SJS and TEN also typically involve lesions on mucosal surfaces. SJS and TEN are thought to be related conditions of varying severity, depending upon percentage of body surface area involved: <10% for SJS, 10–30% for combined SJS/TEN, and >30% of body surface area for TEN [7]. Fortunately, EM, SJS, and TEN are rare, occurring with a rate of 4.2 hospitalizations per million person-years after exposure to an associated medication [8]; rates after vaccinations, as of this writing, are unavailable.

Ball et al previously identified 99 reports of SJS, TEN, and serious reports of EM after vaccination received by the Vaccine Adverse Event Reporting System (VAERS) during July 1990 through September 1999 [9]. Since 1999, over 37 vaccines have been approved for use in the United States [10]. Thus, we searched and described reports of EM, SJS, SJS/TEN, and TEN after vaccination (these specific dermatologic AEs will be referred to collectively as EM/SJS/TEN) reported to VAERS during 1999–2017, and compared the relative frequency of EM/SJS/TEN reported to VAERS by vaccine, to better understand if there are any concerning patterns of reported EM/SJS/TEN (e.g., after a specific vaccine, or among a particular age group) or other emerging safety concerns.

2. Methods

2.1. Data source

AEs occurring after vaccination can be reported to VAERS, a national spontaneous reporting system established in 1990 to monitor AEs that is jointly administered by the Centers for Disease Control and Prevention and the U.S. Food and Drug Administration [11,12]. VAERS receives reports from healthcare providers, vaccine manufacturers, vaccine recipients, and other persons. Reported symptoms and diagnoses are coded using Medical Dictionary for Regulatory Activities (MedDRA) Preferred Terms (PTs) [13]. MedDRA PTs are not necessarily medically confirmed diagnoses, and multiple MedDRA PTs can be assigned to a VAERS report. Federal regulations define a serious report as a report in which one or more of the following conditions is reported: death, life-threatening illness, hospitalization or prolongation of existing hospitalization, permanent disability, congenital anomaly, or birth defect [14]. A report might therefore not be classified as serious, despite describing a clinically severe presentation. VAERS personnel routinely request medical records for non-manufacturer serious reports. Vaccine manufacturers that directly receive reports of AEs typically request and review medical records per regulatory processes [12]; these records are not always available to manufacturers. Serious reports from vaccine manufacturers therefore often do not contain medical records that VAERS personnel can review.

2.2. Descriptive analysis

We searched the VAERS database for U.S. reports of EM/SJS/TEN following vaccination received by VAERS during October 1, 1999, through December 31, 2017 (among reports received by CDC and FDA through April 27, 2018); reports specifying a vaccination date outside the analytic period were excluded. We searched for reports that included the PTs "erythema multiforme," "Stevens Johnson Syndrome," and "toxic epidermal necrolysis." We also searched text fields for the following terms: "skin ulcer," "blister," "blister, infected," and/or "blister, rupture," where "multiforme," "Johnson," and/or "necrolysis" was also present. We combined results from both searches, then deduplicated records.

We reviewed the resulting reports for reports of EM/SJS/TEN that were diagnosed by physicians (reported by a physician, or a physician's diagnosis was documented in available medical records). For reports of symptoms consistent with EM/SJS/TEN, but without a physician's diagnosis, we defined (1) EM as papular lesions, classically with a ringed ("targetoid") appearance, usually beginning peripherally and then spreading to the torso, that could involve the palms of the hands and/or soles of the feet, without mucosal lesions; (2) SJS as peeling or blistering of the skin with lesions on mucosal membranes that involved <10% of total body surface area (BSA); (3) SJS/TEN as signs and symptoms consistent with SJS, but involving between 10 and 30% BSA; and (4) TEN as signs and symptoms consistent with SJS, involving >30% BSA.

We stratified data by condition (EM, SJS, SJS/TEN, and TEN). For each condition, we analyzed reports by sex; age group (<4 years, 4–10 years, 11–18 years, 19–49 years, ≥50 years, and age not reported), roughly corresponding with the recommended schedules for vaccination [15]; seriousness of report (serious or non-serious); and time from vaccination to onset of symptoms. Reports were further analyzed for persons with exposure to a known trigger [8,10] proximal to onset of symptoms (viral or *Mycoplasma* infection, medications (anticonvulsants, sulfa drugs, penicillins, non-steroidal anti-inflammatory drugs (NSAIDs), macrolide antibiotics, and acetaminophen); history of predisposing conditions (past history of EM/SJS/TEN, atopic dermatitis, asthma/reactive airway disorder, allergies to medications, or malignancy) [16]; and whether vaccines were given alone or concomitantly with other vaccines.

2.3. Estimated reporting rates

Estimating reporting rates of AEs after vaccination using data from VAERS is challenging because data about doses of vaccine that were distributed or administered are difficult to obtain. However, we were able to estimate crude reporting rates of EM, SJS, SJS/TEN, and TEN after varicella vaccine using reports received during 2006 through 2016 as the numerator, divided by doses distributed by the manufacturer [17] during the corresponding time period (Merck and Company, Inc., personal communication) as the denominator; rates were estimated as reports per 1 million doses distributed. For influenza vaccine (all types), annual crude reporting rates for EM, SJS, SJS/TEN, and TEN were estimated during 2010 through 2017 using reports received as the numerator, with population estimates and vaccine coverage for that year multiplied as the denominator [18,19]; rates for 2 age groups (1–17 years, ≥18 years) were estimated. From these annual crude reporting rates, median rates of reports per million doses administered were estimated for 2010–2017.

2.4. Data mining (disproportionate reporting)

We used Empirical Bayesian data mining to assess whether vaccine-AE combinations were reported more frequently than expected (when compared to all other vaccine-AE combinations in the VAERS database) using the Multi-Item Gamma Poisson Shrinker (MGPS) algorithm [20]. We analyzed U.S. reports received by VAERS during October 1, 1999 – December 31, 2017 (received as of April 27, 2018). We stratified by age group (<4, 4–10, 11–18, 19–49, ≥50 years, and unreported), and adjusted for sex, and year in which the report was received by VAERS. We analyzed disproportionate reporting for vaccine-EM/SJS/TEN combinations using: (1) the PTs "erythema multiforme," "Stevens Johnson Syndrome," and "toxic epidermal necrolysis"; and (2) PTs included in reports with the words "skin ulcer," "blister," "blister infected," or "blister

rupture" in the narrative, symptoms text, or comments fields, if the words "multiforme", "Johnson", and/or "necrolysis" were also present. We conducted our analyses for all reports, and serious reports only, in Oracle's Empirica™ Signal System [20,21]. The main statistical scores computed were the Empirical Bayes Geometric Mean (EBGM) and its associated 90% confidence interval (EB05, EB95). We used the lower 5% bound of the 90% confidence interval for an EBGM (EB05) of ≥2.0 as the threshold to define vaccine-AE combinations reported at least twice as often as expected, indicating combinations of potential significance [21].

3. Results

3.1. Descriptive analysis

Of 466,027 reports to VAERS during the analytic period [22], we identified 1086 (0.2%) reports of EM/SJS/TEN. Overall, over half (51%) of reported EM occurred among children aged <4 years (Table 1), with a median age of 3 years (range: <1 year to 89 years); among reports of SJS, overall median age was 15 years (range: <1 year to 84 years). Most (91%) reports of EM were non-serious, while most (52%) reports of SJS, and all reports of SJS/TEN and TEN, were serious. Most reports of EM/SJS/TEN described onset of symptoms within 14 days of vaccination; for cases of SJS with known time to onset, 71% described onset of symptoms within 3 days of vaccination.

Few reports of EM/SJS/TEN described a known trigger or stimulus for these conditions near when vaccine was administered (Table 2). No reports of SJS/TEN or TEN reported recent infection (such as with *Mycoplasma* or herpes simplex virus 1). Of persons with a history of EM/SJS/TEN, 4 had a similar reaction to past vaccination: (1) a female aged 15 months with EM after varicella vaccine, who later experienced EM after combined measles, mumps, and rubella (MMR) vaccine; (2) a female aged 2 years with EM after influenza vaccine, who later experienced EM after influenza vaccine; (3) a male aged 10 years with EM after combined diphtheria, tetanus, and acellular pertussis (DTaP), who later experienced EM after a combined diphtheria tetanus booster; and (4) a male aged 27 years with SJS after anthrax vaccine who later experienced SJS after another dose of anthrax vaccine. One patient had an active malignancy: a male aged 78 years who was undergoing radiation therapy for non-small cell lung cancer, who developed EM 4 days after receiving trivalent inactivated influenza vaccine; notably, this patient had been taking phenytoin for 2 months prior to developing EM.

The most commonly administered vaccines described in reports of EM/SJS/TEN were vaccines commonly administered in infancy and childhood: MMR (22%), DTaP (18%), varicella (18%), and 7-valent pneumococcal conjugate (13%) vaccines (Table 3). Among reports describing the administration of only one vaccine, the most common vaccines were smallpox (16%), trivalent inactivated influenza (15%), and varicella (7%) vaccines.

3.2. Reported deaths

We identified 6 reports of persons who developed EM/SJS/TEN after vaccination, and subsequently died (Table 4): 4 received medications known to trigger EM/SJS/TEN prior to or at the time of vaccination. Two patients received varicella vaccine, and 2 patients received inactivated trivalent influenza vaccine.

3.3. Estimated reporting rates

During 2006 through 2016, EM after varicella vaccine was reported to VAERS at an estimated rate of 1.0 per 1 million doses distributed; SJS after varicella vaccine was reported at an estimated rate of 0.1 per 1 million doses distributed. No reports of SJS/TEN or TEN after varicella vaccine were identified. Regardless of age group, estimated median reporting rates of EM, SJS, and TEN to VAERS during 2010 through 2017 after influenza vaccine (all types) were ≤0.1, <0.1, and <0.1 per 1 million doses administered, respectively; no reports of SJS/TEN after influenza vaccines were identified.

3.4. Data mining (disproportionate reporting)

We found elevated data mining statistics (EB05 ≥ 2.0) for "erythema multiforme" following smallpox vaccination among persons aged 19–49 years. When we restricted data mining analyses to serious reports only, or to reports identified through text field

Table 1
Description of U.S. reports of Erythema Multiforme (EM), Stevens Johnson Syndrome (SJS), Toxic Epidermal Necrolysis (TEN), and SJS/TEN to VAERS, 1999–2017.

	EM, n = 984	SJS, n = 89	SJS/TEN, n = 6	TEN, n = 7	Total, N = 1086
Sex, no. (%)					
Male	550 (56)	37 (42)	1 (17)	5 (71)	593 (55)
Female	413 (42)	49 (55)	5 (83)	2 (29)	469 (43)
Not reported	21 (2)	3 (3)	0 (0)	0 (0)	24 (2)
Seriousness, no. (%)					
Death	1 (<1)	0 (0)	1 (17)	4 (57)	6 (1)
Serious, non-death	88 (9)	46 (52)	5 (83)	3 (43)	142 (13)
Non-serious	895 (91)	43 (48)	0 (0)	0 (0)	938 (86)
Age, no. (%)					
<4 years	502 (51)	17 (19)	1 (17)	2 (29)	522 (48)
4–10 years	111 (11)	9 (10)	3 (50)	1 (14)	124 (11)
11–18 years	60 (6)	18 (20)	0 (0)	0 (0)	78 (7)
19–49 years	162 (16)	18 (20)	2 (33)	2 (29)	184 (17)
50+ years	126 (13)	20 (22)	0 (0)	2 (29)	148 (14)
Not reported	23 (2)	7 (8)	0 (0)	0 (0)	30 (3)
Time from vaccination to onset of symptoms, no. (%)					
<1 day	113 (11)	20 (22)	0 (0)	0 (0)	133 (12)
1–3 days	326 (33)	31 (35)	1 (17)	3 (43)	361 (33)
4–6 days	134 (14)	5 (6)	2 (33)	2 (29)	143 (13)
7–14 days	262 (26)	12 (13)	0 (0)	1 (14)	275 (25)
15–30 days	52 (5)	3 (3)	0 (0)	0 (0)	55 (5)
>30 days	16 (2)	1 (1)	0 (0)	0 (0)	17 (2)
Not reported	81 (8)	17 (19)	3 (50)	1 (14)	102 (9)

Table 2
History of hypersensitivity and exposure to known triggers among U.S. reports of Erythema Multiforme (EM), Stevens Johnson Syndrome (SJS), Toxic Epidermal Necrolysis (TEN), or SJS/TEN to VAERS, 1999–2017.

	EM (%), (n = 984)	SJS (%), (n = 89)	SJS/TEN (%), (n = 6)	TEN (%), (n = 7)	Total, N = 1086
Infection, no. (%)					
Yes	4 (<1)	2 (2)	0 (0)	0 (0)	6 (1)
No	980 (>99)	87 (98)	6 (100)	7 (100)	1080 (99)
Medication,[a] *no. (%)*					
Anticonvulsant	1 (<1)	5 (6)	0 (0)	2 (29)	8 (1)
Antibiotics					
Sulfa drugs	4 (<1)	3 (3)	0 (0)	1 (14)	8 (1)
Penicillin	52 (5)	3 (3)	1 (17)	0 (0)	56 (5)
Macrolides	6 (1)	3 (3)	0 (0)	0 (0)	4 (<1)
NSAIDs[b]	11 (1)	6 (7)	0 (0)	0 (0)	8 (1)
Acetaminophen	5 (1)	5 (6)	0 (0)	0 (0)	10 (1)
Existing/history of hypersensitivity,[a] *no., (%)*					
EM-SJS-TEN	6 (1)	5 (6)	0 (0)	0 (0)	11 (1)
Atopic dermatitis	25 (3)	2 (2)	1 (17)	1 (14)	29 (3)
Respiratory allergies[c]	47 (5)	4 (4)	0 (0)	1 (14)	52 (5)
Drug allergy					
Anticonvulsant	0 (0)	1 (1)	0 (0)	0 (0)	1 (<1)
Antibiotics					
Sulfa drugs	8 (1)	2 (2)	0 (0)	0 (0)	10 (1)
Penicillin	27 (3)	6 (7)	1 (17)	0 (0)	34 (3)
Macrolide	7 (1)	0 (0)	1 (17)	0 (0)	8 (1)
NSAIDs	4 (<1)	3 (3)	0 (0)	0 (0)	7 (1)
Acetaminophen	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a] Not mutually exclusive.
[b] NSAID = non-steroidal anti-inflammatory drug.
[c] Includes asthma/reactive airway disease, allergic sinusitis, and allergic bronchitis.

Table 3
Most frequently reported vaccines included in U.S. reports of Erythema Multiforme (EM), Stevens Johnson Syndrome (SJS), Toxic Epidermal Necrolysis (TEN), or SJS/TEN submitted to VAERS, 1999–2017.

All vaccines		Vaccines administered alone	
Vaccine[a]	No. (%)	Vaccine[a]	No. (%)
MMR	237 (22)	Smallpox	80 (16)
DTaP	199 (18)	Inactivated influenza, trivalent	74 (15)
Varicella	196 (18)	Varicella	34 (7)
7-valent pneumococcal conjugate	146 (13)	Pneumococcal polysaccharide	29 (6)
Inactivated influenza, trivalent	128 (12)	Hepatitis A	25 (5)
Haemophilus influenzae b	119 (11)	MMR	25 (5)
Inactivated polio	119 (11)	Hepatitis B	22 (5)
Smallpox	109 (10)	Herpes zoster	22 (5)
Hepatitis A	108 (10)	DTaP	17 (3)
Hepatitis B	67 (6)	4-valent human papillomavirus	17 (3)
13-valent pneumococcal conjugate	50 (5)	7-valent pneumococcal conjugate	16 (3)
Pneumococcal polysaccharide	43 (4)	Live attenuated influenza, trivalent	13 (3)

[a] DTaP = combined diphtheria, tetanus, and acellular pertussis vaccine; MMR = combined measles, mumps, and rubella vaccine; unless administered alone, listed vaccines are not mutually exclusive.

searches, we did not identify disproportional reporting for any vaccine-EM/SJS/TEN combination.

4. Discussion

Historically, EM/SJS/TEN have rarely been reported after vaccination [1]. Consistent with this history, an analysis of VAERS reports during 1990 to 1999 found few reports of SJS and/or TEN after vaccination [9]. Our analysis of VAERS data during 1999 through 2017 identified no new safety concerns, despite the introduction of several new vaccines since 1999. While these data are reassuring, new vaccines continue to be introduced to the market [23,24]. Continued surveillance for increased reporting of EM/SJS/TEN (SJS and TEN in particular) is therefore warranted.

Data from a systematic review by Chahal et al. and case reports from other groups have described median ages of 7 years for EM (range of <1–49 years) and 13–15 years (range of 1–75 years) for both SJS and TEN after vaccination [25,26]. The younger reported median age for EM in our analysis might reflect the younger age at which persons receive most of their vaccinations [15]. Reported sex was generally consistent with previous analyses [8,27].

Reported times from exposure to the suspected trigger, to onset of the rash, vary for SJS and TEN, ranging from a median of 10 days to a median of 3 weeks or longer [28,29]. A median of 6 days from vaccination to onset of EM has been observed [25]. Similarly, we observed varying reported times to onset of symptoms after vaccination but found that most events occurred within 14 days and rarely after 30 days; most SJS with known time to onset began within 3 days of vaccination. Notably, AEs are frequently reported when occurring within a short time after a potentially attributable trigger, regardless if a true association exists [30].

We observed no concerning patterns of reporting of EM/SJS/TEN after any particular vaccine. Reported vaccines seemed to reflect age groups for which vaccines are recommended: EM/SJS/TEN were mostly reported after childhood vaccines among persons

Table 4
U.S. reports to VAERS of Erythema Multiforme (EM), Stevens Johnson Syndrome (SJS), Toxic Epidermal Necrolysis (TEN), or SJS/TEN with subsequent death of the patient, 1999–2017.

Patient	Age, years	Sex	Past medical history	Vaccine (s) received[a]	Diagnosis	Onset, days	Clinical course	Cause of death
1	1	Male	Bronchitis, atopic dermatitis	Hepatitis B, varicella	TEN	2	Receiving amoxicillin/clavulanate and phenobarbital at time of vaccination; developed TEN with desquamation on 50% of BSA.	Sepsis with *E. coli* and *S. aureus* and disseminated intravascular coagulopathy
2	1	Female	Allergies (unspecified), atopic dermatitis	MMR, PNC7, varicella	EM	9	Developed fever of 101° F, vomiting, dehydration, and rash consistent with EM; later presented to emergency room with marked dehydration, suffered from hypovolemic shock, and died.	Unknown (autopsy declined)
3	25	Female	None	Anthrax, typhoid	TEN	13	Few details available; treated for anti-N-methyl-D-aspartate receptor encephalitis, subsequently developed TEN and cardiopulmonary arrest	Cardiopulmonary arrest
4	43	Female	Post-herpetic neuralgia	IIV	SJS/TEN	3	Receiving amoxicillin at time of vaccination; presented with SJS, was diagnosed with SJS/TEN	Respiratory failure
5	71	Male	Multiple myeloma, rheumatic heart disease, gout	IIV	TEN	1	Began allopurinol for gout 8 weeks prior to vaccination; developed TEN with desquamation of 80–90% of BSA.	Not specified
6	82	Male	Chronic kidney disease, type I diabetes	Not reported	TEN	Not reported	Trimethoprim-sulfamethoxazole administered at time of vaccination, subsequently developed TEN	Not specified

[a] BSA = body surface area; F = Fahrenheit; IIV = trivalent inactivated influenza vaccine; MMR = combined measles, mumps, and rubella vaccine; PNC7 = 7-valent conjugated pneumococcal vaccine.

receiving multiple vaccines simultaneously, but mostly reported after smallpox and inactivated influenza vaccines among persons receiving single vaccines (Table 3) [15,31]. Likewise, our data mining analyses identified no notable vaccine-AE combinations for EM/SJS/TEN following vaccination overall. Data mining did identify disproportionate reporting of EM following smallpox vaccination among persons aged 19–49 years. This age distribution is consistent with the age ranges of military personnel and selected civilian populations vaccinated due to concerns of potential bioterrorism and orthopoxvirus outbreaks per U.S. vaccination policy [32–34]. EM is a well-documented complication of smallpox vaccination, and the package insert for ACAM2000™ (live vaccinia virus smallpox vaccine) includes a "boxed warning" alerting about the risk of "EM major" (i.e., SJS), following either primary vaccination or revaccination with live vaccinia virus smallpox vaccine. Overall, our observations are consistent with previous descriptions of EM/SJS/TEN after vaccination [35–37].

While prior episodes of EM/SJS/TEN in a patient might indicate a predisposition to subsequent episodes, the likelihood of such an occurrence is unclear. Of the 4 persons we describe who previously experienced EM or SJS after vaccination, two experienced another episode of EM or SJS after the same vaccine (positive rechallenge). In a case series of nine children with a history of SJS precipitated by *Mycoplasma* infection, one developed a subsequent episode of SJS after a repeat infection with *Mycoplasma* [38,39]. Likewise, persons with a history of EM after infection with herpes simplex virus (HSV) can develop subsequent episodes of EM with recurrence of HSV [40]. However, our analysis (Table 2) and reports by other investigators [41] describe few persons with exposure to known precipitants of EM/SJS/TEN, or a history of allergies or other hypersensitivities. Notably, a patient who experienced SJS after infection with wild-type influenza B virus received multiple seasonal influenza vaccinations with no subsequent episodes of SJS [42]. Together, these observations suggest further exploration into predisposing factors for EM/SJS/TEN, including vaccines, is warranted.

SJS and TEN can be dire conditions, with case fatalities as high as 35% observed for TEN [43]. Consistent with this observation, 5 of the 6 deaths among reports of EM/SJS/TEN after vaccination had either TEN or SJS/TEN (Table 4). However, any association with vaccination is dubious, given that 4 reports described receipt of a medication known to cause SJS or TEN. Additionally, 1 report involved treatment for anti-N-methyl-D-aspartate (NDMA) receptor encephalitis and subsequent TEN; while treatment was unspecified, cyclophosphamide has been used to treat anti-NDMA receptor encephalitis and is known to cause SJS and TEN [44,45]. The 1 report of death after EM described a woman who experienced considerable vomiting, subsequent dehydration, and shock; the degree to which EM contributed to her death is uncertain.

Previous investigators have estimated rates of EM/SJS/TEN after drug exposure as high as 200 per 1 million persons exposed (phenobarbital) [8]. Our estimated reporting rates of EM/SJS/TEN after varicella and influenza vaccines were well below this figure (i.e., the highest estimate was 1.0 report of EM per million doses of varicella vaccine distributed), a notable observation considering that varicella vaccine was one of the most commonly reported vaccines after which EM/SJS/TEN occurred. Despite potentially underestimating rates, our data suggest that EM/SJS/TEN is reported no more frequently after vaccination than after known causes of these conditions. Importantly, while EM/SJS/TEN can occur after vaccination, previous post-marketing analyses and case-control studies demonstrate that vaccination itself does not increase the likelihood of these conditions [46,47].

These data have limitations. As a passive reporting system, VAERS is subject to reporting biases, under-reporting, inconsistent data quality and completeness, and changes in reporting over time; reports to VAERS also lack an unvaccinated comparison group. These limitations generally do not allow VAERS data to determine if a vaccine caused a particular adverse event, including EM/SJS/TEN [11]. Because data on doses distributed or administered are not available for many of the vaccines in this analysis, our ability to estimate reporting rates for EM/SJS/TEN was limited to varicella and influenza vaccines and are likely underestimates. Despite these limitations, results from data mining reflect known associations (i.e., smallpox vaccine and EM) [48,49].

While EM (and to a lesser degree, SJS) is typically benign and self-limiting [1,50], SJS/TEN and TEN can be life-threatening. [43] Surveillance for unexpected, increased reporting of these conditions after vaccination should therefore continue. Fortunately,

our observations and the observations of other investigators [1,9] suggest that EM/SJS/TEN rarely occur after vaccination.

5. Note

This article reflects the views of the authors and should not be construed to represent FDA's views or policies.

6. Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention (CDC), or the US Food and Drug Administration (FDA). Mention of a product or company name does not constitute endorsement by the CDC or FDA.

CRediT authorship contribution statement

John R. Su: Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Penina Haber:** Conceptualization, Formal analysis, Investigation, Writing – review & editing. **Carmen S. Ng:** Data curation, Methodology, Validation, Writing – review & editing. **Paige L. Marquez:** Data curation, Methodology, Validation, Writing – review & editing. **Graça M. Dores:** Conceptualization, Data curation, Formal analysis, Methodology, Validation, Writing – original draft, Writing – review & editing. **Silvia Perez-Vilar:** Conceptualization, Data curation, Formal analysis, Methodology, Validation, Writing – original draft, Writing – review & editing. **Maria V. Cano:** Conceptualization, Investigation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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EXHIBIT 150

Autoimmune disorder | definition of Autoimmune disorder by Medical dictionary

https://medical-dictionary.thefreedictionary.com/Autoimmune+disorder

Autoimmune disorder

Also found in: **Dictionary**, **Thesaurus**, **Encyclopedia**.

Autoimmune disorder

A disorder caused by a reaction of an individual's immune system against the organs or tissues of the body. Autoimmune processes can have different results: slow destruction of a particular type of cell or tissue, stimulation of an organ into excessive growth, or interference in function.

have a pink fit To become angry. Primarily heard in UK. Don't... *Watch Video*

have a cadenza
have a cadenzaTo become angry, agitated, or irritated.... *Watch Video*

Mentioned in: **Discoid Lupus Erythematosus**, **Goodpasture's Syndrome**, **Idiopathic Thrombocytopenic Purpura**, **Immune Complex Test**, **Immunoelectrophoresis**, **Pernicious Anemia**, **Rheumatic Fever**, **Scleroderma**, **Sjögren's Syndrome**, **Systemic Lupus Erythematosus**, **Vasculitis**

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Patient discussion about Autoimmune disorder

Q. Why does the body attack itself in autoimmune diseases? And if it's possible - How come it doesn't happen most of tl time?

A. Some say cell-wall deficient (CWD) bacteria can live inside your cells (were apparently photographed in immune cells electron microscope). See www.marshallprotocol.com and autoimmunityresearch.org (run by the autoimmunity research foundation). Also see bacteriality.com. I have been on the MP for just over a year. It has helped a lot of my symptoms, including lowering my TSH (thyroid) from hashimoto's thyroiditis (autoimmune thyroid condition). I hope that my thyroid w eventually regain all of it's function (still taking some thyroid hormone supplement, but less). The MP is not without "side e which are said to be from bacterial die-off and cell death when the bacteria are killed. It is experimental and should only b undertaken with that in mind. The marshallprotocol.com website is currently moderated by volunteers. There needs to be more research on CWD bacterial colonies and their possible role in autoimmune diseases. Please mention this to your doctor(s).

Q. I heard that omega 3 is good for autoimmune diseases- is that true? I have Rheumatoid Arthritis, and I take all sort o anti inflammatory drugs. And I heard I can take omega 3 and I'll be able to cut down the medication.

A. According to studies Omega 3 fatty acids have anti inflammatory effects and a lot of other helpful qualities. Here is a s articles I found about it. Any way you should consult your doctor maybe for you specific- it won't help. But here it is: http://www.jacn.org/cgi/content/abstract/21/6/495

Q. My boy has diabetes. Recently he was diagnosed with vitiligo. What is it and what can be the reason for this? My boy has diabetes. recently he was diagnosed with vitiligo. Our doctor said that he hopes it not a polyglandular autoimmur syndrome. what is vitiligo and what does this big phrase (polyglandular autoimmune syndrome) mean?

A. Vitiligo is a pigmentation disorder and the major cause of vitiligo is the autoimmunity. Some internal factor cause the destruction of melanocytes cell which produce the melanin a substance responsible for the coloration of skin. this lack of melanin infect results in white patch on skin of hypo pigmentation.

Normally vitiligo is not related with other disease like diabetes. However a little inheritance may include in the occurence of vitiligo.

More discussions about Autoimmune disorder

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EXHIBIT 151

National Institute of Allergy and Infectious Diseases (/)

Autoimmune Diseases

More than 80 diseases occur as a result of the immune system attacking the body's own organs, tissues, and cells. Some of the more common autoimmune diseases include type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus, and inflammatory bowel disease.

Although the causes of many autoimmune diseases remain unknown, a person's genes in combination with infections and other environmental exposures are likely to play a significant role in disease development. Treatments are available for many autoimmune diseases, but cures have yet to be discovered.

Why Is the Study of Autoimmune Diseases a Priority for NIAID?

The chronic and debilitating nature of these diseases, which can lead to high medical costs and reduced quality of life, is a burden on patients and also affects their families and communities.

How Is NIAID Addressing This Critical Topic?

Although researchers have made considerable progress in understanding how the immune system causes organ, tissue, and cell injury in autoimmune diseases, much remains to be learned. By supporting a broad range of basic, preclinical, and clinical research in autoimmune diseases, NIAID enhances understanding of the causes of these diseases, the genetic factors that make people susceptible to them, and the regulatory mechanisms that control the production of self-destructive antibodies.

NIAID-supported research on autoimmune diseases focuses on the immunologic basis of disease, including developing a greater understanding of the fundamental immunologic principles underlying disease onset and progression, developing improved animal models of disease, developing improved diagnostic tools, and identifying and evaluating more effective immune-based treatment and prevention strategies.

To learn about risk factors for autoimmune diseases and current prevention and treatment strategies visit the Medline Plus autoimmune diseases site (https://medlineplus.gov/autoimmunediseases.html).

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Read more about women's health (/node/12045)

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- Systemic Lupus Erythematosus (Lupus) Studies (/node/5609)
- Strategy to Prevent the Onset of Clinically-Apparent Rheumatoid Arthritis (StopRA) (https://www.clinicaltrials.gov/ct2/show/NCT02603146)

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Review

Expert Panel Workshop Consensus Statement on the Role of the Environment in the Development of Autoimmune Disease

Christine G. Parks [1,*], Frederick W. Miller [2], Kenneth Michael Pollard [3], Carlo Selmi [4,5], Dori Germolec [6], Kelly Joyce [7], Noel R. Rose [8] and Michael C. Humble [9]

1 Epidemiology Branch, National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH), Research Triangle Park, NC 27709, USA
2 Environmental Autoimmunity Group, NIEHS, NIH, Bethesda, MD 20892, USA; E-Mail: millerf@mail.nih.gov
3 Department of Molecular and Experimental Medicine, the Scripps Research Institute, La Jolla, CA 92037, USA; E-Mail: mpollard@scripps.edu
4 Division of Rheumatology, Allergy and Clinical Immunology, University of California, Davis, CA 95616, USA; E-Mail: carlo.selmi@unimi.it
5 Rheumatology and Clinical Immunology, Humanitas Clinical and Research Center, Rozzano 20089, Milan, Italy
6 National Toxicology Program, NIEHS, NIH, Morrisville, NC 27560, USA; E-Mail: germolec@niehs.nih.gov
7 Department of History and Politics, Drexel University, Philadelphia, PA 19104, USA; E-Mail: kaj68@drexel.edu
8 John Hopkins Center for Autoimmune Disease Research, Bloomberg School of Public Health, Baltimore, MD 21205, USA; E-Mail: nrose2@jhu.edu
9 Division of Extramural Research and Training, NIEHS, NIH, Research Triangle Park, NC 27709, USA; E-Mail: humble@niehs.nih.gov

* Author to whom correspondence should be addressed; E-Mail: parks1@niehs.nih.gov; Tel.: +10-919-541-2577.

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Abstract: Autoimmune diseases include 80 or more complex disorders characterized by self-reactive, pathologic immune responses in which genetic susceptibility is largely insufficient to determine disease onset. In September 2010, the National Institute of Environmental Health Sciences (NIEHS) organized an expert panel workshop to evaluate the role of environmental factors in autoimmune diseases, and the state of the science

regarding relevant mechanisms, animal models, and human studies. The objective of the workshop was to analyze the existing data to identify conclusions that could be drawn regarding environmental exposures and autoimmunity and to identify critical knowledge gaps and areas of uncertainty for future study. This consensus document summarizes key findings from published workshop monographs on areas in which "confident" and "likely" assessments were made, with recommendations for further research. Transcribed notes and slides were reviewed to synthesize an overview on exposure assessment and questions addressed by interdisciplinary panels. Critical advances in the field of autoimmune disease research have been made in the past decade. Collaborative translational and interdisciplinary research is needed to elucidate the role of environmental factors in autoimmune diseases. A focus on exposure assessment methodology is needed to improve the effectiveness of human studies, and more experimental studies are needed to focus on causal mechanisms underlying observed associations of environmental factors with autoimmune disease in humans.

Keywords: National Institute of Environmental Health Sciences (U.S.); consensus; autoimmune diseases; mechanisms; environmental exposures; epidemiology; exposure assessment

1. Introduction

Autoimmune diseases result from a damaging immune response directed against the body's own tissues. Of the 80 individual autoimmune diseases, common examples include rheumatoid arthritis (RA), Type 1 Diabetes (T1D), and the autoimmune thyroid diseases; others may be rarer, but as a group afflict 5%–9% of the U.S. population [1–5]. Chronic and incurable, these diseases constitute a major public health problem with high individual suffering and societal costs. The majority of autoimmune diseases disproportionately affect women with a few notable exceptions (e.g., Type 1 diabetes) [5,6]. Some are more common or severe in different racial/ethnic populations (e.g., lupus in African Americans) and age-groups (e.g., Type 1 diabetes in children). The reasons for these differences and underlying cause(s) of autoimmune disorders remain largely unknown, but are likely to involve both genetic and environmental factors [2]. This is well illustrated by the concordance rates observed in monozygotic *versus* dizygotic twins, which differ significantly but remain in most instances are well below 50% [7].

Over the years, a number of trans-NIH committees and NIH-supported workshops have examined the role of the environment in the development of autoimmune disease [8–11]. In 2003, the NIEHS co-sponsored the "Environmental Factors in Autoimmune Disease" workshop (along with other NIH partners, the United States Environmental Protection Agency (EPA), and the American Autoimmune Related Diseases Assoc., Inc., Eastpointe, MI, USA), and in 2005 co-sponsored the "Workshop on Lupus & the Environment: Disease Development, Progression and Flares" [10,11]. These produced recommendations for research initiatives on the role of the environment in autoimmune disease. One specific recommendation was to facilitate interactions between specialties

and encourage multidisciplinary approaches to improve overall knowledge of the hazard, mechanisms, and outcomes associated with specific environmental exposures.

To evaluate the state of the science and provide an opportunity for interactions between specialties, the NIEHS convened an "Expert Panel Workshop to Examine the Role of the Environment in the Development of Autoimmune Disease" on 7–8 September 2010. The goal was to bring together an interdisciplinary group of experts from the environmental health science and autoimmune research communities to review the literature and evaluate the state of the science, recommending productive directions for research on environmentally related autoimmune disease via the publication of a consensus statement.

The workshop utilized a format implemented in previous expert panel meetings [12–14]. Participants were selected for three panels examining the role of the environment in the development of autoimmune disease: molecular mechanisms and receptor dynamics; animal models; and epidemiology/human studies. Each panel defined the areas for review and reported their findings, grouped by confidence levels: (1) "Based on existing evidence we are confident of the following…"; and (2) "We consider the following to be likely but require confirmation…". The panels were asked to identify key knowledge gaps and broad themes for future research. Each group determined the scope of environmental factors they would consider, but all included chemical, physical, biological exposures. A fourth panel was tasked with addressing issues in exposure assessment, a topic of importance to the advancement of human studies.

During the second half of the workshop, four trans-disciplinary panels were formed consisting of members from each of the original review panels. Each panel discussed a common set of over-arching question using the same framework as the initial reviews and reported the findings according to confidence level with summary recommendations for broad themes for future investigations.

In this workshop report and consensus document, we summarize the individual panel reviews on mechanisms, animal models, and human studies, published elsewhere in full [15–17]. Because of the volume of literature reviewed, citations here are limited to key publications and examples. This report also summarizes findings from the panel on human exposure assessment and the interdisciplinary panel discussions.

2. Workshop Summaries

2.1. Mechanisms

The specific mechanisms leading to autoimmune diseases and the effects of environmental exposures on those mechanisms remain largely unknown. A variety of experimental studies are beginning to identify mechanisms by which environmental agents may induce or enable tolerance breakdown and/or autoimmune disease. Focusing on environmental exposure-based autoimmunity, the panel examined six major sub-topics summarized in Table 1 [15] including: effects on innate immunity such as Toll-like receptor (TLR) activation by xenobiotics; adjuvant effects and inflammatory responses; B cell activation; direct effect impairing the immune function, such as T-helper 17 (Th17) cells T regulatory (Treg) cells; and modifications of self-antigens.

Table 1. Panel findings on mechanisms involved in the role of environmental factors and development of autoimmune disease.

We Are Confident of the Following	We Consider the Following Likely, but Requiring Confirmation	Broad Themes to Be Pursued in Future Investigations
B cells		
Dysfunctions of B cell tolerance checkpoints are directly correlated with autoimmune disease in murine models; B cells modulate autoimmunity positively and negatively as secretors of antibodies and inflammatory cytokines, as antigen presenting cells to autoreactive T cells, and secretors of anti-inflammatory cytokines such as IL-10; Follicular B cells (B2) are a major source of autoreactive pathogenic antibodies; B cells secreting pathogenic autoantibodies can emerge when somatic hypermutation occurs outside of germinal centers; Sex hormones like estrogen and prolactin can differentially activate autoreactive B cell populations from different subsets (e.g., B2).	B1 cells and marginal zone B cells can modulate autoimmunity by exacerbating it through secretion of autoreactive antibodies and/or by down-modulating it through secretion of anti-inflammatory cytokines; B10 cells appear to exclusively secrete IL-10 may be functionally specialized to carry out a negative regulatory role in inflammation and autoimmunity.	The roles of B1 and marginal zone B cells in autoimmunity; The role of the recently discovered B10 cell population in autoimmunity; The survival/apoptotic pathways that when dysregulated lead to expansion and survival of autoreactive B cells (such as the BAFF/BlyS receptor system and CD40); Tolerance checkpoint mechanisms regulating the formation of high affinity autoreactive B2 cells both in and outside the germinal center; Environmental agents with the potential to disrupt B cell function.
T-helper 17 (TH17) cells		
Dysregulated Th17 cell activity can lead to pathology, as in chronic inflammatory diseases such as asthma or inflammatory bowel disease; Th17 cells are involved in multiple sclerosis (MS), rheumatoid arthritis (RA), Crohn's disease and psoriasis, where they seem to be involved in disease development and relapse.	Smoking is an important risk factor for RA; and nicotine exerts effects via Th17 cells; Aryl-hydrocarbon Receptor (AhR) binding by aromatic hydrocarbons and non-halogenated polycyclic aromatic hydrocarbons favors differentiation of Th17 cells and can exacerbate autoimmunity.	The involvement of environmental agents and exacerbation of autoimmune disease through Th17 cells; Therapeutic modulation of Th17 cells.

Table 1. *Cont.*

We Are Confident of the Following	We Consider the Following Likely, but Requiring Confirmation	Broad Themes to Be Pursued in Future Investigations
Innate Immunity		
The interaction between xenobiotics and Toll-like receptor (TLR) is a major mechanism involved in the interaction of environmental factors with autoimmunity development; Innate immune activation via TLR predisposes to toxic-induced inflammation; Adjuvants activate both innate and adaptive immunity, inducing release of chemokines and inflammatory cytokines; Immunization must be accompanied by a strong adjuvant, such as complete Freunds adjuvant, including the mycobacterium component. Incomplete Freund adjuvant results in production of antibodies, but without occurrence of autoimmune diseases.	Altered innate immune responses and dysregulated TLR signaling are a key step in triggering autoimmune diseases, as in virus-induced animal models of type I diabetes; TLR activation in macrophages may predispose cells to toxin-induced inflammatory cytokine production; Active infection or microbial products of infection can provide the adjuvant effect necessary for the induction of many autoimmune disorders.	Allergenicity, functional mimicry of environmental contaminants and physical/chemical elements resembling TLR ligands; Dysregulation of the regulatory B cell (IL-10 producing, CD5+ B cells) through modulation of TLR signaling; Molecular motifs of adjuvants and their physiological receptors that are associated with clinical manifestation of autoimmunity; Genomic predisposition to innate immunity dysfunctions.
T-regulatory (Treg) cells		
Quantitative and qualitative Treg changes contribute to a breakdown in tolerance; The AhR ligand dioxin 2,4,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induces immunosuppressive T cells expressing specific Treg markers; AhR ligands also affect skewing of the T cell repertoire towards Treg cells indirectly via antigen presenting cells; TCDD induces indoleamine 2,3-dioxygenase (IDO) transcription to skew the T cell repertoire towards FoxP3+ Tregs; Activation of peroxisome proliferator-activated receptor gamma (PPARγ) promotes Treg induction from naïve cells.	Most studies suggest that AhR activation in T cells or in antigen presenting cells may increase Treg production and therefore decrease autoimmunity, but the opposite outcome is also likely and possibly ligand-specific; Context-specific activation of the AhR by specific ligands may result in either increased or decreased Treg activity; Sex hormones play an important role in Treg development and may underlie female predominance of autoimmune diseases.	Specific chemical, infectious, or physical agents capable of modulating Tregs; Environmental modulators of AhR stimulation; Mechanisms of sex-specific Treg changes.

Table 1. *Cont.*

We Are Confident of the Following	We Consider the Following Likely, but Requiring Confirmation	Broad Themes to Be Pursued in Future Investigations
Modification of self-antigens		
The majority of human proteins undergo post-tranlational modification (PTM) and these modifications or lack thereof may lead to tolerance breakdown; PTM may explain the tissue specificity of autoimmune diseases; MS pathogenesis includes PTM that increase the complexity of myelin proteins through the autoimmune response or neurodegenerative processes; In RA, citrullination is an apoptotic PTM that seems to be helpful in opening protein conformation and favoring cleavage processes; In PBC, cholangiocytes do not covalently link glutathione to lysine-lipoyl groups during apoptosis leading to accumulation and exposure to potentially self-reactive antigens, accounting for bile duct specific pathology.	Multiple self-protein modifications (phosphorylation, glycosylation, acetylation, deamidation) can lead to either T or B cell responses to self-antigens; Serum autoantibodies to modified self antigens may bind either modified or unmodified forms and thus be crucial to effector immune reaction in target tissues; Mercury-induced cell death results in formation of a unique and more immunogenic 19 kDa cleavage fragment of fibrillarin.	Mechanisms by which citrullination and glutathionylation lead to tolerance breakdown in susceptible individuals; The role of glucosylation in MS and other autoimmune diseases; Experimental models to prove that autoantigens can be modified to increase their immunogenicity; Technologies to reverse or induce PTM in animal models of autoimmunity.

Table 1. *Cont.*

We Are Confident of the Following	We Consider the Following Likely, but Requiring Confirmation	Broad Themes to Be Pursued in Future Investigations
Modification of DNA methylation		
DNA methylation profiles are associated with environmental factors including prenatal tobacco smoke, alcohol, and environmental pollutants; The importance of DNA methylation in regulating immune function is suggested by two rare congenital diseases, Silver-Russel and Beckwith-Wiedemann syndromes; Changes in DNA methylation in specific peripheral immune cell types are associated with autoimmune diseases.	Phenotypic differences are increased with age in twins in a trend coined as "epigenetic drift", due to different environmental exposures, and may explain late-onset autoimmunity; Specific impairments in epigenetic regulation in immune cells may be responsible for immune-tolerance breakdown through hypo-methylation of genes or involvement of transcription repressors; Recent genome-wide association studies demonstrate that genomics significantly predispose to systemic lupus erythematosus (SLE) onset, but experimental studies indicate that epigenetic mechanisms, especially impaired T and B cell DNA methylation, may be one of these factors.	The functional effects *in vivo* of DNA methylation changes under different environmental and genomic conditions; The development of new therapeutic molecules capable of preventing or counteracting DNA methylation changes in a cell-specific manner; The DNA methylation changes in the target cells and not only in the rapidly accessible effector immune cells.

2.1.1. Effects on Innate Immunity

Two major related mechanisms within innate immunity were discussed: Toll-like receptor (TLR) activation, and the role of adjuvants. The Toll-like receptors (TLRs) are pattern recognition receptors that play a key role in the effectiveness and function of the innate immune system. The panel was confident that TLR activation and signaling is a major mechanism linking environmental factors to development of autoimmunity. TLR deficiency impacts both disease severity and autoantibody profiles in pristane-induced autoimmunity [18]. Moreover, TLR-related pathways are likely to play a role in virally-induced animal models of autoimmune disease (e.g., Type 1 diabetes), and active infections or microbial exposures may provide the necessary adjuvant effect for the induction of many autoimmune diseases. Recommendations for further research included investigation of environmental factors and TLR activation, and TLR-related effects on regulatory B cells [19–21].

Adjuvants are agents that non-specifically stimulate the immune system without direct antigenic effects, including TLR-mediated effects on innate immune response and factors that modulate the adaptive immune response. The panel concluded that adjuvants (e.g., complete Freund's) are important in the development of autoimmune disease. Further research is needed to characterize molecular aspects of adjuvants and receptors involved in autoimmune diseases, and on genetic risk factors that may modify autoimmune responses to environmental adjuvants and triggers of the innate immune response [11,22,23].

2.1.2. B Cell Activation

One of the two major cell types in the adaptive immune response, B cells secrete pathogenic auto-antibodies and can also present antigens to auto-reactive T cells. A breakdown in central tolerance (in the bone marrow) is a major contributor to autoimmunity in many experimental models. Determining the contributions of B cell subtypes in autoimmune disease and the role of environmental factors in biasing their activation is critical. The panel reported a high degree of confidence in the role of follicular B cells and the influence of sex hormones (e.g., estrogens) via this mechanism, and that research is needed to identify effects of environmental exposures on B cell development and function, e.g., environmental estrogens [24–27].

2.1.3. T-Helper 17 Cells

T-helper 17 (Th17) cells, an interleukin 17 (IL17)-producing subset of T-helper cells, play an important role in the adaptive immune response and mechanisms leading to autoimmunity and chronic inflammation. The panel was confident that dysregulation of Th17 cells contributes to chronic inflammatory pathology, and that Th17 cells are involved in development and relapse of several autoimmune diseases [28,29].

Increasing evidence suggests that xenobiotics, allergens and micronutrients can influence Th17 cells at multiple levels. For example, smoking, a risk factor for RA and other autoimmune diseases, exerts effects on Th17 cells through nicotine exposure. Aromatic hydrocarbons and non-halogenated polycyclic aromatic hydrocarbons also induce differentiation of Th17 cells through binding at the Aryl hydrocarbon Receptor (AhR), exacerbating autoimmunity. Given the important role of Th17 cells

on development and exacerbation of autoimmunity, more research is needed on the effects of environmental exposures on Th17 cells [30–32].

2.1.4. T Regulatory (Treg) Cells

T regulatory (Treg) cells play a key role in the maintenance of immune tolerance, and can dampen or suppress activation of the immune system. Experimental studies demonstrate a number of mechanisms through which environmental agents may affect Treg induction or function. The panel was confident that changes in Treg cells play a role in loss of tolerance for self-antigens. Strong evidence supports 2,4,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induction of suppressive T cells with Treg markers, along with mechanisms involving antigen-presentation, through which AhR ligands skew the T cell repertoire towards Treg production [33–35]. The peroxisome proliferator-activated receptor gamma (PPARγ) receptor is activated by a wide variety of environmental chemicals (e.g., phthalate esters), promoting Treg induction [36,37].

Confirmation is needed that the context-specific activation of the AhR by specific ligands may result in either increased or decreased Treg activity. Sex-hormones are likely to regulate Treg development, and may underlie the female predominance of most autoimmune diseases. The panel concluded that studies should focus on environmental factors capable of modulating Treg and AhR activity and also consider the role of chemical mixtures and direct stressors, such as ultraviolet (UV)-light [35,38–40].

2.1.5. Modification of Self-Antigens

Post-translational modification (PTM) is the chemical modification of a protein following its synthesis, e.g., methylation, phosphorylation, acetylation, lipidation, or glycosylation, occurring on 50% to 90% of proteins in the human body. An environmental exposure may alter PTM, affecting immunogenicity of self-proteins and triggering an autoimmune response. PTM may explain tissue specificity of some autoimmune diseases. For example, PTM increases complexity of myelin proteins through autoimmune or neurodegenerative processes in MS. Conversely, lack of PTM during apoptosis alters protein degradation and leads to accumulation of self-reactive antigens related to bile duct specific pathology of primary biliary cirrhosis (PBC) [41,42].

Confirmation is needed that multiple types of environmentally-induced PTM may lead to B and T cell reactivity to self-antigens, and that self-reactive antibodies react to both modified and unmodified forms of antigen. One example is the binding of mercury to fibrillarin to create a modified self-antigen and the generation of a new cleavage fragment due to proteolysis following mercury-induced cell death. Research is also needed on mechanisms by which citrullination (e.g., linking smoking to RA) leads to loss of self-tolerance and studies of PTM biomarkers across the natural history of autoimmune diseases [43–47].

2.1.6. Modifications of DNA Methylation

The field of epigenetics examines the regulation of the genome through modifying mechanisms not involving changes in the nucleotide sequence itself, such as DNA methylation and histone

acetylation. Environmental factors can affect epigenetic gene regulation, and so understanding the role of epigenetic modifications in the development of autoimmunity is an important topic for future study. The panel confidently noted the association of DNA methylation profiles with environmental exposures, including prenatal tobacco smoke, alcohol use, and environmental pollutants (*i.e.*, particulate matter).

Confirmation is needed that ageing-related phenotypic changes arise due to exposures over the lifetime, contributing to development of autoimmune diseases later in life. Studies are needed to show whether loss of tolerance is related to specific exposure-associated impairments in regulation of epigenetic processes and confirming impaired methylation of B and T cell DNA in relation to systemic lupus erythematosus (SLE) risk. Recommendations include research on the *in vivo* effects of DNA methylation under different environmental conditions and target tissue differences in DNA methylation associated with autoimmune diseases [7,48].

2.2. Animal Models

Animal models have been used extensively in the study of autoimmune disease and the role of environmental exposures. The panel focused their attention on studies of non-therapeutic chemical, biological, and physical factors associated with autoimmune outcomes as summarized in Table 2 [17]. A high level of confidence was reached if multiple studies from different laboratories confirmed the same findings. For findings considered likely and requiring further confirmation, there needed to be significant support, including multiple studies from a single laboratory, or repetition of some but not all findings in multiple laboratories.

The panel noted that autoimmune responses to chemical factors are species and strain-specific. Autoimmune animal models (predominately rats and mice) are typically genetically manipulated or inbred strains that spontaneously develop disease or autoimmunity induced by immunization with specific antigens. Some studies of environmental factors in autoimmunity involve the *induction* of autoimmune diseases or autoimmunity in non-susceptible, inbred strains. Due to the genetic complexity of disease susceptibility, autoimmune effects may not be observed. Thus, studies may also investigate environmental effects on models of spontaneous autoimmune disease, in which case the effects of exposure may include *exacerbation* or acceleration of disease expression. Because of the great depth of the literature in this area, the published review on animal models and autoimmune diseases was limited in scope [17]; an additional white paper on the full workshop review session is available by request.

Studies provided conclusive evidence that forms of inorganic mercury ($HgCl_2$, vapor, amalgam) can induce systemic autoimmune disease in rats (transient) and mice [49], and exacerbate or accelerate systemic disease in lupus-prone mice [50]. Several mineral oil components and other hydrocarbons can induce inflammatory arthritis in rats [51]; and one component, 2,5,10,14-tetramethylpentadecane (TMPD or pristine) induces lupus-like disease and inflammatory arthritis in some strains of mice [52]. With a high degree of confidence, the panel also noted a role for specific pathogens (*i.e.*, Streptococcal group A, Coxsackie B virus) and exacerbation of autoimmune thyroiditis by iodine in genetically predisposed animal models.

Table 2. Panel findings studies of animal models in the role of environmental factors and development of autoimmune disease.

We Are Confident of the Following	We Consider the Following Likely, but Requiring Confirmation	Broad Themes to Be Pursued in Future Investigations
Forms of inorganic mercury ($HgCl_2$, vapor, amalgam) induce systemic autoimmune disease in rats (transient) and mice, and exacerbates systemic autoimmune disease in lupus-prone mice; Several mineral oil components and certain other hydrocarbons can induced an acute inflammatory arthritis in some rat strains; The mineral oil component 2,6,10,14-tetramethylpentadecane (TMPD or pristane) induces lupus-like disease and inflammatory arthritis in several strains of mice; For a limited number of pathogens there is a clear association with development of autoimmune diseases; Excess iodine increases the incidence of autoimmune thyroiditis in genetically predisposed animal models.	Gold causes (transient) nephropathy in rats. Gold and silver cause autoimmune responses, but not autoimmune disease, in mice; but the ability of silver and gold to exacerbate spontaneous autoimmune disease requires study; Silica exacerbates autoimmune disease but more studies are needed using more species/strains and a wider range of doses and exposure routes; Trichloroethylene (TCE) exacerbates systemic autoimmunity although responses are often limited and transient. Studies of autoimmune liver disease are needed with additional species/strains and in developmental studies; TCDD exposure during fetal or early neonatal development may promote autoimmunity; Organochlorine pesticides may enhance lupus-like disease in a predisposed mouse strain; Sunlight/ultraviolet (UV) light exposure exacerbates lupus in genetically prone mice.	Studies should be "shaped by what is observed in humans, not by what is possible in mice" [53]; Studies should not be restricted to a "gold standard" animal model. Multiple models should be investigated to reflect human genetic heterogeneity; When using spontaneous disease models it is important to consider whether environmental exposures directly impacts idiopathic autoimmunity, or reflects environmental factor-specific autoimmunity; More studies on the effects of environmental factor exposure on expression of autoimmunity during different stages of life (gestational to adulthood) are needed.

The panel considered a wide range of other associations to be likely, but needing confirmation. Examples include some heavy metals (gold and silver), though more studies are needed of other metals (organic mercury, cadmium, lead, and arsenic) to confirm observed effects. Silica exacerbates autoimmune disease in lupus models, but studies are needed in different species/strains and across a wider range of exposure routes and doses. Evidence is suggestive that trichloroethylene (TCE) can exacerbate systemic autoimmunity in a limited or transient manner, and UV radiation/sunlight is likely to exacerbate lupus in genetically prone mice. Developmental exposures (fetal/neonatal) to TCDD may promote systemic autoimmunity, supporting the idea that early exposures may influence the developing immune system and subsequent development of disease. Findings that organochlorine pesticides (e.g., dichlorodiphenyltrichloroethane [DDT]) enhance autoimmune disease in a susceptible mouse model also require confirmation.

The panel noted several themes for future use of animal models to study environmental autoimmunity and disease. Above all, it was recommended that findings in animal models should not be the only driving force for human studies, which should be "shaped by what is observed in humans, not by what is possible in mice". A single mouse strain cannot encompass the genetic heterogeneity in human populations, so studies should not be limited to "gold standard" animal models. Rather, the effects of environmental exposures should be tested on multiple models, and if necessary, humanized models. Exposure effects should be examined during all stages of life, from gestation to adulthood. When using spontaneous autoimmune models, studies should consider whether exposures exacerbate or accelerate idiopathic autoimmunity or induce more specific "environmentally-associated" forms of autoimmunity. The panel also recommended specific improvements to animal studies, including use of disease markers from easily obtained biological fluids (e.g., blood) to enhance comparisons with human studies.

2.3. Epidemiology/Human Studies

Findings of the epidemiology/human studies review are summarized in Table 3 [16]. The panel restricted their focus to peer-reviewed studies published in the last 30 years using defined Medline searches of the primary literature. Meta-analyses were examined with respect to study identification, inclusion and exclusion criteria, and the methods used to abstract and derive summary estimates. When the design and analysis methodology was deemed acceptable, the study estimate was used to summarize evidence through the period covered by that review; additional studies published subsequent to the meta-analysis were also reviewed.

Diseases of focus included: Crohn's disease (CD), gluten-sensitive enteropathy (GSE, celiac disease), Graves' disease (GD), Hashimoto's thyroiditis (HT), idiopathic inflammatory myopathies (IIM), multiple sclerosis (MS), primary biliary cirrhosis (PBC), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), systemic sclerosis (SSc), type 1 diabetes (T1D), and ulcerative colitis (UC). Additional diseases were examined if a substantial literature existed for a particular exposure e.g., eosinophilia myalgia syndrome [54]. Exposures were grouped in three broad classes: chemicals, physical factors, and biologic agents.

Table 3. Panel findings on human studies on the role of environmental factors and development of autoimmune disease.

We Are Confident of the Following	We Consider the Following Likely, but Requiring Confirmation	Broad Themes to Be Pursued in Future Investigations
Chemicals		
Crystalline silica (quartz) contributes to development of several systemic autoimmune diseases, including RA, systemic sclerosis (SSc), SLE and anti-neutrophil cytoplasmic antibody (ANCA)-related vasculitis. Solvents contribute to development SSc. Smoking contributes to development of anti-citrullinated protein antibody (ACPA)-positive and anti-rheumatoid factor. (RF)-positive RA (with an interaction with the shared eptiope genetic susceptibility factor).	Solvents contribute to development of MS. Smoking contributes to development of seronegative RA, MS, SLE, Hashimoto's thyroiditis (HT), Graves' disease (GD) and Crohn's disease (CD). Current smoking protects against development of ulcerative colitis (UC).	There is insufficient evidence on the role of metals, including those associated with animal models of autoimmunity, e.g., mercury. The identification of single causal agents within groups of exposures is needed (e.g., specific solvents or pesticides contributing to increased risk for the group). Studies are needed on plasticizers (e.g., phthalates and bisphenol A), some of which may be endocrine or immune disruptors, and have been associated with other immune mediated diseases. There is insufficient evidence on the role of cosmetics in autoimmune diseases.
Physical factors		
An inverse association exists between increased ultraviolet radiation exposure and risk of developing MS.	Ionizing radiation contributes to development of HT and GD.	There is insufficient evidence on a possible protective role of ultraviolet radiation on type 1 diabetes (T1D). Prospective data are needed on sun exposure as a risk factor for SLE (prior to early clinical symptoms) and dermatomyositis.
Biologic agents		
Ingestion of gluten contributes to development of gluten-sensitive enteropathy (GSE). Ingestion of certain lots of L-Tryptophan contributes to development of eosinophilia myalgia syndrome. Dietary intake of 1,2-di-oleyl ester (DEPAP)- and oleic anilide-contaminated rapeseed oil contributes to development of toxic oil syndrome.	Epstein-Barr virus (EBV) infection contributes to MS development. Early introduction of complex foods contributes to development of T1D and GSE. Low dietary vitamin D intake and blood levels contribute to development of MS.	Studies are needed on MS and vitamin D in racial/ethnic groups with darker skin (associated with UV-associated vitamin D deficiency), and examining dose-effects. Prospective data are needed on vitamin D and other autoimmune diseases. Additional studies are needed on associations of food chemicals, dyes, or additives. Prospective studies are needed on nitrates/nitrosamines and T1D.

A "confident" association was based on evidence from multiple studies in different populations using different designs; robust evidence of an overall association (*i.e.*, high magnitude risks or based on high quality or established exposure assessment methods); a dose-response relationship, or effect differences by disease subtype or genetic factors supporting biologic plausibility. "Likely" associations were based on similar body of research, but missing key evidence, such as a temporal relationship, less consistent results, or fewer studies.

2.3.1. Chemical Factors

The panel was confident that crystalline silica exposure contributes to development of several systemic autoimmune diseases, including RA, SSc, SLE, and anti-neutrophil-cytoplasmic antibody (ANCA)-associated vasculitis [55,56]. Evidence also supports an association of solvent exposure (in general) and development of SSc [57]. The panel was confident that smoking contributes to anti-citrullinated peptide antibody (ANCA) and anti-rheumatoid factor (RF)-positive RA, interacting with the shared epitope genetic risk factor [58,59].

Evidence also suggests smoking is likely to play a role in development of seronegative RA, MS, SLE, HT, GD, and CD, while protecting against UC [60,61]. Research needs varied by disease type; findings are inconsistent on the relation of smoking with MS and SLE. General solvent exposure may also contribute to development of MS, but more research is needed using improved exposure assessment methods. Major research gaps include studies of metals associated with autoimmunity in animal models and identification of specific causal agents within general groups of exposures (e.g., pesticides or solvents). Based on their endocrine and immune-disrupting qualities, the panel recommended studies of plasticizers in development of autoimmune diseases.

2.3.2. Physical Factors

The panel was confident in the inverse association of higher UV exposure and risk of MS [62]. Ionizing radiation is likely to contribute to development of autoimmune thyroid diseases (HT and GD), though several limitations contribute to a degree of uncertainty. These include studies of medical radiation therapy that did not distinguish HT from general hypothyroidism, and inconsistency in findings from nuclear testing fallout and accidental radiation contamination. Research gaps include the role of UV exposure as a protective factor against T1D and as a risk factor for dermatomyositis and SLE, with prospectively collected data and attention given to the period prior to potential clinical symptoms related to sun sensitivity.

2.3.3. Biological Factors

The panel was confident about the role of gluten in the development of GSE [63]. Strong evidence supports a role of specific environmental exposures in eosinophilia myalgia syndrome [54] and toxic oil syndrome [64].

It was also deemed likely that Epstein-Barr virus (EBV) infection contributes to development of MS, and that early introduction of complex (e.g., solid) foods contributes to development of T1D and GSE. The panel noted that MS may be associated with lower vitamin D intake and blood levels, but

research was recommended in non-Caucasian populations at higher risk of UV-related vitamin D deficiency, and studies of dose-effects. Prospective studies on vitamin D and other autoimmune diseases were also recommended. A research gap was noted on the role of food additives, dyes, and chemicals, and prospective studies on the role of nitrates and nitrosamines in T1D.

Broad recommendations for future epidemiologic research included studies of multiple exposures and chemical mixtures, reflecting the real life complexity of human exposures. More studies are needed on exposure-related risks within specific disease phenotypes and in the context of genetic risk factors, such as the association of smoking with RA in the context of anti-citrullinated peptide antibody positivity and the shared epitope. Research needs also include defining critical windows in the timing of exposures and latencies relative to developmental stage, understanding dose-response relationships, and identifying mechanisms.

2.4. Exposure Assessment in Human Studies

Addressing a unique need recognized by the epidemiologic and clinical research community, the workshop also included a focus group on exposure assessment, bringing together experts in epidemiologic methods and exposure measurement technologies. Most autoimmune diseases are chronic and the relevant timing of exposures is not well established. Because most of the diseases are individually rare, retrospective case-control studies are often the most efficient design, with assessment methods based on questionnaires, relying on self-report and recall. The accuracy of internal dose estimates may be improved using exposure biomarkers, such as serum pesticides or metals; however, many exposures (e.g., silica) do not have easily accessible biomarkers, and for others (e.g., pesticides or metals), current biomarker levels may misclassify exposures during relevant time-periods of disease initiation or progression.

The ability to identify environmental risk factors for autoimmune diseases depends heavily on the availability of rigorous exposure assessment methods that can be applied in different populations and allow comparisons across studies. Standardized questionnaires, such as those offered by the PhenX Toolkit [65], may address this need for some exposures, such as smoking. But studies identifying or confirming risk factors for autoimmune diseases often require greater detail on specific agents, such as solvents or pesticides, which vary by disease. Assessment methods may also need to be tailored to specific populations or settings, such as the methods used to assess occupational silica exposure in women in rural *versus* urban settings [66,67]. A life-course perspective is also critical, given the general lack of knowledge on the relevant time-windows of exposures in human studies and the well-established influence of developmental exposures on the immune system.

New technologies in exposure measurement are being developed with potential applications in autoimmune disease research. Personal measurement technologies may have limited usefulness in assessing exposures years or decades prior to disease onset, but may help validate questionnaires on current or recent exposures or in long-term follow-up studies. Studies are also using new technologies to link geographic exposures with autoimmune disease studies, for example: air pollution and RA [68], UV radiation and dermatomyositis [69].

The panel recommended an integrated approach to improve exposure assessment in human autoimmune disease research. While adequate tools may exist to assess some relevant exposures

(e.g., smoking, silica), they are not widely accessible to researchers and their use typically requires collaboration with experts in assessment methodology. The application of new technologies may improve the accuracy and efficiency of existing methods, for example, using measurement data to validate questionnaires. Analytic methods that utilize complex data resources to model past exposures [70–72], or simultaneously take into account multiple exposures (e.g., Exposure-wide association studies; EWAS, [73,74]), will be important resources for future studies. Applications of information technologies are also needed to create useful databases incorporating biomarkers, questionnaires, measurement studies, and data analysis guidelines for autoimmune researchers. These investments require a focus on the "big picture" and integration across disciplines, with research and infrastructure development that requires support and cooperation across multiple agencies conducting public health and scientific research, such as the Centers for Disease Control and Prevention, National Institutes of Health, Environmental Protection Agency and National Science Foundation.

Specific recommendations for advancing exposure assessment in environmental autoimmune diseases research include: (1) improving sensitivity, specificity, and dose estimates for established risk factors for one or more specific autoimmune diseases (*i.e.*, silica, solvents, UV radiation); (2) a focus on disease risk in high exposure groups (occupational, military, and other risk populations); (3) consideration of highly prevalent or emerging "new" exposures (e.g., obesity, phthalates); and (4) prospective exposure assessment in susceptible populations (e.g., family members autoimmune disease patients, women). Resources are also needed to guide clinicians in the collection and interpretation of environmental exposure data [75]. Information collected in clinical settings is often limited to smoking and current occupation. In order to target specific exposures (e.g., silica or solvents), clinically applicable questionnaires are need to integrate across a wide variety of industries and occupations, including past as well as ongoing exposures.

2.5. Transdisciplinary Breakout Panels

Integrated responses from the four transdisciplinary breakout panels identified a range of specific needs and opportunities advancing research in environmental autoimmunity.

Topic 1—Do animal models recapitulate disease observed in humans following exposure?

In the majority of examples animal models do not entirely mimic human autoimmune diseases. Across the range of 80+ autoimmune diseases, most have complex etiologies, while animal models are designed to minimize complexity to foster an understanding of mechanisms. Exceptions in which animal models recapitulate features associated with environmental autoimmunity/disease in humans include pristane-induced lupus, toxic oil syndrome, Coxsackie virus-induced myocarditis, and L-tryptophan-associated eosinophilia–myalgia syndrome (EMS). The (NZBxNZW)F1 model also recapitulates human SLE (with central nervous system (CNS) involvement, vasculitis, dermatitis), and models of ultraviolet B (UVB) exposure and SLE exacerbation correspond well with the common clinical perception of flares and lupus. In these cases, however, the complexity of the animal model approaches that of the human disease, making a mechanistic understanding more difficult to achieve. A number of models support human data on environmental risk factors for autoimmune diseases, but

need further development and characterization, including models of TCE-induced autoimmunity designed to investigate the relationship of solvents and autoimmune diseases, including SSc.

In several instances, animal models of environmentally induced autoimmune need to be promoted when there are good epidemiological data supporting an association. A prime example is that of silica and silicate exposures, which have been associated with multiple autoimmune diseases in humans. These findings are corroborated by relatively few studies in animal models, and so more work is recommended, particularly for the inhalation route of exposure. Other examples include studies of smoking effects on MS, and early dietary exposures and diabetes. (At the same time, epidemiological studies should also be promoted when good mechanistic data exists based on animal studies (such as mercury-induced SLE-like disease, and effects of mercury and other heavy metals associated with exacerbations)). Panel discussions highlighted a need for genetically diverse animal models of autoimmunity to reflect the heterogeneity of human populations and for studies of gene-environment interactions. Models are also needed that reflect relevant doses and exposure mixtures, and that mimic the sex differences often seen in human autoimmunity.

Topic 2—Do exposures associated with autoimmune disease *in vitro* and in animal models have relevance to exposures in human populations?

Although exposure levels in human studies of autoimmunity are often unclear, animal models generally use higher doses to shorten experimental periods or reveal underlying mechanisms as proof-of-principal. Studies of risk factors associated with human autoimmune disease may provide hints as to what exposure types and doses to evaluate in animal models. There are reasonably good *in vitro* data for some exposures (e.g., mercury) where the exposures achieved following animal dosing *in vivo* may be comparable to levels found in humans [76]. Other relevant associations seen in experimental studies may include EBV associated with MS, mineral oil components with RA/SLE/inflammatory arthritis.

Further research was recommended to explore similarities and differences between animal models and humans in the metabolism, pharmacokinetics, distribution/internal dose, and target organ dose of specific xenobiotics related to autoimmunity. Given the current limitations of exposure assessment in human studies, efforts are needed to determine the validity of biomarkers of exposure, for example methylation arrays and self-protein reactivity arrays to citrullinated or glycosylated proteins.

Topic 3—How do susceptible populations, time frames of exposure, or genetic predisposition contribute to exposure-related autoimmune disease?

Many intrinsic factors (e.g., genetics, gender, age) work together in complex ways to contribute to the development of exposure-related autoimmune disease; these interactions are likely to be both complex within given exposure/disease relationships and variable across different types of exposures and diseases. Animal models provide examples of differences in autoimmune susceptibility by age or genetic background (e.g., mercury [77]). Human studies also provide proof of principle—for example the difference in relationship between smoking, autoantibodies and RA phenotypes depending on the human leukocyte antigen (HLA)-DR4. Animal models suggest the time frame of exposure is likely to be important, for example prenatal/early life TCDD exposures may have different outcomes than

exposures more proximal to autoimmune onset [78,79]. Confirmation is needed in humans that exposure timing (e.g., early life exposures *versus* later life exposures) affects disease risk, which may be difficult given the long latency time and challenges in assessing early life exposures. Other examples that timing or genetic factors are likely to be important include the timing of the introduction of complex foods during infancy and T1D, and UV exposure interacting with genetic risk factors in MS risk [16].

The interactions of age, gender, genetics and windows/timeframes with environmental exposure(s), and the impact of their relative contributions, are critical to a more complete understanding of the development of autoimmune diseases. Recommendations for further investigation included: mechanisms underlying sex or gender differences in autoimmune-effects of solvents and other environmental exposures, and timing of exposures and role of genetic susceptibility for several exposures, including sunlight (e.g., protection against MS), vitamin D, silica, and EBV infection. Findings on genetic susceptibility in human autoimmune diseases, e.g., HLA-DR4 and RA, should be incorporated into animal models (e.g., humanized mice) when possible.

Topic 4—To what extent does ability to quantify environmental exposures limit our ability to identify factors associated with human autoimmune disease?

Limitations in methods and technologies to assess environmental exposures in humans can substantially hinder the detection of exposures related to the development of autoimmune disease. Better exposure data would produce more accurate dose-response estimates and lead to identification of more risk factors. For example, using a less sensitive and specific method for silica exposure made a substantial difference in observed association with lupus [66]. Because autoimmune diseases in humans are relatively uncommon, case-control studies are often needed to achieve sufficient numbers of cases for analyses. Thus, there is a particular need for the development and dissemination of methods to assess historical exposures.

The low incidence of many of the individual diseases (especially in men) presents a logistical challenge for studies in exposure-enriched populations, e.g., occupational cohorts, which often have higher levels of specific exposures and sometimes also include measurement data. Likewise the rarity of some exposures (e.g., high level silica exposure) present challenges in studies conducted in the general population or patient registries.

There is an urgent need to develop cost-effective non-invasive methods to quantify those environmental exposures most likely related to autoimmune disease in both human populations and animal models. New technologies must be developed, and emerging technologies exploited. Recommendations were provided in the context of the panel discussion specifically devoted to the topic of exposure assessment (above).

Topic 5—How well do mechanistic studies *in vivo* or *in vitro* relate to clinical outcomes?

There is a paucity of studies that relate autoimmune disease mechanisms with clinical measures and exposure-associated autoimmune diseases. It was generally agreed that experimental/mechanistic studies are focused on models used for proof of principle, and so findings do not correspond well with what is known in humans. Table 4 reviews knowledge on mechanisms related to three exposures

identified with the greatest confidence as being associated with human disease in this workshop: smoking, silica, and solvents.

One of the most solid examples is the mechanistic relationship between citrullination, a form of post-translational modification and the development of autoantibodies to citrullinated proteins, thought to suggest a causal role for smoking in the development of RA [80]. Experimental data is somewhat limited, however studies of gene-environment interactions and other human studies are providing additional clues to etiologic pathways [81–83]. The role of other smoking-related mechanisms in RA including heat shock gene expression and related autoantibodies is less clear [84,85]. Notably, animal models suggest nicotine-associated delays in development of arthritis [86,87].

Despite the strong and consistent associations of silica with multiple systemic autoimmune diseases, there relatively little evidence on the possible mechanisms underlying this relationship. Silica exposure can exacerbate lupus in rodent models [88]; though the mechanisms by which this occurs are not established, hypotheses include an adjuvant effect. Other evidence coming from human studies includes associations of silica exposure with dysregulation of apoptosis and balances of T-helper/Treg [89,90], and associations of silica disease-specific autoantibodies in a highly exposed population [91].

Lastly, although the solvent/SSc association seen in human studies is consistent, it lacks support from specific animal models of SSc. However, indirect evidence comes from the observed specificity of the solvent association in patients with disease-specific autoantibodies [92]. By contrast, a large body of literature provides evidence of one specific solvent, Trichloroethylene, in immune disease regulation, such as increased IFN-γ and decreased IL-4, and disease aggravation/acceleration in lupus models [93].

Table 4. Evidence and hypothesized mechanisms underlying autoimmune disease associations with smoking, silica, and solvents.

Exposure-Disease Association in Humans	Evidence on *in Vitro* and *in Vivo* Mechanisms
Smoking and seropositive-RA	Post-translational modification—antigen citrullination and anti-cyclic citrullinated peptides (CCP) antibodies [80,82]; Nicotine and Th17 activation [86,87]; Upregulation of heat shock gene expression [84] *; Disease relevant autoantibodies (RF, anti-HSP70) [85] *.
Silica and RA/SLE/SSc/ ANCA-vasculitis	Aggravation of lupus in animal models [88]; Adjuvant effect-apoptotic debris [88]; Dysregulation of apoptosis [90] *; Disease relevant autoantibodies (anti-dsDNA, anti-Ro/SSA, anti-La/SSB antibodies in silica associated SLE) [91] *; Altered CD4+/CD4+ CD25+ T cell ratio [89] *.
Solvents and SSc	Accelerated autoimmunity in animal models [93] SSc disease relevant autoantibodies (anti-Scl-70) [92] Increased IFN-γ, reduced IL-4 [93] *

* Similar observations made in animal studies.

Patterns of T and B cell skewing, antibody and cytokine profiling may be useful biomarkers of autoimmune disease or predisposition, and represent mechanisms linking the environment with disease initiation. Data in human studies on autoantibody profiles in patients, controls and high-risk

populations would allow examine exposure-associations with specific autoantibodies and whether exposures impact development or progression of disease susceptible populations (e.g., due to genetic or autoantibody profiles). Many models have been established to study mechanisms regulating autoimmune diseases, and these need to be tested in terms of their role in exposure-mediated disease.

Inclusion of more mechanistic endpoints in human autoimmune studies will entail important logistical adjustments. The collection of patient samples must provide access to live cells for *in vitro* investigations, e.g., preserving peripheral mononuclear blood cells rather than merely serum samples for future functional assays and phenotyping. This will require greater collaboration and understanding between basic environmental health scientists, autoimmune clinicians, and environmental epidemiologists.

Topic 6—How well do *in vitro* mechanisms relate to *in vivo* mechanisms in animals or effects of human exposures?

There is a growing list of examples (e.g., Hg and silica) showing concordance between mechanistic findings from *in vitro* and *in vivo* studies in laboratory animals. The types of mechanisms for which there is agreement include: apoptosis, co-stimulation, antigen clearance and presentation, cytokines and signaling. In most model systems there is good correlation between *in vitro* and *in vivo* outcomes, for example AhR modulation [94,95]. At the same time, *in vitro* systems are often too limited to recapitulate observations from animal models.

At the same time, there is inadequate data on whether most exposure-related autoimmune mechanisms are found in humans at relevant exposure levels, though there are some suggestive data regarding cytokines, lymphocyte subsets, DNA methylation and other epigenetic factors, e.g., for silica and air pollution [90,96–98]. Oxidative stress, specific environmental chemical receptors, and environmentally-induced TLR activation likely play a role in development of human disease [20,99]. While *in vitro* evidence suggests AhR ligands can affect T cell differentiation [34,100,101], additional animal *in vivo* and human studies are needed to determine the importance of these findings. Analyses of different molecular and biological outcomes following AhR activation with different classes of ligands (e.g., dioxins, PCBs, dietary flavonoids) should also be pursued as a model for context-specific environmental chemical signaling.

3. Summary and Conclusions

3.1. Overall Advances in this Field

Critical advances in the field of autoimmune disease research include a growing understanding of the contribution of antigen specific T cell subsets, B cell antibody repertoire, and antigen presentation. Specific to the role of environmental factors in autoimmune disease etiology, there has been an improved understanding of the role of specific signaling molecules (e.g., TLRs, AhR). Other advances include the emergence of new technologies for assessing molecular markers (e.g., gene, methylation, and antibody arrays), genetic manipulations in animal models to define mechanisms and potential use of the GWAS (genome-wide association studies) repository. A key finding from human studies includes the identification an environmental exposure (cigarette smoke), which interacts with genetic factors to promote specific RA phenotypes and for which there are relevant mechanistic data, which

provides a model for future studies of environmental autoimmunity integrating exposure, genotype, and phenotype.

3.2. Conclusions and Recommendations

More "translational" epidemiological studies of environmental autoimmunity are needed and should be guided by mechanisms defined in model systems and *vice versa*. An integrated, multidisciplinary approach is critical, and programs should be established to provide opportunities for collaboration and improve communication between epidemiologists, exposure scientists, and basic cellular/molecular biologists, *i.e.*, fostering of interdisciplinary research through forums, funding and training. Funding opportunities need to be specifically targeted towards autoimmunity and environmental factors. Better coordination across the diverse disciplines and agencies conducting autoimmune research may help to encourage collaborations. Such coordinated efforts may also promote a more cohesive body of knowledge through studies of multiple autoimmune diseases with similar underlying mechanisms, and shared genetic or environmental risk factors.

An important need for human autoimmune research is the availability of high-quality, validated measurement tools. Similar to efforts to characterize the genome, new technologies should be harnessed to address the critical need to characterize human environmental exposures. An environment-wide association (*i.e.*, "exposome") database linked to common questionnaires would facilitate epidemiological studies. More data are also needed on the contribution of psychosocial factors, infections, complex mixtures and susceptibility factors to the development of autoimmune diseases. Biomarkers identified by mechanistic studies should be applied to epidemiologic research in the context of relevant exposure measures. Investments in high quality exposure measures and biological markers will increase the ability to identify environmental contributions to the etiopathogenesis of autoimmune diseases.

Finally, a consensus-based approach should be developed to define autoimmune phenotypes (rather than diseases), which may improve comparability between human studies and animal models. The focus on studying diseases defined by classification criteria may limit interpretation of animal model data and the ability to identify human exposure cohorts using the broadest disease definitions. Conversely, there is a need for animal models to better represent phenotypes that occur in human diseases (e.g., CNS-lupus). Some environmental exposures may cause diseases characterized by a mixture of outcomes or multiple phenotypes that do not fit standard diagnostic criteria. Outbreak investigations should collect data to characterize the emerging phenotypes, and include the preservation and archiving of biological specimens. Long-term follow-up of affected individuals is critical to assess phenotypes that might develop with long latency.

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Parks and Humble organized and wrote the initial manuscript draft, with individual subject matter contributions of Germolec, Joyce, Miller, Pollard, Rose, and Selmi.

Conflicts of Interest

The authors declare no conflict of interest.

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EXHIBIT 153

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The World Incidence and Prevalence of Autoimmune Diseases is Increasing

Aaron Lerner[1,*], Patricia Jeremias[2], Torsten Matthias[2]

[1]B. Rappaport School of Medicine, Technion-Israel Institute of Technology, Haifa, Israel
[2]AESKU.KIPP Institute, Wendelsheim, Germany
*Corresponding author: aaronlerner1948@gmail.com

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Abstract Epidemiological data provide evidence of a steady rise in autoimmune disease throughout Westernized societies over the last decades. Multiple publications exist, describing past or actual incidences/prevalence of individual autoimmune diseases, however, long term studies on selected populations are scarce. Aims: to calculate the % increases per year of autoimmune diseases frequencies worldwide, analyze the differential increases per country and disease, and identify geoepidemiological trends. Methods: A systematic review was performed to identify incidence and prevalence of autoimmune diseases. 30 Studies from the last 30 years were identified using Medline, Google, and Cochrane Library databases. Only long-term regional or national follow-ups are reported. Results: The means ± s.d. of the net % increased /year incidence and prevalence of autoimmune diseases worldwide were 19.1±43.1 and 12.5±7.9, respectively. Rheumatic, endocrinological, gastrointestinal and neurological autoimmune diseases revealed the following annual % increases per year: 7.1, 6.3, 6.2, and 3.7, respectively. In all of these, differences between old vs new frequencies were highly significant ($p < 0.0001$). Comparing various autoimmune diseases, celiac disease increased the most and the highest increase in incidence, comparing old to new surveys is allocated to myasthenia gravis. Despite considerable variations between the countries, celiac, type 1 diabetes and myasthenia gravis frequencies increased the most in Canada, Israel and Denmark, respectively. Frequencies of the autoimmune diseases increased significantly in the West and North when compared to East and South, respectively. Conclusions: Despite multiple reports on autoimmune diseases frequencies, long-term longitudinal follow-ups are scarce. Incidences and prevalences have increased significantly over the last 30 years. Rheumatic, endocrinological and gastrointestinal autoimmune diseases in Israel, Netherlands, USA and Sweden increased the most. These observations point to a stronger influence of environmental factors as opposed to genetic factors on autoimmune disease development.

Keywords: *autoimmune disease, incidence, prevalence, surge, geoepidemiology*

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1. Introduction

Increasing evidence is accumulating for a steady rise in the frequency of autoimmune diseases (AD), in the last decades [1]. In fact, the rise in ADs parallels the surge in allergic and cancer conditions while infections are less frequent in the Western societies, creating the basis for the hygiene hypothesis [2]. Multiple sclerosis (MS), type 1 diabetes (IDDM), inflammatory bowel diseases (mainly Crohn's disease) (IBD), systemic lupus erythematosus (SLE), primary biliary cirrhosis, myasthenia gravis (MS), autoimmune thyroiditis (AT), hepatitis and rheumatic diseases (RA), bullous pemphigoid, and celiac disease (CD) are several examples [3,4]. Their relationship to socioeconomic status, their rapid increase in developed countries and observations in selected migrant populations, indicate some form of environmental impact, rather than long-term genetic influences which are driving these recent evolutionary processes [1,2,3,4,5]. Among many others, three major environmental factors, strongly related to socioeconomical status are suspected to drive these phenomena: infections, ecology and nutrition. The purpose of the present review is to calculate the surge per year of AD frequencies worldwide and analyze the differential increases of AD per country and disease identifying geoepidemiological trends. There is a plethora of publications on the incidences/prevalences of ADs in medical literature, most of them describe past or actual frequencies of individual ADs, however, long term comparative follow-up studies on selected populations, in individual countries are scarce.

2. Methods

A Medline search was performed using the following search words: autoimmune disease or syndrome, incidence, prevalence or frequency, spanning the period 1985-2015.

Special emphasis was given to the identification of increase/surge or decrease in incidence/prevalence worldwide. Suitable publications were identified by periodical scans of PubMed but also Google, and the Cochrane Library databases, were screened. Original papers, in the English language, especially those on epidemiology of autoimmune diseases commonly encountered, were identified. Foreign language papers with English abstracts were also identified. Only long-term regional or national longitudinal follow-ups are reported. Hospital archived data, specific high-risk groups or case reports, were excluded. Entities were validated and used when directly stating at least one of the abovementioned epidemiological indices. Chi-square test or Fisher`s exact test were used for categorical data and p-values less than 0.05 were considered as statistically significant. Statistical analyses were performed using the statistics software MedCalc version 15.6.1.

3. Results

30 studies were identified. The means ± s.d. of the net % increased /year incidence and prevalence of ADs worldwide were 19.1±43.1 and 12.5±7.9, respectively (Figure 1). Of interest, grouping the different ADs to disease categories, the highest net % increase per year was noted in the rheumatologic (7.1), followed by endocrine (6.3), gastrointestinal (6.2) and neurological diseases (3.7). In all of these, differences between old vs new frequencies were highly significant (p< 0.0001) (Figure 2). The table inserted in Figure 2 details the diseases and the countries included in the 4 disease categories.

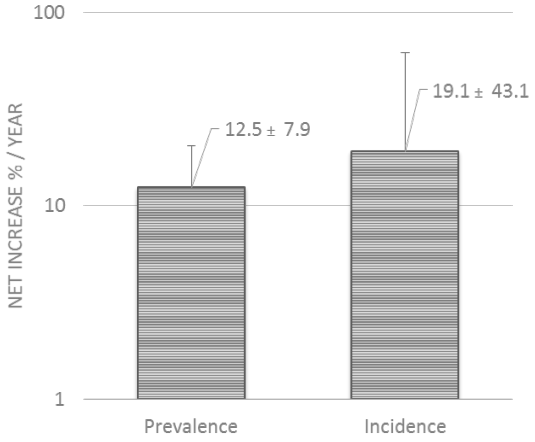
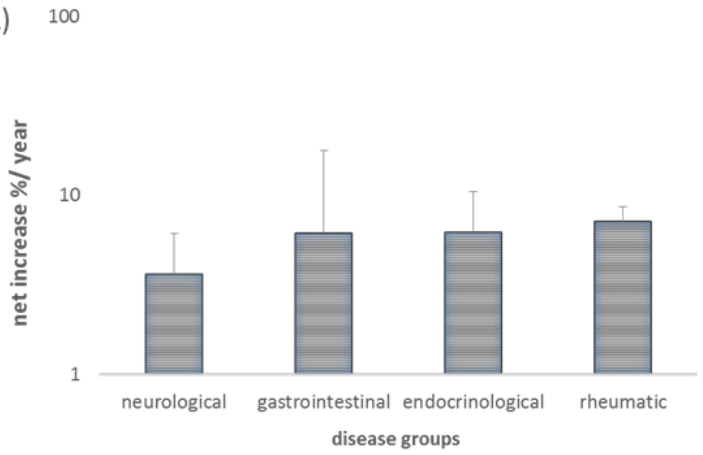


Figure 1. The net % increase/year of the incidence and prevalence of autoimmune diseases worldwide

(A)



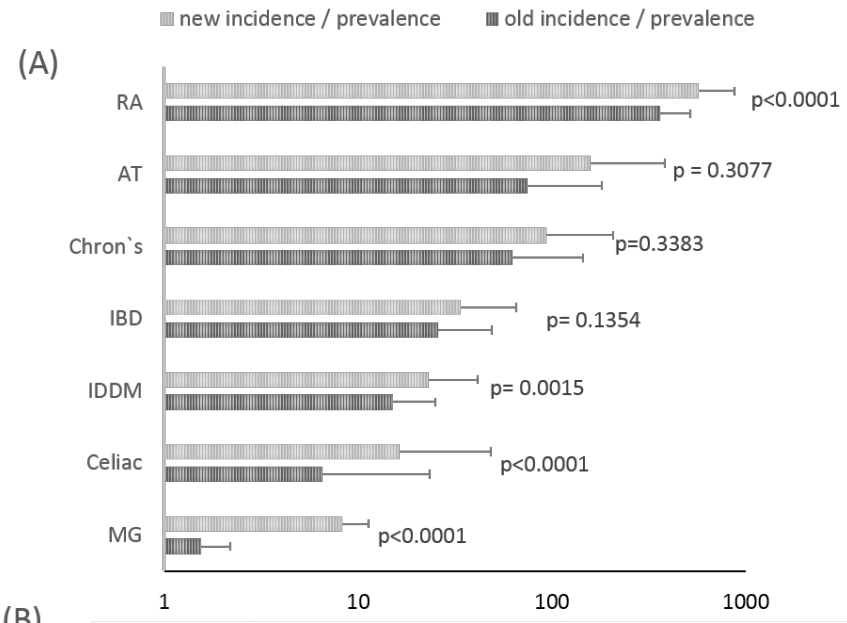
(B)

disease kind	statistical significance (p; old vs. new)	Mean net increase % /year	countries	diseases
neurological	<0.0001	3.7 ±2.5	Finland, Denmark, Norway, Italy, Spain	MS, Myasthenia Gravis
gastrointestinal	<0.0001	6.2 ±11.5	Denmark, Canada, Sweden, USA, Finland, Israel, Netherlands, UK, Czech, Scotland, Spain, Estonia, New Zealand	Autoimmune Hepatitis, IBD, Chron`s, Celiac Disease
endocrinological	0.02	6.3 ±4.2	Brazil, Canada, Israel, Serbia, Europe	Autoimmune thyroiditis, IDDM
rheumatic	0.02	7.14 ± 1.5	Canada, UK	SARD, RA, SLE

Figure 2. (A) The net %/year increases of diseases' categories. (B) The table below is detailing the different diseases and countries surveyed

The net increase/year of various diseases in specific countries and old vs. new surveys of incidence/prevalence of various autoimmune diseases, are shown in Figure 3A *and 3B*, respectively. Figure 3A shows the total net increase of Old vs. New surveys of incidence/prevalence of various autoimmune diseases, (mean time interval RA 14.5, AT 13.5, Chron`s 11.5, IBD 11.0, IDDM 17.8, Celiac 16.8 and MG 27 years, respectively), During the reported time interval. CD increased the most (26.3/year %) and the higher increase in incidence, comparing old to new surveys is allocated to MG (432%) (Figure 3A and Figure 3B). Geoepidemiologically, the following countries had high to low % increases /year of AD frequencies: Israel, Netherlands, USA, Sweden, UK, Finland, Canada, and Denmark with 12.9, 10.0, 8.8, 8.4, 7.8, 7.6, 7.3 and 6.3 %, respectively. There was no statistically significant difference between children and adults in the increase of AD incidence/ prevalence (p=0.8036). Figure 4 shows, as an example, two frequent ADs like CD and IDDM and one, less frequent one like MG surveys in various countries. A considerable variation is noticed between the countries. CD, IDDM and MG

frequencies increased the most in Canada, Israel and Denmark, respectively.



(B)

Disease	Countries	Survey
RA	Canada	1995-2010
AT	Brazil, Denmark	1992-2012
Chron`s	Sweden, USA	1991-2007
IBD	Canada	1994-2005
IDDM	Canada, Israel, Serbia, Europe	1982-2010
Celiac	Canada, Denmark, Finland, Israel, Netherlands, USA, Sweden, UK, Czech Republic, Scotland, Spain, Estonia, New Zealand	1950-2011
MG	Denmark, Norway, Italy, Japan	1950-2006

Figure 3. (A) Old vs. New surveys of incidence/prevalence of various autoimmune diseases. (B) The list of various diseases in specific countries and the years' ranges

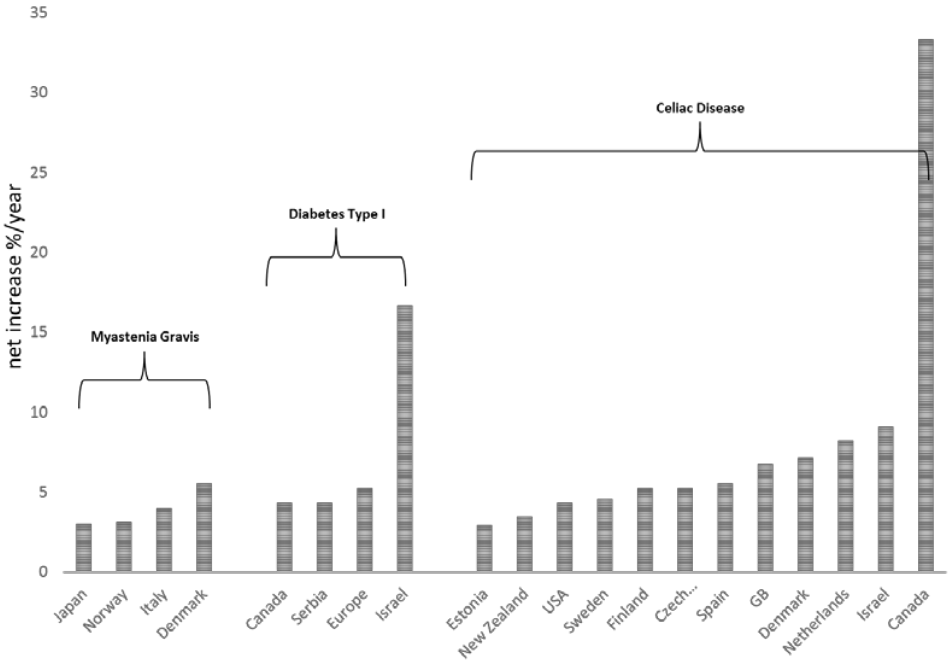


Figure 4. The net increase %/year of 3 autoimmune diseases in the surveyed countries

The geoepidemiologic trend of the net increase %/year of the various ADs is described in Figure 5. Frequencies of the ADs increased significantly in the West and North when compared to East and South, respectively.

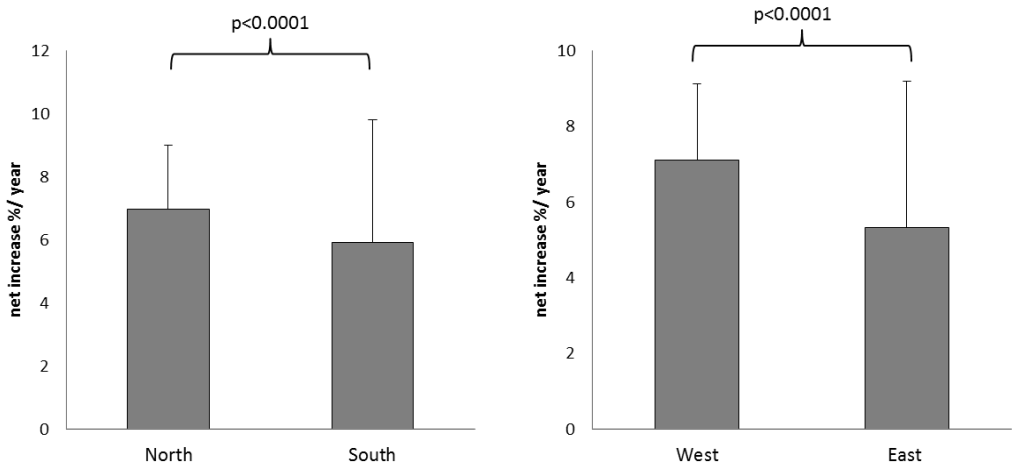


Figure 5. The geoepidemiology of the net increase %/year of autoimmune diseases

4. Discussion

Population-based estimates of the incidence/prevalence of ADs in different countries are crucial for investigating possible etiologies or influencing environmental factors or underlining risk factors.

Quantification of the likely healthcare burden and the planning of future strategies to face the ongoing epidemic of ADs is of no less importance. More so, the beneficial aspects of early recognition of ADs are well known. Multiple AD related complications can be prevented or treated when the disease is diagnosed early enough. The increased awareness responsible for the surge in diagnosed patients, needs to continue together with a lower threshold for screening high risk populations using a cheap, non-invasive and reliable antibody biomarker to prevent delays in diagnosing such a common but preventable disease [6,7,8].

The net increased incidences/prevalences of ADs in the Northern and Western countries, compared to the Southern and Eastern ones follows the global geoepidemiological trends of autoimmune diseases [9,10]. By reviewing available literature, it can be deduced that frequency of ADs have increased significantly over the last 30 years. The recent outbreak of autoimmune diseases in industrialized countries has brought into question the factors contributing to this increased incidence. Given the constancy of genetics, growing attention has focused on environmental factors, and in particular, the western lifestyle [3,4,11]. Indeed, over the last few decades significant changes in western dietary habits, environmental surroundings and pollution exposure, infectious habitat and stress load, have led to a parallel rise in autoimmune diseases. Thus, ADs occupies an important place in this environmental mosaic of autoimmunity, allergy and cancer surges [2].

The present study can serve as a platform for geoepidemiological inquiries, concerning offending/ defending environmental changes. If CD is taken as an example, how comes the surge in the disease frequency was much higher in Canada, Israel and Netherlands, compared to the low rates in Estonia and New Zealand? Considering the diseases, what environmental forces contributed to the higher surge of CD and MG, compared to IBD and IDDM? Comparing the disease categories, why are rheumatic disease surges higher than the neurological ones? Is it the infectious or stress loads? The intestinal disbiosis allocated to specific diseases? Driving openers of the intestinal tight-junctions? Or is it the increased public/professional awareness or the improved diagnosis? A vast list of questions and so few answers.

The present study has multiple biases and drawbacks. The follow up surveys were not done by the same team nor by the same methodology, some of the relevant studies might have escaped detection, the genetic makeup and the environment are different in the screened countries, the environmental forces are dynamic and most probably changed during the last decades, the actual public and professional awareness to autoimmunity and the diagnostic bio-markers have evolved tremendously.

Despite those biases, the present study highlights the significant surge in AD incidence and prevalence, detailing the variations in disease entities and the corresponding countries and substantiate the geoepidemiological trends. The present literature survey is not aiming to investigate etiologies or environmental factors affecting autoimmune induction or progression. It is expected that an improved knowledge of the worldwide distribution of autoimmune disorders will help to understand the role of different genetic factors and different environmental influences involved in autoimmunogenesis. At a public level the epidemiological studies are necessary to assess the social and economic burdens impacting the health systems in the different countries, and worldwide.

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Review Article

Complex syndromes of chronic pain, fatigue and cognitive impairment linked to autoimmune dysautonomia and small fiber neuropathy

Yehuda Shoenfeld[a,b,*], Varvara A. Ryabkova[b], Carmen Scheibenbogen[c], Louise Brinth[d], Manuel Martinez-Lavin[e], Shuichi Ikeda[f], Harald Heidecke[g], Abdulla Watad[a,h], Nicola L. Bragazzi[i], Joab Chapman[a], Leonid P. Churilov[b], Howard Amital[a]

[a] Zabludowicz Center for Autoimmune Diseases, Sheba Medical Center, Tel-Aviv, Israel
[b] Laboratory of the Mosaics of Autoimmunity, Saint Petersburg State University, Russia
[c] Institute for Medical Immunology, Charité—Universitätsmedizin Berlin, Berlin, Germany
[d] Department of Nuclear Medicine, Herlev Gentofte Hospital, Gentofte Hospitalsvej 1, 2900 Hellerup, Denmark
[e] Rheumatology Department, National Institute of Cardiology, Juan Badiano 1, 14080 Mexico City, Mexico
[f] Intractable Disease Care Center, Shinshu University Hospital, Matsumoto 390-0802, Japan
[g] CellTrend GmbH, Luckenwalde, Brandenburg, Germany
[h] Section of Musculoskeletal Disease, Leeds Institute of Molecular Medicine, University of Leeds, NIHR Leeds Musculoskeletal Biomedical Research Unit, Chapel Allerton Hospital, Leeds, UK.
[i] Postgraduate School of Public Health, Department of Health Sciences (DISSAL), University of Genoa, Genoa, Italy

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Abstract

Chronic fatigue syndrome, postural orthostatic tachycardia syndrome, complex regional pain syndrome and silicone implant incompatibility syndrome are a subject of debate among clinicians and researchers. Both the pathogenesis and treatment of these disorders require further study. In this paper we summarize the evidence regarding the role of autoimmunity in these four syndromes with respect to immunogenetics, autoimmune comorbidities, alteration in immune cell subsets, production of autoantibodies and presentation in animal models. These syndromes could be incorporated in a new concept of autoimmune neurosensory dysautonomia with the common denominators of autoantibodies against G-protein coupled receptors and small fiber neuropathy. Sjogren's syndrome, which is a classical autoimmune disease, could serve as a disease model, illustrating the concept. Development of this concept aims to identify an apparently autoimmune subgroup of the disputable disorders, addressed in the review, which may most benefit from the immunotherapy.

1. Introduction

It has been repeatedly noticed in the history of medicine that several diseases which initially are considered being separate nosological entities, with time appeared to be forms or components of a single disease.

A group of complex disorders associated with fatigue and autonomic dysfunction are in dispute, including chronic fatigue syndrome (CFS), postural orthostatic tachycardia syndrome (POTS), complex regional pain syndrome (CRPS) and silicone implant incompatibility syndrome (SIIS). There is a lack of consensus on the etiology and pathogenesis of each of them. However, these disorders share common features, which suggest that underlying alterations of the immune system take place in their pathogenesis. Constellation of typical symptoms may be very similar between these disorders. In this paper we analyze the evidence of autoimmune processes in each of the above-mentioned conditions, describe a common symptom cluster and propose possible mechanisms (namely small fiber neuropathy (SFN) and anti-G protein coupled receptors (anti-GPCR) autoantibodies (AAb)), underlying these seemingly unrelated symptoms. We provide evidence, that these mechanisms could contribute to the development of the similar symptoms in a

Abbreviation list: AAb, autoantibody; AChR, acetylcholine receptor; AdR, adrenergic receptor; ASIA, autoimmune syndrome induced by adjuvants; EBV, Epstein-Barr virus; ESR, erythrocyte sedimentation rate; CFS, chronic fatigue syndrome; CRP, C-reactive protein; CRPS, complex regional pain syndrome; GPCR, G protein-coupled receptors; HPV, human papilloma virus; IVIG, intravenous immunoglobulin; MBP, myelin basic protein; POTS, postural orthostatic syndrome; SjS, Sjogren's syndrome; SFN, small fiber neuropathy; SIIS, silicone implant incompatibility syndrome

* Corresponding author at: Zabludowicz Center for Autoimmune Diseases, Sheba Medical Center, Tel-Hashomer 52621, Israel.
E-mail address: shoenfel@post.tau.ac.il (Y. Shoenfeld).

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classical autoimmune disease – Sjogren's syndrome (SjS), which seems to share some mechanisms of pathogenesis with the described complex medical conditions. Given potential autoimmune contribution to the pathogenesis of CFS, POTS, CRPS and SIIS, we also address the efficiency of therapy targeting autoimmunity in their management.

2. Small fiber neuropathy and anti-G protein-coupled receptors autoantibodies

SFN is a subtype of neuropathy characterized by selective involvement of unmyelinated or thinly myelinated sensory fibers [1]. Its pathogenesis includes a wide range of immune-mediated, metabolic, toxic, hereditary and genetic disorders [2]. However, SFN in otherwise healthy children and young adults most often appears inflammatory, involving autoreactive B-cells [3]. With respect to autoimmunity, SFN has been reported in association with Sjogren's syndrome, celiac disease, systemic lupus erythematosus, rheumatoid arthritis, diabetes mellitus type 1, inflammatory bowel disease, sarcoidosis and paraneoplastic syndrome [1,2,4]. Some data also suppose the association with Hashimoto's thyroiditis [5,6]. Clinical symptoms of SFN may manifest as isolated sensory disturbances, isolated autonomic disorders, and mixed conditions [7]. Intravenous immunoglobulin therapy has been used increasingly with significant efficacy in the treatment of patients with apparently autoimmune SFN in two large retrospective series with similar response rates (77% and 83% of patients) [8,9]. Since 25–90% of SFN cases remain idiopathic [10], the nature of this condition requires further study.

AAb against GPCR have been reported in the last 20 years with increasing frequency in various medical conditions from neurological and cardiovascular diseases to vascular transplant rejection [11]. The fundamental characteristic of these AAb is their ability to bind cell receptors and activate (agonist autoantibodies) or inhibit (antagonist autoantibodies) intracellular signaling pathways that are normally triggered by endogenous ligands [12]. There is evidence that these antibodies belong to a functional network of natural AAb, which are present in the sera of healthy individuals in low titers, but dysregulated and probably causative in various diseases including autoimmune ones [12,13].

3. Chronic fatigue syndrome

CFS, which is also known as myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS), is a complex disease which presents with pronounced disabling fatigue, mental and physical post-exertional malaise, pain, sleep disturbances and cognitive impairment [14]. Diagnostic criteria additionally emphasize symptoms of immune system dysregulation, autonomic nervous system dysfunction and metabolic disturbances [15]. Some typical symptoms, combined in five groups, are outlined in Table 1.

Some evidence for the immune system disturbances, and in particular for the autoimmune mechanisms in CFS are summarized in Table 2. There is also an animal model of immunologically induced CFS (systemic injection of poly-I:C, virus-mimicking synthetic double-stranded RNA, which is an toll-like receptor 3 agonist) [16,17]. Interestingly, that the activation of the poly(I:C)-induced toll-like receptor 3 signaling pathway also results in the aggravation of lupus nephritis and development of autoimmune diabetes in mice [18].

3.1. SFN and anti-GPCR AAb in CFS

Definite and probable small fiber neuropathy, defined as an epidermal nerve fiber density below the 5th centile and between the 5th and 15th centile, was detected correspondingly in 30% and 13% of patients with CFS and low biventricular filing pressures of the heart during exercise [19]. These findings could underlie the pathophysiology of autonomic dysfunction in CFS.

Table 1
Common symptoms of chronic fatigue syndrome, postural orthostatic tachycardia syndrome, complex regional pain syndrome and silicone implant incompatibility syndrome, grouped into several categories.

	Autonomic symptoms	Sensory symptoms	Sleep problems, affective and cognitive symptoms	General fatigue	Inflammatory symptoms	Ref.
CFS	Dizziness, coldness of the limbs, orthostatic intolerance, increased perspiration, abdominal discomfort, nausea	Arthralgia, myalgia, headaches, light and smell hypersensitivity	Memory impairment, sleep disorders, depression, anxiety	+	Fever, lymphadenopathy, flu-like symptoms, weight loss, morning stiffness	[14,15,104]
POTS	Palpitation, nausea, dizziness, syncope and near syncope, gastrointestinal dysmotility, sicca complaints	Distal changes in sensation, visual disturbances, phonophobia	Cognitive impairment, insomnia, depression, anxiety	+	Not reported	[105–108]
CRPS	Regional edema, vasomotor and trophic changes; sometimes vasovagal syncope, nausea, constipation, indigestion, dysphagia and lack of appetite, bladder and sphincter disfunction.	Regional allodynia, hyperalgesia	Elements of a dysexecutive syndrome, sometimes global cognitive impairment, depression, anxiety	+	Regional signs of inflammation	[52,109–111]
SIIS	Orthostatic intolerance, gastrointestinal dysmotility, sicca complaints	Breast pain, paresthesia, myalgias, arthralgias	Sleep disturbances, cognitive impairment, depression	+	Lymphadenopathy	[77,112]

A significant overlap between the disorders can be noticed.

Table 2
Some evidence for the role of the immune mechanisms in the development of chronic fatigue syndrome, postural orthostatic tachycardia syndrome, complex regional pain syndrome and silicone implant incompatibility syndrome at least in subgroups of patients.

	CFS	POTS	CRPS	SIIS
Role of the triggers of autoimmunity for the onset of health problems	Infection of various pathogens, which are also known as risk factors for autoimmune diseases (EBV, CMV, HHV 6, parvovirus B19, Enteroviruses, Chlamydia pneumonia, Borrelia burgdorferi) [113–115].	Frequent onset in the setting of an infection, vaccination, physical trauma, concussion, pregnancy and surgery [107].	Immunoglobulin profiles consistent with antecedent infections by parvovirus B19 (59–94% of patients) [116], and campylobacter (up to 42% of patients) [117], which are associated with autoimmune reactions	Silicone [77]
Immunogenetic predisposition	SNP in genes related to cytokine signaling and others located in HLA and immunoglobulin loci [118,119].	HLA DQB1*06:09 (41%); A*33:03-B*58:01-C*03:02-DRB1*13:02-DQB1*06:09 haplotype (35.3%) [120]	Association with HLA-*A3,* B62, B7, DQ8, DQ1, DR13 and DR2 [121–124].	HLA-DR53 (68%); HLA-DQ2 (57%) [125]. DQA1*0102 (42%) [126].
Association with autoimmune diseases	HT, MS, SjS [127–129].	HT, SLE, SjS, RA, celiac disease [130,131].	RA [132].	Undifferentiated connective tissue disease [77], SjS, scleroderma, RA [133].
Immune cells subsets alterations	↑ CD21 +, CD 24 + and CD19 + B cells; ↑ activated B cells (CD5 +) and T cells (CD26 + HLA-DR +), ↑ activated CD8 + T cells (CD38 + HLA-DR +); ↓ CD45RA + CD4 + T cells	↑ total T cells, α/β double negative T cells, unswitched memory B cells; ↓decreased HLA-DR-to-CD69 ratio, ↑ IL-2 receptor expression in T cells [134].	↑long-lived central memory CD4 + and CD8 + T lymphocytes with increased activation of pro-inflammatory signaling pathway [135].	The capsule around the implants contain inflammatory cells that are predominantly Th1/Th17 cells, whereas T reg in the capsules are defective [136].
Inflammatory markers	Slightly but significantly ↑ CRP compared to the healthy controls [137,138].	↑ serum IL-6 compared to HC [139]	↑ serum TNFa and IL-6 compared to healthy controls [132,140]; ↑ IL1, IL6, TNFa in the cerebrospinal fluid compared to the disease controls [141]	↑ CRP in 2 months after implantation compared to the controls [142]
Autoantibodies besides autoantibodies to GPCR	ANA (4–68%), AAb against gangliosides (43%), phospholipids (38%), cardiolipin (4–95%), cytoplasmic intermediate filaments (35%), heat shock protein 60 (24%), citrullinated protein (28%), glial fibrillary acid protein of astrocytes and antibodies against neo-antigens formed by oxidative or nitrosative damage) [25,143–145].	AAb against nicotinic ganglionic AChR (16–20%) [107], ANA (25%), anti-phospholipid AAb (7%) [130], anti-thyroglobulin/anti-thyroperoxidase AAb (33%) [146]. AAb against 40 unique human heart membrane proteins and against 72 unique lipid raft proteins (proteomic approach) [147,148].	ANA (33%); antineuronal AAb (7.3%) [149]	ANA (5–87%) [112,150,151]; anti-cardiolipin AAB, ANCA, IgM-RF, anti-SSA/SSB, anti-dsDNA, anti-Scl-70 and anti-silicone AAb [77,152,153]

AAb autoantibodies, EBV Epstein-Barr virus, CMV Cytomegalovirus, CFS chronic fatigue syndrome, CRP C reactive protein, CRPS complex regional pain syndrome, HHV6 Human Herpes virus 6, HT Hashimoto's thyroiditis, IBD inflammatory bowel disease, ITP immune thrombocytopenic purpura, MS multiple sclerosis, POTS postural orthostatic tachycardia syndrome, RA rheumatoid arthritis, SjS Sjogren's syndrome, SIIS silicone implant incompatibility syndrome, SLE systemic lupus erythematosus, SNP single nucleotide polymorphisms.

AAb against GPCR are of particular interest in CFS. Higher AAb levels against M1, M3 and M4 muscarinic AChR and β2 AdR are found in CFS patients compared to controls [20,21]. The anti-M1 AChR AAb are associated with muscle weakness [21]. Elevated anti-β2 AdR AAb correlate with the immune activation including the presence of activated HLA-DR+ CD8+ T cells, elevated antinuclear antibodies, anti-thyroperoxidase AAb and IgG1–3 level [20]. This correlation can be attributed to the fact that β AdR are expressed by lymphocytes and regulate activation, differentiation, cytokine and antibody production [22]. Loebel et al. [20] observed a significant decline of anti-β2 AdR and anti-M4 AChR AAb following rituximab treatment in clinical responders. Scheibenbogen et al. have shown in a pilot study that immunoadsorption can effectively remove anti-ß2 AdR and anti-M3/M4 AChR AAb in ME/CFS and can result in a rapid moderate to marked symptom improvement [23]. Since β2 AdR are the primary adrenergic receptors that causes vasodilation in humans and anti-β2 AdR AAb were shown to be elevated also in POTS, one could assume they affect vascular regulation in CFS.

3.2. Treatment targeting autoimmunity in CFS

Positive effect of immunoadsorption is described above. Significant clinical improvements of ME/CFS symptoms were observed in two patients with long-standing ME/CFS who received adjuvant chemotherapy including cyclophosphamide for breast cancer, also in one ME/CFS patient who received chemotherapy including cyclophosphamide for Hodgkin lymphoma [24].

Three pilot ME/CFS patients without oncological comorbidities were thereafter treated with six intravenous infusions of cyclophosphamide 4 weeks apart, in two of these with a significant clinical response and an open-label, phase 2 trial with cyclophosphamide in 40 ME/CFS patients is ongoing [24]. Data on efficacy of intravenous immunoglobulin (IVIG) and rituximab remain controversial [25,26]. However, it could reflect heterogeneity of the patients in the trials with negative results with regard to the presence of AAb [27].

4. Postural orthostatic tachycardia syndrome

POTS is a heterogeneous form of autonomic dysfunction characterized by abnormal increment in heart rate (> 30 bpm within 10 min or above 120 bpm) upon assumption of the upright posture [28,29]. This increment is companied by the symptoms of orthostatic intolerance (light-headedness, blurred vision, cognitive difficulties, generalized weakness) and sympathetic autonomic dominance (palpitations, chest pain, tremulousness), which are relieved by recumbency [29]. The blood pressure remains normal as opposed to orthostatic hypotension. However, some symptoms are apparently not secondary to orthostatic intolerance Table 1. The role of autoimmunity in pathophysiology of POTS is supported by several aspects, summarized in Table 2. Interestingly, different studies reported comorbidity and high prevalence of SFN [30], CFS [31], POTS [32] and autoimmune thyroiditis [33] in joint hypermobility syndrome and other conditions associated with inherited dysplasia of connective tissue. The first animal model of autoimmune POTS is described below.

4.1. SFN and anti-GPCR AAb in POTS

SFN was detected in 20%, 38%, 45% and 50% of patients with POTS in four different studies [34–37]. Low intraepidermal nerve fiber density correlated with reduced myocardial postganglionic sympathetic innervation detected by the scintigraphy with ^{123}I-metaiodobenzylguanidine [37]. The possible explanation for abnormal increment in heart rate on the background of sympathetic denervation is a denervation hypersensitivity phenomenon [37].

AAb against GPCR were reported in the majority of patients with POTS in small cohorts: anti-β1AdR AAb (in 14/14 and in 11/17 POTS patients) [38,39], anti-β2AdR AAb (in 7/14 and in 12/17 POTS patients) [38,39], anti-angiotensin II type I receptor AAb (in 12/17 POTS patients) [40], anti-M1 and M2 AChR AAb (in 14/16 and 11/16 POTS patients) [41] and anti-α1AdR AAb (14/14 and 8/17 POTS patients) [38,39]. In a recent study of 55 POTS subjects, 89% and 51% of patients were found to have elevated AAb against α1AdR and M4 AChR respeclively. The functional effects of anti-GPCR AAb in POTS were verified in different bioassays: anti-β1AdR, anti-β2AdR and anti-M3 AChR AAb demonstrate agonistic activity and anti-α1AdR AAb act as partial antagonists [38,39]. Therefore, excessive increase in heart rate in response to the excessive vasodilation may be at least partially AAb-induced in POTS. Regarding the relevant animal model, Li et al. [42] co-immunized rabbits with peptides from the α1-AdR and β1-AdR to examine the role of adrenergic AAb in vivo in the tilt table test. The main findings of this recent study are that the adrenergic AAb induced a POTS-like phenotype in rabbits, including exacerbated orthostatic tachycardia and adrenergic receptor dysfunction that was suppressed by selectively clearing the AAb in vivo.

4.2. Treatment targeting autoimmunity in POTS

There have been no prospective trials of plasmapheresis in POTS to date. The response rate to IVIG in patients with POTS and seropositivity for one or more AAb that have been associated with autonomic dysfunction was 88,4% (23/26) in one study [9]. Antiphospholipid AAb and novel Sjögren's AAb were often present in these patients and correlated with a high response rate to IVIG administration. One clinical trial of IVIG in POTS is ongoing [43]. Plasmapheresis, IVIG and subcutaneous immunoglobulin were shown to be effective in several case reports of POTS mostly in cases coupled with other immunological disturbances [44–49].

5. Complex regional pain syndrome

CRPS is a enigmatic painful condition typically developing after injury or surgery to a limb [50,51]. CRPS is divided into type I and type II, depending on the presence of definable nerve lesion, which is absent in type I. CRPS was recognized for a long time as a pain condition with regional sensory, motor and autonomic abnormalities in the affected limb [52]. However, more recent data provide evidence for the systemic symptoms of CRPS. Thus, an increased heart rate with decreased heart rate variability in the rest and a reduction in cardiac output with an increase in total peripheral resistance during tilt test were shown in CRPS, suggesting a general autonomic imbalance [53]. These findings resemble the hemodynamic pattern of elderly individuals with the reduction of the dynamic capacity of cardiac autonomic regulation [54]. Other regional and systemic manifestations are presented in Table 1. The evidence for autoimmunity in CRPS comes from different aspects, outlined in Table 2. Animal models also provided evidence for autoimmune mechanisms of CRPS pathogenesis. Passive transfer of IgG from CRPS patients to mice with the limb trauma normally preceding the development of CRPS, enhanced mechanical hyperalgesia, edema and wound area substation P level [55]. Manifestation of allodynia, postural unweighting, and vascular changes in tibial fracture/cast immobilization model of CRPS are all attenuated when the model produced in the muMT mice that do not produce mature B cells [56]. The passive transfer of IgM but not IgG antibodies purified from CRPS model in wild-type mice reconstituted nociceptive sensitization in CRPS model in muMT mice [57] CRPS-related IgM AAb could lead to the pain via a direct interaction with their targets, or via the activation of complement by the deposition of antibodies [50].

5.1. SFN and anti-GPCR AAb in CRPS

CRPS has been proposed to be partly SFN because of the clinical similarity between the two medical conditions both in humans and in

animal models [58]. Several pathological studies have found a decrease in epidermal nerve fibers and in sweat gland and vascular innervation in skin biopsies of patients with CRPS, which was in line with the small fiber afferent pathway dysfunction revealed by quantitative sensory testing [59]. Alterations in skin innervation were seen in approximately 20% of CRPS-I patients with standart skin biopsy evaluation procedure in a more recent study, which confirmed the previous results [60].

Anti-α1 AdR AAb, anti-β2 AdR AAb and anti-M2 AChR AAb, which demonstrated receptor agonist activity in functional assays, were reported to be positive in the majority of CRPS patients but not in healthy controls [61,62]. α1 AdR are expressed by skin cells, nerves, and immune cells, and their activation may directly cause CRPS pain and symptoms of dysautonomia [62]. Both β2 AdR and M2 AChR have been reported to take part in the modulation of pain and inflammation [61]. In particular, intradermal injection of epinephrine produces a dose-dependent mechanical hyperalgesia, which is attenuated significantly by intradermal pretreatment with propranolol, a β-AdR antagonist [63]. M2 AChR on peripheral nerve endings were shown to be responsible for nociceptor desensitization [64]. There is a challenging question of the matching between the AAb presented in the sera of patients and the symptoms which are mostly regional. The upregulation of inhibitory M2 AChR in the dorsal root ganglion neurons after limb trauma ipsilateral to the nerve injury could be responsible for the "symptoms localization" through the interference of AAb binding in the physiological balance of acetylcholine and M2 AChR [65,66]. The possible mechanism underlying anti-M2 AChR AAb production in CRPS has been recently revealed. Limb nerve trauma was shown to release a potent proalgesic, immunodominant myelin basic protein (MBP) fragment, and the sequence database analyses reveal a structural homology of this proalgesic MBP fragment with the M2 AChR [66]. However, other AAb could be also responsible for regionalized trophic changes and allodynia in CRPS. Increased IgM deposition in the skin of the affected hindpaw was detected in animal model suggesting the presence of auto-antigens in skin tissue [56], which is supported by the case reports of Langerhans antigen presenting cell proliferation in CRPS-affected skin [67]. Further keratin 16 (KRT16) was identified to be elevated in abundance in the skin of mouse which underwent limb fracture and appeared to be reactive with IgM in sera from fracture mice as well as sera from CRPS patients [68]. This suggests that, despite the ubiquitous distribution of keratin 16, it may be a marker for regional autoimmunity [68]. Besides AAb, cell-mediated mechanisms could also contribute to the pathogenesis of CRPS. In the course of Wallerian degeneration, the repeated exposure of the cryptic MBP epitopes (in particular proalgesic MBP fragment), which are normally sheltered from immunosurveillance, may induce the MBP-specific T cell clones and a self-sustaining immune reaction, which may together contribute to the transition of acute pain into a chronic neuropathic pain state [69].

5.2. Treatment targeting autoimmunity in CRPS

No convincingly effective treatments exist for CRPS. Data on IVIG therapy remains controversial [50]. Plasma exchange therapy has been shown effective in reducing pain in CRPS patients, but larger trials are required to confirm these results [70]. Corticosteroid treatment was shown to cause decreased proinflammatory TNFa and increased anti-inflammatory IL1-RA concentrations in the skin of patients, which were paralleled by pain reduction [71]. In CRPS model mice treated with rituximab, the manifestation of allodynia, postural unweighting, and vascular changes were all attenuated [56]. The role of cytokines is supported by the efficacy of biological therapy: administration of a TNF-α antibody (infliximab) may produce notable reductions in CRPS symptoms in some patients [72]. However, when the entire group of patients with CRPS was assessed, independent of the individual patient responses, reduction in clinical signs of regional inflammation (based on total impairment level sumscore: ISS) was not demonstrated in infliximab treated group, although quality of life significantly improved compared to the placebo group [73]. IL-1 receptor type 1 blockade with the IL-1 receptor antagonist anakinra was reported to be effective both for prevention and for treatment of CRPS in the animal model of the passive transfer of this syndrome [74].

6. Silicone implant incompatibility syndrome

Since the introduction of silicone breast implants to the market in 1962, they has been the subject of international debate [75]. At least 49 studies in PubMed and Medline databases were identified, which deal with a clinical syndrome resulting from silicone implants insertion [76]. The typical manifestations bears considerable similarities to those of the medical conditions described above (Table 1). This condition received during the last 50 years several different names: human adjuvant disease, siliconosis, SIIS and it has also been described in the context of autoimmune/inflammatory syndrome induced by adjuvants (ASIA) [77,78]. Many patients with SIIS also fulfil the criteria for CFS/ME, fibromyalgia, sarcoidosis and/or undifferentiated connective tissue disease [77]. The indications of autoimmunity in SIIS are summarized in Table 2. Furthermore, it has been shown in animal models that silicone implantation induce an adjuvant effect and increase the susceptibility to and/or exacerbate autoimmune diseases [77].

6.1. SFN and anti-GPCR AAb in SIIS

No direct evidence of silicone gel toxicity to peripheral nerves was observed when gel was injected directly into or around the sciatic nerve of rats, although an inflammatory response followed by fibrosis was present [79]. No articles, to our knowledge, have been published specifically on SFN in SIIS. In one study the authors diagnosed a polyneuropathy syndrome in 83 of the 100 patients with SIIS based on history and physical examination [80]. EMG and nerve conduction studies were performed in 93 patients with 44 normal and 49 abnormal results. Given that EMG results are normal in SFN [3], one could suggest that SFN contributed to the sensory and autonomic disturbances in SIIS.

The study of AAb against GPCR receptors in SIIS is ongoing. In the cohort of 11 patients 9 females were positive for ≥1 AAb against GPCR and the results for the remaining 2 females were between positive and negative values ("at risk")[Personal communication]. In particular the following AAb were detected: anti-α1 AdR AAb (9/11 positive), anti-α2 AdR AAb (6/11 positive), anti-β2 AdR AAb (4/11 at risk), anti-M2 AChR AAb (6/11 positive), anti-M3 AChR AAb (6/11 positive, 5/11 at risk), anti-M4 AChR AAb (5/11 positive), anti-M5 AChR AAb (2/11 positive), anti-EtAR AAb (1/11 positive, 3/11 at risk), anti-angiotensin II receptor type 1 (2/11 positive, 2/11 at risk).

6.2. Treatment targeting autoimmunity in SIIS

Improvement induced by the removal of the inciting agent is one of the major diagnostic criteria for ASIA syndrome [78]. The explantation of the silicone breast was shown to improve silicone-related complaints in 60–80% of the patients, according to the recent review article [77]. In patients who had developed well-defined autoimmune diseases, however, the improvement was only infrequently observed without additional immunosuppressive therapy [81]. In some cases of SIIS patients respond to the medical management with various agents including hydroxychloroquine, steroids, methotrexate and plasmapheresis without the need for explantation [76].

7. Sjogren's syndrome as a real-life model of the established concept

SjS, chronic systemic inflammatory disorder, is among most common rheumatic diseases and may present as a primary condition or

as in association with other autoimmune disorders, such as rheumatoid arthritis, systemic lupus erythematosus, systemic sclerosis and primary biliary cirrhosis. SjS may have diverse manifestations that can affect virtually any organ system and arise from multiple mechanisms not restricted to exocrine gland dysfunction and lymphocytic infiltration of other organs, but also including hyperactivation and dysregulation of the adaptive and innate immune system [82,83]. This is one of the reason which make SjS an ideal model to study autoimmune diseases [84]. With respect to the several medical conditions with the overlapping symptoms, which are discussed above, fatigue is the most common systemic symptom of SjS (70–80% of patients) [83]. Neurological manifestations (including autonomic, sensory, affective and cognitive symptoms, listed in Table 1) also occur in ~70% of patients with SjS altogether [83]. This spectrum demonstrates the possibility of both peripheral and central nervous system involvement. Common clinical and laboratory aspects have been also observed between SjS and ASIA syndrome [85]. The onset of SjS is often linked to infectious agents exposure (mainly viruses) and the cases of SjS possibly associated with adjuvants (including silicone) have been described [85]. Viral infection, especially with the Epstein–Barr virus (EBV), take a prominent place among environmental factors, which trigger the development of primary SjS by causing a damage of epithelium and stimulation of the innate and adaptive immune systems [86]. EBV viral load and EBV-directed antibodies can be found in the saliva, salivary biopsies and blood of SjS patients in amounts greater than found in normal individuals [87]. Additionally, SjS patients are known to have an increased risk of development of EBV-associated lymphomas [87]. The breakdown of the host immune regulation, B-cell immortalization and stimulation of B-cell proliferation are among the mechanisms which have been recognized in both primary SjS and EBV driven malignancies [86]. Primary SjS - considering the same target cells (B-cells), molecular mimicry between the main primary SjS AAb (Ro-60) and viral protein (EBNA-1) and tropism to the same glandular structures – seems to be particularly associated with EBV infection among other autoimmune diseases, for many of which the link between the disease and EBV virus have been demonstrated [88] SjS has been also associated with another lymphotropic virus with immunostimulating effect, namely human T lymphotropic virus type I (HTLV-1), in several studies [89]. The HTLV-1 infects predominantly not only T cells but also B cells and myeloid cell lineage inducing cell activation and proliferation [90]. NF-κB pathway plays a critical role in regulating the survival, activation and differentiation of innate immune cells and inflammatory T cells [91]. HTLV-1 encodes the pleiotropic transactivator protein Tax-1 and Tax-1-mediated deregulation of the NF-κB pathway play a major role in HTLV-1 cellular transformation [92]. Green et al. [93] demonstrated in 1989 that HTLV-I tax transgenic mice showed SjS-like sialadenitis. The documentation of a lymphocytic infiltration and the Tax-1 expression in the salivary gland of patients with dry mouth infected by the virus are the main evidences that salivary gland destruction in HTLV-1 infection is linked to the immunostimulatory properties of this virus [94]. Regarding adjuvant materials, the analysis of data from 500 subjects exposed to adjuvants from the ASIA syndrome international registry showed that among the well-defined immune diseases, reported by 69% of patients, SjS was the second most common disease, following undifferentiated connective tissue disease (16.8% and 38.8% of all reported autoimmune diseases respectively) [95]. These findings are further supported by US FDA Breast Implant Postapproval Studies, which is by far the largest study of breast implant outcomes. According to this study, silicone implants are associated with higher rates of several autoimmune diseases, of which SjS had the highest Standardized incidence ratio [SIR] of 8.14 [96]. Another compound with adjuvant properties - alum - induces a SjS-like disorder in the NZM2758 mice, that is characterized by chronic salivary gland dysfunction and the presence of lymphocytic infiltrates within the salivary glands [97]. Although there were no differences in the levels of anti-Ro/La autoantibodies in sera of alum and phosphate-buffered saline treated groups in this study, the alum group showed higher antinuclear antibodies reactivity. It was noticed, that the pathogenesis of SjS shares similar mechanisms to ASIA with the up-regulation of innate and adaptive immune responses [98]. At least in a subgroup of patients with CFS, POTS, CRPS and SIIS the onset of the disease also appear to be related to some triggers with immunostimulatory effects (Table 2).

7.1. SFN and anti-GPCR AAb in SjS

SjS is the most common systemic autoimmune disorders linked to SFN [8]. While the first line treatment of SFN in SjS is aimed at symptom management, IVIG, as reported in small case series, may provide additional relief to patients with progressive or refractory symptoms [83]. These data suggest the role of immune mechanisms in the development of SFN in SjS, although virtually nothing is known about how systemic autoimmune diseases affect small fibers [8]. In respect to anti-GPCR AAb, evidence has been accumulated in the last two decades arguing for a role of AAb against M3 AChR in the development of SjS [99]. These AAb are functional and generally demonstrate antagonist-like activity, as summarized recently by Yu et al. [99]. According to this updated review of the issue, passive transfer of anti-M3 AChR AAb recognizing conformational epitopes, but not linear epitopes, into mice can impair the pilocarpine-induced secretion of saliva. These AAb also inhibit both carbachol- and nerve-evoked mouse bladder and colon contraction [99], which suggests their potential contribution to gastrointestinal and bladder dysfunction in SjS.

8. Conclusions

In this paper we focus on the evidence for autoimmunity in CFS, POTS, CRPS and SIIS, the common manifestations of these medical conditions, probable mechanisms underlying these manifestations and some therapeutic modalities targeting the immune system. We suggest that autonomic dysfunction, at least in a subset of patients, could develop due to the presence of AAb against GPCR, which were reported in each of the discussed disorders. This assumption is additionally supported by the evidence that one of these AAb (anti-M3 AChR AAb) is responsible for autonomic dysfunction in such a well-known autoimmune disease as SjS. It has been also recently shown, that the levels of anti-GPCR AAb are significantly higher in the serum of adolescent girls with the complaints which are common for POTS and CRPS after vaccination with human papillomavirus vaccine compared to unvaccinated controls [100]. These findings suggest that the increased production of anti-GPCR AAb, which were reported to be present in the sera of healthy individuals, albeit in lower amounts [13], reflects the hyperstimulation of the immune system. Regarding clinical manifestations, patients with all medical conditions described in this review demonstrate symptoms, typical both for peripheral autoimmune autonomic dysfunction and for central nervous system involvement, which is also characteristic of SjS. One could propose a role for anti-GPCR AAb not only in the development of dysautonomia, but also in the pathogenesis of the central nervous system related symptoms, since AdR and muscarinic AChR are expressed both in the peripheral and central nervous system. Indeed, a PET study demonstrated a reduction of neurotransmitter receptor binding in brains of CFS patients with high levels of anti-M1 AChR AAb in the sera [101]. These results suggest the possibility of the AAb interacting directly with the muscarinic AChR in the brain, although the cognitive function of CFS patients in this study did not differ from healthy controls. The other cause of dysautonomia and sensory disturbances, namely SFN, is also relevant for some patients with each of the medical conditions, which we address, as well as for some patients with Sjogren's syndrome. Meanwhile, SFN and anti-GPCR AAb could be interlinked. Primary sensory neurons normally express α1, α2 and β2 AdR, the expression of which is altered after injuries of peripheral nerve fibers or inflammatory processes [102]. α_1 AdR and M2 AChR are expressed also on nerve fibers distributed to the

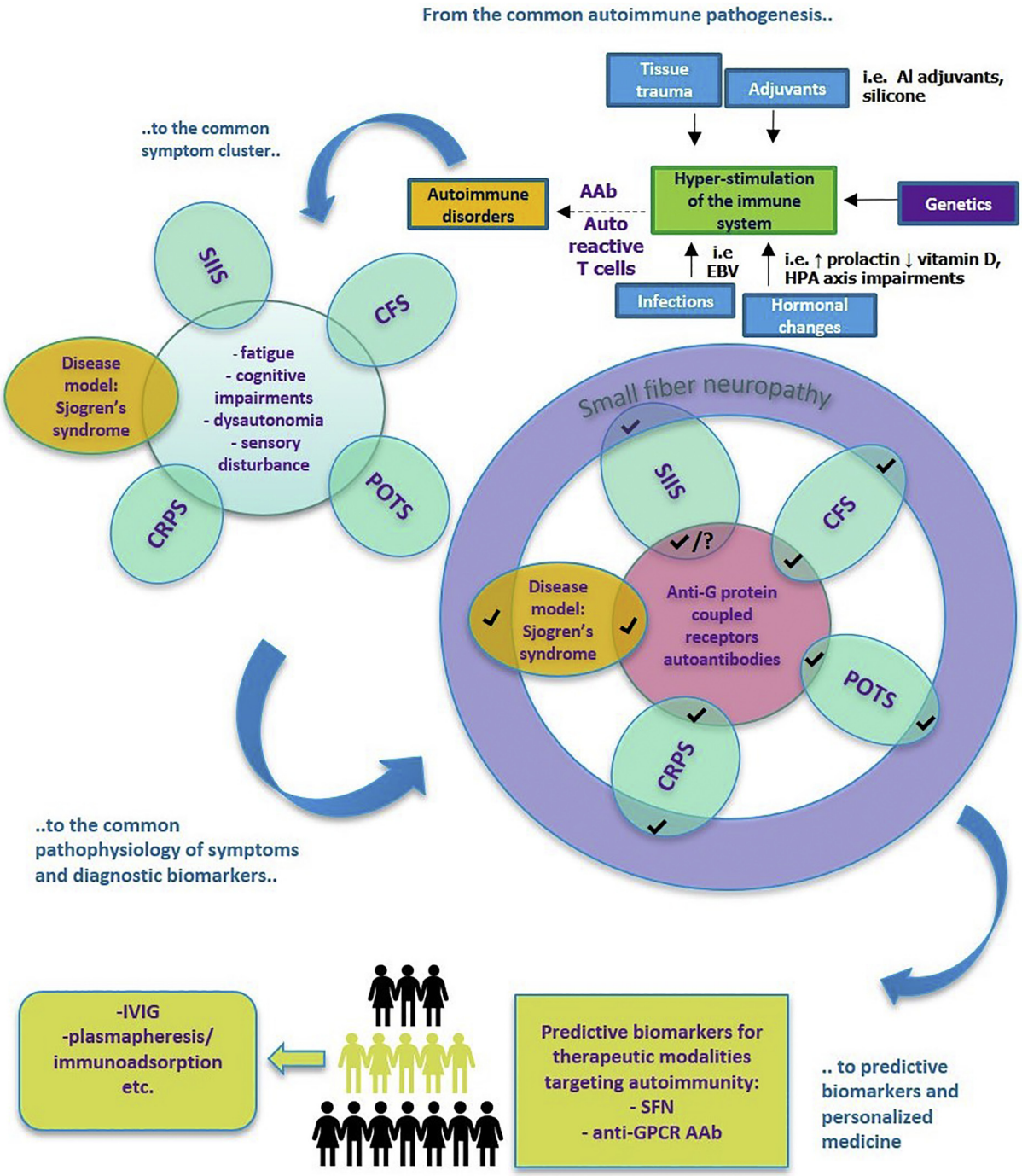

Fig. 1. Autoimmune aspects, prevalent clinical presentations and common diagnostic parameters of the overlapping clinical entities: chronic fatigue syndrome, postural orthostatic tachycardia syndrome, complex regional pain syndrome, silicone implant incompatibility syndrome.

In most cases the onset of several enigmatic medical conditions, namely CFS, POTS, CRPS and SIIS appears to be related to some triggers, which are known to provoke hyperstimulation of the immune system in the pathogenesis of autoimmune diseases, and in particular in the pathogenesis of Sjogren's syndrome. Other evidence for autoimmunity in the aforementioned medical conditions are also outlined in this review. A symptom cluster, common for all these disorders, was further identified, which includes fatigue, cognitive impairment, dysautonmia and sensory disturbance, and thus clearly demonstrates the involvement of both peripheral and central, somatic and autonomic nervous system. We subsequently propose the role of SFN and anti-GPCR AAb in the development of these common symptoms, which are also typical for Sjogren's syndrome. SFN was reported in a considerable proportion of cases of almost each of the described disorders (suspected in SIIS) and anti-GPCR AAb were detected in all of them. Further research is needed, if SFN and anti-GPCR AAb could serve as potential diagnostic biomarkers for these disorders, whose complaints are often subjective. Notably, that both SFN and anti-GPCR AAb have been described in Sjogren's syndrome. Finally, SFN and anti-GPCR AAb in the disorders addressed in the review appear to have a potential value as predictive biomarkers of benefit from the therapeutic modalities, which target autoimmunity. A pilot study provide the evidence that anti-β2 AdR and anti-M4 AChR AAb could determine the positive effect of the immunoadsorption in CFS [23]. In another recent study 77% of patients with apparently autoimmune SFN and dysautonomia have been described as the responders to IVIG [8].

AAb autoantibodies, ASIA autoimmune syndrome induced by adjuvants, CFS chronic fatigue syndrome, CRPS complex regional pain syndrome, EBV Epstein-Barr virus, IVIG intravenous immunoglobulin, POTS postural orthostatic syndrome, SFN small fiber neuropathy.

skin [64,103]. Further studies are necessary to clearly define the subgroups of patients with apparent autoimmune nature of CFS, POTS, CRPS and SIIS, who would potentially benefit most from the therapy targeting autoimmunity (e.g. immunoadsorption/plasmapheresis, IVIG, biological agents etc.). In our opinion, anti-GPCR AAb and SFN could serve as probable biomarkers for these subgroups. The brief summary of the established concept is shown in Fig. 1.

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Author contributions

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EXHIBIT 155

Myalgic Encephalomyelitis/Chronic Fatigue Syndrome

What is ME/CFS?

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a disabling and complex illness.

People with ME/CFS are often not able to do their usual activities. At times, ME/CFS may confine them to bed. People with ME/CFS have overwhelming fatigue that is not improved by rest. ME/CFS may get worse after any activity, whether it's physical or mental. This symptom is known as post-exertional malaise (PEM). Other symptoms can include problems with sleep, thinking and concentrating, pain, and dizziness. People with ME/CFS may not look ill. However,

- People with ME/CFS are not able to function the same way they did before they became ill.
- ME/CFS changes people's ability to do daily tasks, like taking a shower or preparing a meal.
- ME/CFS often makes it hard to keep a job, go to school, and take part in family and social life.
- ME/CFS can last for years and sometimes leads to serious disability.
- At least one in four ME/CFS patients is bed- or house-bound for long periods during their illness.

Anyone can get ME/CFS. While most common in people between 40 and 60 years old, the illness affects children, adolescents, and adults of all ages. Among adults, women are affected more often than men. Whites are diagnosed more than other races and ethnicities. But many people with ME/CFS have not been diagnosed, especially among minorities.

As noted in the IOM report:

- An estimated 836,000 to 2.5 million Americans suffer from ME/CFS.
- About 90 percent of people with ME/CFS have not been diagnosed.
- ME/CFS costs the U.S. economy about $17 to $24 billion annually in medical bills and lost incomes.

Some of the reasons that people with ME/CFS have not been diagnosed include limited access to healthcare and a lack of education about ME/CFS among healthcare providers.

- Most medical schools in the United States do not have ME/CFS as part of their physician training.
- The illness is often misunderstood and might not be taken seriously by some healthcare providers.
- More education for doctors and nurses is urgently needed so they are prepared to provide timely diagnosis and appropriate care for patients.

Researchers have not yet found what causes ME/CFS, and there are no specific laboratory tests to diagnose ME/CFS directly. Therefore, doctors need to consider the diagnosis of ME/CFS based on in-depth evaluation of a person's symptoms and medical history. It is also important that doctors diagnose and treat any other conditions that can cause similar symptoms. Even though there is no cure for ME/CFS, some symptoms can be treated or managed.

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EXHIBIT 156

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Review

Myalgia and chronic fatigue syndrome following immunization: macrophagic myofasciitis and animal studies support linkage to aluminum adjuvant persistency and diffusion in the immune system

Romain K. Gherardi[a,b,*], Guillemette Crépeaux[a,c], François-Jérome Authier[a,b]

[a] Inserm U955 E10 (Biology of the neuromuscular system), Paris Est Créteil University, 94010 Créteil, France
[b] Neuromuscular Pathology Expert Centre, Henri Mondor University hospital, Assistance Publique-Hôpitaux de Paris, 94010 Créteil, France
[c] Ecole Nationale Vétérinaire d'Alfort, Maisons-Alfort, France

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ABSTRACT

Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a multifactorial and poorly undersood disabling disease. We present epidemiological, clinical and experimental evidence that ME/CFS constitutes a major type of adverse effect of vaccines, especially those containing poorly degradable particulate aluminum adjuvants. Evidence has emerged very slowly due to the multiplicity, lack of specificity, delayed onset, and frequent medical underestimation of ME/CFS symptoms. It was supported by an epidemiological study comparing vaccinated *vs* unvaccinated militaries that remained undeployed during Gulf War II. Affected patients suffer from cognitive dysfunction affecting attention, memory and inter-hemispheric connexions, well correlated to brain perfusion defects and associated with a stereotyped and distinctive pattern of cerebral glucose hypometabolism. Deltoid muscle biopsy performed to investigate myalgia typically yields macrophagic myofasciitis (MMF), a histological biomarker assessing longstanding persistency of aluminum agglomerates within innate immune cells at site of previous immunization. MMF is seemingly linked to altered mineral particle detoxification by the xeno/autophagy machinery. Comparing toxicology of different forms of aluminum and different types of exposure is misleading and inadequate and small animal experiments have turned old dogma upside down. Instead of being rapidly solubilized in the extracellular space, injected aluminum particles are quickly captured by immune cells and transported to distant organs and the brain where they elicit an inflammatory response and exert selective low dose long-term neurotoxicity. Clinical observations and experiments in sheep, a large animal like humans, confirmed both systemic diffusion and neurotoxic effects of aluminum adjuvants. Post-immunization ME/CFS represents the core manifestation of "autoimmune/inflammatory syndrome induced by adjuvants" (ASIA).

1. Introduction

Vaccines and clean water have played major roles in fighting life-threatening infectious diseases. During the past century, vaccination allowed the eradication of smallpox, almost eradication of poliomyelitis and considerable decline of measles and mumps [1]. Large vaccination coverage has been shown to avoid the resurgence of several infectious diseases by reducing the number of people who can transmit the pathogens [1]. Vaccines represent the most cost-effective method of infectious disease control and appear as globally safe. However, the risk of adverse effects inherent to any effective pharmaceutical product exists for vaccines as well. Despite low signalling rates, adverse effects following immunization (AEFI) deserve special attention because (i) unlike conventional medicines, vaccines are administered to healthy

Abbreviations: AEFIs, Adverse Effects Following Immunization; Al, Aluminum; ASIA, Autoimmune/inflammatory Syndrome Induced by Adjuvants; BCG, Bacille Calmette-Guérin; CCC, Canadian Consensus Criteria; CRPS, Complex Regional Pain Syndrome; FDG-PET, FluoroDeoxyGlucose-Positron Emission Tomography; GWI, Gulf War Illness; HANS, HPV vaccination Associated Neuro-immunopathetic Syndrome; HAV, Hepatitis A Virus; HBV, Hepatitis B Virus; HPV, Human Papilloma Virus; IBS, Irritable Bowel Syndrome; ICD, International Classification of Diseases; i.m., intramuscular; LPS, LipoPolySaccharide; ME/CFS, Myalgic Encephalomyelitis/Chronic Fatigue Syndrome; ME-ICC, ME International Consensus Criteria; MMF, Macrophagic MyoFasciitis; MRL, Minimal Risk Level; MS, Multiple Sclerosis; PGW, Persian Gulf War; POTS, Postural Orthostatic Tachycardia Syndrome; s.c., subcutaneous; SEID, Systemic Exertion Intolerance Disease; SPECT, Single-Photon Emission Computerized Tomography; Th2, T helper 2; TT, Tetanus Toxoid

* Coresponding author at: Neuromuscular Pathology Expert Centre, Henri Mondor University hospital, 94010 Créteil, France.
E-mail address: romain.gherardi@aphp.fr (R.K. Gherardi).

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subjects; (ii) an unprecedented expansion of vaccination programs has been announced by America's biopharmaceutical research companies, with more than 260 new vaccines currently being developed [2]; and (iii) because AEFI could probably be largely avoided by the optimization of both vaccinology products and practices based on the understanding of AEIs pathophysiological mechanisms and risk factors [3]. A main concern about vaccine safety relates to the adjuvant compounds that are used in most vaccines to elicit strong and lasting immunization [4,5]. Particular attention has been paid to aluminum salts that were empirically introduced in vaccines by Alexander Glenny in 1926 and constitute the main class of adjuvants licensed for human and animal use worldwide [6,7].

There are two main aluminium (Al) salts used as vaccine adjuvants. Al oxy-hydroxide (AlOOH, Alhydrogel®), commonly called Al hydroxide, is composed of nanoparticles of about 2.2 nm × 4.5 nm × 10 nm which spontaneously form micron-sized aggregates having a nano-fibrous appearance at electron microscopy; while Al hydroxyphosphate (AlOHPO4, Adju-Phos®), commonly called Al phosphate, is amorphous [8]. Both adjuvants strongly potentiate the production of antibodies with very little production of cytotoxic T lymphocytes. The mechanisms underlying their adjuvant effect have only been intensely explored in recent years and remain incompletely understood [9]. Al hydroxide is a stable hydrated gel with a positive surface charge and high antigen adsorption capacities driven by hydrostatic interactions and hydroxyl group exchanges with phosphate of the ligand. Al phosphate has a negative charge, fewer hydroxyl groups, and lower adsorption capacities. The biodisposition kinetics of the two adjuvants are also significantly different: Al hydroxide is solubilized at a much slower rate, and is more avidly internalized and less toxic to phagocytic cells than Al phosphate [8].

The present review will focus on the possible implication of Al adjuvant-containing vaccines in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS). Our own experience in the field comes from clinical phenotyping of a large cohort of patients in whom an unusually long persistence of Al hydroxide is detected within immune cells at site of previous immunization, forming a specific lesion called macrophagic myofasciitis (MMF) [10–14]. In 2011, Yehuda Shoenfeld used this MMF-syndrome as one paradigm of the condition he named "autoimmune/inflammatory syndrome induced by adjuvants" (ASIA) [5].

2. ME/CFS definitions

ME/CFS is a common, often severely disabling, costly and still unexplained condition [15]. In the USA between 836,000 and 2.5 million people have ME/CFS at a cost estimated between 17 and 24 billion dollars annually [15,16]. ME/CFS ranks as "very poor" in terms of health-related quality of life [17]. ME/CFS follows a prolonged course over years, with relapses and remissions. Patients experience a substantial loss of physical and mental functional capacity and may become wheelchair dependent, housebound or bed-bound. ME/CSF is associated with an increased risk of developing B-cell non-Hodgkin lymphoma [18].

A variety of names and definitions have been proposed to designate and characterize a similar constellation of symptoms of unknown cause affecting all the major systems and organs of the body [19].

The term "Myalgic encephalomyelitis" was first included by the WHO in their International Classification of Diseases (ICD) in 1969 and ME is still listed as a neurologic disease under ICD G93.3. Besides muscle pain and exercise intolerance, ME patients often present with neurologic dysfunction [20] which was acknowledged by the Oxford criteria in 1991 that emphasized the mental fatigue and neurological background of the disease [21]. The so-called CFS was subjected to a revised definition from the CDC in 1994 [22], including profound incapacitating fatigue of unknown cause lasting more than 6 months with at least four concomitant symptoms including myalgia, arthralgia, headache, memory or concentration impairment, unrefreshing sleep, post-exertional malaise, and tender lymph nodes (Table 1) [22]. The 94 CDC (Fukuda) criteria are still widely used but they put exclusive emphasis on the trivial term "fatigue" that proved to be detrimental to patients being seen as suffering from a psychiatric or psychological illness. To address this limitation, the 2003 Canadian Consensus Criteria (CCC) document interchangeably used "ME" and "CFS", the illness being referred to as "ME/CFS" [23]. In this document, fatigue but also post-exertional malaise, sleep dysfunction and myalgia/arthralgia were included as major criteria. The symptoms lasting more than 6 months had to be associated with at least two cognitive/neurological manifestations, and at least one symptom from two of the following categories: (1) autonomic manifestations, (2) neuro-endocrine manifestations, or (3) immune manifestations [23]. In 2011, a novel classification derived from the CCC reported as ME International consensus criteria (ME-ICC), abandoned the term CFS [24], and the condition became recognized as a major health problem in children [25]. In 2015, the Institute of Medicine [15] also suggested to replace the confusing name CFS by Systemic Exertion Intolerance Disease (SEID), and proposed a case definition that included the following 4 symptoms: (1) substantial reduction or impairment in the ability to engage in pre-illness levels of […] activities; (2) post-exertional malaise, (3) unrefreshing sleep; (4) cognitive impairment and/or orthostatic intolerance. As a whole SEID diagnostic criteria are less specific than CDC 94, CCC and ME-ICC criteria [26], since they do not exclude psychiatric disorders "except in the unlikely event that all symptoms can be accounted for by them" [15].

Table 1
The 1994 CDC definition for chronic fatigue syndrome

CDC 1994 definition (Fukuda) criteria
1. Profound fatigue for 6 or more consecutive months that is not due to ongoing exertion or other medical conditions associated with fatigue
2. The fatigue significantly profoundly interferes with daily functioning and work
3. The individual concurrently has four or more of the following symptoms:
• Post-exertion malaise lasting more than 24 h
• Unrefreshing sleep
• Significant impairment of short-term memory or concentration
• Muscle pain
• Pain in the joints without swelling or redness
• Headache of a new type pattern or severity
• Tender lymph node in the neck or the armpit

In summary, the variety of proposed case definitions and their apparent discrepancies may indicate that no firm consensus on nomenclature or classification has yet been reached among different countries and researchers to designate and characterize this complex and heterogeneous neuroimmune condition with multisystemic dysregulation.

3. ME/CFS overlaps with other conditions

ME/CFS frequently overlaps with other syndromes of unknown etiology including fibromyalgia, irritable bowel syndrome (IBS), postural orthostatic tachycardia syndrome (POTS) and other syndromes [27].

Fibromyalgia share many clinical manifestations with ME/CFS, including myalgia, fatigue, headache, impaired memory, decreased concentration and sleep disturbances. This large clinical overlap has fuelled a debate on whether ME/CFS and fibromyalgia are distinct entities or merely represent phenotypic variations of a single disease including more severe fatigue in one side of spectrum (ME/CFS) and more myalgia in the other (fibromyalgia). To date, the question remains unsolved [27]. Until a global consensus is reached, and since WHO officially classifies fibromyalgia among musculoskeletal disorders (WHO ICD10M79.7) and not among neurologic diseases like ME/CFS, we still distinguish the two conditions by using the 1990 ACR criteria for fibromyalgia, that are based on tenderness on pressure (tender points) in at least 11 of 18 specified sites and the presence of widespread pain [28]. Thus, by using both 1994 CDC criteria for ME/CFS

(Fukuda) and 1990 ACR criteria for fibromyalgia [28], ME/CFS patients may exhibit, or not, fibromyalgia co-morbidity (see below). However, new criteria for fibromyalgia have been proposed with the support of Lilly Research Laboratories in 2010, revised in 2011 and 2016 [29], abandoning tender points testing and thus becoming much more inclusive [30]. The 2016 criteria include (1) generalized pain, defined as pain in at least 4 of 5 regions; (2) symptoms present at a similar level for > 3 months; (3) widespread pain and other symptoms of sufficient severity (according to a widespread pain index and a severity scale score); (4) diagnosis of fibromyalgia being valid irrespective of other diagnoses, i.e. diagnosis of fibromyalgia does not exclude the presence of other clinically important illnesses.

IBS is one of the most common functional bowel disorders. It is characterized by abdominal discomfort and disordered bowel habits. About 50% of IBS patients exhibit co-mobidity: 14% have ME/CFS, and 35-90% of ME/CFS patients have IBS [31,32].

POTS is characterized by excessive increase in heart rate (> 30 beats/min) with positional change from laying to standing up. It is linked to autonomic nervous system dysfunction. POTS is often co-morbid with ME/CFS, especially in young patients [33]. About 40% of ME/CFS patients may suffer from POTS [34] and 64% of patients with POTS fulfill criteria for ME/CFS [35].

Complex Regional Pain Syndrome (CRPS) is another chronic pain and dysautonomic condition. CRPS affects the arms, hands, legs, or feet, and manifests by intense burning pain with hyperalgesia, and dramatic changes in skin temperature, color, or texture which can no longer be explained by an initiating noxious event [36]. CRPS can be associated with body perception disturbances and movement disorders.

4. ME/CFS and vaccines

Idiopathic ME-CFS shares similarities with post-infectious fatigue syndromes [37], but no pathogen has been shown to be present in all affected patients [38], leading to the emerging view that similar symptoms could be triggered by a variety of different pathogens and toxic compounds [38–42]. Among possible triggering factors, vaccines and their multiple components have long been suspected to play a role [43,44].

4.1. First strong signal: HBV vaccine

Following the first campaign of immunization against Hepatitis B virus (HBV) in Canada, a nurse declared in 1992 on Canadian television she had acquired ME/CFS after receipt of the vaccine. Viewers were asked to report any similar experience. The name of 69 such individuals were forwarded to the Department of National Health and Welfare who committed an *ad hoc* working group to examine the question. The committee confirmed the temporal associations between immunization against HBV and ME/CFS onset but recommended to allocate no funds for research on a possible causal link on the basis of arguments such as: (1) the absence of tight time-clustering between immunization and onset of symptoms; (2) the small proportion of overall Canadian ME/CFS patients vaccinated against HBV within "*3 months*" prior to onset of symptoms; and (3) the lack of biological plausibility [45]. Since that time numerous patients with histories of ME/CFS occurring at various times after immunization against HBV have been reported in the literature [11,46,47], and the aluminium hydroxide adjuvant used in HBV vaccines was shown to be poorly degradable and to persist and disseminate in the immune system for much longer time than previously believed [7,48]. Retrospectively, these novel data and those collected for other Al-containing vaccines (see below) cast doubt on the validity of temporal and plausibility arguments used by the Canadian *ad hoc* committee.

4.2. Limitations of epidemiological studies in the assessment of ME/CFS as an AEFI

In 2012, the Institute of Medicine [49] indicated "the evidence was inadequate to accept or reject a causal relationship for the vast majority of vaccine adverse effects they examined", due to the limited number of satisfactory epidemiological studies. Admittedly, recognizing ME/CFS as an AEFI and assessing the causal role of multiple immunizations in ME/CFS are challenging tasks because all identified limitations of AEFI epidemiological studies are at play in this setting. According to IOM [50] these limitations (in italic letters) include:

- *Lack of long-term follow-up studies*, precluding detection of delayed effects;
- *Small sample sizes,* precluding detection of rare occurrences;
- *Lack of evaluation of multiple vaccinations as a whole*;
- *Lack of symptoms specific to vaccination;*
- *Multiple symptoms occurring in combination*, at high risk of being trivialized and not being recognized as forming an entity, as pointed in the lay press for ME/CFS symptoms in pre-licensure HPV vaccine safety trial [51];
- *Underreporting inherent to passive surveillance systems*, ME/CFS symptoms often being not considered dramatic enough to desserve reporting; time lapsed since exposure being highly variable and often very long, thus blurring the picture; ME/CFS being ignored/unbelieved to be possibly linked to vaccine [51]; and poor diligence in reporting being the general rule [52];
- *High vaccination rates and multiple vaccine administrations* precluding comparison with control groups that did not receive the vaccines;
- *Restricted population in vaccine trials* yielding results that may not be generalizable to the general population (e.g. trials including children or individuals without risk factors);
- *Changes in vaccine technology* precluding safety experience based on earlier vaccines to be generalizable to substantially different new vaccines (see novel Al adjuvants, below).

4.3. Insights from the Gulf War Illness in deployed and non-deployed veterans

Fortunately, privileged epidemiological studies linked to the Gulf War Illness (GWI) compared vaccinated *vs* un-vaccinated individuals, pointing out a link between multiple vaccine administration and ME/CFS [53]. At least one quarter of Gulf War Veterans returning from the Persian Gulf War (PGW) in February 1991 has reported a variety of chronic symptoms that vary somewhat among individuals but share striking similarities with ME/CFS [53,54]. As defined by the CDC, cases of multisymptom GWI must have chronic symptoms from at least two of the following three groups: (1) fatigue; (2) mood/cognition (feeling down or depressed, memory problems, difficulty concentrating, trouble finding words, problems falling or staying asleep); and (3) musculoskeletal (joint pain, muscle pain) [55]. Due to biases that regularly occurred in the course of investigations, there is still uncertainty over the exact causal factors of GWI but, among other factors, multiple vaccinations administered within a short period of time have been repeatedly suspected [56].

A cross-sectional study from UK showed strong association of CDC-defined GWI with multiple vaccinations administered during deployment (odds ratio OR 5.0; 95% confidence interval 2.5 to 9.8) [57]. Moreover, "consistent, specific, and credible relations" were found between the reported number of inoculations and health indices in UK Gulf war veterans [58]. Both vaccination against biological warfare agents (anthrax and plague with pertussis as an adjuvant to boost immune responses) and multiple routine vaccinations were associated with the CDC multisymptom syndrome in the UK Gulf War cohort [59]. In Canada, a significant association has been reported between "non-routine immunizations" and several symptom-defined outcomes in Gulf

war veterans [60]. In USA, declines in long-term subjective health of Gulf War veterans were associated with receipt of anthrax vaccine and veterans who reported more severe reactions to vaccines were more likely to report declines in subjective health [61]. The U.S. licensed anthrax vaccine (AVA; Biothrax®) is adsorbed on aluminum hydroxide and can induce MMF [62]. In addition, the vaccine has been suspected to contain squalene because of detection of circulating squalene antibodies in affected veterans [63] but no relationship was found by the Department of Defense between squalene antibodies and chronic symptoms [64]. Therefore, the role of vaccines in GWI has remained elusive, more focus being put on the role of wartime chemical exposures, such as pyridostigmine bromide used as prophylaxis against chemical warfare attacks or personal pesticide use [54].

Toxicant exposures specific to PGW, however, could hardly be incriminated in veterans that were not deployed or who served elsewhere than in the Persian Gulf, and developed, at lower rate, chronic symptoms similar to GWI. Lea Steele [53] specifically studied possible effects of vaccines in veterans from Kansas who did not serve in the PGW (non-PGW veterans): compared to unvaccinated veterans, non-PGW veterans reporting vaccine administration had significantly more fatigue/sleep problems, pain symptoms, neurologic/cognitive/mood symptoms, and gastrointestinal symptoms. Vaccinated (n = 208) *vs* unvaccinated (n = 187) non-PGW veterans had significantly much higher prevalence of GWI as defined by both Kansas criteria (*i.e.* chronic symptoms occurring in at least 3 domains: 11.5% *vs* 3.7%, OR 3.78 [1.50-9.54]) and CDC criteria (OR 2.04 [1.15–3.60]). In contrast to PGW veterans in whom GWI possibly resulted from “clusters of causes” and “combination of effects”, no other cause than self-reported receipt of vaccines was found in non-PGW veterans [53].

5. ME/CFS in Al adjuvant-induced macrophagic myofasciitis

Accuracy of self-reported receipt of vaccine is classically open to question [53], but this is not the case when vaccine files are available and when a highly specific histological marker can unambiguously assess previous immunization as it is the case in patients with MMF [10].

5.1. Macrophagic myofasciitis: a biomarker of Al adjuvant biopersistency

5.1.1. Characterization in humans

Macrophagic myofasciitis (MMF), first described in 1998 [65], is a specific Al hydroxide-induced granuloma detected at site of previous vaccine injection [10]. The lesion is characterized by sheets of large macrophages that constantly enclose submicron to micron-sized agglomerates of aluminum nano-crystals in their cytoplasm, intermingled with lymphocytic infiltrates [10]. This immunologically active lesion is not associated with giant cell formation [66].

The first 75 patients reviewed in 2003 [67] had mainly received Al hydroxide through HBV (84%), Tetanus Toxoid-containing vaccines (TT 58%) or Hepatitis A virus vaccines (HAV 19%), usually administered in combination. The proportion of vaccine types changed markedly with time: our last 70 patients, collected from january 2013 to june 2018, of whom 56 had complete vaccine files, had mainly received, as their last immunization or within 10 years before biopsy, TT-containing (86%, 48/56), followed by HBV (27%, 15/56), HAV (11%, 6/56), HPV (11%, 6/56), and meningitis C (< 1%, 1/56) vaccines. TT-containing vaccines (mainly Diphtheria-Tetanus-Poliomyelitis, Diphtheria-Tetanus-Acellular Pertussis, and Diphtheria-Tetanus-Acellular Pertussis-Poliomyelitis), administered alone or in various combinations with non-TT vaccines, was the most recently administered vaccine in 64% of cases. These data clearly indicate that MMF is not specifically associated with HBV vaccine.

MMF is rarely detected despite the huge number of immunized individuals, but, shortly after its initial description, striking increase of MMF detection rate was noted by all French specialized centers [10,68–70]. Prominent detection of MMF in France from 1993 was likely due to: (1) the recommended replacement at the beginning of the decade of the subcutaneous (s.c.) by the intramuscular (i.m) route of immunization; (2) the huge immunization campaign against hepatitis B conducted in France, with 90 millions of doses sold in ten years, two thirds of which being administered to adults; and (3) the usual choice of the deltoid muscle for biopsy in adults in France while this muscle is not preferentially chosen in other countries [10]. For example, only 10% of adult muscle biopsies have been performed in the deltoid muscle in one US center, MMF being detected in 1% of these biopsies [14]. Despite the usual routine choice of an incongruent biopsy site precluding detection of most MMF cases, the lesion could be occasionally documented in Australia [71], Brazil [72], Germany [73–76], India [77], Ireland [78], Israel [79], Italy [80,81], Portugal [13,82,83], Saudi Arabia [84], Spain [85], UK [86,87], USA [13,62,88,89], and several other countries (personal communications to RK Gherardi), thus indicating that MMF is not specific to France.

5.1.2. Clearance of the lesion in animal models

The lesion has been reproduced experimentally in mice, rats, monkeys and sheep [10,90–92]. In sharp contrast to the quick elimination of soluble Al injected intravenously [93], intramuscular injection of isotopic Al hydroxide is associated with much slower elimination of Al in urine, accounting for 6% of the injected dose 28 days after injection in rabbits [94]. Based on data of this unique experimental toxicokinetic study, the duration for complete translocation of solubilized Al ions from the injected site to blood was calculated to be 5.5 months for Al hydroxide [95]. Consistently, experimental MMF invariably shrinks over time [91]. In monkeys, MMF induced by DTP vaccine injection - corresponding to 14- to 21-fold the human DTP-equivalent dose of adjuvant- was entirely cleared out from the injected muscle before 6 months (Al phosphate 100%, Al hydroxide 25%) and between 6 and 12 months (Al hydroxide 25%) after immunization [90]. Similarly to these animal models the vast majority of humans appear to clear out the adjuvant from the injected muscle within months, but in a small proportion of them MMF may be observed up to > 15 years after vaccination (see below).

Thus, longstanding MMF should be considered as a biomarker assessing difficulty of some individuals to clear out the adjuvant from their body [96]. For this reason we recommend to perform biopsy seeking MMF at least 18 months after the last immunization to allow the assessment of the unusually long persistence time of the lesion.

5.1.3. Genetic susceptibility factors

It is generally assumed that subpopulations of humans exist that are more sensitive to certain chemical or particulate exposures than the average population. Individual susceptibility factors usually reflecting specific “genes x environment” interactions likely explain why only a small proportion of vaccinees develop adverse effects. The exact ground of such individual susceptibility may include: (1) genetically-determined impairment of cellular defenses against the prooxidant effects of Al [70]; (2) HLA subgroups, such as HLA-DRB1*0, known to favour autoimmune responses [98]; (3) ageing and/or genetically- based inter-individual differences in production of the chemoattractant MCP1/CCL2 necessary for dissemination of immune cells loaded with Al adjuvant particles [99,100, 101]; (4) individual difficulty to clear out Al adjuvant from immune cells [48]. The size of experimental MMF lesions in rats markedly differs according to the genetic background [91] and in humans conspicuous inter-subject variability exists for aluminum elimination [97].

An intracellular mechanism called xeno/autophagy is instrumental in solubilisation and biodisposition of internalized mineral particles [102], as well as in metal toxicity [103] and many crucial functions in the immune and central nervous system. Promising preliminary data have been obtained by DNA screening of 34 genes directly involved in the xeno-autophagy machinery (in collaboration with Baharia Mograbi, IRCAN, Nice University, France), suggesting that MMF may reflect

genetically-determined inability of some individuals to efficiently dispose of injected aluminum adjuvants (patent deposited), paving the way for development of genetic tests predicting an increased risk of intolerance linked to adjuvant retention.

5.2. Macrophagic myofasciitis syndrome: a post-vaccinal ME/CFS

5.2.1. Clinical characterization

From 1994 to 2012, we have seen 583 adult patients with MMF in the Paris Est University Hospital Henri Mondor, Créteil, France [104], including patients duely registered in the Henri Mondor hospital clinical database (350 registered from 1994 to 2018) and additional patients followed by other centers but punctually referred to us for testing, biopsy, biobanking, certificate delivery, or legal expertise. There is no national MMF registry, but a total number of 445 MMF cases had been officially notified to the French national pharmacogilance system on October 31, 2015. Except cognitive dysfunction data which were specifically collected in our center but not captured at the national level, local and national sources showed similar characteristics of MMF patients, as also confirmed by series from Portugal [13] and USA [14].

The Henri Mondor hospital 1994-2018 MMF database indicates the following characteristics of patients: mean age 52.5 years, female predominance (71%), mean of 5 (range 1–12) Al-containing vaccine shots in the 10 years preceding biopsy (vaccine files available in 236/350), mean Al adjuvant persistence time 71 months (range 9–237 months, assessed by histology and Morin stain for Al), mean delay from clinical onset to biopsy 67 months, main symptoms including myalgia/arthralgia (92%, 309/335), fatigue (86%, 298/347) and cognitive complaints (82%, 154/187). Other symptoms include headaches (49%), dyspnea (51%), abdominal pain (30%), ocular symptoms (34%), thoracic pain (32%), urinary symptoms (21%), and fever (23%) [67].

Patients had received intramuscular administration of Al-containing vaccine prior to the onset of muscular symptoms [13,67]. The delay before first symptoms could substantially vary, ranging from 0 to 72 months in our initial series. Median delay from last vaccination to first myalgia was 11 months, 30% of patients complaining of myalgias within 3 months, 31% from 3 to 12 months, 19% from 12 to 24 months, and 20% after 24 months [67].

In our center, one third of myalgic patients that have received Al hydroxide-containing vaccines in the past 10 years had MMF at deltoid muscle biopsy [105]. Myalgia onset commonly occured after an exercise of unusual intensity, often in lower limbs with progressive extension upward, becoming diffuse at time of biopsy. Myopathic electromyogram and CK elevation are found in less than one half of patients [67].

Fatigue usually had deep impact on the daily life and most of affected patients had rapidly get out of their work after a few months. Cognitive alterations were found to be stereotyped at systematic testing, impacting attention, working and visual memory, and inter-hemispheric connexions, and were neither attributable to chronic pain nor to depression [106–108]. Standard brain MRI was usually normal but functional neuroimaging alterations were conspicuously found, including: (1) focal brain perfusion defects assessed by SPECT (single-photon emission computerized tomography), well correlated to both attention/memory alterations and inter-hemispheric dysconnexion [109]; (2) a characteristic pattern of posterior cerebral glucose hypometabolism assessed by FDG-PET (fluorodeoxyglucose -positron emission tomography) scanner, involving occipital cortex, hippocampus and cerebellum, and predictive of MMF detection at muscle biopsy [110–114].

The main symptoms of patients with MMF, *i.e.* arthromyalgia, fatigue and cognitive complaints, could occasionally occur in isolation for some time. However, a large majority of patients have multiple symptoms with international criteria for ME/CFS being fulfilled in at least 50% of cases [11,13]. The condition also meets the CDC criteria of multistymptom GWI [5].

5.2.2. Consistency analysis

Occurrence of myalgia/arthralgia, chronic fatigue and cognitive alterations in patients with longstanding MMF is very unlikely to represent chance association and rather forms a consistent syndrome. This is assessed by:

(1) *post-immunization* onset of symptoms;
(2) *similar structure of symptoms observed in independent French and foreign MMF series* [10,13,14,70];
(3) *significant association between "myalgia" and "MMF"* in patients who had deltoid muscle biopsy performed in French myopathologic centers before publication of the cause of MMF [10];
(4) *significant clinical differences depending on the presence/absence of MMF in deltoid muscle biopsy among myalgic vaccinees:* in sharp contrast to non-MMF myalgic patients, only a minority of MMF patients had fibromyalgia according to ACR 1990 criteria ($\geq$ 11 tender fibromyalgic points) (55.5 *vs* 16.6%, $p < 0.04$), and MMF patients had much more CNS involvement as assessed by delayed evoked potentials (38.5 *vs* 5.7%, $p < 0.01$). These data indicate that MMF and non-MMF patients differ by more than the simple detection of MMF [105];
(5) *significant association between "chronic fatigue" and "MMF" in a case-control study* ordered by the French drug agency: fatigue was both "more frequent and more severe in patients with MMF than without MMF in deltoid muscle" [115]; in sharp contrast to diseased controls, MMF patients had strikingly little medical antecedents, further indicating that cases differ from controls by more than the simple detection of MMF [115];
(6) *highly consistent functional neuroimaging changes (SPECT and FDG-PET scanner) in MMF patients, not attributable to chronic pain or depression,* indicating that MMF is detected in an homogeneous subset of patients with stereotyped condition [109–114];
(7) *perfect similarity of the MMF syndrome with the GWI multisymptom complex* [116] defined by CDC [55] which has been uniquely associated with vaccine exposure in military personnel non-deployed in the Persian gulf [53].

In summary, MMF is typically detected in adult patients with a homogeneous subset of ME/CFS of post-vaccinal onset.

6. ME/CFS and related conditions following HPV vaccines administrations

6.1. Current debates

In the absence of deltoid muscle biopsy that could determine if longstanding MMF was present or not, a number of papers have reported combinations of myalgia, arthralgia, chronic fatigue, cognitive dysfunction, unrefreshing sleep and neurovegetative alterations (meeting international criteria for ME/CFS, fibromyalgia, POTS, CRPS, or described as somatoform manifestations) temporally associated with administration of multiple injections of Al adjuvant-containing vaccines, in Australia [117], Canada [118], Denmark [119,120], Italy [121], Israël [47,122], Japan [123–125], Mexico [126], and USA [127]. Whether or not, such temporal associations may indicate possible causal link has been the matter of continuous controversy.

Controversy first emerged following the HBV vaccine campaign, running in parallel for ME/CFS [47] and multiple sclerosis (MS) [128–130], which occasionally occurred in combination [131] following immunization. Most epidemiological studies failed to substantiate the unprecedented increase of post-HBV immunization MS claims [129,130], but these short term studies overlooked the "t" factor. The possibility of delayed onset of clinical symptoms after HBV immunization was pointed out by Hernán [128]: in a unique case–-control study conducted on the long-term in the British population he found an increased risk of MS (OR 3.1; CI 1.5–6.3) in the 3 years

following HB vaccination. Interestingly, there was no increased risk during the first year after immunization (OR 1.8; CI 0.5–6.3), as reported in previous short-term studies, but the increased risk of developing MS became obvious at 2 and 3 years after immunization (OR 4.1; CI 1.3–13.6). This result is in keeping with the reported increase of overall incidence of MS in France following the HBV immunization campaign [129].

Then, controversy culminated with the debate about HPV vaccines safety [132]. Similarly to HBV vaccine that was associated to a disproportionately high level of AEFIs signalling compared to other vaccines, e.g. 5 fold for MS signaling [130], HPV vaccine programs in different parts of the world were associated to a 10 fold higher incidence of AEFIs signalling compared to other vaccines [125,133]. Post-HPV vaccine AEFIs approximately accounted for 1 per 1000 inoculations in Spain [132] and 1 per 1000 vaccinees in Canada [134]. However, HPV vaccine security has been endorsed by international regulatory health agencies [135,136] (EMA, WHO) and the Cochrane collaboration [137]. Nevertheless, criticisms were made, pointing out conflicts of interest with the industry and disclosing numerous methodological flaws in both HPV vaccine safety studies themselves and the systematic reviews grounding institutional reassuring claims [138-142].

6.2. Limitations of epidemiological studies on ME/CFS in HPV vaccine receivers

For the present review, we examined in detail the two studies indicating no evidence that the overall occurrence of CFS in HPV vaccinated girls was different from that expected in the same age groups [144,145].

Donegan *et al.* [144] analyzed the occurrence of ME/CFS in UK girls immunized the bivalent HPV vaccine (Cervarix®) that contains Al hydroxide mixed with 3-O-desacyl-4'-monophosphoryl lipid A.

In the first part of their study, *i.e.* "observed *vs.* expected analysis", Donegan *et al.* [144] considered that underreporting levels could range from 0% to 90%. However underreporting of adverse drug reactions is higher, with a median rate of 94% found across 37 studies in a systematic review, including a maximal level of 98% in UK [146], similar underreporting levels also applying to vaccines [147]. Moreover, the highest (90%) underreporting hypothesis tested by Donegan *et al.* [144] was associated with striking above-the-threshold signal of ME/CFS in Cervarix^{R} receivers. The authors did not retain this result and preferred to focus on lower ($\leq$75%) levels of underreporting that were not associated with increased signal. This is a highly debatable choice given the documented failure in the assessment of ME/CFS symptoms in HPV vaccine receivers [51], vaccine damage and ME/CFS being concepts to which the medical establishment remains generally hostile.

In the second part of their study, *i.e.* "self-controlled case series", Donegan *et al.* [144] has estimated the risk of ME/CFS in the year after the *first* Cervarix^{R} injection (first of three given in 6 months) and paid no attention to other Al-containing vaccines. However, the reported time to adverse effects after the first HPV vaccine dose ranged from 1 day to 51 months (mean 10.7$\pm$11.6 months) in a series of 72 Japanese girls [125] and from 1 day to 43 months (mean 14.0 + 11.6 months) in another series of 35 girls [148]. In the same way, median time of first symptom onset was "11 to 12 months after the *last* Al hydroxide-containing vaccine administration" in our ME/CFS cases [105], making likely that a substantial number of ME/CFS cases possibly linked to Al hydroxide-containing Cervarix^{R} injection have been missed in the Donegan study, even when the risk window was extended to 18 months (*i.e.* about 12 months after the *last* injection, a time after which nearly 40 % of our MMF patients developed their first symptoms). At last, the authors felt there was "*no reason to suspect that girls with fatigue syndrome would be less likely to receive HPV vaccination*", thus precluding a healthy vaccinee bias. This does not respect good practices in the field of vaccine safety science. It is well established that underlying conditions that predispose to medical outcomes suspected to be vaccine adverse effects are linked to lower vaccine uptake [149]. For example, uptake of HPV vaccine dropped in from 82.3% to 39.4% when norvegian girls were diagnosed with ME/CFS prior to immunization [145]. This is an important bias, emphasized by experts from both the Japan Institute of Pharmaco-vigilance [138] and from the CDC who stated "*studies that do not control adequately* [healthy vaccinee bias] *will tend to underestimate any real risks associated with vaccination*" [149].

Feiring *et al.* [145] studied the quadrivalent HPV vaccine (Gardasil®) which is adjuvanted by amorphous Al hydroxy-phosphate sulfate (Merck proprietary AAHS) that significantly differs from Al hydroxide [150]. The study found that people more at risk of developing ME/CFS tended to avoid the vaccine. Despite this healthy vaccinee bias, the authors remained confident in the reliability of their finding of no increase of ME/CFS in vaccinated *vs* non-vaccinated girls (after adjustment for age), because there was no higher increase of ME/CFS in girls than in boys while only girls had received the HPV vaccine. Indeed, a similar increased rate of ME/CFS was found in girls and boys during the studied years, and remained unexplained. Possible implication of other vaccines administered to both genders in this increase has apparently not been evaluated. Another critical point resided in the challenge of discriminating comorbid conditions from ME/CFS. Reported Gardasil® adverse effects have been fragmented into multiple subcategories, such as POTS, CRPS, somatoform syndrome, dysautonomic syndrome, ME/CFS, fibromyalgia, HPV vaccine syndrome, and HPV vaccination associated neuro-immunopathetic syndrome (HANS). It may be, therefore, misleading to compare idiopathic ME/CFS to the HPV vaccine syndrome since its symptoms are only partially co-morbid with ME/CFS. The variety and inaccurate designations of HPV vaccine adverse events has been viewed as a major obstacle in reporting [143,151].

Of note, MMF syndrome, which is caused by an adjuvant substantially different from AAHS, is much less polymorphic than the HPV vaccine syndrome, both POTS and CRPS being nearly never documented in the setting of MMF. In the setting of HPV vaccine, the traditional observational epidemiological approach has been complicated by the lack of a case definition for the multiple symptoms that constitute the signal, making highly desirable novel epidemiological approaches [151].

For example, the Uppsala Monitoring Center developed a novel data-driven cluster analysis of HPV vaccine reports in Vigibase®, the WHO international database, that identified natural groupings based on terms used to report AEFI. The analysis revealed clusters of serious AEFI more frequently reported in HPV vaccine reports compared to non-HPV vaccine reports in the same sex and age band. They included headache, dizziness, fatigue and syncope that sometimes contained diagnostic labels of POTS, CRPS and CFS but most often lacked explicit diagnoses, pointing out marked underestimation of the signal by traditional post-marketing safety evaluation [151].

In Japan, Osawa analysed temporality of the AEFI on the population level, and showed that the peak of post-vaccination syndrome onset followed the peak period of HPV vaccination and that novel cases were not seen after 14 months from withdrawal of the government recommendation for HPV vaccination [125].

Another approach was based on careful analysis of HPV vaccine randomized trials by Martinez-Lavin [140], who among several disquieting results, pointed out the shocking fact that pre-licensure randomized trials were almost always made against Al adjuvants-containing comparators - not inert placebos. The only one quadrivalent HPV vaccine double blind trial using inert saline placebo showed 0.4% (5/1165) of serious adverse events in HPV-vaccinated subjects *vs* none (0/584) in the inert placebo group [140]. None of these effects were considered vaccine-related, but a potential role of Al adjuvants was further suggested by the largest Gardasil® trial in which receipt of the 9-valent vaccine that contains 500 μg Al adjuvant AAHS was associated with significantly higher rates of both local and systemic compared to

the 4-valent vaccine that only contains 225 μg AAHS [140]. Such a safety imbalance between the two Gardasil® vaccines suggesting a dose-effect was recently confirmed by a FDA report showing higher rates of injection-site reactions, multiple sclerosis and spontaneous abortions with the 9-valent vaccine [152].

7. Insights from experimental studies

In addition to limitations of epidemiological approaches, inadequate understanding of biologic mechanisms underlying vaccine adverse effects is a major factor hindering assessment of causality [49]. This led the Institute of Medicine to declare "*the value of dialogue between both epidemiologic and mechanisms approaches cannot be overstated. These conversations between different types of research can be difficult, but the results are worth it*" [49].

Indeed, the history of vaccines has been largely built on an empirical basis during the last century. This was specifically the case for the Al adjuvants that were first introduced in vaccines in 1926 but remained administered a very low rate to the general population until 1985 when they began to be massively injected along with the introduction of Al-containing DTP, HiB, HBV, HAV, pneumoccocus, meningoccocus, HPV and other vaccines [153]. This was done without clear knowledge of the injected Al adjuvant fate and, since that time, very little effort has been made to clarify the question [7].

Therefore, the classical hypotheses on the injected Al adjuvant fate were tested in mouse models in our lab.

7.1. Old dogma turned upside down

It was classically believed that once injected in the tissue, Al adjuvants and the vaccine antigens adsorbed at their surface form an extracellular depot at site of injection, then progressive solubilisation of the particulate adjuvant was thought to take place, mediated by Al chelating acids present in the interstitial fluid, causing gradual desorption of the vaccine antigen and the observed adjuvant effect [94]. In the frame of this pre-conception it was claimed that Al adjuvant inocuity could be inferred from the little amount of injected Al and rapid elimination of soluble Al in the urine [94]. None of these dogmatic hypotheses proved to be correct when experimentally tested in our lab.

We first showed that, in contrast to previous belief, Al hydroxide particles injected in muscle are not solubilized in the interstitial fluid, and vaccine derived Al is not quickly eliminated in urine: instead, this nearly insoluble particulate compound is quickly captured by monocyte/macrophage lineage cells [99] and persists within these cells from many months after injection in animals [91] to up to > 15 -years in some human beings with MMF. As stated above (Section 5.1.2) and below (Section 7.2.2.) results of the sole experimental study on the toxicokinetics of Al adjuvants [94] are incompatible with rapid biodisposition and renal elimination of vaccine-derived Al [7]. In addition, theoretical models based on Flarend's pre-conceptions and short-term results are flawed [7]. For example, Mitkus *et al.* [95] proposed a model to assess the risk of Al vaccines in infants, by reference to an oral minimal risk level (MRL) extrapolated from animal studies. They only considered solubilized Al, with erroneous calculations of absorption duration. Systemic Al particle diffusion and neuro-inflammatory effects were omitted. The MRL they used was both inappropriate (oral Al lactate *vs.* injected Al adjuvant) and too high regarding recent animal studies indicating that MRL should be reduced by at least 7 fold [7]. In summary, systematic analysis of the available "reference" studies has revealed complete failure to support their reassuring claims, and make mandatory novel experimental studies of Al adjuvant toxicokinetics conducted on the long term and in a sufficient number of animals, under the aegis of health agencies [7].

We also showed that, in contrast to the classical depot formation hypothesis, Al particles do not stay entirely localized in the injected tissue in mice, but, instead, can disseminate within immune cells to the regional lymph nodes and then to more distant sites and to the brain [99,154] where they persist as long as in the injected muscle [155]. The distant organs showing collections of Al particle-loaded cells include the regional lymph nodes, spleen and liver, and the brain in which they enter in using a CCL2-dependent Trojan horse mechanism and from which they do not recirculate [99]. In line with our studies, it has been shown that removal of the vaccine injection site as early as 2 hours after administration has no appreciable effect on the immunological response in rats, thus indicating that the adjuvant exerts its effect remote from the injection site which invalidates the depot formation theory [156]. This is an important point since there appears to be a fine balance between the efficacy of Al adjuvants and their potential toxicity, and these may be one and the same effect [157]. Obviously, the potential toxicity of Al adjuvants depend on whether the bioactive nanomaterial remains localized at injection points or rather scatters and accumulates in distant organs and tissues [99,154]. The latter appears to take place since systemic diffusion of Al adjuvants reported in mice [99,154,155] was also documented in sheep that developed Al-induced granulomas persisting at injection sites associated to similar large macrophage infiltrates with increased Al levels in the draining lymph nodes [92].

We finally showed that, in contrast to previous belief that innocuity of Al adjuvants can be inferred from the low quantities of Al^{3+} injected with vaccines ("the dose makes the poison" paradigm), neurotoxic effects of Al hydroxide particles (Alhydrogel®) respond to a non-linear dose response curve with selective toxicity of the lowest tested dose [158]. Compared to high concentrations, that were associated with spontaneous formation of large particle aggregates and surprisingly caused no toxicity, the lowest Alhydrogel® concentration selectively caused cerebral Al accumulation, microglial activation and long-term neurotoxicity in mouse. Interestingly, the toxic low concentration uniquely formed small 'bacteria-size' agglomerates that were presumably easier to capture and to transport to distant sites [158]. It is, therefore, likely that toxicity of particulate adjuvants taken up by immune cells obeys the specific rules of small particle toxicology rather than any simplistic dose-response relationship.

In summary, our experimental results suggest that capture and long-term Al hydroxide biopersistency within phagocytic cells is a prerequisite for its neuromigration and neurotoxicity in mouse.

7.2. Comparing toxicology of different forms of Al and different types of exposure is incorrect

It is often stated that the intake of oral Al is higher than the quantity of Al injected with vaccines which, therefore, could cause no harm. This superficial statment ignores the marked differences of Al fate in the two situations.

7.2.1. Oral Al (initial value 100%)

In case of healthy intestinal barrier, 99.7% of oral Al is eliminated in faeces and only 0.3% can cross the barrier, in an atomic form, and become bound to blood transporters like transferrin, albumin and citrate. Then > 0,2% is quickly eliminated in the urine [93,159] and the remaining < 0.1% is distributed to the whole body. Of note the body spaces comprise 41% of cells (35 trillions cells) and 59% of extracellular spaces [160]. Preferential Al deposition occurs in bone extracellular matrix, but other organs may show deposits, mainly extracellular deposits [161]. Intoxication may occur on the long term especially in case of combination of high intake with intestinal barrier alteration and/or renal insufficiency.

7.2.2. Injected Al hydroxide (initial value 100%)

In contrast to oral Al, 100% of the initial adjuvant dose crosses the natural barrier with the needle and reaches the internal milieu. In case of Al hydroxide, Al is in a poorly soluble particulate form [8], and more than 6% of the injected Al is quickly eliminated in the urine [94], the remaining 94% being avidly captured by macrophages and transported

to distant organs where Al particles remain mainly intracellular [99]. Thus, in contrast to oral Al, very little of the injected Al diffuses in the extracellular spaces, the bulk of it being selectively and highly concentrated in a small fraction of the phagocytic cells (one of the 200 cell types of the body), representing about 3% of the body weight [162]. This incorporation in phagocytic cells limits extracellular solubilization of the particles, and induces long-term cell survival [163]. The very slow solubilization rate of Al adjuvant particles, especially Al hydroxide [7], makes determination of Al blood levels nearly useless to assess Al adjuvant toxicity. When a single dose of adjuvant corresponding to 0.85 mg Al is administered i.m. to an adult human, an increase in the plasma Al concentration of 0.8% is expected, that would be masked by the Al background if an isototpic ^{26}Al-labelled adjuvant is not used [94]. For the same reason, the spontaneous cumulative urinary excretion of Al is quasi-flat for Al hydroxide a few days after injection [7]. Thus, Al adjuvants do not usually cause massive intoxication by soluble Al similarly to what was previously documented in patients with renal failure undergoing dialysis with Al-containing water. Instead, the particulate Al adjuvants exclusively concentrate in immune cells, a very small part of the human body, in which they chronically exert their immunostimulatory adjuvant effects [164,165], until eventual dispostion. If one estimates that the diffusion space of a locally i.m. injected adjuvant could hardly exceeds 1% of the body space before solubilization of the particle, rough calculation indicates that an oral dose of Al should be about 1 million fold higher than the vaccine dose to induce the same level of Al in phagoctic antigen presenting cells.

Specialized toxicologists are now aware that comparing toxicological properties of different forms of Al (soluble *vs* particulate) administered by different routes (oral *vs* i.m.) is incorrect and, therefore, inadmissible [166]. This constitutes another reason to dismiss "the dose makes the poison" rule to adress toxicity of Al hydroxide adjuvant particles.

7.3. From Al toxicity to chronic immune stimulation

Several experimental studies of the literature have documented the potential neurotoxicity of Al adjuvants. In a seminal study, Alhydrogel® adjuvant, subcutaneously injected in mice at doses relevant to the dose received by US veterans with GWI, induced motor deficits and cognitive alterations associated with motor neuron death and a significant increase of reactive astrocytes indicative of an inflammatory process [167]. Subsequently, toxicity on the adult or developing mouse brain of either Al adjuvant or whole Al-containing vaccines has been reported in Canada [168–170], Israël [171–172] and France [158]. Of note, small animal studies showing toxic effects of Al adjuvants are often suspected to be irrelevant to the human situation but this is not the case of large animal models. Therefore, it should be emphasized that Spanish veterinarians have reported that multiple Al-containing vaccine administrations in sheep can induce a biphasic neurologic disease including initial meningo-encephalomyelitis with behavioral alterations followed by progressive spinal neurodegenerative changes, offering an invaluable model to understand the human ASIA [173–174]. Moreover, multiple injections of the adjuvant alone (Al hydroxide), compared to saline placebo, was sufficient to induce both diffusion of Al and granulomas to draining lymph nodes [92], and the behavioral changes observed in sheep ASIA, including restlessness, aggressiveness, stereotypies, dissociation from the group, and lethargic states (Asin J & Llujan L, personal communication). Whole Al vaccine injections resulted in even more pronounced immunological effects than Al adjuvant alone [92,175]. Both the Al adjuvant alone and the whole vaccine groups showed increased biologic unwellness markers, such as high circulating levels of cortisol, the stress hormone, in winter time.

Pathophysiology of ME/CFS remains poorly understood, but the classical hypothesis that ME/CFS patients may suffer from an inappropriate clearance of either pathogens or toxic compounds with immuno-stimulating effects [41] causing "protracted immune stimulation that fails to switch off" [176] and leading to eventual immune system "burnout" fits well with recent evidence that ME/CFS patients are flush with cytokines until around the three-year mark, at which point the immune system becomes exhausted and cytokine levels drop [177]. Consistently with patients with longstanding ME/CFS studied by Hornig *et al.* [177], MMF patients typically exhibit immune system "burnout" assessed by significant drop of blood IL1b, IL1ra, IL4, IL10, IL12, IL17 and FGFb, at the exception of the major monocyte chemoattractant CCL2 which is selectively increased [101].

It has been demonstrated that, even in the absence of initial CNS inflammation, brain microglia respond to peripheral inflammation by increasing their production of MCP-1/CCL2 which attracts circulating CCR2-expressing monocytes [178]. This monocyte influx drives peripheral inflammatory states-associated sickness behaviour, manifesting by fatigue, mood disorders, cognitive dysfunction and sleep disturbances [179]. In the setting of immunization, MCP1/CCL2 expression is upregulated by Al hydroxide [180], which likely polarizes response to vaccine towards T helper 2 immune responses [181], and favour Al adjuvant-loaded cells incorporation to the brain [99]. Al hydroxide particles elicit inflammation by activating the so-called NALP3 inflammasome [182] and NALP3 inflamasome activation mediates fatigue-like behaviour in mice via neuroinflammation [183]. The hallmark of this activation is the release of IL1beta, which was detected in both brain immune cells and neurons loaded with Al hydroxide particles in our mouse experiments [99]. In the same way, chronic pain syndromes arise from hypersensitization within the dorsal horn of the spinal cord and microglia activated by an adjuvant like CFA administered at the periphery has been shown to initiate hypersensitization through release of IL1b and other inflammatory cytokine [184]. Thus, Al adjuvants that enter the CNS can amplify activation of microglial cells triggered by peripheral inflammation which is known to elicit fatigue and pain. The immune system also plays a pivotal role in modulating learning and memory, and hippocampal synaptic plasticity is particularly sensitive to neuroinflammation [185]. It has been consistently shown that neonatal administration of Al hydroxide-containing HBV vaccine induces a T helper 2 (Th2) immune response in the periphery, while increasing IL-1β, IL-6, and TNF-α in the hippocampus and hampering hippocampal synaptic plasticity, whereas neonatal Bacille Calmette-Guérin (BCG) vaccination induces opposite effects [186]. Of note, Al hydroxide and Al phosphate are strong Th2 adjuvants that can likely act in synergy with known factors of a Th1 to Th2 shift of the adaptive T cell responses, including mental stress, excess sympathetic stimulation, excess glucocorticoids, high female hormones levels, immunosuppression, chronic infection or overwhelming microbial burden [187–189]. Long-term Th2 shift has long been suspected to underpin clinical manifestations of GWI [190], and, consistently, immune activation with a Th2 shift has been documented in the cerebrospinal fluid of ME/CFS patients [191].

7.4. Future directions deserving investigation: innate immune memory and microbiome

7.4.1. Innate immune memory

In almost all MMF patients, ME/CFS manifests after multiple immunizations. The impact of multiple vaccinations on the immune system has been rarely investigated but represents a critical question [3]. Increasing attention is currently paid to memory-like characteristics of innate immune cells, including peripheral monocytes/macrophages and brain microglia, called trained innate immunity [192]. It was long believed that, in contrast to cells of the adaptive immune system, monocytes and macrophages do not have immunological memory, mounting an identical naïve response each time they are stimulated. Recent studies have demonstrated that, in fact, the innate immune system can adopt a long-term activated phenotype by previous encounters with various microbial or vaccine stimuli. Thus upon infection or vaccination, monocytes/macrophages can be functionally

reprogrammed so as to display an enhanced response against unrelated infections [193]. For example, as also described above [186], BCG vaccination prevents tuberculosis but also induces non-specific beneficial effects, against certain forms of malignancy and unrelated pathogens and autophagy plays a key role in these nonspecific effects [194]. Besides the beneficial effects of trained innate memory, however, deleterious effects may well occur through sequential immune stimuli causing microglial priming favouring neurodegeneration [195], or through the induction or maintenance of autoimmune and auto-inflammatory diseases in case of inappropriate activation or individual susceptibility [196]. To our knowledge, this question has not been investigated yet in ME/CFS and ASIA.

7.4.2. *Microbiome dysbiosis*

Immunosuppression that is typically associated with longstanding ME/CFS, as confirmed in MMF patients (see Section 7.3), makes possible that opportunistic development of as yet unidentified pathogens or, more likely, microbiote dysregulation could contribute to or perpetuate ME/CFS [197]. As stated above (Section 5.1.3), longstanding MMF reflects limitation of cellular disposition of particles by the auto/xenophagy machinery [48]. Individual limitation of auto/xenophagy processing, linked to genetic traits or to aging [198], may impede macrophage clearance of adjuvant particles and increase the inflammatory response but it may similarly affect clearance and immune response to intracellular microbes, as previously documented in intestinal epithelial cells of patients with Crohn's disease and other IBDs linked to microbiome dysbiosis [199–201]. In keeping with this view, patients with MMF often suffer from abdominal discomfort and IBD and their general symptoms may occasionally improve after antibiotic therapy [202]. Both compassionate L-carnitine administration used to stimulate mitochondrial function and dietetic measures with probiotics intake seem to be of some benefit in many cases. The fact that clinical symptoms typically occur after immunization in both humans and sheep suggests that vaccines and their adjuvants, similarly to the different pathogens previously implicated at the origin of ME/CFS cases, may interact with various stressors to trigger cascading events that compromise immunologic, metabolic, neuroendocrine and neuro-vegetative functions and push the body toward a state of illness (see Fig. 1). Possible implication of microbiome dysbiosis in these events is suggested by epipharyngitis documented in Japanese girls with HPV vaccine-induced ME/CFS [124], and by epidemiological evidence that French girls immunized against HPV have a slight but significant increased risk of developing IBD [203]. Of note, Al *per se* [204–205] and mental stress [206] are established factors of chronic intestinal inflammation. Moreover, it has been clearly shown in mice that given microbiome strains are important providers of natural adjuvants necessary to elicit immune response to influenza vaccine [207]. Therefore, it is not excluded that persistent microbiome species could induce the immunological alterations previously reported in ME/CFS patients [197], and search for an imbalance of microbiome communities in ASIA patients with or without biopsy-proven MMF could well prove contributory in the future [208].

8. Post-immunization ME/CFS as a core manifestation of ASIA

Yehuda Shoenfeld had the great merit to coin the concept of ASIA in 2011 [5]. Striking clinical similarities between GWI, MMF syndrome and ME/CFS had been previously reported [4], but Yehuda Shoenfeld extended the concept to the deleterious effects of every compound with adjuvant properties, including pathogens themselves and non-vaccinal adjuvant particles. Microbial adjuvants naturally present in pathogens were among the first vaccine adjuvants, including mycobacterial walls used in Freund's complete adjuvant which immunostimulating molecules are muramyl dipeptide and the tréhalose dimycolate, and gram negative bacteria the endotoxin of which is called lipopolysaccharide (LPS) and its adjuvant derivative monophosphoryl lipid A. Silicone particles represent the main non-vaccinal mineral particles with adjuvant properties [209]. Patients with leaky silicone breast implants develop siliconosis consisting in release of silicone particles that allows them, similarly to microbial or vaccine adjuvant particles, to be taken up by macrophages and transported to lymphoid organs and manifesting by a disease complex similar to ME/CFS [40,210,211].

Yehuda Shoenfeld has admitted that most ASIA patients have ME/CFS [19]. There has been a tendancy, however, to extend the ASIA concept to immune diseases beyond ME/CFS, to include autoimmune diseases of post-vaccinal onset, such as Sjogren syndrome [212], narcolepsy [213], antiphospholipid syndrome [214], and primary ovarian failure [215], as well as lymphoma [216]. It is true that idiopathic ME/CFS (up to around 60 %) may suffer from autoimmune responses [217,218] and that ASIA shares similarities with undifferentiated connective tissue disease [219]. It is also true that ME/CFS is associated with an increased risk of lymphoma [18], and that the dramatic rate of immune disorders observed in italian militaries, including lymphomas, leukemias and autoimmune diseases, has been linked to suboptimal vaccine practices, such as injecting 5 vaccine shots simultaneously [3]. In this setting, the Italian Senate committee has calculated that the cumulated amount of non-antigenic vaccine compounds received by Italian militaries-including Al adjuvants (7.65 mg corresponding to 2.57 mg Al), 44 excipients and 47 contaminants- was always above the official security threshold [3]. However, extending too much the scope of ASIA is at high risk of blurring the core picture. The initially proposed definition of ASIA is probably too loose and, therefore, remains a matter of debate despite the extreme practical usefulness of recognizing that similar clinical presentations may be observed in patients exposed to a variety of immunostimulatory compounds.

Whether post-vaccinal ME/CFS represents an authentic autoimmune disease as suggested by the term ASIA is also still incompletely settled. On the one hand, a number of autoantibodies have been reported in patients with idiopathic POTS and ME/CFS [218]. In a subset of these patients auto-antibodies may be specifically directed against neurotransmitter receptors present in the sympathetic nervous system, including ß2 adrenergic receptors, and muscarinic 3 and 4 acetylcholine receptors, and may likely play a role in clinical manifestations as suggested by immunoabsorption studies [220]. In the same way, prolonged B-cell depletion with anti CD20 rituximab has been associated with sustained clinical responses in a subgroup of patients with idiopathic ME/CFS [221]. On the other hand, we found no mention in the literature of detection of specific anti-neuroceptor auto-antibodies in post-vaccinal ME/CFS and very little in post-vaccinal POTS [222]. Our MMF patients inconstantly presented with low titers of common circulating autoantibodies, mainly antinuclear antibodies that were detected in about 30% of patients, assessing low grade autoimmunity. In addition, a minority of MMF patients (10-20%) had a well-defined concurrent autoimmune disorder (MS, thyroiditis, dermatomyositis, etc) [67]. We do not remember to have seen MMF cases evolving from pure initial ME/CFS to full blown specific autoimmune disease. It is therefore not excluded that specific autoimmunity only occurs in a subset of post-immunization ME/CFS patients, presumably due to either individual susceptibility to develop an autoimmune disease or to a specific, possibly opportunistic, antigen challenge. For example, it has been suggested that persistent microbiome pathogens could induce immunological alterations previously reported in idiopathic ME/CFS patients, including altered NK cell functions, clonal T-cells, and autoantibodies [197]. Todate, the role of specific auto-antibodies against neurotransmitter receptors, though representing a fascinating new issue in ME/CFS, remains elusive in post-immunization ME/CSF.

Nevertheless, the ASIA concept has gained growing popularity in human and veterinary medical communities, with more than 4000 reported cases in the literature [223], pointing out that a critical need has been met by ASIA in routine practice of human and veterinary medicine [173,223].

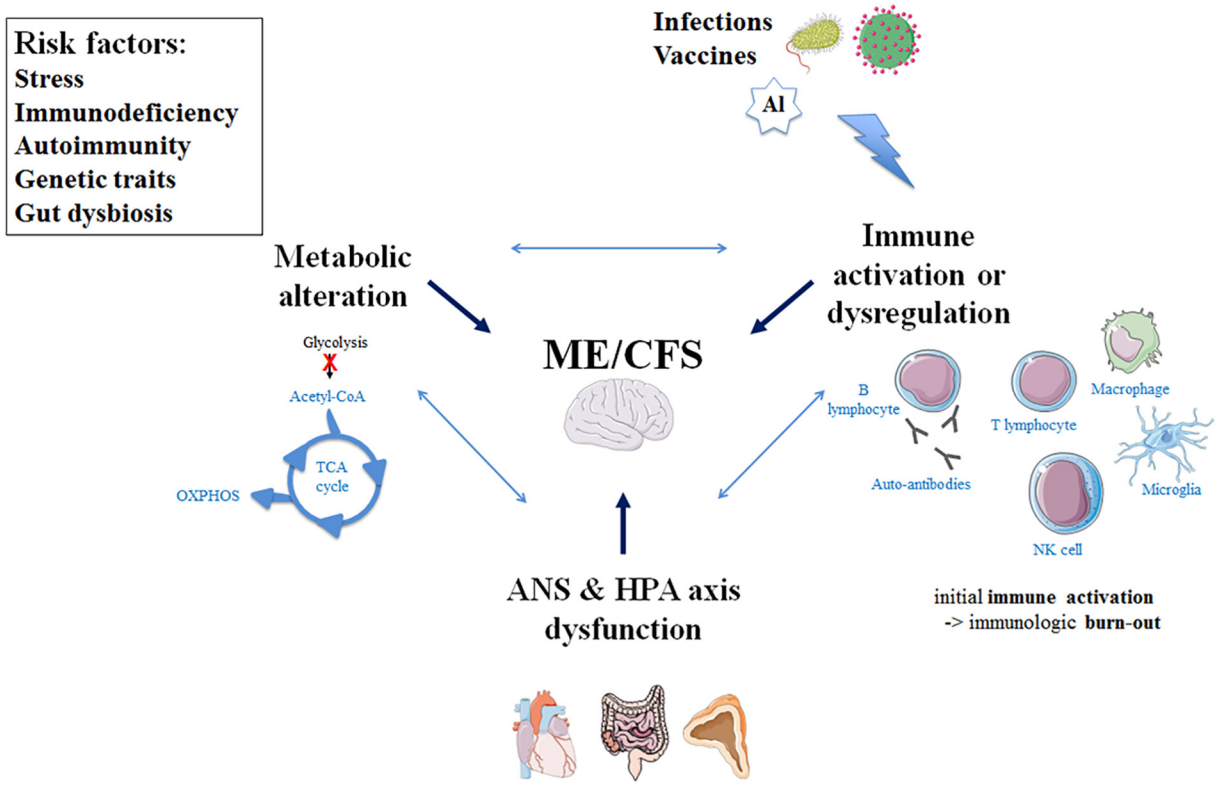

Fig. 1. Schematic representation of the ME/CFS pathophysiology following challenge with infectious or adjuvanted vaccine particles (redrawn from Sotzny *et al.*, 2018 [207]).
Aluminum adjuvant-containing vaccines, similarly to persistent natural pathogens, can induce immune system dysregulation and metabolic, neuroendocrine and autonomic nervous system disturbances at the origin of ME/CFS symptoms. Contribution of a variety of risk factors is likely.
Abbreviations: Acetyl-CoA: acetyl coenzyme A, ANS: autonomic nervous system, ME/CFS: Myalgic Encephalomyelitis/Chronic Fatigue Syndrome, NK: natural killer, OXPHOS: oxidative phosphorylation, TCA: tricarboxylic acid.
Drawings by Servier medical art® https://smart.servier.com/

9. A tentative pathophysiological model

Post-infectious fatigue is recognized to occur in about one in ten people infected with Epstein-Barr virus or *Coxiella burnetti*, the causative agent of Q fever, and in a number of patients infected by enteroviruses, *Borrelia burddorferi*, and other infectious agents. Long-term persistency of the infectious agent has been repeatedly shown to cause prolonged immune activation and ME/CFS-like syndrome [224–227]. Both human MMF [10] and relevant small and large animal models [92,99,154,155,173] indicate that the same holds true for ME/CFS following administration of Al adjuvants that persist unusually long within immune cells throughout the immune system.

ME/CFS has an extremely complex pathophysiology affecting multiple systems. The reader is referred to excellent extensive papers on each of the impacted systems [228–231] and on their interplays [232]. Basically ME/CFS is associated with: (1) immune system abnormalities including impaired natural killer cell function and/or T cell function, increased and then decreased production of inflammatory cytokines [177], and occasional increase in some autoantibodies [218]; (2) cellular metabolism abnormalities with impaired ability to produce energy from oxygen, glucose, fatty acids, and amino acids, associated with mitochondrial dysfunction and reduced oxidative metabolism; these changes cause abnormal response to exercise and mimick an hibernation state [233]; (3) neuroendocrine and neurovegetative disturbances including dysregulation of the hypothalamic-pituitary-adrenal axis (HPA axis) and, particularly in adolescents, orthostatic intolerance with blood pressure or heart rate regulation abnormalities.

Fig. 1, redrawn from Sotzny *et al.* 2018 [218], summarizes the differents changes driven by immune dysregulation that may be caused by vaccine or natural adjuvants in susceptible individuals and likely form the core ASIA pathophysiology (Fig. 1).

10. Conclusion

Adjuvant safety is an “important and neglected field” [234], suffering from both misconception of Al adjuvant toxicokinetics [7] and lack of population-based studies evaluating associations between exposure to Al adjuvants and clinical outcomes [235].

ME/CFS is a multifactorial condition of major public health and clinical importance. Evidence that ME/CFS may represent an important type of AEFI has emerged very slowly due to the multiplicity, apparent lack of specificity, delayed onset, and frequent medical underestimation of symptoms, all characteristics ranging among the main explanations for the “inherent methodological limitations of epidemiological studies” in the field of vaccine safety (see above Section 4.2.). Fortunately, however, a well-conducted epidemiological study comparing vaccinated vs unvaccinated individuals has provided strong evidence of post-immunization ME/CFS [53]. In depth clinical analysis of patients with post-immunization ME/CFS has revealed highly consistent cognitive and functional neuroimaging alterations. Biologic plausibility of an association between particulate adjuvant administration and ME/CFS was supported by long-term Al adjuvant persistency in immune cells of affected individuals (assessed by MMF detection at muscle biopsy), and by Al adjuvant transportation to distant organs documented in small and large animal models, with long-standing immunostimulating and low dose neurotoxic effects.

These data, fitting the ASIA concept, have already grounded right to compensation for damages in USA and France where the highest

administrative court ruled compensation for 8 of our patients that had received mandatory vaccination for professional reasons. We hope they will trigger solid additional epidemiologic and basic research studies on long-term Al adjuvant fate and toxicity, individual susceptibility factors, and satisfactory alternatives to Al adjuvants. Several efficient and biodegradable adjuvants devoid of noxious metals have been already identified [236–238].

Competing interests statement

This review paper compiles previous results from our INSERM group obtained with the help of public sources of funding (Région Ile-de-France, Agence Nationale de Sécurité du Médicament) and from patients associations and charities including Association Française contre les Myopathies (AFM), Entraide aux Malades de Myofasciite à Macrophages (E3M) and Children's Medical Safety Research Institute (CMSRI/Dwoskin Foundation). Neither AFM nor E3M nor CMSRI played any role in the design, data analysis, interpretation of results and writing of this or any other papers from our group. None of the authors received payment from these non-profit organizations. Romain Gherardi and François-Jérôme Authier have occasionally done expert testimony and have deposited one patent relevant to the field of vaccine safety.

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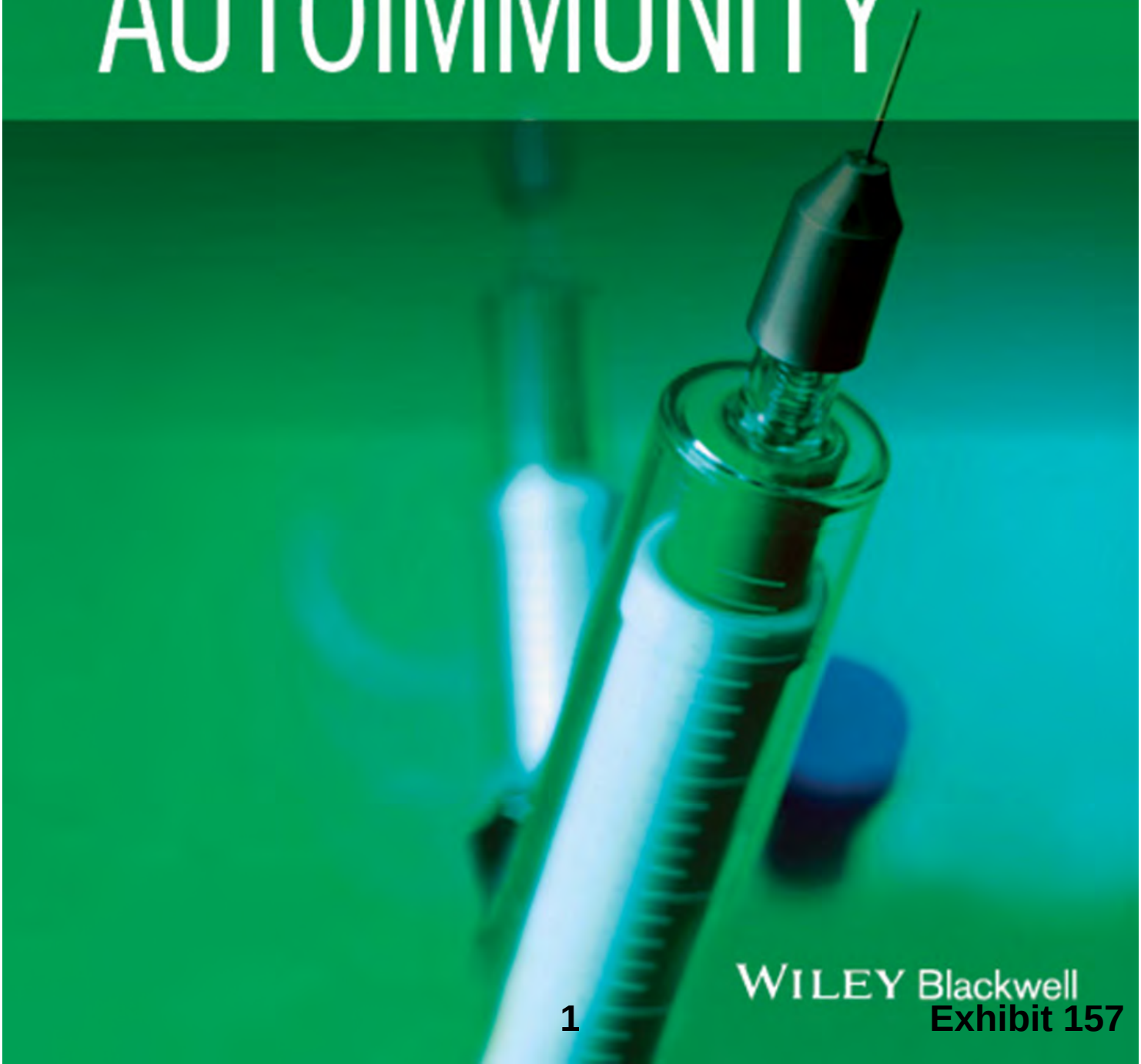
EXHIBIT 157

Edited by
Yehuda Shoenfeld, Nancy Agmon-Levin
and Lucija Tomljenovic

VACCINES & AUTOIMMUNITY



WILEY Blackwell

36 Infections, Vaccinations, and Chronic Fatigue Syndrome

Hussein Mahagna, Naim Mahroum, and Howard Amital

Department of Medicine B, Sheba Medical Center, Tel Hashomer, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

Introduction

Chronic fatigue syndrome (CFS) is a heterogeneous disorder affecting more than 267 people in ever 100 000 (Abbi and Natelson, 2013; Moss-Morris *et al.*, 2013). It has been estimated that in the United States, approximately 1 million people suffer from CFS symptoms (Reynolds *et al.*, 2004). Women are nearly twice as likely to be affected as men (Appel *et al.*, 2007). Similar prevalence rates have been found in different geographic locations and in diverse ethnicities (Steele *et al.*, 1998). The pathophysiology and etiology of CFS are unknown, since there are no characteristic physical signs or diagnostic laboratory abnormalities (Whistler *et al.*, 2003).

CFS patients suffer from disabling fatigue, headaches, concentration difficulties, and memory deficits (90%). Additional symptoms, such as sore throat (85%), tender lymph nodes (80%), skeletal muscle pain and feverishness (75%), sleep disruption (70%), psychiatric problems (65%), and rapid pulse (10%), are often observed (Appel *et al.*, 2007; Friedberg, 2010; Lewis *et al.*, 2013; Werker *et al.*, 2013). Due to these complaints, patients often face familial, social, and vocational crises (Bell *et al.*, 2001).

The diagnosis of CFS is complex, due to its similarity to other ill-defined disorders presenting similarly, such as fibromyalgia syndrome (FMS), Gulf War syndrome (GWS), and Sjögren syndrome (SjS) (Sirois and Natelson, 2001; Abbi and Natelson, 2013; Lewis *et al.*, 2013; Werker *et al.*, 2013).

Fukuda *et al.* (1994) described the significant overlap between CFS and FMS, considering CFS a subclass of prolonged fatigue. They proposed a method for its proper diagnosis: a patient must present with four or more symptoms contemporary for at least 6 months. Characteristics excluding patients from CFS are: active medications, past or current major depressive disorders, alcohol abuse, and severe obesity. However, some of the criteria are difficult to interpret, and opinions differ about the classification of chronic fatigue cases with a history of psychiatric illnesses (Matthews *et al.*, 1988). It is important to stratify patients with suspected CFS by the assessment of four criteria: (i) coexisting medical or neuropsychiatric condition not explaining the chronic fatigue; (ii) level of fatigue, including subjective and performance aspects; (iii) total duration of fatigue; and (iv) level of overall functional performance (Fukuda *et al.*, 1994). All of these evaluations can be performed with available instruments, Medical Outcomes Study Short Form 36, and Sickness Impact Profile (Bergner *et al.*, 1981; Ware and Sherbourne, 1992; Schwartz *et al.*, 1993; Piper *et al.*, 1998; Piper and Cella, 2010).

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Etiology

CFS was first described in the 1980s, when it was thought to be a consequence of a viral or bacterial infection. One of the first suspected pathogens was Epstein–Barr virus (EBV), because patients often have higher titers of IgM to the EBV viral capsid antigen (Lerner *et al.*, 2004). In addition, antibodies against cytomegalovirus (CMV) and human herpes virus 6 (HHV-6) are also detected more often in CFS patients (Ablashi *et al.*, 2000; Lerner *et al.*, 2004; Yao *et al.*, 2010), although other reports have failed to repeat these results (Soto and Straus, 2000).

Another virus family studied as a possible cause of CFS is the enteroviridae, because RNA copies were detected in the muscle biopsies of CFS patients but not in a healthy control group (Bowles *et al.*, 1993; Lane *et al.*, 2003). Other studies have failed to demonstrate positive serological tests or PCR for enteroviridae, however (Straus 1996). Parvovirus B19 is considered to be one of most probable causes of CFS, because there are some case reports of patients with a chronic course of fatigue following infection, fulfilling the criteria for CFS diagnosis. In one of these studies, the stress index was significantly associated with development of fatigue during the acute phase of parvovirus B19 infection, and also with chronic fatigue and arthritis occurring 1–3 years following the acute parvovirus B19 infection, with an odd ratio (OR) of 25.7 (95% CI: 1.7–121.9; $p = 0.005$) (Lane *et al.*, 2003; Kerr, 2005; Kerr and Mattey, 2008). In addition, a higher prevalence of Mycoplasma infections has been reported in CFS patients than in healthy subjects (Nijs *et al.*, 2002).

Although the pathophysiology of the disease is not yet well known, molecular mimicry and autoimmune processes have been suggested to be involved, because of reports of post-infectious onset and the presence of autoantibodies. This is also thought to be true of the role of vaccinations in the onset of the illness.

Vaccinations and CFS

It is known that vaccinations can cause self-limiting fatigue and flu-like symptoms. CFS has been reported to emerge following vaccination in several reports, including after measles, mumps, and rubella (MMR), Pneumovax, influenza, hepatitis B virus (HBV), tetanus, typhoid, and poliovirus vaccines (Devanur and Kerr, 2006). A case of CFS onset following the double effect of exposure to silicone and HBV vaccine was reported. It was suggested that the breast implants and vaccination acted as facilitators and triggers for the emergence of CFS onset in the patient (Agmon-Levin and Shoenfeld, 2008). Such reports have made researchers concerned regarding the role of vaccinations in the onset of CFS and the safety of their use in CFS patients.

HBV is one of the most controversial vaccines with regard to the potential risk of inducing CFS. Several researchers advocate for a contributory role of the vaccine in the development of CFS (House, 1992; O'Sullivan, 1992; Delage *et al.*, 1993; Agmon-Levin and Shoenfeld, 2008), while others claim the vaccine is safe, with minimal adverse effects (Zuckerman, 2004).

In Norway, a vaccine against *Neisseria meningitides* group B was administered to teenagers in 1988–89. Relative risk for CFS and myalgic encephalomyelitis was calculated in a case–control study in 2007, with 201 cases diagnosed at one of two hospitals and 389 controls. The adjusted OR for these two conditions was 1.06 (95% CI: 0.67–1.66) for subjects who received the active vaccine as opposed to those who did not (Magnus *et al.*, 2009).

Another study reported a case of CFS associated with aluminum overload, suggesting that vaccination involving aluminum-containing adjuvants could trigger a cascade of immunological events, which are associated with immune-disrupting conditions, including CFS and macrophagic myofasciitis (Exley *et al.*, 2009). Previous studies indicated that, although aluminum-based adjuvants may persist at the site of injection for years ("vaccine tattoo"), this does not reflect the existence of a diffuse inflammatory muscular disease and is not associated with a specific clinical disease (Siegrist, 2003, 2005).

Several studies have investigated the safety and efficacy of vaccines in CFS patients. One double-blind randomized study checked the safety of oral poliovirus vaccination. It was not found to be clinically contraindicated in CFS patients, but there was evidence of minimally altered immune reactivity to the live vaccine virus; the objective responses to the vaccine revealed differences between patients and controls, increased poliovirus isolation, earlier peak proliferative responses, lower T cell subsets on certain days post-vaccination, and a trend for reduced gamma-interferon in the CFS vaccine group (Vedhara *et al.*, 1997).

One study tested the effect of staphylococcal toxoid on patients with fibromyalgia and CFS. In this double-blind randomized study, treatment with staphylococcus toxoid injections over 6 months led to significant improvement in patients with FMS and CFS. The proportion of patients with a symptom reduction of ≥50% showed on an intention-to-treat analysis as 32/49 (65%) responders in the vaccinated group, compared to 9/49 (18%) in the placebo group ($p < 0.001$) (Zachrisson *et al.*, 2002).

Regarding influenza vaccination, it appears to provide protective antibody levels without worsening CFS symptoms or causing excessive adverse effects (Sleigh *et al.*, 2002). Recently, a study aimed at comparing the humoral and cellular immune responses following influenza vaccination found that CFS patients have comparable outcomes to healthy controls. Putative aberrations in immune responses in CFS patients were not evident for immunity toward influenza. Standard seasonal influenza vaccination is thus justified and, when indicated, should be recommended for patients suffering from CFS (Prinsen *et al.*, 2012).

In conclusion, except for several case reports, there are no studies that indicate vaccines might have a deleterious effect in patients with CFS. However, it is possible that various vaccines or exposures to various pathogens might take part in the induction of CFS.

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EXHIBIT 158

Centers for Disease Control and Prevention

Vaccine Safety

Guillain-Barré Syndrome

Guillain-Barré syndrome (GBS) is a rare disorder where the body's immune system damages nerve cells, causing muscle weakness and sometimes paralysis. While its cause is not fully understood, the syndrome often follows infection with a virus or bacteria. Each year in the United States, an estimated 3,000 to 6,000 people develop GBS. Most people fully recover from GBS, but some have permanent nerve damage.

GBS is rare.

Anyone can develop GBS, but people older than 50 are at greatest risk. In addition, about two-thirds of people who get GBS do so several days or weeks after they have been sick with diarrhea or a lung or sinus illness. Infection with the bacteria Campylobacter jejuni, which causes gastroenteritis (including symptoms of nausea, vomiting and diarrhea), is one of the most common risk factors for GBS. People also can develop GBS after having the flu or other infections such as cytomegalovirus and Epstein Barr virus. On very rare occasions, people develop GBS in the days or weeks after getting a vaccination.

To study whether a new vaccine might be causing GBS, CDC would compare the usual rate of GBS to the observed rate of GBS in persons getting vaccinated. This helps to determine whether a vaccine could be causing more cases.

GBS and the link to flu vaccine

In 1976, there was a small increased risk of GBS after swine flu vaccination, which was a special flu vaccine for a potential pandemic strain of flu virus. The National Academy of Medicine, formerly known as Institute of Medicine, conducted a scientific review of this issue in 2003 and found that people who received the 1976 swine flu vaccine had an increased risk for developing GBS. The increased risk was approximately one additional case of GBS for every 100,000 people who got the swine flu vaccine. Scientists have several theories about the cause, but the exact reason for this link remains unknown.

There have been several studies of the risk of GBS after flu vaccine and CDC monitors for GBS during each flu season. The data on an association between seasonal influenza vaccine and GBS have been variable from season-to-season. When there has been an increased risk, it has consistently been in the range of 1-2 additional GBS cases per million flu vaccine doses administered.

Studies suggest that it is more likely that a person will get GBS after getting the flu than after vaccination. It is important to keep in mind that severe illness and death are associated with flu, and getting vaccinated is the best way to prevent flu infection and its complications.

CDC and FDA closely monitor the safety of all vaccines.

CDC and the Food and Drug Administration (FDA) are committed to ensuring that all vaccines are as safe as possible. Once vaccines are licensed in the United States, they are continuously monitored through several safety systems and programs. Learn more about the nation's vaccine safety efforts.

Exhibit 158

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Related Links

- Guillain-Barré Syndrome Fact Sheet
- Guillain-Barré Syndrome (GBS): Questions & Answers
- Meningococcal Vaccine Safety

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Guillain–Barré syndrome after Gardasil vaccination: Data from Vaccine Adverse Event Reporting System 2006–2009

Nizar Souayah[a,*], P.A. Michas-Martin[c], Abu Nasar[d], Nataliya Krivitskaya[a], Hussam A. Yacoub[a], Hafiz Khan[e], Adnan I. Qureshi[b]

[a] Department of Neurology, University of Medicine and Dentistry of New Jersey, Newark, NJ 07103, United States
[b] Zeenat Qureshi Stroke Research Center, University of Minnesota, Minneapolis, MN 55454, United States
[c] New York Medical College, Valhalla, NY 10595, United States
[d] Department of Surgery, Columbia University Medical Center, New York, NY 10032, United States
[e] Department of Epidemiology & Biostatistics, Robert Stempel College of Public Health & Social Work, Florida International University, Miami, FL 33199, United States

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ABSTRACT

Using data from Vaccine Adverse Event Reporting System, we identified 69 reports of Guillain–Barré Syndrome (GBS) after Gardasil vaccination that occurred in the United States between 2006 and 2009. The onset of symptoms was within 6 weeks after vaccination in 70% of the patients in whom the date of vaccination was known. The estimated weekly reporting rate of post-Gardasil GBS within the first 6 weeks (6.6 per 10,000,000) was higher than that of the general population, and higher than post-Menactra and post-influenza vaccinations. Further prospective active surveillance for accurate ascertainment and identification of high-risk groups of GBS after Gardasil vaccination is warranted.

1. Introduction

On June 8, 2006, a quadrivalent vaccine (Gardasil) that provides protection against four human papilloma virus (HPV) variants associated with approximately 70% of cervical cancers and more than 90% of genital warts was licensed for use in the United States in women between 9 and 26 years of age [1]. The availability of cervical cancer vaccine has elicited enormous enthusiasm from the medical community, particularly after the publication of several reports showing the effectiveness of the vaccine in preventing precancerous cervical lesions caused by HPV-16 and HPV-18 [2–4]. However, several reports derived from analysis of data from the Vaccine Adverse Events Reporting System (VAERS) pointed to several cases of post-Gardasil vascular and neurological complications [5]. Since Guillain–Barré syndrome (GBS) is considered one of the most common neurological sequelae of vaccination, we investigated the occurrence of GBS after vaccination with Gardasil to provide further information about the characteristics and temporal profile of these occurrences. We also compared the occurrence of GBS in patients vaccinated with Gardasil to that following other vaccinations, and to the incidence of GBS in the general population.

2. Methods

We used data derived from the Vaccine Adverse Event Reporting System (VAERS) supplemented by data from the Center for Biologics and Research under the freedom of information act. The VAERS is a national cooperative program instituted by the Centers for Disease Control and Prevention (CDC) and the Food and Drug Administration (FDA). The VAERS collects information about adverse events that occur after the administration of licensed vaccines in the United States. The database records the demographics and clinical characteristics of the events, results of diagnostic tests, and outcome using a non-standardized format. We searched the database between June 2006 and September 2009 using the following keywords and subject terms: “Guillain–Barré syndrome”, “acute polyneuropathy”, “Miller–Fisher syndrome”, “paraparesis”, “paraplegia”, “paralysis”, “flaccid paralysis”, “weakness”, and “numbness”. The clinical findings and diagnostic test results were reviewed by a board-certified neuromuscular specialist to identify events that met the diagnostic criteria of GBS defined by progressive arm and leg weakness and areflexia. The diagnosis was supported by the presence of elevated cerebrospinal

* Corresponding author at: 90 Bergen Street, DOC 8100, Newark, NJ, 07103, United States. Tel.: +1 973 972 8577; fax: +1 973 072 8738.
E-mail address: souayani@umdnj.edu (N. Souayah).

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fluid protein concentration with fewer than 10 cells/mm^2 or by electrodiagnostic study findings consistent with primary demyelination. Cases of Miller–Fisher syndrome, a variant of GBS, were also included in the study.

The occurrence of GBS within 6 weeks of Gardasil vaccination was suggestive of a causal association [6,7]. The estimated reporting rate of GBS cases identified after vaccination with Gardasil was compared to that after vaccination with the meningococcal (Menactra) and the influenza vaccines. We chose the Menactra vaccine, licensed by the FDA in January 2005, as a comparative control since it is used in a similar patient population, including children approximately 11 years of age or older. Since Menactra and Gardasil vaccines are indicated for the same patient population, they are frequently co-administrated. The reporting rates of GBS associated with Menactra and influenza vaccines, both of which have been associated with GBS, were used to provide the relative values for comparative purposes [6–10].

The crude reporting rate of occurrence of GBS was estimated by dividing the number of events reported within 6 weeks by the total number of vaccinations administered each year. We used the first 2 weeks after Gardasil vaccination to determine peak weekly incidence of GBS based on previous observations that the highest incidence of GBS occurred within the first 2 weeks of seasonal influenza vaccination [9,11]. The total number of vaccinations administered each year was derived from either estimates provided by the CDC or the National Health Interview Survey. The incidence of GBS in the general population was estimated based on a literature review. We searched the medical literature using MEDLINE, BIOSIS, and Cochrane databases for pertinent publications from 1966 to 2009 using the keywords mentioned above. The weekly incidence of GBS was derived from the annual incidence and based on events that were reported within 2 and 6 weeks of vaccination.

We estimated the total number of reported events, life-threatening events, emergency department visits, hospitalizations, and disability per 100,000 vaccinated subjects for Gardasil, Menactra, and influenza vaccines using the VAERS database.

3. Results

There were 69 reported cases of GBS after vaccination with Gardasil in the United States between June 2006 and September 2009. The mean patient's age (±standard deviation) was 16.7 (±6.2) years. GBS occurred after administration of Gardasil as a single vaccine in 47 (68%) subjects, in combination with Menactra in 18 (26%) subjects, and in combination with vaccines other than Menactra in 4 (6%) subjects. Among 48 patients with a known date of occurrence, 34 (70%) GBS cases occurred within 6 weeks of vaccination with Gardasil. The number of days between vaccine administration and symptoms onset was unknown in 21 cases. The distribution of time interval between Gardasil vaccination and occurrence of GBS showed a peak within the first 2 weeks post-vaccination (Fig. 1). Of the 34 patients who developed GBS within 6 weeks post-vaccination, 25 (74%) developed symptoms within the first 2 weeks. The probability of observing an asymmetrical distribution over the 6 weeks by chance alone was low ($p = 0.0002$). Hospitalization after Gardasil vaccination occurred in 42 (61%) subjects. Disability, defined by a substantial disruption of subject's ability to conduct normal life function, occurred in 12 (17%) subjects. Death was not reported in any of the cases.

Between June 2006 and September 2009, 52 cases of GBS were reported following Menactra vaccination. GBS occurred after the administration of Menactra as a single vaccine in 18 (34.6%) cases and in combination with other vaccines in 34 (65.4%) cases. GBS occurred within 6 weeks after Menactra vaccination in 42 (80.1%) subjects and after 6 weeks of vaccination in 10 (19.2%) subjects.

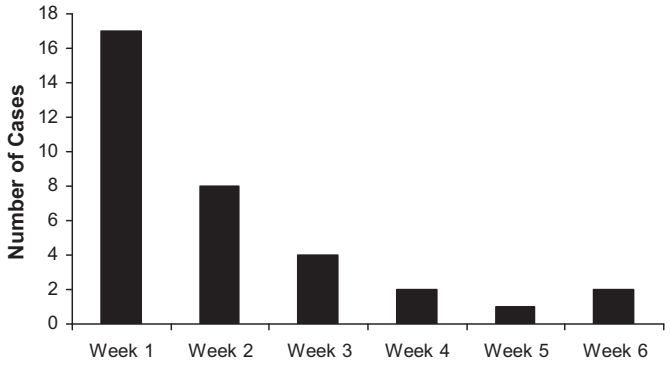

Fig. 1. The incidence of Guillain–Barré syndrome within 6 weeks after Gardasil vaccination.

In two subjects, the date of vaccination was unknown. Of the 42 patients who developed GBS within 6 weeks post-vaccination, 26 (62%) developed GBS within the first 2 weeks.

Between June 2006 and September 2009, 166 cases of GBS were reported following the influenza vaccine. GBS occurred after the administration of the influenza vaccine as a single vaccine in 145 (87%) cases and in combination with other vaccines in 21 (13%) cases. The onset of GBS occurred within 6 weeks after vaccination in 132 (80%) subjects and after 6 weeks of vaccination in 14 (8%) subjects. In 20 (12%) subjects, the date of vaccination was unknown.

According to the CDC, as of September 1, 2009, approximately 26 million Gardasil doses were distributed in the United States [12]. This corresponds to approximately 8.6 million subjects vaccinated with Gardasil since its approval in the United States. The estimated annual reporting rate of GBS after vaccination with Gardasil was approximately 80.23 per 10 million patients. The average weekly reporting rate of GBS within the first 6 weeks after vaccination with Gardasil was 6.6 events per week per 10 million subjects. The average weekly reporting rate within the first 2 weeks after Gardasil vaccination was higher and estimated to be 14.5 cases per week per 10 million subjects.

According to the CDC, approximately 22.6 million doses of Menactra have been distributed in the United States between June 2006 and September 2009 [13,14]. Consequently, the annual reporting rate of GBS occurrence post-Menactra vaccination is approximately 23 cases per 10 million treated patients. The average weekly reporting rate within the first 6 weeks after vaccination with Menactra was three events per week per 10 million treated patients. The average weekly reporting rate within the first 2 weeks after Menactra vaccination was 5.7 cases per week per 10 million patients treated.

From June 2006 to September 2009, it is estimated using the National Health Interview Survey that approximately 173 million influenza vaccines were administered in the United States [15]. The annual reporting rate of GBS following the influenza vaccination is estimated to be 9.6 cases per 10 million patients treated. The average weekly reporting rate within the first 6 weeks after vaccination with influenza was 1.3 events per week per 10 million patients treated.

In the general population, the annual incidence of GBS is reported to be 34–134 cases per 10 million [16]. This corresponds to an average weekly incidence of 0.65–2.57 cases per week per 10 million.

The average weekly reporting rate of post-Gardasil GBS within the first 6 weeks of vaccine administration was higher than the estimated incidence of GBS in the general population: 29 cases per 10 million within the first 2 weeks of Gardasil vaccination compared to 1.3–5 per 10 million cases in the general population. According to the VAERS, there were more life-threatening events, emergency department visits, hospitalizations, and disability

Table 1
Events reported after Gardasil, Menactra, and influenza vaccine administration between June 2006 and September 2009 (VAERS).

Events	Gardasil vaccination treated persons (*n*)	Menactra vaccination treated persons (*n*)	Influenza vaccination treated persons (*n*)
Total reports	13,115	4903	13,801
Total reports per 10^5 subjects	152.5	21.7	8
Life-threatening	269	71	352
Life-threatening per 10^5 subjects	3.1	0.31	0.2
Emergency department visits	5851	1813	5021
Emergency department visits per 10^5 subjects	68	8	3
Hospitalizations	1444	305	1326
Hospitalizations per 10^5 subjects	16.8	1.3	0.76
Disability	456	46	284
Disability per 10^5 subjects	5.3	0.2	0.16
GBS per 10^5 subjects	69	52	166

reports associated with the Gardasil vaccine compared with Menactra and influenza vaccines between June 2006 and September 2009 ($p < 0.0001$) (Table 1).

4. Discussion

An increased risk of GBS associated with Gardasil vaccination may cause public concern, influence public policy on vaccination, and reduce utilization of vaccination [17]. There was nearly a 2.5- to 10-times greater risk of acquiring GBS within 6 weeks after Gardasil vaccination when compared with the general population. Compared to Menactra, the VAERS database reported more post-Gardasil GBS within the first 2 weeks post-vaccination (29 cases versus 11.4 cases per 10 million vaccinated subjects). Gardasil vaccination was associated with approximately 8.5-times more emergency department visits, 12.5-times more hospitalizations, 10-times more life-threatening events, and 26.5-times more disability than the Menactra vaccination (Table 1).

The distribution of time interval between Gardasil vaccination and onset of GBS suggested a peak occurrence within the first 2 weeks post-vaccination followed by a decline in occurrence in the third week and a value close to the risk observed in the general population in the fourth to sixth weeks (Fig. 1). A similar pattern of association between GBS and influenza vaccine was observed by Lasky et al. [9] and Haber et al. [11] with more GBS cases reported in the second week after vaccination. The highest relative risk of GBS onset after respiratory infection, a potential triggering factor, was also reported in the first 2 weeks after infection [18].

Molecular mimicry and other immune system stimulation mechanisms may play a role in mediating GBS after Gardasil vaccination. Some vaccines, such as the Gardasil vaccine, may be more likely to trigger GBS because of the high antigenicity of the vaccince's recombinant proteins, antigenicity of components of the vaccine other than the recombinant proteins (especially aluminum), and the genetic predisposition of vaccinated subjects to develop vaccine-induced autoimmunity [19–21]. The Gardasil vaccine leads to a 40-fold increase in HPV antibodies compared with the physiological antibody level triggered by a natural HPV infection [22]. The antibody titer against the HPV genotypes 16 and 18 may remain 11-times higher than those induced by a natural infection 5.5 years after vaccination [23]. Polyclonal sera from Gardasil recipients cross neutralize HPV 45 pseudovirions inducing a cross protection against persistent infection with HPV 45 [21,23]. Immediate and long-term elevation in non-physiological immunogenicity with cross reactivity with other antigens may explain the higher occurrence of GBS in genetically predisposed subjects. Amorphous aluminum hydroxyphosphate sulfate is used as an adjuvant to Gardasil vaccine. Although aluminum has been associated with post-vaccination macrophagic myofasciitis and heavy metal ingestion has been associated with a single case of GBS, the role of aluminum in the genesis of GBS is not clear [24–27].

Our study has some limitations that need to be addressed prior to interpreting the data. Case ascertainment was based on VAERS reports, a passive surveillance system that is subject to underreporting, differential reporting, ascertainment bias, and variability in report quality and completeness [11]. The actual reporting rate of GBS following vaccination is probably higher because the reporting system is voluntary, and it is therefore likely that an undefined proportion of GBS events were not reported to the VAERS. On the other hand, over-diagnosis of GBS following vaccination should be taken into consideration due to lack of a standard definition and diagnostic criteria. Therefore, besides the true increase of risk of GBS with vaccination, an increase in reports to the VAERS may be due to an increase in efficiency of reporting, vaccine coverage, and background rate of GBS.

Despite the limitations of our study, there is evidence that GBS was reported more frequently within 6 weeks of Gardasil vaccination compared with other vaccines and the general population. Because of possible underestimation incurred during ascertainment using passive surveillance, a prospective active surveillance for accurate ascertainment and identification of high-risk groups is warranted.

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EXHIBIT 160

NEUROMUSCULAR DISEASE

Volume 11, Number 1
September 2009

Guillain-Barré Syndrome after Vaccination in United States: Data From the Centers for Disease Control and Prevention/Food and Drug Administration Vaccine Adverse Event Reporting System (1990–2005)

Nizar Souayah, MD, Abu Nasar, MS,† M. Fareed K. Suri, MD,* and Adnan I. Qureshi, MD**

Abstract

Background:
There are isolated reports of Guillain-Barré syndrome (GBS) after receiving vaccination.

Objective:
To determine the rates and characteristics of GBS after administration of vaccination in United States

Methods:
We used data for 1990 to 2005 from the Vaccine Adverse Event Reporting System, which is a cooperative program of the Centers for Disease Control and Prevention and the US Food and Drug Administration.

Results:
There were 1000 cases (mean age, 47 years) of GBS reported after vaccination in the United States between 1990 and 2005. The onset of GBS was within 6 weeks in 774 cases, >6 weeks in 101, and unknown in 125. Death and disability after the event occurred in 32 (3.2%) and 167 (16.7%) subjects, respectively. The highest number (n = 632) of GBS cases was observed in subjects receiving influenza vaccine followed by hepatitis B vaccine (n = 94). Other vaccines or combinations of vaccines were associated with 274 cases of GBS. The incidence of GBS after influenza vaccination was marginally higher in subjects <65 years compared with those ≥65 years (P = 0.09); for hepatitis vaccine, the incidence was significantly higher ($P < 0.0001$) in the <65 group. Death was more frequent in subjects ≥65 years compared with those <65 years ($P < 0.0001$).

Conclusions:
Our results suggest that vaccines other than influenza vaccine can be associated with GBS. Vaccination-related GBS results in death or disability in one fifth of affected individuals, which is comparable to the reported rates in the general GBS population.

Key Words: vaccination, Guillain-Barré, syndrome, morbidity, mortality, influenza vaccine

(*J Clin Neuromusc Dis* 2009;11:1–6)

INTRODUCTION

Although vaccination substantially reduces morbidity and mortality from many infections, cases of Guillain-Barré syndrome (GBS) after vaccination have been reported. Concern about developing postvaccination GBS arose first during the 1976 to 1977 "swine influenza" vaccine season.[1] A statistically significant elevation of GBS within weeks after vaccination was found in swine flu vaccinees relative to nonvaccinees.[2] Subsequently, there has been a rising interest whether other vaccines also increase the risk of GBS. Without evidence of clear relationship of cause and effect, few studies have attempted to address a linkage between GBS and vaccines other than influenza vaccine.[3,4] The aim of this study is to determine the occurrence of GBS after vaccination for a variety of diseases and to provide further information about the characteristics and temporal profile of these occurrences.

METHODS

We used the data derived from the Vaccine Adverse Event Reporting System (VAERS), which is a cooperative program instituted by Centers for Disease Control and

From the *Epidemiological and Outcomes Research Division, Zeenat Qureshi Stroke Research Center, and the Department of Neurology, University of Medicine and Dentistry of New Jersey, Newark, NJ; and †Department of Surgery, Columbia University, New York, NY.

Reprints: Nizar Souayah, MD, 90 Bergen Street, DOC 8100, Newark NJ, 07103 (e-mail: souayani@umdnj.edu).

NEUROMUSCULAR DISEASE

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Prevention and the US Food and Drug Administration. VAERS collects information about adverse events that occur after the administration of US-licensed vaccines. GBS cases reported after vaccination in the United States were recorded between 1990 and 2005. The occurrence of GBS within 6 weeks of vaccination was considered suggestive of causal association.[1,2] This was based on the fact that in studies that showed an increased risk of GBS after vaccination, most of the risk was within 6 weeks after vaccination.[1,5]

RESULTS

There were 1000 cases of GBS reported after vaccination in the United States between 1990 and 2005. The mean age was 47 years. Four hundred ninety-seven subjects (49.7%) were male, 466 (46.6%) were female, and in 37 (3.7%), subjects' gender was unknown. The mean number of GBS after vaccination is 62.5 cases per year (± 19.67). The trends in GBS incidence were stable over the study time. GBS occurred either after the administration of a single vaccine (or combination of two vaccines administered as a single vaccination) or a combination of two or more different vaccines (Table 1). The onset of GBS was within 6 weeks in 773 subjects, in 102 subjects, and unknown in 125. Death and disability after the event occurred in 32 (3%) and 167 (16.7%) subjects, respectively.

The highest percentage of GBS with a single vaccine was observed in subjects receiving influenza vaccine. Of these 632 cases, 511 (81%) developed GBS within 6 weeks of vaccination, 53 (8%) > in 68 others (11%), the time between GBS and vaccination was unknown. Death occurred in 29 (4%) of these cases and disability in 98 (15%). Based on data obtained from the National Health Interview Survey from 1997 to 2005, it is estimated that an average of 54 million patients are vaccinated with influenza vaccine each year. Thus, the incidence of postinfluenza vaccination GBS is approximately 0.75 per million vaccinations. Hepatitis B vaccination was the second most frequently linked with postvaccination GBS with 94 cases. Of these, 55 subjects (59%) developed GBS within 6 weeks of vaccination,

TABLE 1. Guillain-Barré Syndrome Associated With Vaccination*

Vaccines	Total No. of Cases	Number of Cases Observed Within 6 Weeks After Vaccination	Number of cases Observed >6 Weeks After Vaccination	Number of Cases With Unknown Time Interval Between Vaccination and Event	Death	Disability
Influenza virus vaccine	632	511 (81%)	53 (8.4%)	68 (11%)	29 (4%)	98 (15%)
Hepatitis B vaccine	94	55 (59%)	14 (15%)	25 (27%)	1 (1%)	21 (22%)
Tetanus and diphtheria toxoids, adult	32	28 (88%)	2 (6%)	2 (6%)	0	5 (16%)
Measles–mumps–rubella virus vaccine live	24	19 (79%)	4 (17%)	1 (4%)	0	4 (17%)
Pneumococcal vaccine polyvalent	23	14 (61%)	1 (4.3%)	8 (35%)	0	6 (26%)
Single vaccine other than the mentioned vaccines	73	43 (59%)	14 (19%)	16 (22%)	0	13 (18%)
Combination of two or more vaccines	122	103 (84%)	14 (12%)	5 (4%)	2 (2%)	20 (16%)
Total	1000	773 (77%)	102 (10%)	125 (13%)	32 (3%)	167 (17%)

*Data from the Vaccine Adverse Event Reporting System (VAERS).

14 (15%) >6 weeks, and in 25 (27%) unknown. One case of death (1%) and 21 cases of disability (22%) were reported after GBS after hepatitis vaccination. Based on data obtained from the National Health Interview Survey from 2000 to 2005, it is estimated that an average of 2.4 million patients are vaccinated with influenza vaccine each year. Thus, the incidence of posthepatitis B vaccination GBS is approximately 2.4 per million vaccinations.

Tetanus and diphtheria toxoids vaccination (TD) was the third most frequently reported cause of postvaccination GBS with a single vaccine. Of a total of 32 subjects who developed GBS after TD vaccination, 28 (88%) developed GBS within 6 weeks of vaccination. Disability was reported in five subjects (16%).

Postvaccination GBS occurred in 24 subjects after measles, mumps, and rubella virus vaccination. In 19 of them (79%), GBS occurred within 6 weeks of vaccination and four (17%) sustained disability. Pneumococcal vaccine was associated with GBS in 23 subjects. Fourteen of them (61%) developed GBS within 6 weeks postvaccination and six (26%) developed disability.

Seventy-three subjects developed postvaccination GBS after a single vaccine other than the vaccines mentioned previously. Forty-three of them (59%) developed GBS within 6 weeks of vaccination. Seventeen (23%) developed postvaccination disability.

Postvaccination GBS occurred in 122 subjects after the administration of a combination of two or more vaccines. In 103 of them (84%), GBS occurred within 6 weeks after vaccination. Death and disability occurred in, respectively, two (2%) and 20 (19%) subjects.

The percentage of posthepatitis vaccine GBS and postinfluenza vaccine was significantly higher in subjects <65 years compared with those ≥65 years (Table 2). Death was more frequent in subjects ≥65 years compared with <65 years ($P < 0.0001$).

The distribution of vaccine-associated GBS according to the date of reported onset of the disease in our study demonstrated a peak in the first 2 weeks postvaccination for influenza vaccine as well as for hepatitis vaccine and other vaccines administered as a single vaccine or in combination. Then, the number of postvaccination GBS declines in the third week postvaccination to reach a baseline at 6 weeks postvaccination (Fig. 1). This pattern is similar to the previously reported pattern of distribution postinfluenza vaccine-associated cases of GBS.[5,6]

DISCUSSION

The possibility of an association between GBS and vaccination, even without establishing causality, may cause public concern that could influence public policy on vaccination and perhaps reduce vaccination coverage.[7] Any possible association should be carefully evaluated. If there is a true linkage between GBS and vaccination, current vaccination policies should be modified to target subjects at high risk, in whom the benefit of vaccination outweighs the risk of postvaccination GBS. If no evidence of causal relationships is found, the public should be informed and the actual vaccination policies should be reinforced.[7]

The incidence of GBS in the general population has been reported to be uniform

TABLE 2. Postvaccination Guillain-Barré Syndrome Stratified by Age

	Total No. of Cases	Number of Cases Aged ≤65 Years	Number of Cases Aged >65 Years	Number of Cases of Unknown Age	*P* Value (>65 versus younger)
Influenza virus vaccine	632	408 (65%)	179 (28%)	45 (7%)	0.09
Hepatitis B vaccine	94	75 (80%)	2 (2%)	17 (18%)	<0.0001
Death	32	12 (38%)	17 (535)	3 (9%)	<0.0001

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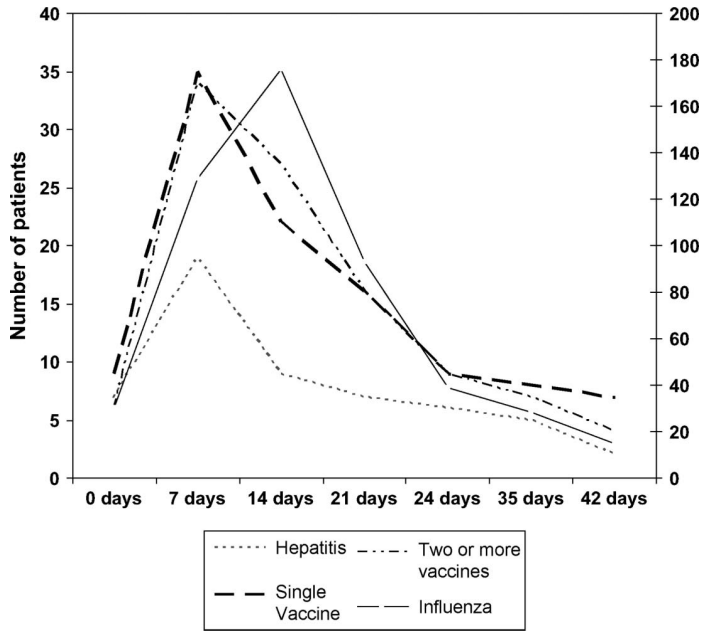

FIGURE 1. Guillain-Barré syndrome reported after vaccination, 1990 to 2005; distribution of cases in the first 42 days after vaccination.

between 0.6 and four cases per 100,000 person-year.[8] Although the estimated incidence of postinfluenza vaccination is not different from the incidence in the general population, the occurrence of most cases within 6 weeks after vaccination support the common understanding that GBS is more strongly associated with vaccination for influenza than for vaccination for other diseases. However, it is also apparent that influenza vaccine is not the only one that presents a risk. We hypothesize that the GBS observed after vaccination may arise by either molecular mimicry or nonspecific activation of the immune system. Some vaccines may be more likely to trigger GBS because of high dose or high antigenicity of its endotoxin, antigenicity of other components of the vaccine (other than the endotoxin), and genetic predisposition of the vaccinated subjects to develop vaccine-induced autoimmunity.[9,10]

In our study, the distribution of postvaccination GBS according to the date of onset of the disease follows a pattern with a peak occurrence within the first 2 weeks postvaccination followed by declining occurrence starting in the third week to reach a baseline in the fourth to sixth week (Fig. 1). This was true for all of the vaccines in our study, not just influenza vaccine. A similar pattern was observed by Lasky et al when they studied the association between GBS and influenza vaccines.[5] The probability to observe this distribution over 6 weeks with such degree of imbalance by chance alone was considered to be low.[5] When Haber et al[6] analyzed data from the VAERS for GBS related to influenza vaccine, they also noted the peak of GBS onset in the second week

Although the incidence of GBS after influenza vaccination is low (the risk of GBS increases by one case per million person vaccinated)[5], the swine influenza experience suggests that continued monitoring of GBS occurrence after vaccination would be prudent.[3,6,7,11]

Within 1 year after GBS occurrence in the general population, 4% to 15% of subjects die and up to 20% are disabled despite advances in patient management.
study, 3.2% of subjects died and 16.7% were disabled, which figures are comparable to those reported rates in the general GBS population.

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Because the VAERS data do not record the total number of vaccinated subjects in the general population for each vaccine, it was not possible to estimate the incidence of postvaccination GBS for most vaccines in our study.

Our study has some limitations because it is based on data from VAERS, a passive surveillance system. Like all passive surveillance systems, VAERS may be subject to underreporting, differential reporting, ascertainment bias, and variability in report quality and completeness.[6] Thus, besides the true increase of risk of GBS with vaccination, an increase in reports to VAERS may be the result of an increase in efficiency of reporting, vaccine coverage, and background rate of GBS. However, the objective of our study was to identify the occurrence of GBS without attempting to determine incidences of GBS. We think that our report provides adequate information on occurrence of GBS with the available vaccines from 1990 to 2005 in the United States. Vaccination dramatically reduced fatalities caused by many infections including influenza infection.[12] In the prevaccination era, the mortality from diphtheria was 8.654%, tetanus 81.3%, hepatitis B 0.35%, pneumococcal infection was 10.3%, measles 0.08%, mumps 0.024%, and rubella 0.036%.[12] An estimated >20,000 influenza-associated deaths occurred during each of 11 different US epidemics from 1972 to 1973 through 1994 to 1995, and 40,000 influenza-associated deaths occurred during each of six of these 11 epidemics.[5] The total number of deaths reported in our study (32 deaths) is much lower than death related to one influenza epidemic. The number of cases of most vaccine-preventable diseases is at an all-time low; hospitalizations and deaths have also shown striking decreases.[12] Although our study may suggest that some cases of GBS are caused by vaccination, the very low incidence of vaccine-associated GBS, the significantly increased complications from influenza virus infection as well as from other viral and bacterial infections, and the rise of drug resistance to infections make vaccination the first-line strategy for many viral and bacterial infection prevention.[13–16]

CONCLUSIONS

Our results suggest that vaccines other than influenza vaccine can be associated with GBS. Vaccination-related GBS results in death or disability in one fifth of affected individuals, which is comparable to the reported rates in general GBS population. However, because of the lack of a case cohort control group in our study, which was based on data from passive surveillance system, further controlled studies are needed to confirm the association of GBS with vaccines other than influenza vaccine.

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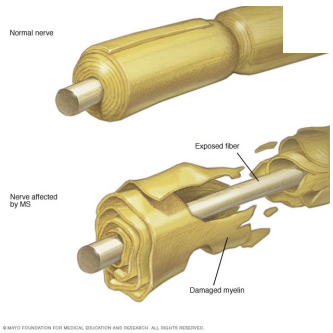
EXHIBIT 161

Multiple sclerosis

Overview

Multiple sclerosis (MS) is a potentially disabling disease of the brain and spinal cord (central nervous system).

In MS, the immune system attacks the protective sheath (myelin) that covers nerve fibers and causes communication problems between your brain and the rest of your body. Eventually, the disease can cause permanent damage or deterioration of the nerves.



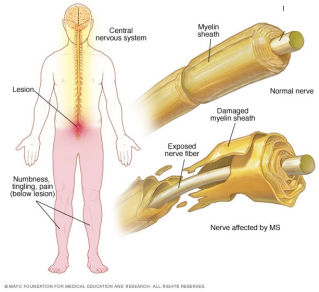
Multiple sclerosis

Signs and symptoms of MS vary widely and depend on the amount of nerve damage and which nerves are affected. Some people with severe MS may lose the ability to walk independently or at all, while others may experience long periods of remission without any new symptoms.

There's no cure for multiple sclerosis. However, treatments can help speed recovery from attacks, modify the course of the disease and manage symptoms.

Symptoms

Multiple sclerosis signs and symptoms may differ greatly from person to person and over the course of the disease depending on the location of affected nerve fibers. Symptoms often affect movement, such as:



Myelin damage and the nervous system

- Numbness or weakness in one or more limbs that typically occurs on one side of your body at a time, or your legs and trunk
- Electric-shock sensations that occur with certain neck movements, especially bending the neck forward (Lhermitte sign)
- Tremor, lack of coordination or unsteady gait

Vision problems are also common, including:

- Partial or complete loss of vision, usually in one eye at a time, often with pain during eye movement

- Prolonged double vision
- Blurry vision

Multiple sclerosis symptoms may also include:

- Slurred speech
- Fatigue
- Dizziness
- Tingling or pain in parts of your body
- Problems with sexual, bowel and bladder function

When to see a doctor

See a doctor if you experience any of the above symptoms for unknown reasons.

Disease course

Most people with MS have a relapsing-remitting disease course. They experience periods of new symptoms or relapses that develop over days or weeks and usually improve partially or completely. These relapses are followed by quiet periods of disease remission that can last months or even years.

Small increases in body temperature can temporarily worsen signs and symptoms of MS, but these aren't considered true disease relapses.

At least 50% of those with relapsing-remitting MS eventually develop a steady progression of symptoms, with or without periods of remission, within 10 to 20 years from disease onset. This is known as secondary-progressive MS.

The worsening of symptoms usually includes problems with mobility and gait. The rate of disease progression varies greatly among people with secondary-progressive MS.

Some people with MS experience a gradual onset and steady progression of signs and symptoms without any relapses, known as primary-progressive MS.

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Multiple sclerosis care at Mayo Clinic

Cannabis for MS: Can it help treat symptoms?

Multiple sclerosis: Can it cause seizures?

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Causes

The cause of multiple sclerosis is unknown. It's considered an autoimmune disease in which the body's immune system attacks its own tissues. In the case of MS, this immune system malfunction destroys the fatty substance that coats and protects nerve fibers in the brain and spinal cord (myelin).

Myelin can be compared to the insulation coating on electrical wires. When the protective myelin is damaged and the nerve fiber is exposed, the messages that travel along that nerve fiber may be slowed or blocked.

It isn't clear why MS develops in some people and not others. A combination of genetics and environmental factors appears to be responsible.

Risk factors

These factors may increase your risk of developing multiple sclerosis:

- **Age.** MS can occur at any age, but onset usually occurs around 20 and 40 years of age. However, younger and older people can be affected.
- **Sex.** Women are more than two to three times as likely as men are to have relapsing-remitting MS.
- **Family history.** If one of your parents or siblings has had MS, you are at higher risk of developing the disease.
- **Certain infections.** A variety of viruses have been linked to MS, including Epstein-Barr, the virus that causes infectious mononucleosis.
- **Race.** White people, particularly those of Northern European descent, are at highest risk of developing MS. People of Asian, African or Native American descent have the lowest risk.
- **Climate.** MS is far more common in countries with temperate climates, including Canada, the northern United States, New Zealand, southeastern Australia and Europe.
- **Vitamin D.** Having low levels of vitamin D and low exposure to sunlight is associated with a greater risk of MS.
- **Certain autoimmune diseases.** You have a slightly higher risk of developing MS if you have other autoimmune disorders such as thyroid disease, pernicious anemia, psoriasis, type 1 diabetes or inflammatory bowel disease.
- **Smoking.** Smokers who experience an initial event of symptoms that may signal MS are more likely than nonsmokers to develop a second event that confirms relapsing-remitting MS.

Complications

People with multiple sclerosis may also develop:

- Muscle stiffness or spasms
- Paralysis, typically in the legs
- Problems with bladder, bowel or sexual function
- Mental changes, such as forgetfulness or mood swings
- Depression

- Epilepsy

More Information

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EXHIBIT 162

The prevalence of MS in the United States

A population-based estimate using health claims data

Mitchell T. Wallin, MD, MPH, William J. Culpepper, PhD, Jonathan D. Campbell, PhD, Lorene M. Nelson, PhD, Annette Langer-Gould, MD, PhD, Ruth Ann Marrie, MD, PhD, Gary R. Cutter, PhD, Wendy E. Kaye, PhD, Laurie Wagner, MPH, Helen Tremlett, PhD, Stephen L. Buka, ScD, Piyameth Dilokthornsakul, PharmD, PhD, Barbara Topol, MS, Lie H. Chen, DrPH, and Nicholas G. LaRocca, PhD, on behalf of the US Multiple Sclerosis Prevalence Workgroup

Correspondence
Dr. Wallin
mitchell.wallin@va.gov

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Abstract

Objective

To generate a national multiple sclerosis (MS) prevalence estimate for the United States by applying a validated algorithm to multiple administrative health claims (AHC) datasets.

Methods

A validated algorithm was applied to private, military, and public AHC datasets to identify adult cases of MS between 2008 and 2010. In each dataset, we determined the 3-year cumulative prevalence overall and stratified by age, sex, and census region. We applied insurance-specific and stratum-specific estimates to the 2010 US Census data and pooled the findings to calculate the 2010 prevalence of MS in the United States cumulated over 3 years. We also estimated the 2010 prevalence cumulated over 10 years using 2 models and extrapolated our estimate to 2017.

Results

The estimated 2010 prevalence of MS in the US adult population cumulated over 10 years was 309.2 per 100,000 (95% confidence interval [CI] 308.1–310.1), representing 727,344 cases. During the same time period, the MS prevalence was 450.1 per 100,000 (95% CI 448.1–451.6) for women and 159.7 (95% CI 158.7–160.6) for men (female:male ratio 2.8). The estimated 2010 prevalence of MS was highest in the 55- to 64-year age group. A US north-south decreasing prevalence gradient was identified. The estimated MS prevalence is also presented for 2017.

Conclusion

The estimated US national MS prevalence for 2010 is the highest reported to date and provides evidence that the north-south gradient persists. Our rigorous algorithm-based approach to estimating prevalence is efficient and has the potential to be used for other chronic neurologic conditions.

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From the Department of Veterans Affairs Multiple Sclerosis Center of Excellence (M.T.W., W.J.C.); Georgetown University School of Medicine (M.T.W.), Washington, DC; University of Maryland (W.J.C.), Baltimore; University of Colorado (J.D.C., P.D.), Aurora; Stanford University School of Medicine (L.M.N., B.T.), CA; Southern California Permanente Medical Group (A.L.-G., L.H.C.), Pasadena; Departments of Internal Medicine and Community Health Sciences (R.A.M.), Max Rady College of Medicine, Rady Faculty of Health Sciences, University of Manitoba, Winnipeg, Canada; University of Alabama at Birmingham (G.R.C.); McKing Consulting Corp (W.E.K., L.W.), Atlanta, GA; Faculty of Medicine (Neurology) and Centre for Brain Health (H.T.), University of British Columbia, Vancouver, Canada; Brown University (S.L.B.), Providence, RI; and National Multiple Sclerosis Society (N.G.L.), New York, NY.

Go to Neurology.org/N for full disclosures. Funding information and disclosures deemed relevant by the authors, if any, are provided at the end of the article.

United States Multiple Sclerosis Prevalence Workgroup (MSPWG) coinvestigators are listed in appendix 2 at the end of the article.

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Glossary

AHC = administrative health claims; **CI** = confidence interval; **ICD-9** = *International Classification of Disease-9th revision*; **KPSC** = Kaiser Permanente Southern California; **MEPS** = Medical Expenditure Panel Survey; **MS** = multiple sclerosis; **NMSS** = National Multiple Sclerosis Society; **OP** = Optum; **TH** = Truven Health; **VA** = Department of Veterans Affairs.

Chronic disease morbidity is challenging to assess within the United States because it lacks a unified health system, and limited infrastructure exists for identifying and tracking patients across their lifespan. Options for determining incidence and prevalence estimates include surveys, registries, or administrative health claims (AHC) datasets. Each method has strengths and limitations; however, the increasing availability of large AHC datasets has made this approach efficient and cost-effective.[1]

Multiple sclerosis (MS) is the most common progressive neurologic disease of young adults worldwide.[2,3] Current estimates suggest that 300,000 to 400,000 individuals are affected in the United States, but this is based largely on revisions of estimates from older data.[2–6] These estimates do not reflect the changing demographics of the United States or potential changes in the ascertainment of MS due to modifications in the diagnostic criteria and new treatment options. Moreover, studies in neighboring Canada have reported steep increases in the prevalence of MS over the past few decades across several provinces.[7–9]

Because of the challenges in estimating MS prevalence for the United States, the National Multiple Sclerosis Society (NMSS) formed the Multiple Sclerosis Prevalence Workgroup, made up of scientists and policy advocates with the goal of producing a scientifically sound and economically feasible national MS prevalence estimate. By applying a validated case algorithm for MS to multiple large AHC datasets, we aimed to generate a robust national MS prevalence estimate for the adult population, stratified by age, sex, and region.

Methods

Setting and data sources

The United States includes 48 contiguous states, Hawaii, and Alaska. The 48 contiguous states range from ≈24.52° N to 49.28° N latitude and from ≈66.95° W to 124.77° W longitude. The US population encompassing all 50 states is steadily growing, having increased from 309.3 million in 2010 to 325.8 million in 2017.[10] In the United States, health insurance may be obtained from several private or public (government) sources, and a proportion of the population is uninsured. We acquired several AHC datasets representing the US private and government-sponsored insurance programs, reasoning that nearly all persons with MS, except the uninsured, Native Americans using the Indian Health Service, and the incarcerated, would receive health services through one of these programs. Each included the adult population (≥18 years) and their health care use for the years 2008 to 2010. The breakdown of the population within specific health insurance programs varies by income, sex, disability, and age group.[1]

Private insurance

In 2016, most adults <65 years of age (73%) obtained their health care coverage from private insurance plans.[11] Because the AHC datasets available for the private insurance sector vary in terms of geographic coverage and the types of providers represented, we accessed 3 datasets: Optum (OP), Truven Health (TH), and Kaiser Permanente Southern California (KPSC). These 3 private health datasets represent a broad sample of all such plans in the US insurance market. Two of these datasets (OP and TH for 2008–2010) were used in the prevalence calculations and collectively capture ≈35% of the privately insured in the United States.

Public insurance

Low-income adults and those with particular disabilities may obtain health care coverage through government-funded Medicaid plans. In 2016, 96% of US adults ≥65 years of age were enrolled in government-funded Medicare.[11] In the public sector, the Centers for Medicare & Medicaid Services datasets captured all eligible persons (100%) enrolled in Medicare or Medicaid across the United States (>50 million individuals).[12] All active-duty military enrollees receive their health care from the Department of Defense, and based on current eligibility criteria, ≈30% to 40% transfer to the Department of Veterans Affairs (VA) health care and benefit system when they leave active duty. To assess the military sector, we used the VA database, which included all persons enrolled in the VA health care system. Collectively, these datasets provided health care information for >125 million persons.

The AHC datasets varied with respect to the information captured. Therefore, we developed a common data dictionary and variable list for this study. These included a denominator file for all enrollees, including dates of insurance eligibility, sex, year of birth, and geographic region of residence. Because race and ethnicity were not available in all data sources, they were not considered in this analysis. We also accessed data on health care encounters in the inpatient and outpatient settings, as well as prescription drug claims. Each inpatient and outpatient encounter included ≥1 diagnostic codes, recorded with the ICD-9 system, as well as the date of the encounter. In the ICD-9 system, MS is uniquely identified by the 340 code. For inpatient encounters, we used the date of admission. Prescription drug claims included the name of the medication and the date of release.

Diagnostic algorithm for MS

As described elsewhere,[13] we developed and tested several algorithms to identify people with MS using AHC datasets compared with physician-adjudicated MS cases as the reference standard. The optimal algorithm in terms of sensitivity, specificity, and simplicity required the accumulation of ≥3 MS-related hospitalizations, outpatient visits, or prescription release encounters for an MS disease-modifying therapy in any combination within a 1-year period. For prescription drug claims, we considered only disease-modifying therapies approved by the US Food and Drug Administration for MS by 2010, including the interferon betas, glatiramer acetate, natalizumab, and fingolimod.[14] To avoid misclassification, claims for natalizumab were not included if the individual also had an ICD-9 code for inflammatory bowel disease, another disorder for which this medication is approved.

When tested among individuals with at least 1 MS claim, the sensitivity of the MS algorithm was 86% to 92% and the specificity was 66% to 83%, depending on the dataset.[13] When tested in a Canadian population that included individuals with and without any MS claims (i.e., general population), the sensitivity was 96.0% and the specificity was 99.5%.[13]

Prevalence estimates

For the TH, OP, VA, and KPSC datasets, enrollees who also had Medicare coverage were removed from both the numerators and the denominators within each dataset to prevent double counting. The annual prevalence within a given dataset was demarcated as all those who met the MS case definition divided by the annual population at risk, defined as all enrollees ≥18 years of age at the beginning of the calendar year and with health plan eligibility for a total of 6 months within the calendar year. Because individuals with MS may have variable contact with the health system, once an enrollee met the case definition and remained eligible for care, he or she was considered a case thereafter. Applying the algorithm to each dataset, we determined the prevalence at the end of the 3-year study period by identifying all persons who met the case definition in any 1 of the 3 study years who were still alive and eligible for care in the last year of the study period (2010) and dividing this by the population at risk in 2010. These 3-year prevalence estimates were stratified by sex, age (18–24, 25–34, 35–44, 45–54, 55–64, 67–74, and ≥75 years) and US Census region (North, East, South, and West). They were directly age and sex standardized to the 2010 US Census.[10] Confidence intervals (CIs) were calculated for the final total number of cases using binomial CIs ($\pm 1.96 \times \sqrt{(NPQ}$, where P and Q are the proportions of cases and noncases and N is the estimated US population in 2010). The 95% CIs were then adjusted for the rate per 100,000, and the inflation factors were calculated with a fixed-effects model. Verification of the prevalence estimates was performed for each dataset by 2 independent reviewers (W.E.K. and L.W.) for quality control.

To obtain a national US prevalence estimate for MS, we undertook several analytic steps. First, we treated estimates from OP and TH as random samples drawn from the same underlying population (the commercially insured), so their age- and sex-stratified estimates were pooled with the use of a random-effects model to represent the US private insurance populations. Because KPSC was included in the general population denominator in the West region, it was not included in these calculations. Sensitivity analyses examining the effects of including or excluding KPSC from the West region were conducted, and the findings did not differ significantly (data not shown). Second, we used data from the US Census to determine the total size of the US population in each age and sex stratum and the proportion with private, public, and military health insurance coverage.[15] Medicaid, Medicare, and military veteran prevalence estimates fully captured these populations.[10,15] The stratum-specific estimate was multiplied by the total insured US population in that stratum to determine the number of individuals affected. In 2010, 16% of the US population was uninsured, but the proportion uninsured varies across conditions.[11,16] To account for the uninsured MS population, we used data from both the Sonya Slifka cohort study[17] and the NMSS, which reported a 5.0% uninsured rate within the MS population before the initiation of the Affordable Care Act. Thus, the number of individuals affected in each stratum was summed, inflated by 5.0% to account for the uninsured across all strata, and then divided by the total US population to generate a summary prevalence estimate for the United States.

The term cumulative prevalence applies to our case finding approach within datasets in that once an individual meets the MS case definition for a given year, that person is counted as a case for subsequent years through 2010 if he or she remains active in the health plan. This method of case ascertainment effectively represents a limited-duration (3-year) prevalence. Ultimately, the prevalence estimate of interest is lifetime prevalence, which is the proportion of a population that at some point in life (up to the time of assessment) has developed MS. In chronic, predominantly relapsing diseases such as MS that start in early adult life, individuals may forgo contact with the health system for extended periods. Thus, long periods of observation (minimum 10 years) are needed to approach lifetime prevalence in the assessment of AHC datasets, as described previously for systemic lupus erythematosus[18] and as widely recognized in the cancer literature.[19] As noted elsewhere,[13] by using AHC datasets available from Intercontinental Marketing Services, the VA, and the province of Manitoba over the period of 2000 to 2016, we determined the proportion of cases missed by using a 3-year vs 10-year cumulative prevalence estimate. On the basis of these findings, undercount adjustment factors for the 10-year cumulative prevalence were required and were estimated to range from 1.37 (lower bound, 95% CI 1.13–1.66) to 1.47 (upper bound, 95% CI 1.23–1.76). We applied these factors to derive estimates for the 2010 prevalence of MS cumulated over 10 years.[13]

Complementary analysis

The cumulative prevalence of MS grows at variable rates and eventually levels off when an algorithm is applied to a given health system AHC dataset.[7,13] We have shown that the average annual growth in the MS prevalence rate between 2010 and 2017 for 2 AHC datasets was 2.3%/y.[13] This growth rate can be applied to the 2010 prevalence estimates to obtain a more recent prevalence figure.

SAS version 9.4 (SAS Institute Inc, Cary, NC) and SPSS version 22 (IBM Inc, Armonk, NY) were used to conduct the statistical analyses.

Standard protocol approvals, registrations, and patient consents

The study was approved by the Institutional Review Board at the VA Medical Center–Baltimore/University of Maryland Medical Center, KPSC, Colorado Multiple Institutional Review Board, Stanford University, and Quorum Review. Standard contracts and data use agreements were obtained for the analysis of all datasets.

Data availability

The datasets for this study were purchased and are owned by the NMSS. There are no current sharing agreements, and data are held under a data use contract with the NMSS.

Results

The characteristics of the AHC datasets during the study period are summarized in table 1; in total, they captured 125 million persons ≥18 years of age. The 3 private insurance datasets collectively captured 58 million individuals, approximately half of the privately insured US adult population (age 18–64 years). Collectively, the public (government) insurance sources captured all 68 million individuals nationwide who are insured through these plans.

The prevalence estimates reported refer to the adult population. The age- and sex-stratified prevalence estimates for MS in 2010 cumulated over 3 years (2008–2010) and number of cases from these datasets are displayed in tables e-1 through e-5 (available from Dryad, https://doi.org/10.5061/dryad.pm793v8). The prevalences for OP and TH are remarkably similar, while the VA sex-stratified estimates are highest among the datasets. Denominator data from the 2010 US Census are shown in table e-6 (available from Dryad, https://doi.org/10.5061/dryad.pm793v8).

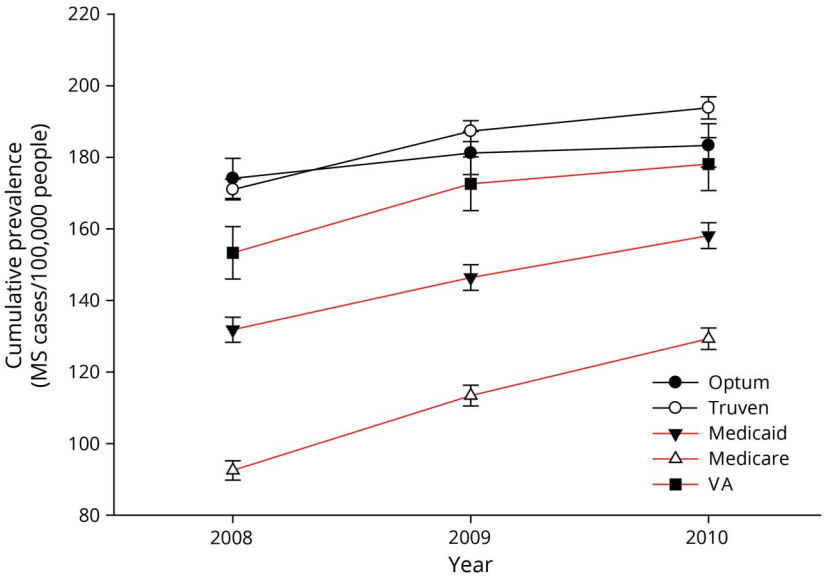
The annual cumulative prevalence of MS in the United States for 2008 to 2010 using the MS algorithm is displayed for the 2 private health insurance datasets (OP and TH) and the 3 government insurance datasets (Medicaid, Medicare, and VA) in figure 1. The average annual increase in prevalence

Table 1 Characteristics of AHC datasets used for the US MS prevalence estimate

Health claims database (URL)	Adult enrollees 2008–2010, n	Health plan characteristics	Geographic coverage	Variables in database
OP (optum.com)	15 million	Employer-based, fee-for-service, preferred provider, or capitated health plans; United Health Care	All 50 states	Demographic data, hospital admissions, outpatient claims, emergency room claims, prescription medications
TH Market Scan (marketscan.truvenhealth.com)	40 million	Mix of private health insurance companies	All 50 states	Demographic data, hospital admissions, outpatient claims, behavioral health claims, emergency room claims, prescription medications
KPSC (share.kaiserpermanente.org)	2.7 million	Health maintenance organization with integrated clinical and hospital network	Southern California	Demographic data, hospital admissions, outpatient claims, emergency room claims, prescription medications, clinical records data, laboratory data, imaging data
VA (va.gov)	8.5 million	National government health care program for military veterans; outpatient clinics, medical centers, rehabilitation facilities, nursing homes, and service offices	All 50 states	Demographic data, hospital admissions, outpatient claims, emergency room claims, prescription medications, clinical records, laboratory data, imaging data
Medicaid (national) (cms.gov)	27.9 million	State-based health insurance program supplemented by federal funding to provide for those in poverty or with designated disabilities	All 50 states	Demographic data, hospital admissions, outpatient claims, emergency room claims, prescription medications
Medicare (national) (cms.gov)	31.2 million	Federal health insurance program for the elderly (≥65 y) and disabled	All 50 states	Demographic data, hospital admissions, outpatient claims, emergency room claims, prescription medications

Abbreviations: AHC = administrative health claims; KPSC = Kaiser Permanente Southern California; MS = multiple sclerosis; OP = Optum; TH = Truven Health; VA = Department of Veterans Affairs.

Figure 1 Average annual prevalence of MS in the United States for 2008, 2009, and 2010 per 100,000 population for private (OP and TH, black lines) and government (Medicaid, Medicare, and VA, red lines) health insurance datasets



Multiple sclerosis (MS) cases acquired from the MS algorithm in year 1 are displayed, along with new cases added cumulatively in subsequent years. OP = Optum; TH = Truven Health; VA = Department of Veterans Affairs.

over this 3-year period for all datasets was 6.3% (SD 3.8%). The 2010 MS prevalence estimate cumulated over 3 years for the combined datasets was 199.84 (95% CI 199.83–199.85), corresponding to 470,053 people with MS.

After adjustment for the uninsured and application of the lower-bound inflation factor to account for undercounting due to the limited period of observation, the estimated 2010 prevalence for MS cumulated over 10 years was 265.1 per 100,000 (95% CI 264.3–265.8), corresponding to 623,437 people with MS. Similarly, after adjustment for the uninsured and application of the upper-bound inflation factor, the 2010 prevalence for MS cumulated over 10 years was 309.2 per 100,000 (95% CI 308.1–310.1), representing a total of 727,344 people with MS. The 2010 MS prevalence estimates cumulated over 10 years, 95% CIs, and number of cases in the United States stratified by age and sex (lower and higher estimates) are shown in table e-7 (available from Dryad, https://doi.org/10.5061/dryad.pm793v8). Figure 2 shows the lower and figure 3 displays the higher 2010 MS prevalence estimates cumulated over 10 years in the United States stratified by age and sex. The overall female:male prevalence ratio for 2010 was 2.8. The peak age-specific MS prevalence was 55 to 64 years, followed by 65 to 74 years.

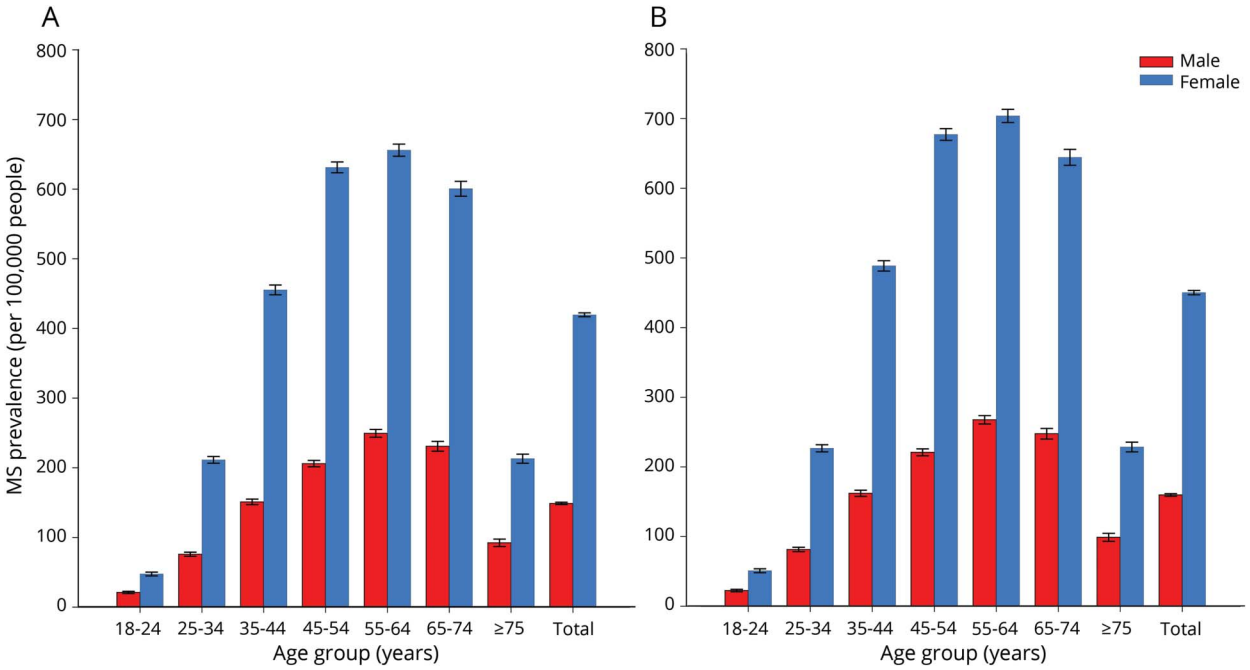
Tables 2 and 3 show the 2010 MS prevalence estimates cumulated over 10 years, 95% CIs, and number of cases in the US by age, sex, and geography (lower and higher estimates). Figure 3 illustrates the higher estimate for 2010 cumulated over 10 years by Census regions, along with corresponding sex ratios. The prevalence in the northern Census regions of the US (Northeast and Midwest) was statistically significantly higher than in the southern Census region as evidenced by nonoverlapping 95% CIs.

Discussion

We report a current national prevalence estimate for MS in the adult population by using a validated algorithm across 5 large US AHC datasets, which also accounted for the uninsured population and for the limited (3-year period) of observation. Our estimates were based on a case-finding strategy that identified MS cases annually combined with the 2010 Census for denominator data. Overall, nearly 45% of the US population was assessed, including 100% of those with publicly funded insurance. In 2010, our higher 10-year prevalence estimate was 309.2 per 100,000 population, representing 727,344 adults affected by MS. This higher 2010 estimate is based on the adjustment (3-year vs 10-year) seen in a single health insurance payer system covering the entire population. The lower-level estimate is based on a dataset adjustment (3-year vs 10-year) for a government insurance carrier for a segment of the population. Our approach accounted for the demographics of the national population, the sporadic follow-up for a chronic disease with young-adult onset, the different insurance providers within the health care system, and the uninsured.

When coupled with prior estimates of the prevalence of MS in the US, our findings suggest that there has been a steady rise in the prevalence of MS over the past 5 decades, that the prevalence of MS remains higher for women than men, and that a north-south geographic gradient still persists.[2] The earliest published national US MS prevalence estimate for the

Figure 2 2010 Prevalence for MS cumulated over 10 years in the United States per 100,000 population by age and sex



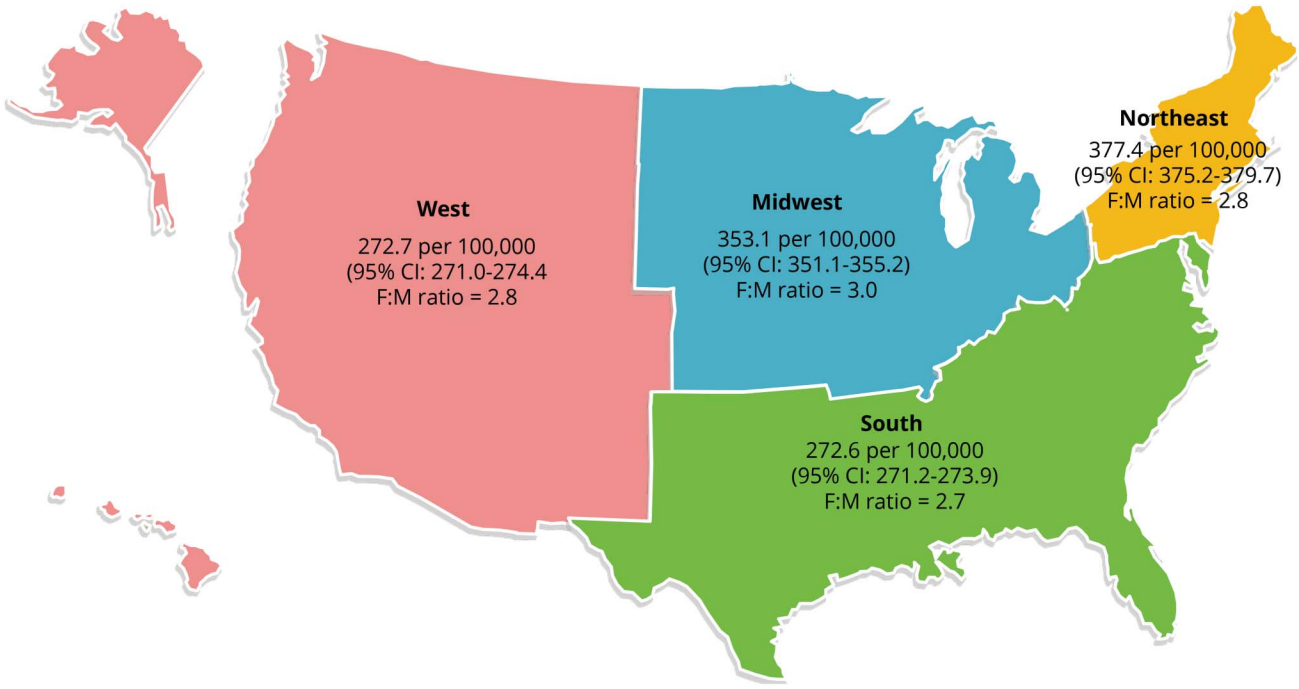
Higher and lower estimates adjusted to 2010 US Census based on combined datasets from the multiple sclerosis (MS) algorithm inclusive of the following: Truven, Optum, Department of Veterans Affairs, Medicare, and Medicaid (full data available for all age and sex estimates in data table e-7 available from Dryad, https://doi.org/10.5061/dryad.pm793v8). (A) Lower-estimate and (B) higher-estimate 2010 MS prevalence in the United States per 100,000 population.

adult and pediatric populations combined was 58 per 100,000 for the year 1976,[4] corresponding to 123,000 cases with a 1.7 female:male ratio. That study used surveys of hospitals, physicians, and patients to estimate prevalence. Subsequent researchers used this prevalence of 58 per 100,000 as a baseline and estimated the number of physician-diagnosed MS cases in the United States as 300,000 by 1990 after factoring in population changes and more contemporary regional prevalence estimates.[5] Using the National Health Interview Survey, investigators calculated a national MS prevalence rate of 85 per 100,000 for the period of 1989 to 1994.[20] The corresponding female:male ratio was reported as 2.6:1, much higher than the 1976 sex ratio.[4] Authors of a later study reported a national annual period prevalence of 0.21% over a 10-year period (1998–2009), with cases with MS identified by at least 1 ICD-9 340 code in the Medical Expenditure Panel Survey (MEPS).[21] This corresponded to 572,312 patients with MS. More recently, a team with a more restrictive algorithm over 5 years (2008–2012) using the PharMetrics commercially insured claims database produced an annual period MS prevalence estimate of 149 per 100,000 with 403,630 individual cases,[22] which is reasonably consistent with our 3-year estimate in 2010 of 470,053 cases. The corresponding female:male ratio was 3.1. The higher prevalence estimate of the MEPS database over a longer ascertainment period than that of the PharMetrics study emphasizes the need to account for undercounting with limited observation periods. This also accounts for the fact that our prevalence figure is most in line with the 10-year MEPS database estimate but with the advantage of a formally validated algorithm and a broader demographic sample.

We did not assess the prevalence of MS in children, and this should be considered when our findings are compared to those reported in other populations. If we use our age-stratified rates for 2010 (low and high estimates), they fall within the range of the 2006 MS prevalence estimates in Manitoba, Canada,[7] for all 10-year age groups. Similarly, the age-stratified prevalence estimates for Northern Ireland, United Kingdom, in 2004 were slightly higher than our age-specific estimates for the following age deciles: 25 to 34, 25 to 44, and 45 to 54 years.[23] MS prevalence in older age groups was slightly higher in the 2010 US population. Compared to the 2008 age-stratified prevalence estimates from British Columbia, Canada,[8] our US rates were variably higher (2%–30%) for all age categories. Thus, our age-stratified estimates are in line with recent North American and European figures. If we were to assume that the prevalence of MS in children were zero, although this is a conservative assumption, our 2010 prevalence estimates would range from 218.6 (low estimate) to 234.5 (high estimate) per 100,000 population.

Thus, our prevalence results are consistent with recent reports of MS prevalence from other regions that examined the entire population. Canada has observed dramatic increases in the prevalence of MS, with an estimate of 266.9 per 100,000 in Nova Scotia[9] for the year 2010 and a prevalence of 179.9 per

Figure 3 High-estimate 2010 prevalence of MS in the United States per 100,000 population (2010 US Census) by Census region with 95% CI and F:M prevalence ratio



CI = confidence interval; F:M = female:male; MS = multiple sclerosis.

100,000 in the province of British Columbia in 2008.[8] In European regions, the prevalence of MS has been variable, with northern regions having higher estimates. For example, a 2005 national prevalence of 154.5 per 100,000 (European Standard Population) was reported from Denmark.[24] Over the past decade, France has had national prevalence estimates generally <94.7 per 100,000 (standardized to French population).[25] South American prevalence studies are largely regional, but a 2005 study from Panama produced a crude prevalence of 5.2 per 100,000,[26] substantially lower than North American estimates.

Prevalence is the product of the incidence rate and the average duration of a condition. Incidence rates for MS have been generally stable or have slightly increased over the past 4 to 5 decades in white populations but are higher in selected racial groups.[27–29] Therefore, the rising prevalence estimates for MS across the Western world (i.e., populations of predominately European ancestry) largely reflect the aging of the population with improved survival.[7] In addition, the diagnostic criteria for MS have evolved, and an earlier diagnosis of MS is possible with the use of neuroimaging, and this is likely contributing to the increased prevalence observed.[30,31]

With the wide availability of electronic datasets within health care systems and new techniques to analyze data, there has been a rise in the number of morbidity and mortality studies on a global scale.[3] However, the methods for identifying cases and other variables of interest have not been standardized. To ensure that our approach is transparent and to support comparisons to future work in the United States and other jurisdictions, we have reported the annual MS prevalence rates for our combined and individual datasets.

A strength of our study is the validation of our algorithm against an available gold standard, that is, medical records in multiple datasets and health systems.[13] Our approach also accounted for the complexity of the current national health care system.[1] To address the need for accurate morbidity and mortality data for neurologic conditions, a national surveillance system approach compiling electronic AHC datasets and vital statistics would be a logical way forward. The Neurological Disease Surveillance System that was authorized by the US Congress in 2016 could adopt this relatively time- and cost-efficient approach.[32]

On the basis of observed increases in prevalence with our VA and Intercontinental Marketing Services datasets after 2010, we estimated that the prevalence of MS in 2017 cumulated over 17 years would range from 337.9 per 100,000 population (n = 851,749 persons with MS) to 362.6 per 100,000 population (n = 913,925 persons with MS). These 17-year cumulative estimates approach lifetime prevalence for MS within the bounds of our AHC datasets. However, these estimates should be viewed with caution because they assume that the factors that have contributed to the rising prevalence observed in the United States as of 2010 based on coding records have persisted to 2017 and that substantial changes in

Table 2 2010 Prevalence for MS cumulated over 10 years in the United States per 100,000 population by age, sex, and geography

Lower-estimate 2010 MS prevalence in the US per 100,000 population									
	West		Midwest		Northeast		South		Total
Age and sex groups (y)	Cases, n	Prevalence (95% CI)	Cases, n	Prevalence (95% CI)	Cases, n	Prevalence (95% CI)	Cases, n	Prevalence (95% CI)	Prevalence (95% CI)
Women 18–24	1,276	36.0 (33.6–38.4)	1,506	46.8 (44.0–49.6)	1,716	63.9 (60.3–67.5)	2,652	47.1 (45.0–49.3)	47.4 (46.1–48.8)
Men 18–24	759	19.8 (18.2–21.5)	599	17.9 (16.2–19.7)	832	30.1 (27.6–32.5)	1,123	19.2 (17.8–20.5)	21.0 (20.1–21.8)
Women 25–34	8,921	179.4 (174.9–183.8)	10,254	241.0 (235.4–246.6)	9,130	258.3 (252.0–264.7)	14,769	193.6 (189.8–197.3)	211.2 (208.8–213.6)
Men 25–34	2,935	56.8 (54.3–59.2)	3,442	80.0 (76.8–83.3)	3,548	102.0 (97.9–106.0)	5,618	74.5 (72.1–76.8)	75.8 (74.4–77.3)
Women 35–44	17,235	356.0 (349.7–362.4)	23,328	537.7 (529.4–546.0)	20,554	543.1 (534.2–552.0)	33,574	428.6 (423.1–434.1)	455.3 (451.8–458.8)
Men 35–44	6,254	126.7 (122.9–130.5)	7,476	174.1 (169.4–178.9)	6,834	187.8 (182.5–193.1)	10,473	136.4 (133.3–139.5)	151.0 (149.1–153.1)
Women 45–54	28,460	561.2 (553.3–569.0)	35,392	705.2 (696.4–714.0)	33,093	762.3 (752.5–772.2)	47,026	561.2 (555.1–567.3)	631.2 (627.3–635.1)
Men 45–54	8,765	175.0 (170.6–179.4)	11,889	240.9 (235.7–246.1)	10,775	260.5 (254.6–266.4)	14,107	175.6 (172.1–179.1)	205.9 (203.6–208.2)
Women 55–64	27,055	640.1 (630.9–649.2)	31,080	745.1 (735.2–755.0)	26,874	752.2 (741.4–763.0)	39,796	564.0 (557.4–570.6)	656.0 (651.6–660.3)
Men 55–64	9,964	248.8 (242.9–254.6)	11,753	295.5 (289.1–301.9)	10,018	304.8 (297.6–311.9)	12,451	193.2 (189.1–197.2)	249.4 (246.6–252.2)
Women 65–74	14,304	573.1 (561.8–584.3)	17,410	684.9 (672.8–697.1)	15,373	703.6 (690.2–716.9)	23,247	517.8 (509.8–525.7)	600.5 (595.2–605.8)
Men 65–74	5,376	241.2 (233.5–248.9)	5,972	270.2 (262.0–278.4)	4,867	265.4 (256.5–274.4)	7,232	186.0 (180.9–191.2)	230.8 (227.2–234.3)
Women ≥75	4,685	204.7 (197.7–211.7)	5,838	220.8 (214.0–227.6)	6,565	276.9 (268.9–285.0)	6,987	174.6 (169.7–179.5)	213.0 (209.7–216.2)
Men ≥75	1,749	110.4 (104.2–116.6)	1,583	95.6 (89.9–101.2)	1,288	89.4 (83.6–95.3)	2,084	80.5 (76.3–84.6)	92.2 (89.6–94.9)
Total	137,734	254.1 (252.5–255.7)	167,521	329.1 (327.2–331.0)	151,467	351.8 (349.6–353.9)	221,138	254.0 (252.7–255.3)	288.2 (287.4–289.0)

Abbreviations: CI = confidence interval; MS = multiple sclerosis.
Lower estimates adjusted to 2010 US Census on the basis of combined datasets from the MS algorithm inclusive of the following: Truven, Optum, Department of Veterans Affairs, Medicare, and Medicaid.

the distribution and survival of the population at risk of MS have not occurred. These assumptions include the continued high longevity of patients with MS and the general population, stable incidence of MS, and similar coding practices from 2010 to 2017. Extrapolated estimates have been modeled for MS and systemic lupus erythematosus within the Canadian health care system[17,33] by assuming stable incidence, which has been consistently reported across Canada. In the United States, recent data from the Centers for Disease Control and Prevention show a slight decrease in life expectancy for the US population in 2016,[34] and the demographic composition of the US population is also changing.[35] Future studies can use these methods to reassess the prevalence of MS and to examine how these factors affect the findings.

There are limitations to our approach. First, we did not include children, the Indian Health Service, the US prison system, or undocumented US residents in our prevalence estimates. However, these segments of the population are relatively small or, in the case of children, would contribute few cases, and many individuals would be detected by other health systems, including the Medicare insurance program, at some point later in life. Furthermore, diagnosing pediatric MS is challenging; the performance of our proposed algorithm would need to be tested in this population given the recognized differences in relapse rates, more prominent cognitive impairment that may affect health care use, and reported differences in performance of algorithms across the pediatric and adult populations in other chronic diseases.[36–38] This

Table 3 2010 Prevalence for MS cumulated over 10 years in the United States per 100,000 population by age, sex and geography

Higher-estimate 2010 MS prevalence in the US per 100,000 population									
	West		Midwest		Northeast		South		Total
Age and sex groups (y)	Cases, n	Prevalence (95% CI)	Cases, n	Prevalence (95% CI)	Cases, n	Prevalence (95% CI)	Cases, n	Prevalence (95% CI)	Prevalence (95% CI)
Women 18–24	1,369	38.6 (36.1–41.2)	1,616	50.2 (47.2–53.3)	1,841	68.6 (64.7–72.5)	2,846	50.6 (48.2–52.9)	50.9 (49.5–52.3)
Men 18–24	814	21.3 (19.5–23.1)	642	19.2 (17.4–21.1)	893	32.3 (29.6–34.9)	1,205	20.6 (19.1–22.0)	22.5 (21.6–23.4)
Women 25–34	9,572	192.5 (187.7–197.3)	11,003	258.6 (252.6–264.6)	9,796	277.2 (207.4–294.0)	15,846	207.7 (203.7–211.7)	226.7 (224.1–229.2)
Men 25–34	3,149	60.9 (58.2–63.5)	3,694	85.9 (82.4–89.3)	3,807	109.4 (105.1–113.7)	6,029	79.9 (77.4–82.4)	81.4 (79.8–82.9)
Women 35–44	18,493	382.0 (375.2–388.9)	25,030	577.0 (568.1–585.8)	22,055	582.7 (573.2–592.2)	36,024	459.9 (454.0–465.8)	488.5 (484.8–492.3)
Men 35–44	6,711	136.0 (131.9–140.0)	8,022	186.9 (181.8–191.9)	7,333	201.5 (195.8–207.2)	11,237	146.4 (143.0–149.7)	162.1 (159.9–164.3)
Women 45–54	30,537	602.1 (593.7–610.5)	37,975	736.7 (747.3–766.2)	35,509	818.0 (807.4–828.5)	50,458	602.2 (595.7–608.7)	677.2 (673.0–681.4)
Men 45–54	9,404	187.8 (183.1–192.5)	12,756	258.4 (252.9–264.0)	11,562	279.5 (273.2–285.8)	15,136	188.4 (184.7–192.1)	220.9 (218.5–223.4)
Women 55–64	29,030	686.8 (677.0–696.6)	33,349	800.0 (788.8–810.1)	28,835	807.1 (795.5–818.7)	42,701	605.2 (598.0–612.3)	703.8 (699.1–708.5)
Men 55–64	10,691	266.9 (260.7–273.2)	12,611	317.0 (310.2–323.9)	10,750	327.0 (319.3–334.7)	13,360	207.3 (202.9–211.6)	267.6 (264.6–270.6)
Women 65–74	15,349	614.9 (602.8–627.0)	18,680	734.9 (721.9–748.0)	16,495	754.9 (740.6–769.2)	24,945	555.6 (547.0–564.1)	644.3 (638.6–650.0)
Men 65–74	5,769	258.8 (250.5–267.1)	6,408	290.0 (281.1–298.8)	5,222	284.8 (275.2–294.4)	7,760	199.6 (194.1–205.1)	247.6 (243.8–251.4)
Women ≥75	5,027	219.6 (212.1–227.2)	6,264	236.9 (229.6–244.2)	7,043	297.2 (288.5–305.8)	7,497	187.3 (182.1–192.6)	228.5 (225.0–232.0)
Men ≥75	1,877	118.5 (111.8–125.1)	1,699	102.6 (96.5–108.6)	1,382	95.9 (89.7–102.2)	2,236	86.4 (81.9–90.8)	98.9 (96.1–101.8)
Total	147,792	272.7 (271.0–274.4)	179,749	353.1 (351–355.2)	162,523	377.4 (375.2–379.7)	237,279	272.6 (272.2–274.0)	309.2 (308.3–310.1)

Abbreviations: CI = confidence interval; MS = multiple sclerosis.
Higher estimates adjusted to 2010 US Census on the basis of combined datasets from the MS algorithm inclusive of the following: Truven, Optum, Department of Veterans Affairs, Medicare, and Medicaid.

work was out of the scope of the present project but should be pursued in future studies. Second, those with MS not followed in the traditional health care system (e.g., alternative medicine or cash health care practices that bypass health insurance reimbursement) would be missed by our method. This would result in an underestimate of MS cases. Third, we had 10 years of data for the VA health care system and the province of Manitoba but shorter lengths of data for other health systems to assess period effects on prevalence. While a decade of data would have been optimal for all health system datasets, the high costs of obtaining >3 years of data for all insurance pools were prohibitive. Finally, we have not characterized the racial or ethnic demographics of our MS population in this report because race and ethnicity were not uniformly collected in the AHC datasets used. Racial and ethnic differences in MS susceptibility may be a factor contributing to the geographic differences in prevalence in US Census regions.[28] Strengths of our approach included the large sample, which captured one-third of the US population; broad healthcare system representation; and the use of a validated case-finding algorithm that performed consistently across different health systems.[13]

The US national MS prevalence estimate for 2010 is the highest reported to date and provides a contemporary understanding of the disease burden. Our rigorous algorithm-based approach to estimate prevalence is efficient and can be reproduced in other health systems. We would advocate for this approach to be used for other chronic neurologic

conditions. Further work is needed to better understand the current differences in MS prevalence by race and to evaluate possible regional differences in health care use and disease morbidity for MS.

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Disclosure

M. Wallin has served on data safety monitoring boards for the National Institutes of Neurological Disease and Stroke–NIH, is a member of the NMSS Health Care Delivery and Policy Research study section, and receives funding support from the NMSS and the Department of Veterans Affairs Merit Review Research Program. W. Culpepper has research funding from the NMSS, receives support from the VHA MS Center of Excellence, and is a member of the NMSS Health Care Delivery and Policy Research study section. J. Campbell has consultancy or research grants over the past 5 years from the Agency for Healthcare Research and Quality, ALSAM Foundation, Amgen, AstraZeneca, Bayer, Biogen Idec, Boehringer Ingelheim, Centers for Disease Control and Prevention, Colorado Medicaid, Enterprise Community Partners Inc, Institute for Clinical and Economic Review, Mallinckrodt, NIH, NMSS, Kaiser Permanente, PhRMA Foundation, Teva, Research in Real Life Ltd, Respiratory

Appendix 1 Authors

Name	Location	Role	Contribution
Mitchell T. Wallin, MD, MPH	Department of Veterans Affairs Medical Center; Georgetown University, Washington, DC	Author	Study concept and design, critical review, statistical analyses, revision of the manuscript for content, approval of final manuscript
William J. Culpepper, PhD	Department of Veterans Affairs Medical Center, Washington, DC; University of Maryland, Baltimore, M D	Author	Study concept and design, statistical analyses, critical review, preparation of draft manuscript, approval of final manuscript
Jonathan Campbell, PhD	University of Colorado, Denver, CO	Author	Statistical analyses, critical review of the manuscript for content, approval of final manuscript
Lorene M. Nelson, PhD	Stanford University, Stanford, CA	Author	Study concept and design, critical review, revision of the manuscript for content, approval of final manuscript
Annette Langer-Gould, MD	Kaiser Permanente Southern California, Los Angeles, CA	Author	Study concept and design, statistical analyses, critical review, revision of the manuscript for content, approval of final manuscript
Ruth Ann Marrie, MD, PhD	University of Manitoba, Winnipeg, Manitoba, Canada	Author	Study concept and design, critical review, revision of the manuscript for content, approval of final manuscript
Gary R. Cutter, PhD	University of Alabama, Birmingham	Author	Statistical analyses, study concept and design, revision of the manuscript for content, approval of final manuscript
Wendy Kaye, PhD	McKing Consulting Corp, Atlanta, GA	Author	Statistical analyses, critical review of the manuscript for content
Laurie Wagner, MPH	McKing Consulting Corp, Atlanta, GA	Author	Statistical analyses, critical review of the manuscript for content
Helen Tremlett, PhD	University of British Columbia, Vancouver, BC, Canada	Author	Study concept and design, critical review of the manuscript for content, approval of final manuscript
Steven L. Buka, ScD	Brown University, Providence, RI	Author	Critical review of the manuscript for content, approval of final manuscript
Piyameth Dilokthornsakul, PharmD, PhD	University of Colorado, Aurora, CO	Author	Statistical analysis, critical review of the manuscript for content
Barbara Topol, MS	Stanford University, Stanford, CA	Author	Statistical analysis, critical review of the manuscript for content
Lie H. Chen, DrPH	Southern California Permanente Medical Group, Pasadena, CA	Author	Statistical analysis, critical review of the manuscript for content
Nicholas G. LaRocca	National Multiple Sclerosis Society, New York, NY	Author	Study concept and design, critical review of the manuscript for content, approval of final manuscript

Appendix 2 Coinvestigators and members of the US Multiple Sclerosis Prevalence Workgroup

Name	Location	Role	Contribution
Albert Lo, MD, PhD	Brown University, Providence, RI	Coinvestigator	Study concept and design
Robert McBurney, PhD	Accelerated Cure Project, Boston, MA	Coinvestigator	Study concept and design
Oleg Muravov, PhD	Agency for Toxic Substances and Disease Registry Division of Health Studies, Atlanta, GA	Coinvestigator	Study concept and design
Bari Talente, Esq	National Multiple Sclerosis Society, Washington, DC	Coinvestigator	Study concept and design
Leslie Ritter	National Multiple Sclerosis Society, Washington, DC	Coinvestigator	Study concept and design

Effectiveness Group, and Zogenix Inc. L. Nelson receives grants from the Centers for Disease Control and Prevention, NIH, and NMSS and contracts from the Agency for Toxic Substances and Diseases Registry. She receives compensation for serving as a consultant to Acumen, Inc and is on the Data Monitoring Committee of Neuropace. A. Langer-Gould was site principal investigator for 2 industry-sponsored phase 3 clinical trials (Biogen Idec and Hoffman-LaRoche) and 1 industry-sponsored observation study (Biogen Idec). She receives grant support from the NIH, National Institutes of Neurological Disease and Stroke, Patient-Centered Outcomes Research Institute, and the NMSS. R. Marrie is supported by the Waugh Family Chair in Multiple Sclerosis and receives research funding from Canadian Institutes of Health Research, Research Manitoba, Multiple Sclerosis Society of Canada, Multiple Sclerosis Scientific Foundation, Consortium of MS Centers, and NMSS. She also serves on the Editorial Board of *Neurology*. G. Cutter is a member of Data and Safety Monitoring Boards for AMO Pharmaceuticals, Apotek, Horizon Pharmaceuticals, Modigenetech/Prolor, Merck, Merck/Pfizer, Opko Biologics, Neurim, Sanofi-Aventis, Reata Pharmaceuticals, Receptos/Celgene, Teva Pharmaceuticals, National Heart, Lung, and Blood Institute (Protocol Review Committee), and National Institute of Child Health and Human Development (Obstetric Pharmacy Research Unit oversight committee) and on the Consulting or Advisory boards for Atara Biotherapeutics, Argenix, Bioeq GmBH, Consortium of MS Centers (grant), Genzyme, Genentech, Innate Therapeutics, Klein-Buendel Inc, Medimmune, Medday, Novartis, Opexa Therapeutics, Roche, Savara Inc, Somahlution, Teva Pharmaceuticals, Transparency Life Sciences, and TG Therapeutics. W. Kaye receives funding from the Agency for Toxic Substances and Disease Registry, the NMSS, and the Association for the Accreditation of Human Research Protection Programs. L. Wagner receives funding from the Agency for Toxic Substances and Disease Registry and NMSS. H. Tremlett is the Canada Research Chair for Neuroepidemiology and Multiple Sclerosis. She receives research support from the NMSS, the Canadian Institutes of Health Research, the Multiple Sclerosis Society of Canada, and the Multiple Sclerosis Scientific Research Foundation. In addition, in the last 5 years, she has received research support from the Multiple Sclerosis Society of Canada, the Michael Smith Foundation for Health Research, and the UK MS Trust, as well as travel expenses to attend conferences; all speaker honoraria are either declined or donated to an MS charity or to an unrestricted grant for use by her research group. S. Buka receives research funding from the NIH and the NMSS. P. Dilokthornsakul receives research funding from Pfizer Thailand and the Thai Traditional Medical Knowledge Fund. B. Topol receives grant support from the Centers for Disease Control and Prevention and the NMSS and contracts from the Agency for Toxic Substances and Diseases Registry. L. Chen reports no disclosures relevant to the manuscript. N. LaRocca is employed full-time by the NMSS. Go to Neurology.org/N for full disclosures.

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Neurology®

The prevalence of MS in the United States: A population-based estimate using health claims data

Mitchell T. Wallin, William J. Culpepper, Jonathan D. Campbell, et al.

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Out-of-pocket costs are on the rise for commonly prescribed neurologic medications

Neurology® 2019;93:688. doi:10.1212/WNL.0000000000008353

In the article "Out-of-pocket costs are on the rise for commonly prescribed neurologic medications" by Callaghan et al.,[1] first published online May 1, 2019, the 2004 out-of-pocket costs for MS medications (mean [SD]) in the Abstract and Results should be $33 ($50) rather than $15 ($23), and the 2004 median/IQR in the Results should be $25 ($20–$32) rather than $14 ($10–$16). The authors regret the errors.

Reference

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The prevalence of MS in the United States

A population-based estimate using health claims data

Neurology® 2019;93:688. doi:10.1212/WNL.0000000000007915

In the article "The prevalence of MS in the United States: A population-based estimate using health claims data" by Wallin et al.,[1] first published online February 15, 2019, the text regarding the lower bound for MS prevalence in a paragraph in Results should read: "After adjustment for the uninsured and application of the lower-bound inflation factor to account for undercounting due to the limited period of observation, the estimated 2010 prevalence for MS cumulated over 10 years was 288.2 per 100,000 (95% CI 287.4–289.0), corresponding to 623,437 people with MS." This is correctly represented in table 2. The authors regret the error.

Reference

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Epidemiology of NMOSD in Sweden from 1987 to 2013

A nationwide population-based study

Neurology® 2019;93:688. doi:10.1212/WNL.0000000000008382

In the article "Epidemiology of NMOSD in Sweden from 1987 to 2013: A nationwide population-based study" by Jonsson et al.,[1] in figure 5, the incidence of NMOSD in Australia and New Zealand should have been 0.37/1,000,000 person-years (CI: 0.35–0.39). The figure should also have included A and B labels for the panels and a label for the first panel's x-axis, "Incidence rate (per 1.000.000 individuals)." The authors and the editorial office regret the errors.

Reference

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EXHIBIT 163

MultipleSclerosis.net

MS Statistics

By Editorial Team • January 8, 2013

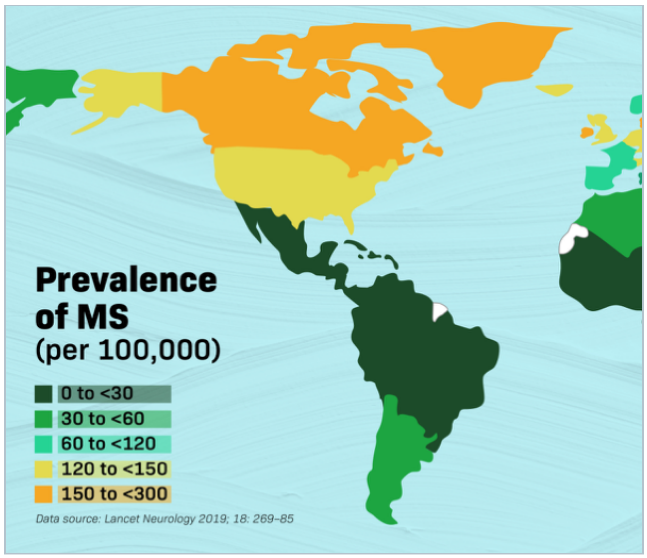
Epidemiology is the branch of medicine that looks for patterns in how a disease like multiple sclerosis (MS) affects certain groups of people. It answers questions such as, "Who gets MS?" Understanding who gets a disease helps doctors make important discoveries about the condition and sheds light on its possible causes.

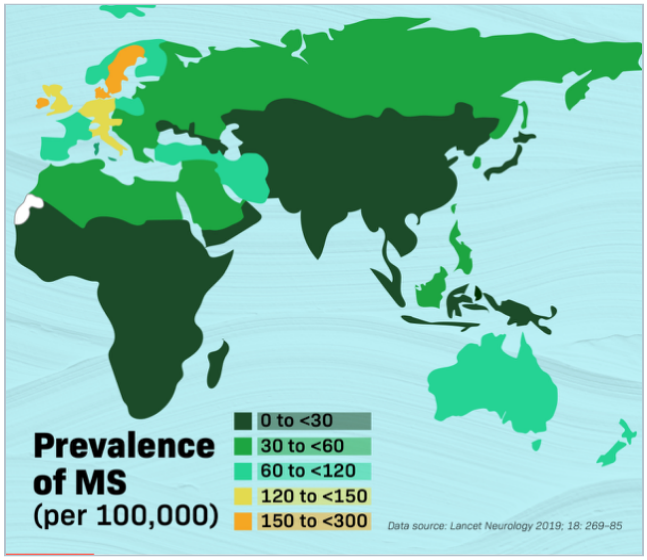
Where people with MS live

Multiple sclerosis is more common in people from Europe, the U.S., Canada, New Zealand, and parts of Australia. It is less common among people in Asia, and Africa. It is less common in tropical areas near the equator. In high-altitude regions far from the equator, cases of MS increase. The color-coded map below gives you an idea of the number of people with MS around the world.

Between 750,000 and 1 million people in the U.S. over the age of 18 are living with MS. There are more people living with MS in the Northeast and Midwest than in the South.[1,2]

Figure 1. Multiple sclerosis rates around the world





Is MS more common in females than males?

Like other autoimmune diseases, MS is much more common in females than males. Women develop multiple sclerosis 3 times more often than men. Some studies show that women with MS are more likely to have other autoimmune conditions such as psoriasis, thyroid disease, and inflammatory bowel disease.[1,3]

What ages are affected by MS?

MS can affect people of any age. The average age when symptoms appear ranges from 28 to 31 years old. MS symptoms may appear as early as age 15 or as late as 45. It rarely occurs in childhood or as late as the 70s. MS appears about 5 years earlier in women than in men.[3]

Relapsing-remitting MS tends to start around 25 to 29 years, and progress to secondary progressive MS in the 40s. Primary progressive MS tends to start later, in the late 30s to early 40s.

Does race or ethnicity impact who gets MS?

Multiple sclerosis is more common among Caucasians, especially if they live far from the equator. People of Danish descent and those living in the Orkney Islands have some of the highest rates of MS in the world. It is rare among some ethnic groups, such as Inuit, Yakutes, Hungarian Romani, Norwegian Lapps, Australian Aborigines, and Maoris.[2,3]

Factors such as household income, education, and employment do not seem to play a role in who gets MS.[4]

Is MS becoming more common?

The rates of MS have increased worldwide since 1955. The first report of MS in the U.S. was published in 1976 and found 123,00 cases. A study from 1990 found 300,000 cases of MS. This compares to the estimated 750,000 to 1 million cases in 2019. Another study found that rates of MS increased by about 10 percent between 1990 and 2016.[1,3,4]

Cause remains unknown

Scientists do not know what is causing this increase in the number of people with MS. Some studies point to certain genes that may be inherited. Others believe viruses and environmental factors such as vitamin D, sunshine, and place of birth may play a role. Some studies show smoking and obesity increase in the risk of developing MS.[3]

Improving diagnosis and care

Improvements in medicine also mean that doctors can diagnose MS more accurately.

The North American Registry for Care and Research in Multiple Sclerosis formed in 2017 to help scientists pool information about MS patients. The registry will help build knowledge about MS over time to improve diagnosis and care. Currently, 22 hospitals in the U.S. and Canada participate in the database.[5]

Written by: Jonathan Simmons & Jessica Johns Pool | Last revised: December 2019.

View References

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EXHIBIT 164

Acute disseminated encephalomyelitis

Silvia Tenembaum, MD; Tanuja Chitnis, MD; Jayne Ness, MD, PhD; and Jin S. Hahn, MD;
for the International Pediatric MS Study Group*

Abstract—Acute disseminated encephalomyelitis (ADEM) is an immune-mediated inflammatory disorder of the CNS characterized by a widespread demyelination that predominantly involves the white matter of the brain and spinal cord. The condition is usually precipitated by a viral infection or vaccination. The presenting features include an acute encephalopathy with multifocal neurologic signs and deficits. Children are preferentially affected. In the absence of specific biologic markers, the diagnosis of ADEM is still based on the clinical and radiologic features. Although ADEM usually has a monophasic course, recurrent or multiphasic forms have been reported, raising diagnostic difficulties in distinguishing these cases from multiple sclerosis (MS). The International Pediatric MS Study Group proposes uniform definitions for ADEM and its variants. We discuss some of the difficulties in the interpretation of available literature due to the different terms and definitions used. In addition, this review summarizes current knowledge of the main aspects of ADEM, including its clinical and radiologic diagnostic features, epidemiology, pathogenesis, and outcome. An overview of ADEM treatment in children is provided. Finally, the controversies surrounding pediatric MS and ADEM are addressed.

NEUROLOGY 2007;68(Suppl 2):S23–S36

Acute disseminated encephalomyelitis (ADEM) is an immune-mediated inflammatory disorder of the CNS, which is commonly preceded by an infection, and predominantly affects the white matter of the brain and spinal cord.[1-4] Several terms can be found in the literature to describe patients with ADEM, reflecting the more prominent aspects of the disease:

> "Postinfectious or postvaccinial encephalomyelitis, postinfectious multifocal encephalitis," when the triggering events were considered.
>
> "Acute perivascular myelinoclasia, perivenous encephalitis, disseminated vasculomyelinopathy," when emphasizing the histopathologic features and distribution of lesions.
>
> "Acute demyelinating encephalomyelitis, hyperergic encephalomyelitis, postvaccinal perivenous encephalitis, postencephalitis demyelination," relating to the probable immunopathogenetic mechanism.[5-16]

Based on our current clinicopathologic understanding of the disease, ADEM is probably the most appropriate nosologic designation, as the precipitating event may be absent and the pathogenesis of the disease is unclear.

In the absence of specific biologic markers, the diagnosis of ADEM is based on the clinical and radiologic features. Although ADEM usually has a monophasic course, recurrent or multiphasic forms have been reported, raising diagnostic difficulties in distinguishing these cases from multiple sclerosis (MS). This article reviews what we currently know about ADEM, including diagnostic features, pathogenesis, treatment, and outcomes, and includes a proposed definition of this disorder.

Epidemiology. ADEM can occur at any age, but it is more common in pediatric patients than in adults. Rare cases in older adults have been reported,[17] although careful exclusion of other diseases should be applied in these cases. The diagnosis is often made in the setting of a defined viral illness or vaccination. Although there appears to be no gender predominance in ADEM,[18,19] a male predominance has been described in two pediatric cohorts, with reported female:male ratios of 0.6[20] and 0.8.[21] as opposed to a 2:1 female preponderance frequently described for MS. The mean age at presentation in children ranges from 5 to 8 years.[21-23]

A seasonal distribution in the winter and spring months has been found in studies conducted in the United States.[19,20] A recent study conducted in San Diego County, CA, estimated the mean incidence of ADEM as 0.4/100,000/year among persons less than 20 years of age living in that region.[19] Five percent of these patients had received a vaccination within 1 month prior to the ADEM event, and 93% reported signs of infection in the preceding 21 days. There are no clear studies of worldwide distributions of ADEM. Some regional cases are linked to specific vaccines, as in the case of the Semple rabies vaccine, smallpox vaccine, and older forms of the measles vaccine.

Clinical presentation. ADEM is classically described as a monophasic disorder which typically be-

*Members of the International Pediatric MS Study Group are listed in the Appendix.

From the Department of Pediatric Neurology (S.T.), National Pediatric Hospital, Dr. J.P. Garrahan, Buenos Aires, Argentina; Massachusetts General Hospital for Children (T.C.), Brigham & Women's Hospital, Harvard Medical School, Boston; Department of Pediatrics (J.N.), University of Alabama at Birmingham and Children's Hospital of Alabama; and Pediatric Neurology Division (J.H.), Stanford University Medical Center, CA.

Disclosure: The authors report no conflicts of interest.

Address correspondence and reprint requests to Dr. Silvia Tenembaum, Pediatric Neurologist, Pediatric Multiple Sclerosis Clinic, Department of Neurology, National Pediatric Hospital "Dr. J. P. Garrahan," Buenos Aires, Argentina; e-mail: silviatenembaum@hotmail.com, solier@ciudad.com.ar

Table 1 *Demographic characteristics, presenting features, and outcome findings from published ADEM series between 2000 and 2004*

	Murthy et al.,[20] USA, 2002, n = 18	Dale et al.,[18] England, 2000, n = 35	Hynson et al.,[22] Australia, 2001, n = 31	Hung et al.,[51] Taiwan, 2001, n = 52*	Tenembaum et al.,[21] Argentina, 2002, n = 84	Gupte et al.,[26] England, 2003, n = 18	Mikaeloff et al.,[39] France, 2004, n = 119†	Idrissova et al.,[107] Russia, 2003, n = 90‡	Leake et al.,[19] USA, 2004, n = 42§	Anlar et al.,[23] Turkey, 2003, n = 46
Mean age, y (range)	7.5 (2.5–22)	7.4 ± 0.65 (3–15)	5.9 (2–16)	6.7 (0.7–16)	5.3±3.9 (0.4–16)	8.6 ± 1.2 (2.5–16)	7.1 ±4.3¶ (0.7–16)	9.8 ± 0.5 (2–16)	6.5 (0.8–18)	8 (1–15)
Male, %	61	54	42	56	64	61	56¶	54	57	63
Mean follow-up, y (range)	1.8 (0.2–5)	5.8 ± 0.8 (1–15)	1.5	>1.5	6.6 (1–19)	1.2 ± 0.2 (0.25–4)	2.9 ±3 (0.5–14.9)	Mean NR (1–5)	Mean NR (1–5)	Mean NR (1–12)
Preceding illness, %	72	74	71	100	74	50	51¶	100	93	46
Altered mental status, %	45	69	74	72	69	33	75¶	44	66	46
Ataxia/cerebellar, %	NR	51	65	4	50	50	NR	52	50	28
CN deficits (includes vision), %	23	89	45	13	44	50	55¶	24	>50	28
Seizures, %	17	17	13	47	35	11	NR	34	19	10
Full recovery, %	72	57	81	71	89	61	92	43–70‡	86 (2 deaths)	64
Residual focal neurologic deficits, %	16	29	13	8	11	22	NR	4	10	30
Behavior or cognitive problems, %	NR	20	6	15	4	11	NR	15	50	10
Recurrent or multiphasic course, %	6	20	13	2	10	11	29†	12	29 (7 MS)	33

* Hung et al. (2001) separated postinfectious encephalomyelitis (n = 38) from ADEM (n = 13) based on the number of MRI lesions, at least three for ADEM. No difference in mental status, though 70% in both groups.

† Mikaeloff et al. (2004) initially gave the diagnosis of ADEM to 119 patients (out of 296 with demyelinating event) but reclassified all of them as MS if any recurrence. As some patients may be considered multiphasic ADEM, we kept the original 119 in analysis. However, in table 1, "¶" provides data from only the 85 monophasic cases.

‡ In the series of Idrissova et al., MRI was only performed in the 14 children with more severe clinical course. They reported full recovery only if no fatigue was present. However, neurologic disability was identified by telephone contact.

§ Leake et al. (2004) reclassified as MS 7% of the relapsing forms of ADEM.

ADEM = acute disseminated encephalomyelitis; NR = not reported; MS = multiple sclerosis.

gins within 2 days to 4 weeks after an antigenic challenge. Approximately 70 to 77% of patients report a clinically evident antecedent infection or vaccination during the prior few weeks.[21,22,24] The typical symptoms and signs of ADEM include a rapid onset encephalopathy associated with a combination of multifocal neurologic deficits. A prodromal phase with fever, malaise, headache, nausea, and vomiting may be observed shortly before the development of meningeal signs and drowsiness. The clinical course is rapidly progressive and usually develops over hours to maximum deficits within days (mean, 4.5 days).[21]

The initial neurologic features are determined by the location of the lesions within the CNS. Table 1 summarizes the demographic distribution and presenting features in recently published case studies of patients with ADEM. Frequent neurologic symptoms and signs described in various combinations include unilateral or bilateral pyramidal signs (60 to 95%), acute hemiplegia (76%), ataxia (18 to 65%), cranial nerve palsies (22 to 45%), visual loss due to optic neuritis (7 to 23%), seizures (13 to 35%), spinal cord involvement (24%), impairment of speech (slow, slurred, or aphasia) (5 to 21%), and hemiparesthesia (2 to 3%), with invariable involvement of mental status, ranging from lethargy to coma.[19-23] Although certain signs and symptoms may be observed in both pediatric and adult cases, such as changes in mental status, ataxia, motor deficits, and brainstem involvement, other features appear to be age related.[25] Long-lasting fever[22] and headaches[19,21-23,26] occur more frequently in children with ADEM, while sensory deficits predominate in adult patients.[17] Seizures are rarely observed in adult patients with ADEM,[17] and are mainly seen in children younger than 5 years. One study has documented prolonged focal motor seizures in 70% of the younger patients, with 82% of these patients going on to status epilepticus.[21]

Peripheral nervous system (PNS) syndromes such as acute polyradiculoneuropathy[24,27,28] may occur in ADEM but are considered rare in childhood ADEM cases. The combination of PNS and CNS features may be more common in adults and was noted in 43.6% of one cohort of adult patients.[29]

There is a wide variation in the severity of the illness. Occasionally, ADEM can present as a subtle disease, with nonspecific irritability, headache, and somnolence, or may show a rapid progression of symptoms and signs to coma and decerebrate rigidity.[30] Respiratory failure secondary to brainstem involvement or severely impaired consciousness occurs in 11% to 16% of cases.[21,30]

MRI features. Neuroimaging is extremely important in establishing the diagnosis of ADEM. MRI

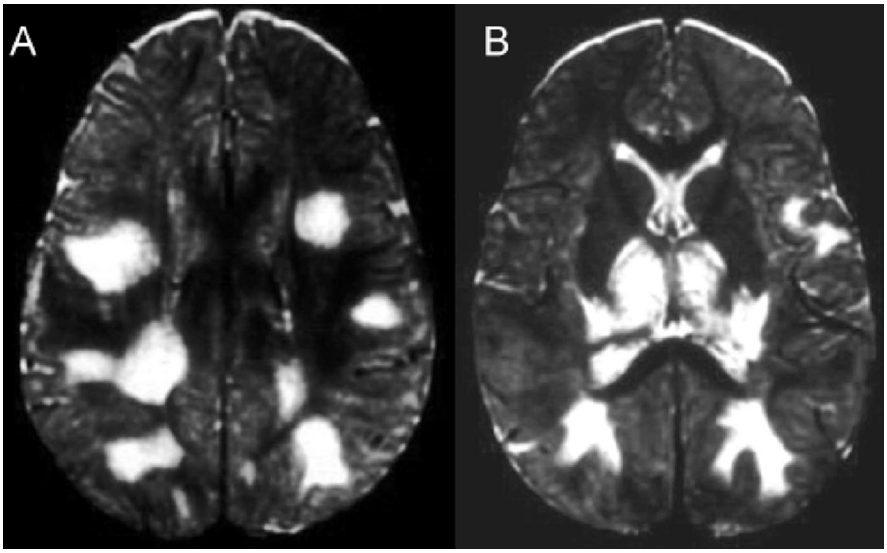

Figure 1. Acute disseminated encephalomyelitis with small lesions. (A) Axial T2-weighted MRI showing bilateral, poorly marginated hyperintense lesions in central, periventricular, and juxtacortical white matter, (B) also involving both thalami and internal capsules, in a 17-month-old boy, 2 weeks after measles vaccination.

abnormalities are most frequently identified on T2-weighted and fluid-attenuated inversion recovery (FLAIR) sequences as patchy, poorly marginated areas of increased signal intensity. Lesions in ADEM are typically large, multiple, and asymmetric. They typically involve the subcortical and central white matter and cortical gray-white junction of both cerebral hemispheres, cerebellum, brainstem, and spinal cord.[30] The gray matter of the thalami and basal ganglia are frequently involved, typically in a symmetric pattern.[21,31] The periventricular white matter is also frequently involved, being described in 30 to 60% of cases.[20,22,30] Lesions confined to the corpus callosum are less common. However, large demyelinating lesions of the adjacent white matter may extend into the corpus callosum and cross into the contralateral hemisphere.

Four patterns of cerebral involvement have been proposed to describe the MRI findings in ADEM[21]: 1) ADEM with small lesions (less than 5 mm; figure 1); 2) ADEM with large, confluent, or tumefactive lesions, with frequent extensive perilesional edema and mass effect (figure 2); 3) ADEM with additional symmetric bithalamic involvement (figure 3); and 4) acute hemorrhagic encephalomyelitis (AHEM), when some evidence of hemorrhage can be identified in the large demyelinating lesions (figure 4). The MRI pattern does not appear to correlate with any particular outcome or disability, as observed in a large pediatric cohort,[21] since most lesions tend to resolve on follow-up imaging studies.[21,32] However, this classification may be useful when considering the differential diagnosis of ADEM and may potentially help to identify those children for whom the initial ADEM-phenotype is really the first manifestation of MS.

The incidence of gadolinium enhancing lesions on T1-weighted sequences is quite variable in ADEM and may depend on the stage of inflammation.[31,33,34] Gadolinium enhancing lesions have been described in 30 to 100% of patients.[21,35,36] The pattern of enhancement is variable; complete or incomplete ring-shaped (figure 5), nodular, gyral, or spotty patterns have been described.[36-38] Meningeal enhancement of the brain or spinal cord is unusual.

Spinal cord involvement in ADEM has been described in 11 to 28%.[18,21-23,39] The typical spinal cord lesion is large and swollen, showing variable enhancement, and predominantly affects the thoracic region.

Sequential MRI scanning during the follow-up period plays an important role in establishing the diagnosis of ADEM. Monophasic ADEM is not associated with the development of new lesions. Complete resolution of MRI abnormalities after treatment has been described in 37 to 75% of patients with ADEM, and partial resolution in 25 to 53% of

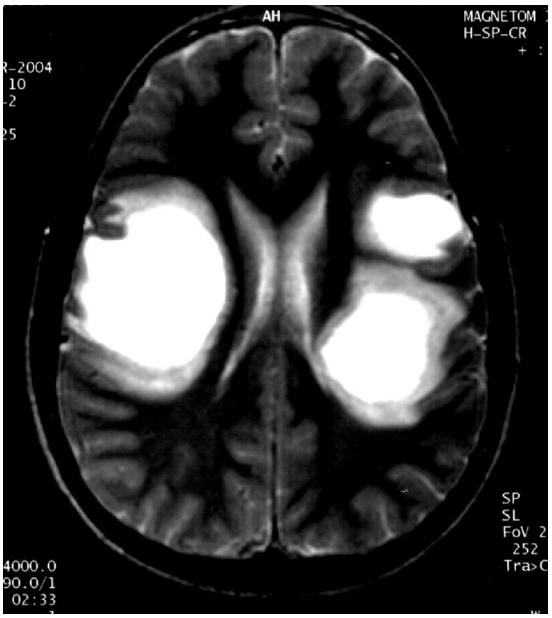

Figure 2. Acute disseminated encephalomyelitis with tumefactive lesions. Axial T2-weighted image demonstrating extensive, tumefactive, and bihemispheric lesions with perilesional edema, in a 13-year-old boy.

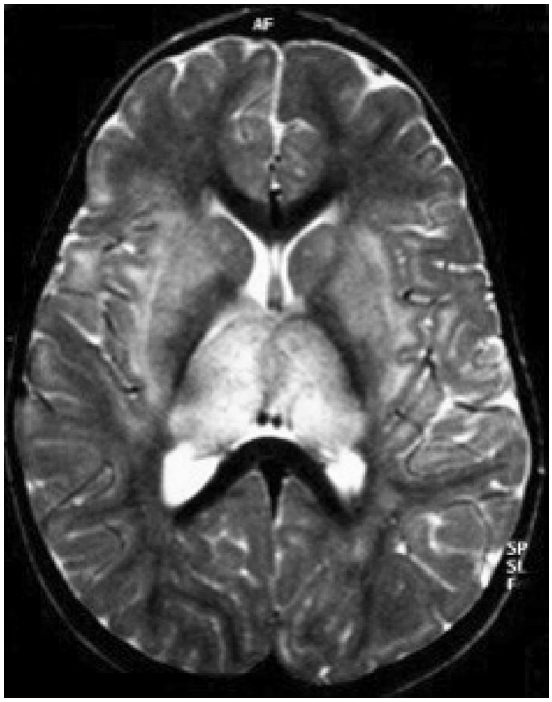

Figure 3. Acute disseminated encephalomyelitis with bithalamic involvement. Axial T2-weighted MRI showing symmetric increased signal in both thalami, with additional involvement of the insula and subcortical white matter, in an 18-month-old boy, 3 weeks after having mumps.

patients.[18,21,24,33,35] Resolution of MRI abnormalities within 6 months has been positively associated with a final diagnosis of ADEM in one study.[40] There are no clear criteria documenting how long to continue to image patients with one ADEM event. However, the authors suggest reassessing the patient with at least two additional MRI studies after the first normal MRI, over a period of 5 years from the initial episode, as the appropriate way to confirm the absence of ongoing accrual of lesions.

Advanced neuroimaging techniques. Low levels of *N*-acetylaspartate (NAA) and elevated lactate levels within regions of prolonged T2-MRI signal, without increase in choline, have been observed with quantitative proton MR spectroscopy during the acute stages of ADEM.[41,42] These abnormal signals resolved after normalization of clinical and MRI findings. Diffusion and perfusion weighted MRI show a diffusion pattern with reduced, normal, or increased diffusion coefficients, or reduced or normal perfusion within ADEM lesions.[43,44] A global and bilateral decreased cerebral metabolism has been demonstrated by PET scanning in a case where CT scan had only showed a focal demyelinating lesion.[45]

SPECT using 99m Tc-HMPAO has consistently shown areas of hypoperfusion that are more extensive than the MRI lesions.[46-48] The time course of SPECT abnormalities also reflects the clinical course more accurately than MRI. In spite of the resolution of MRI lesions, SPECT with acetazolamide detects persistent cerebral circulatory impairment that may contribute to the neurocognitive and language deficits observed in some patients with ADEM.[49,50]

Monophasic and multiphasic ADEM. Although ADEM is classically described as a monophasic disorder, several studies have described ADEM relapses, occurring at the following rates: 1/18 (5.5%),[20] 1/14 (7%),[51] 8/84 (10%),[21] 4/31 (13%),[22] 7/46 (15%),[23] 7/35 (20%),[18] and 9/42 (21%).[19] It should be noted that different diagnostic criteria for relapses were used in these different studies, which may in part account for the variability. In addition, the mean length of follow-up reported in some of these ADEM series varied considerably: 18 months,[22] 22 months,[23] 5.3 years,[18] 6.6 years,[21] and again may contribute to the interstudy variability.[52]

The final outcome of multiphasic ADEM has been described in detail in two pediatric series with long-term follow-up.[18,21] In one study, no long-term impairment was observed in 86% of multiphasic ADEM patients.[18] Similarly, eight children with multiphasic ADEM, who remained relapse-free after a follow-up of 3 to 16 years (mean 8.2 years), had a median EDSS score of 1 (range 0 to 2.5).[21] Serial brain-spinal MRI performed in these patients revealed complete or almost complete resolution of demyelinating lesions without evidence of new active lesions.

Acute hemorrhagic leukoencephalitis. Acute hemorrhagic leukoencephalitis (AHL), AHEM, and acute necrotizing hemorrhagic leukoencephalitis (ANHLE) of Weston Hurst are variants of an acute, rapidly progressive, and frequently fulminant inflammatory hemorrhagic demyelination of CNS white matter. It is usually triggered by upper respiratory tract infections. Death from brain edema is common within 1 week of onset of the encephalopathy, but increasing evidence of favorable neurologic outcomes has been published with early and aggressive treatment using various combinations of corticosteroids, immunoglobulin, cyclophosphamide, and plasma exchange.[21,53-55]

AHL, AHEM, and ANHLE are considered hyperacute subforms of ADEM and were observed in 2% of children in a large cohort.[21] Lesions on MRI tend to be large, with perilesional edema and mass effect.[56,57] Diffusion-weighted imaging disclosing areas of restricted diffusion in the affected areas of the brain has been recently published,[57] and this finding might be due to acute vasculitis with subsequent vessel occlusion in AHL.

Controversies in diagnosis based on published studies: Rationale for proposed definitions. ADEM should be adequately defined and distinguished from other diseases affecting the white matter. In particular, a diagnostic challenge lies in distinguishing multiphasic forms of ADEM from MS. This is especially important, not only for prognostic

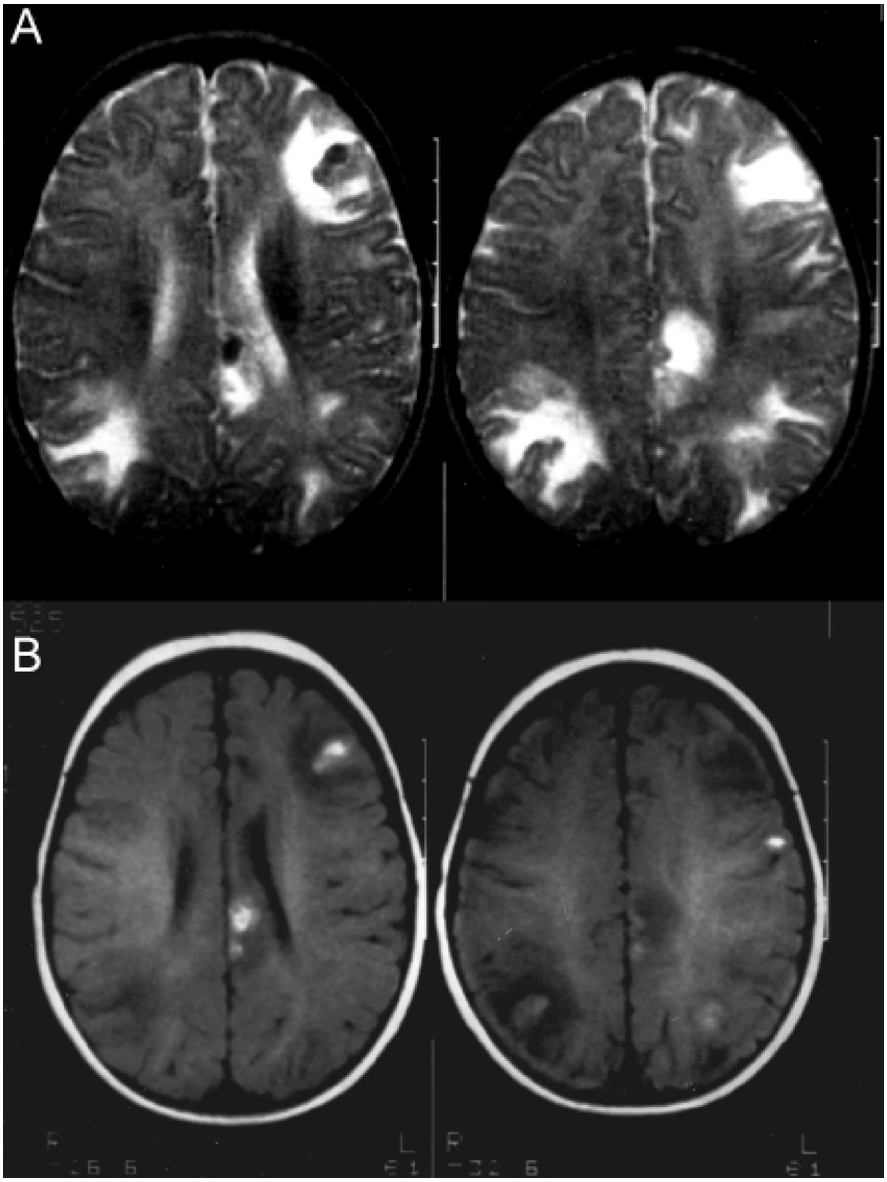

Figure 4. Acute hemorrhagic encephalomyelitis. (A) Axial T2-weighted MRI with prominent bilateral hyperintense lesions, with areas of very low signal, corresponding to breakdown products of hemoglobin, in a 5-month-old boy, 2 weeks after pertussis vaccination. (B) Axial T1-weighted MRI of the same case, showing spontaneous hyperintense signal inside the large hypointense lesions.

purposes, but for therapeutic purposes, since a diagnosis of MS, at least in adult patients, carries the recommendation for early treatment with immunomodulators.

Historically, different definitions of ADEM have been used in published cases of pediatric and adult patients.[17-23,26,39,51] The lack of a uniform definition and clear clinical and neuroimaging diagnostic criteria has led to the classification of other neurologic conditions as ADEM. Due to this lack of uniformity, it is difficult to compare neuroimaging aspects or outcomes, establish prognostic factors, or compare percentages of patients with ADEM that evolve into MS. For example, the proportion of patients initially diagnosed with ADEM who go on to be diagnosed with MS ranges from 9.5%[19] to 27%.[18] However, two children from a cohort of seven diagnosed with "multiphasic ADEM" had monosymptomatic relapses—optic neuritis in one, and a brainstem syndrome in the other—suggestive of MS.[18] Conversely, a recently published study applied the concept that "any second

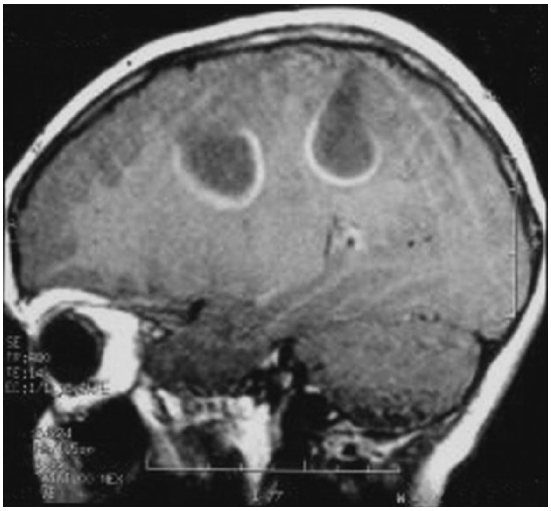

Figure 5. Sagittal T1-weighted imaging demonstrating two lesions with open-ring enhancement in an 8-year-old girl, 1 week after an upper respiratory viral infection.

attack after an initial diagnosis of ADEM had to be reclassified as MS,"[39] and reported a frequency of second attacks as high as 29%. Thus, the use of a uniform definition may help to distinguish ADEM from other lifelong demyelinating conditions and provide a foundation for consistent prospective outcome studies. Nevertheless, the long-term outcome and evidence of multiple recurrent demyelinating events are required conditions to clearly delineate MS from ADEM.

Unusual cases of ADEM have been described in patients with demyelinating lesions confined to the brainstem, when the presentation was more indicative of a clinical isolated syndrome (CIS) with brainstem involvement or brainstem encephalitis.[58-60] A case of atypical acute disseminated encephalomyelitis is described in a 3-year-old girl, with a longitudinal lesion restricted to the spinal cord in the absence of brain lesions, consistent with longitudinal myelitis.[61] Unfortunately, the report does not provide imaging of the brainstem to better explain the child's alteration of consciousness. A neurodegenerative picture with progressive decline in mental and motor skills was reported in an 11-month-old baby following a meningoencephalitis.[62] This infant was misdiagnosed as having ADEM because the MRI showed subtle areas of hyperintense signal in the frontal and parieto-occipital white matter that seemed to be transitional areas of myelination or delayed myelination.[63] A recent report describes a patient with recurrent simple and complex partial seizures, who then progressed to intractable epilepsia partialis continua and cognitive decline.[64] Although this case represented a classic picture of chronic Rasmussen's encephalitis, because the patient started symptoms after a viral illness and the initial MRI disclosed hyperintense lesions (although predominantly involving cortical and subcortical structures), a diagnosis of ADEM was suspected. Furthermore, when the seizures recurred after 3 months from onset, the patient was misdiagnosed with multiphasic ADEM.

A variety of terms and definitions have been used to describe patients with ADEM who relapse. Recurrent, relapsing, pseudorelapsing, bi- or multiphasic ADEM have all been applied using different criteria[18,21,65-74]: time from the first event varies from less than 4 to more than 8 weeks; neurologic deficits are defined as same or different; individuals are either monosymptomatic or polysymptomatic; and finally MRI lesions are described as either in the same or different areas.

ADEM definitions. To avoid misdiagnosis and develop a uniform classification, the International Pediatric MS Study Group (Study Group) proposes that the following three terms be applied to variations of ADEM (see Krupp et al.,[74a] in this conference report):

ADEM: A first clinical event with a polysymptomatic encephalopathy, with acute or subacute onset, showing focal or multifocal hyperintense lesions predominantly affecting the CNS white matter; no evidence of previous destructive white matter changes should be present; and no history of a previous clinical episode with features of a demyelinating event. If a relapse takes place within 4 weeks of tapering steroid treatment or within the first 3 months from the initial event, this early relapse is considered temporally related to the same acute monophasic condition and would replace the terms "steroid dependent ADEM" or "pseudorelapsing ADEM."

Recurrent ADEM: New demyelinating event fulfilling diagnostic criteria for ADEM, occurring at least 3 months after the initial ADEM event and at least 4 weeks after completing steroid therapy, showing the same clinical presentation and affecting the same areas on MRI as the initial ADEM episode.

Multiphasic ADEM: Refers to one or more ADEM relapses, including encephalopathy and multifocal deficits, but involving new areas of the CNS on MRI and neurologic examination. Relapses take place at least 3 months after initial ADEM attack and at least 4 weeks after completing steroid therapy.

Differential diagnosis. Acute encephalopathy and disseminated demyelination of the CNS in children represent a diagnostic challenge for pediatric clinicians and neurologists. Many inflammatory and noninflammatory disorders may have a similar clinical and radiologic presentation and should be considered in the diagnostic evaluation.

If an acute encephalopathy is suspected based on history and physical examination, the first priority should be to rule out an acute bacterial or viral infection of CNS, and to start empiric antibacterial and antiviral treatment. A gadolinium-enhanced MRI of the brain and spinal cord (to better define the disease burden) and a lumbar puncture should be performed as soon as possible. Evidence of an inflammatory process (CSF pleocytosis, elevated CSF proteins and immunoglobulin index, gadolinium enhancement on MRI) should be determined in addition to screening for viral, bacterial, or fungal infectious agents (See "Differential diagnosis and evaluation of pediatric MS" in this conference report for complete outline of the workup for infectious causes of acute encephalopathy). In the absence of clear evidence of an infectious cause, the neuroimaging findings should define the regional distribution of the demyelinating-inflammatory process.

Neuroimaging at the time of the initial event may be useful in the diagnosis. When the MRI shows large focal tumor-like lesions, one should consider brain tumors, Shilder disease, Marburg variant of MS, and brain abscess.[75-77] An MRI pattern with symmetric bithalamic involvement may be seen in children with acute necrotizing encephalopathy, deep cerebral venous thrombosis, hypernatremia, and extrapontine myelinolysis, as well as in children with ADEM after Japanese B encephalitis vaccination.[78-84] Basal ganglia involvement may be consistent with organic aciduria, poststreptococcal ADEM, or infantile bilateral striatal necrosis.[85,86]

The presence of complete ring-enhanced lesions in the cerebral white matter is unusual in ADEM, and brain abscess, tuberculomas, neurocysticercosis, toxoplasmosis, and histoplasmosis should be excluded.[36]

The diagnosis of MS should be considered in cases of recurrent or multiphasic demyelination, and is discussed in detail later in this review.

Treatment and management. There is no standard therapy for ADEM. Most treatment approaches have employed some form of nonspecific immunosuppressant therapy similar to that used for MS and other autoimmune diseases, including steroids, IV immunoglobulin (IVIg), or plasmapheresis. Most of the data describing treatment for ADEM are derived from case reports and small series. To date, there have been no randomized, controlled trials for the treatment of ADEM in either children or adults.

Steroids. Steroid treatment has been the most widely reported therapy for ADEM, typically at high doses. However, there has been great variety in the specific steroid formulations employed, routes of administration, dosing, and tapering regimens. The earliest report describing steroid treatment for ADEM was published in 1953 using ACTH.[87] Later reports in the pre-MRI era described successful use of prednisone, corticotropin, or dexamethasone with marked improvement of symptoms in both adult and pediatric patients with ADEM.[12,88] Several patients in these reports had recurrence of their symptoms when the steroid therapy was discontinued and improved when steroids were reinstituted.

Most pediatric groups describing their high dose steroid treatment in detail have used IV methylprednisolone (10 to 30 mg/kg/day up to maximum dose of 1 g/day) or dexamethasone (1 mg/kg) for 3 to 5 days[18,21,22,26,89,90] followed by oral steroid taper for 4 to 6 weeks with full recovery reported in 50 to 80% of patients.[18,21,22] In the only comparison of specific corticosteroid regimens, methylprednisolone-treated patients had significantly better outcome with respect to EDSS scores compared to those treated with IV dexamethasone.[21] Outcome may also be influenced by the length of steroid taper since an increased risk of relapse has been reported with steroid taper of 3 weeks or less.[18,23]

High-dose steroid treatment is not without risk. Gastric perforation and death due to gastrointestinal bleeding related to methylprednisolone treatment for ADEM has been reported.[91] Hyperglycemia, hypokalemia, high blood pressure, facial flushing, and mood disorders have also been reported in association with high-dose corticosteroid treatment. It is advisable to provide gastric ulcer prophylaxis while patients are on high dose steroids, in addition to a careful monitoring of blood pressure, urine glucose, and serum potassium.

Immunoglobulin. IVIg has been used successfully in a variety of autoimmune diseases although its effectiveness in MS is limited. There are multiple case reports of IVIg being used successfully alone[92,93] or in combination[61] with corticosteroids in both pediatric and adult cases of ADEM, but there have been no studies which have directly compared IVIg with steroids, plasmapheresis, or other immunomodulatory treatments. In some cases, IVIg was administered after failed IV pulse steroid therapy[94-96] or in cases of recurrent demyelination.[68,70] Reported dosing for IVIg has been quite consistent, using a total dose of 1 to 2 g/kg, administered either as a single dose or over 3 to 5 days. In general, IVIg is well tolerated in the pediatric population. There have been isolated case reports of repeated IVIg administration to treat recurrent episodes of demyelination,[97] although it is questionable whether these cases were definitely MS.

Plasma exchange. The use of plasma exchange in ADEM has been reported in only a small number of cases, typically severe cases when steroid treatment has failed. A recent series[98] examined the outcome following plasma exchange for 59 patients with a variety of CNS demyelinating conditions, including 10 cases of ADEM, and found that 40% of patients (including the ADEM group) had moderate to marked improvement following plasma exchange. In this cohort, a mean number of seven exchanges was performed (range 2 to 20) although a breakdown by demyelinating disease type was not given. In the literature, there were reports of six pediatric ADEM cases treated with plasma exchange. Four of these patients were reported as having a complete recovery,[99-101] one had a residual left hemiparesis,[102] and the outcome for one patient was not described.[103]

Plasma exchange may serve to remove the autoantibodies that are presumably triggering the demyelination in ADEM, but may also shift the dynamics of B- and T-cell interaction within the immune system. There is some evidence from case reviews that plasma exchange may be more effective when given early in the disease course.[104] However, due to the need for trained personnel with specialized equipment and central venous access for multiple treatments over a period of days to weeks, plasma exchange has often been used as a last resort. Symptomatic hypotension, severe anemia, and heparin-associated thrombocytopenia have been described in association with plasma exchange.[98] The role and timing of this intensive treatment for ADEM deserves further investigation; however, for the foreseeable future plasma exchange will likely continue to be used as a rescue therapy in ADEM when other modalities fail.

Other therapies. To our knowledge, there have been no published reports of interferon-β or glatiramer acetate used in the acute stage of ADEM although there are anecdotal descriptions of interferon-β use for episodes of recurrent demyelination consistent with multiphasic ADEM. Some

improvement has been reported with cyclophosphamide use in adult ADEM patients who responded poorly to methylprednisolone,[17] but we are unaware of any published reports of cyclophosphamide, azathioprine, or other cytostatic drug use in pediatric ADEM.

Outcome and prognosis. *Untreated ADEM.* Limited data exist about the natural history of ADEM in the post-MRI era. In the available case studies, there is considerable diversity with respect to antecedent infections, clinical presentation, and neuroimaging findings, further complicating outcomes analysis. Classification of recurrence is a major inconsistency as there is considerable disagreement about when to classify recurrent demyelination as multiphasic ADEM vs defining all recurrent demyelination as MS. Case series from Japan,[105] India,[106] and Russia[107] suggest that the natural history of ADEM in most children is one of gradual improvement over several weeks, with 50 to 70% of patients experiencing full recovery. Improvement in serial MRIs was also shown in seven Japanese patients with untreated postinfectious encephalitis, although three patients had residual lesions on MRI.[105] Seven of 21 patients with partial recovery in the South India group[106] had more extensive white matter lesions compared to MRIs of children with complete recovery. No other factors, including antecedent infections, correlated with outcome.

In contrast, the Moscow group stratified 90 pediatric ADEM patients with respect to antecedent infections (33% rubella, 29% varicella, 22% with unknown viral antecedent) and recurrence (11% were classified as multiphasic ADEM, most with preceding upper respiratory symptoms). Diagnosis was based on clinical symptoms following a prior viral infection. MRI was routinely obtained only in the multiphasic group. Outcome varied with antecedent infections with a good outcome reported in 70% of the ADEM cases without definite infection vs 54% and 43% normal outcome reported for post-varicella and post-rubella ADEM, respectively. Specific recovery times were described as approximately 3 weeks for post-rubella ADEM and up to 12 weeks for multiphasic ADEM, with intermediate but more variable recovery time in the post-varicella and unknown ADEM groups.[107] Taken together, these reports suggest that approximately two-thirds of patients make a complete recovery without specific treatment, but that recovery may require weeks.

Treated ADEM. Table 1 summarizes the outcome information in recently published case series of 15 or more patients with ADEM. Over half the patients treated had a good recovery with minimal or no deficit. The most common problems seen following ADEM were focal motor deficits ranging from mild clumsiness and ataxia to hemiparesis or blindness. Behavioral and cognitive problems were identified in 6 to 50% of children, but are likely underreported in some series. Less frequent late effects included development of seizures following ADEM resolution.

Most patients were treated with high-dose steroids, although some patients were treated with IVIg (with or without steroids), and plasmapheresis was used in some severe steroid-resistant cases. Following initiation of treatment, rapid improvement was sometimes seen within hours although recovery typically evolved over days. More severely affected children (sometimes obtunded and mechanically ventilated) often required weeks or months to improve and were often treated with multiple immunosuppressant regimens, making it unclear whether the treatment influenced outcome or whether these patients improved on their own. Complete recovery was reported for some of these severe cases, albeit less frequently. The prognosis of ADEM in adult patients has been uniformly reported as favorable.[17]

Neurocognitive outcome. More attention is being given to subtle neurocognitive deficits following CNS demyelination in childhood, including ADEM.[50,108] Even children thought to have full recovery demonstrated subtle neurocognitive deficits in attention, executive function, and behavior[50] when reevaluated more than 3 years after ADEM, although these deficits were not as severe as those reported for pediatric patients with MS.[109] One study compared 19 children with ADEM to a normal age- and sex-matched control group and found that patients younger than 5 years at ADEM diagnosis had significantly lower IQ and educational achievement when evaluated at 3.9 years (mean) since illness, while the older-onset patients had slower verbal processing, having been evaluated at 2.2 years (mean) after presentation.[108] Behavioral problems were also more prominent in the young-onset ADEM group. Additional studies are required to further characterize neurocognitive deficits following ADEM. These studies will help to guide assessments in individual patients and will facilitate appropriate educational interventions.

It appears that symptom resolution is more rapid in steroid or IVIg-treated patients. However, due to the heterogeneity of the patient populations and treatment regimens, it is difficult to draw any specific conclusions about the impact of treatment relative to long-term outcome. Multicenter prospective trials with consistent diagnostic criteria, treatment protocols, and uniform data collection are critical to improve our knowledge regarding management of children and adolescents with cognitive deficits.

ADEM and MS. MS in children can initially present with symptoms and signs that are indistinguishable from ADEM. However, subsequent neurologic events or changes on MRI typical of MS lead to the diagnosis of MS.[18,19,110] The possibility that a child may develop MS is a concern for parents and clinicians, particularly in cases of recurrent or multiphasic ADEM. MS in children can also present

with CIS that more closely resemble typical neurologic events seen in adults with MS. CIS differs clinically from ADEM, and is defined as either a monofocal or multifocal demyelinating event in the absence of fever or encephalopathy (except in cases of brainstem syndromes).

Our current consensus definition of pediatric MS states that a first event of ADEM is not considered the first event required for a diagnosis of MS, nor can it be used to determine dissemination in time and space. In these children, a second demyelinating event not meeting criteria for recurrent or multiphasic ADEM would qualify as an initial event, after which subsequent MRI changes or new demyelinating episodes would lead to a diagnosis of MS. While studies suggest that children with an initial ADEM event are at higher risk for the eventual development of MS,[18,19,110] the actual risk of MS following ADEM remains unclear. Identifying prognostic indicators including biomarkers are needed to further clarify the relationship between ADEM and subsequent risk of an MS diagnosis.

At present, there are no clear prognostic factors that determine if a child with a first event of either ADEM or CIS will eventually develop MS. The risk of developing MS after ADEM has been reported as 0%,[21] 9.5%,[19] to 27%[18] and 28%[39] by different studies. It should be noted that in these studies, varying criteria were used to define pediatric MS, and differing lengths of follow-up were used, which may contribute to the wide range in incidence. As a general trend, ADEM carries a lower risk of developing MS than CIS events. A study examining patients with a first demyelinating event, including CIS-like and ADEM events, showed that overall, 57% developed MS as defined by two demyelinating events.[39] Of patients with an initial diagnosis of ADEM, 28% developed MS. Of those with initial CIS-like events, 86% with optic neuritis and 50% with an initial brainstem syndrome developed MS. Overall, positive predictive factors for the development of MS were age at onset 10 years or older (hazard ratio [HR], 1.67; 95% CI), MS-suggestive initial MRI (HR 1.54), or optic nerve lesion (HR 2.59). A lower risk of developing MS was found in patients with myelitis (HR 0.23) or mental status change (HR 0.59) at presentation.[39] Twenty-nine percent (34 of 119) of children with a prior diagnosis of ADEM developed MS, while 75% (134 of 177) of children with a first event consistent with CIS developed MS. Although these clinical findings are helpful and serve as a guide, a definitive diagnosis of MS cannot be made based on these data. Moreover, use of standardized criteria to define MS in future studies would greatly enhance interpretation.

Radiologic parameters provide supportive evidence for the diagnosis of MS; however, they cannot be used as predictors for the development of the disease, since many features thought to be unique to MS are also seen in cases of ADEM.[20] Lesions in the corpus callosum, periventricular white matter, and deep gray matter structures were seen more commonly in patients who developed MS.[22] However, in other series the same features have been documented in typical cases of ADEM.[18,20]

Oligoclonal IgG bands in the CSF were found to be positive in 64 to 92% of pediatric MS cases, and in 0 to 29% of ADEM cases,[18,21,22,111] but this difference was not statistically significant[18] and so cannot be used as a reliable marker of MS. Thus far, immunologic testing has not yielded a reliable marker for the development of MS from initial demyelinating events in children.

To date, there are no clear clinical or radiologic parameters that predict which cases of ADEM or CIS will develop MS.[112,113] Early treatment of MS is strongly advocated in adult patients, and has been shown to be beneficial in reducing long-term disability.[114,115] Moreover, use of beta-interferon-1a in pediatric MS patients has recently been shown to be safe and tolerable.[116,117] However, the risk of an inaccurate diagnosis of MS, which carries a lifetime burden and requires ongoing treatment, is generally thought to outweigh the risk of delaying diagnosis in order to be certain of the diagnosis of MS. The proposed definition of pediatric MS may eventually require modification as more information is gathered regarding the predictability of developing MS after an initial demyelinating event. Clinical prognostic indicators or a biomarker that predicts the development of MS after an initial demyelinating event in childhood is needed to facilitate an early and accurate diagnosis of pediatric MS.

Pathogenesis. ADEM is characterized histologically by perivenular infiltrates of T cells and macrophages, associated with perivenular demyelination. Although ADEM shares common pathologic features with MS, distinct pathologic criteria distinguishing the two diseases have not been defined. There are no systematic studies comparing the histopathology of ADEM and MS, although such studies would undoubtedly yield important information on the relationship between these two diseases. A variety of pathologic features have been described in biopsy and autopsy samples from ADEM and AHEM patients. An autopsy from a 5-year-old boy with fatal ADEM grossly described diffuse brain edema, uncal and tonsillar herniation.[19] Multifocal perivascular lymphocytic infiltrates associated with fibrin deposition within vascular lumens and adjacent demyelination were observed. There was diffuse anoxic-ischemic neuronal degeneration and intersitial edema. Viral inclusion bodies were not seen in H-E sections. A brain biopsy performed in a 10-year-old girl with severe AHEM demonstrated subcortical WM with perivascular hemorrhagic necrosis with subacute inflammation consisting of macrophages, neutrophils, and rare lymphocytes. No evidence of viral, bacterial, fungal, or parasitic infection was noted. Although ADEM is typically described as demyelination with relative preservation of axons, axonal damage has been identified in the brains of

Table 2 *Incidence of vaccination-associated ADEM*

Vaccination forms	Reported incidences of ADEM
Measles	
Live measles vaccine	1–2/million[131] (compared to 20–30/million incidence of measles virus–induced encephalitis)
Rabies	
Neural vaccine (Semple)	1/300–1/7,000[128]
Duck embryo vaccine	1/25,000[3]
Non-neural human diploid cell	<1/75,000
Japanese B encephalitis	
Inactivated mouse-brain derived JEV 1993–1999	0.2/100,000 (Japan)[132]; 0/813,000 (USA)[132]
Smallpox	
New York City Board of Health strain of vaccinia 2002–2004	3/665,000 [135] (reporting encephalitis or myelitis)
Diphtheria/pertussis/tetanus	0.9/100,000[131]
Hepatitis B	Eight cases of CNS inflammation within 10 weeks [133]Four cases of partial myelitis within 3 months[134]

ADEM = acute disseminated encephalomyelitis.

some patients.[118,119] Lesions largely involve the white matter, but can also involve the cortex and deep gray matter structures. The CSF is characterized by elevated protein and white blood cells. Oligoclonal bands are an acute manifestation in up to 30% of patients with ADEM,[18] and may be transient. Elevated CSF levels of the pro-inflammatory cytokines IL-6, IL-10, and TNFα have been described.[120,121]

Acute hemorrhagic and acute necrotizing hemorrhagic leukoencephalitis (AHEM, AHL, ANHLE) of Weston Hurst shares some inflammatory histologic features with ADEM; however, demyelination is often more widespread throughout the CNS and is associated with a pronounced neutrophilic infiltrate. ANHLE is characterized by destruction of small blood vessels associated with acute hemorrhage and fibrin deposition.[122] CSF analysis reflects the hemorrhagic nature of this disease with elevations in protein, RBC, and WBC counts.

ADEM may be classified as either postvaccinial or postinfectious; however, in many cases no clear antecedent history of either is present. Rare cases of ADEM have been described following organ transplantation.[123-127] Postinfectious forms of ADEM typically begin within 2 to 21 days after an infectious event; however, longer intervals have also been described. Viral infections commonly associated with ADEM include influenza virus, enterovirus, measles, mumps, rubella, varicella zoster, Epstein Barr virus, cytomegalovirus, herpes simplex virus, hepatitis A, and coxsackievirus. Bacterial triggers include *Mycoplasma pneumoniae, Borrelia burgdorferi*, *Leptospira*, and beta-hemolytic *Streptococcus*. Acute hemorrhagic leukoencephalomyelitis (AHLE) typically follows influenza or upper respiratory infection. The only epidemiologically and pathologically proven association of ADEM with vaccinations is with the Semple form of the rabies vaccine.[3,128-130] Other vaccinations associated with ADEM include hepatitis B, pertussis, diphtheria, measles, mumps, rubella, pneumococcus, varicella, influenza, Japanese encephalitis, and polio.[19,21,131-136] Vaccines produced in CNS tissue including the Semple form of the rabies vaccine carry a higher risk of ADEM. It is important to note that in general, vaccination forms with high rates of complications are no longer in use. Some reported incidences of encephalomyelitis associated with various forms of vaccination are listed in table 2.

The pathogenesis of ADEM is unclear; however, given its histologic features and typically monophasic course of disease, it has been likened to the animal model experimental autoimmune encephalomyelitis (EAE). EAE is an autoimmune demyelinating disease, which can be induced in a variety of animal species by immunization with myelin proteins or peptides. Moreover, the postvaccinial form of ADEM associated with the Semple rabies vaccine, which contains rabies virus–infected neural tissue, reinforces this analogy to EAE. Viral or bacterial epitopes resembling myelin antigens have the capacity to activate myelin-reactive T cell clones through molecular mimicry,[137] and can thereby elicit a CNS-specific autoimmune response. Thus, it has been suggested that microbial infections preceding ADEM elicit a cross-reactive anti-myelin response through molecular mimicry. Alternatively, ADEM may be caused by the activation of existing myelin-reactive T cell clones through a nonspecific inflammatory process.

Theiler murine encephalomyelitis virus–induced demyelinating disease (TMEV-IDD) model is induced by direct CNS infection of the neurotropic TMEV picornavirus, initially resulting in an immune-mediated reaction primarily involving TMEV-specific CD4 and CD8 T cells.[138,139] However, during the chronic stages of disease, T cell reactivity to host myelin peptides has been observed, indicating

epitope spreading has occurred secondary to T cell responses to myelin breakdown products, resulting in an autoimmune response.[140] Both microglia[141] and dendritic cells[142] from the CNS of TMEV-infected mice are able to present myelin peptides to naïve T cells, thereby facilitating epitope spreading to nonviral, host myelin antigens. The TMEV model highlights the phenomenon of epitope spreading secondary to a destructive CNS viral infection resulting in a secondary autoimmune response and chronic inflammation. Although this model superficially bears some resemblance to ADEM, it is important to note that overwhelming evidence has shown that ADEM is not due to direct viral infection of the CNS, but is a secondary immune-mediated phenomenon. Epitope spreading is likely to be an important phenomenon in chronic inflammatory diseases such as MS, but involvement in ADEM is unknown.

Sequences in myelin basic protein have been shown to resemble several viral sequences, and in some cases, cross-reactive T cell responses have been demonstrated. Examples of cross-reactive T cells with MBP antigens include HHV-6,[143] coronavirus,[144] influenza virus hemagglutinin,[145] and EBV.[146] Proteolipid protein (PLP) shares common sequences with *Haemophilus influenza*.[147] Semliki forest virus (SFV) peptides mimic myelin oligodendrocyte glycoprotein (MOG).[148] Enhanced myelin basic protein (MBP)-reactive T cell responses have been demonstrated in patients with postinfectious forms of ADEM.[149,150] Elevated titers of anti-myelin antibodies in sera from patients with ADEM have recently been demonstrated as compared to patients with MS or viral encephalitis.[151] Previous studies have demonstrated enhanced anti-MBP antibody titers in patients with postvaccinial ADEM following vaccination with the Semple rabies vaccine.[152,153] One of these studies demonstrated elevated anti-MBP antibody titers in ADEM samples compared with MS samples. Although there is controversy surrounding the characterization of anti-myelin antibody responses in MS, studies in ADEM have consistently shown detectable levels, suggesting differences in pathogenesis. Collectively, these studies suggest that enhanced T and B cell myelin responses play a role in the pathogenesis of both postinfectious and postvaccinial ADEM; however, further studies are required to determine causal relationship.

ADEM was associated with the class II alleles HLA-DRB1*01 and HLA-DRB*03 in a Russian study.[107] A similar study from Korea showed an association of ADEM with HLA-DRB1*1501, as well as HLA-DRB5*0101.[154] The same Korean study showed an association of HLA-DRB3*0202 and HLA-DQB1*0502 with acute necrotizing forms of encephalopathy. The gene mostly frequently linked with MS is HLA DRB1,[155] with DR2[15,156] being the most frequently involved allele. Similar associations have been found in the pediatric MS population.[157] Thus, class II alleles may play a role in MS as well as ADEM; however, the disparity between the alleles associated with the two diseases suggests differences in pathogenesis.

Research/future directions. ADEM often poses both a diagnostic and prognostic dilemma for clinicians. In the acute stage, there should be a low suspicion of infection before initiation of corticosteroid or immunosuppressive therapy. Diagnostic tests that increase the rapidity of an accurate diagnosis are recommended. Over the long term, one of the most pressing questions of a child presenting with ADEM, particularly recurrent or multiphasic forms of ADEM, is the potential risk for conversion to MS. Although ADEM and MS share many similar pathologic features, prognosis is drastically different. Therefore, identification of a biomarker that can predict the development of MS after an ADEM event is critical.

Additional studies are required to understand the worldwide epidemiology and distribution of ADEM. These studies may give insight into the pathogenesis of the disease and potential preventative measures. Early identification of triggers for ADEM, such as specific batches of vaccines, is facilitated by stringent monitoring mechanisms.

Current treatments for ADEM generally lead to acceptable outcomes; however, further studies are required to investigate the use of additional agents, particularly for refractory or multiphasic cases. There is a paucity of literature on the use of chemotherapeutic agents for ADEM, although anecdotal use is prevalent. In addition, use of β-interferons for multiphasic forms of ADEM requires further investigation.

The use of standardized definitions for ADEM and MS in children and adolescents will help to facilitate future studies, regarding the prognosis, pathogenesis, and treatment of these two diseases.

Appendix

The International Pediatric MS Study Group: Lauren Krupp, MD (chair), Brenda L. Banwell, MD, Anita Belman, MD, Dorothee Chabas, MD, PhD, Tanuja Chitnis, MD, Peter Dunne, MD, Andrew Goodman, MD, Jin S. Hahn, MD, Deborah P. Hertz, MPH, Nancy J. Holland, EdD, RN, MSCN, Douglas Jeffery, MD, PhD, William MacAllister, PhD, Raul Mandler, MD, Maria Milazzo, RN, MS, CPNP, Jayne Ness, MD, PhD, Jorge Oksenberg, PhD, Trena L. Pelham, MD, Daniela Pohl, MD, PhD, Kottil Rammohan, MD, Mary R. Rensel, MD, Christel Renoux, MD, Dessa Sadovnick, PhD, Steven Robert Schwid, MD, Silvia Tenembaum, MD, Cristina Toporas, Emmanuelle Waubant, MD, PhD, Bianca Weinstock-Guttman, MD.

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Acute disseminated encephalomyelitis

Silvia Tenembaum, Tanuja Chitnis, Jayne Ness, et al.

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EXHIBIT 165

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Review

Post-vaccination encephalomyelitis: Literature review and illustrative case

William Huynh [a], Dennis J. Cordato [b,*], Elias Kehdi [a], Lynette T. Masters [c], Chris Dedousis [d]

[a] Department of Neurology, Liverpool Hospital, Liverpool, New South Wales, Australia
[b] Department of Neurology, Bankstown-Lidcombe Hospital, Eldridge Rd, Bankstown, 2200, New South Wales, Australia
[c] Mayne Radiology, St George Private Hospital, Kogarah, New South Wales, Australia
[d] Department of Aged Care, St George Hospital, Kogarah, New South Wales, Australia

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Abstract

Acute disseminated encephalomyelitis (ADEM) is an inflammatory demyelinating disease of the central nervous system that is usually considered a monophasic disease. ADEM forms one of several categories of primary inflammatory demyelinating disorders of the central nervous system including multiple sclerosis, optic neuropathy, acute transverse myelitis, and neuromyelitis optica (Devic's disease). Post-infectious and post-immunisation encephalomyelitis make up about three-quarters of cases, where the timing of a febrile event is associated with the onset of neurological disease. Post-vaccination ADEM has been associated with several vaccines such as rabies, diphtheria–tetanus–polio, smallpox, measles, mumps, rubella, Japanese B encephalitis, pertussis, influenza, hepatitis B, and the Hog vaccine. We review ADEM with particular emphasis on vaccination as the precipitating factor. We performed a literature search using Medline (1976–2007) with search terms including "ADEM", "acute disseminated encephalomyelitis", "encephalomyelitis", "vaccination", and "immunisation". A patient presenting with bilateral optic neuropathies within 3 weeks of "inactivated" influenza vaccination followed by delayed onset of ADEM 3 months post-vaccination is described.

Keywords: Influenza vaccination; Optic neuropathy; Acute disseminated encephalomyelitis; Multiphasic disseminated encephalomyelitis; Corticosteroid therapy

1. Introduction

Acute disseminated encephalomyelitis (ADEM) is an inflammatory demyelinating disease of the central nervous system (CNS) that is usually considered a monophasic disease, but a relapsing variant (distinct from multiple sclerosis) is well recognised – multiphasic disseminated encephalomyelitis (MDEM).[1] Post-infectious and post-immunisation encephalomyelitis make up about three-quarters of cases, where the timing of a febrile event is associated with the onset of neurological disease.[2] Although the two syndromes are distinguished by their precipitant, clinically and pathologically they are very similar. ADEM forms one of several categories of primary inflammatory demyelinating disorders of the CNS. Others include multiple sclerosis (MS), optic neuropathy, acute transverse myelitis, and neuromyelitis optica (Devic's disease).[1]

ADEM has an estimated annual incidence of 0.8 per 100,000 with a median age of onset of 6.5 years.[2] Although ADEM can occur at any age, it is more common in children. Optic neuropathy and ADEM are rare complications associated with vaccinations.[1,3–5] Most case reports describe patients experiencing a unilateral or bilateral optic neuropathy or ADEM, rather than simultaneous onset of both conditions. Optic neuropathy has also recurred in one patient after repeat administration of the influenza vaccination.[4] The presumptive mechanism is immune-mediated demyelination although immune-complex mediated

* Corresponding author. Tel.: +61 2 9553 7444; fax: +61 2 9553 7455.
E-mail address: DCordato@Southern-Neurology.com.au (D.J. Cordato).

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vasculopathy has also been postulated.[5] Spontaneous recovery or improvement after corticosteroids has been reported for post-vaccination optic neuropathy and ADEM[3] although permanent visual loss has also been described.[5]

We present a review of ADEM with particular emphasis on vaccination as the precipitating factor. We searched the literature using Medline (1976–2007), EMBASE (1980–2007) and PubMed (www.ncbi.nlm.nih.gov/sites/entrez) with the following search terms: "ADEM", "acute disseminated encephalomyelitis", "post-infectious encephalomyelitis", "encephalomyelitis", "encephalitis", "multiphasic disseminated encephalomyelitis", "vaccination", "immunisation", "influenza", and "post-influenza". We then describe a patient presenting with bilateral optic neuropathies within 3 weeks of an influenza vaccination and delayed onset of ADEM 3 months post-vaccination.

2. Review of the literature

2.1. Case definitions for ADEM

The following case definitions for ADEM have been extracted from Sejvar et al. and the Brighton Collaboration Encephalitis Working Group.[6]

The case definitions are structured in three different levels of diagnostic certainty.

a) ***Level 1 of diagnostic certainty***

i. Demonstration of diffuse or multifocal areas of demyelination by histopathology.

OR

ii. Focal or multifocal findings referable to the central nervous system, including one or more of the following:
- Encephalopathy (see case definition for encephalitis for specification of encephalopathy),
- Focal cortical signs (including but not limited to: aphasia, alexia, agraphia, cortical blindness),
- Cranial nerve abnormality/abnormalities,
- Visual field defect/defects,
- Presence of primitive reflexes (Babinski's sign, glabellar reflex, snout/sucking reflex),
- Motor weakness (either diffuse or focal; more often focal),
- Sensory abnormalities (either positive or negative; sensory level),
- Altered deep tendon reflexes (hypo- or hyperreflexia, asymmetry of reflexes), or
- Cerebellar dysfunction, including ataxia, dysmetria, cerebellar nystagmus,

AND

iii. MRI findings displaying diffuse or multifocal white matter lesions on T2-weighted, diffusion-weighted (DWI), or fluid-attenuated inversion recovery (FLAIR) sequences (± gadolinium enhancement on T1 sequences),

AND

iv. Monophasic pattern to illness (absence of relapse within a *minimum* of 3 months of symptomatic nadir).

b) ***Level 2 of diagnostic certainty***

i. Focal or multifocal findings referable to the central nervous system (as outlined in the Level 1 of diagnostic certainty section),

AND

ii. Magnetic resonance imaging (MRI) findings (as outlined in the Level 1 of diagnostic certainty section),

AND

iii. Insufficient follow-up time achieved to document absence of relapse within a minimum period of 3 months following symptomatic nadir.

c) ***Level 3 of diagnostic certainty***

i. Focal or multifocal findings referable to the central nervous system (as outlined in the Level 1 of diagnostic certainty section),

d) ***Exclusion criteria for all levels of diagnostic certainty***

i. Presence of a clear alternative acute infectious or other diagnosis for illness,
ii. Recurrence or relapse of illness at any point following a 3 month period of clinical improvement from symptomatic nadir, or
iii. If known, MRI findings or histopathologic data inconsistent with the diagnosis of ADEM.

2.2. Post-vaccination encephalomyelitis

The initially termed "neuroparalytic accidents" gained recognition in 1853 after the widespread introduction of Jenner's smallpox (actually cowpox) vaccine, and in 1885 with Pasteur's rabies vaccine.[1] Post-vaccination ADEM is associated with several vaccines including those for rabies, diphtheria–tetanus–polio, smallpox, measles, mumps, rubella, Japanese B encephalitis, pertussis, influenza, hepatitis B, and the Hog vaccine.[1,7–10] For most vaccines, incidence rates are as low as 0.1 to 0.2 per 100,000 vaccinated individuals.[2] ADEM following immunisation seems to occur significantly more frequently after primary vaccination as compared to revaccination.[11] Post-vaccination encephalomyelitis accounts for less than 5% of present

cases of ADEM.[1] It should be emphasized to parents, patients, health care providers, and all others concerned with immunization safety, that encephalomyelitis or ADEM – or any other adverse event – that follows administration of an inactivated component or live vaccine may be temporally *associated with*, but is not necessarily *the result of*, administration of a vaccine.[6] Furthermore, the time interval from immunisation to the onset of the clinical event is not considered part of the definition itself.

Sejvar et al. reviewed the neurological adverse events associated with the smallpox vaccination in the USA between 2002 and 2004. They found 214 such occurrences with only 3 of these suspected of being ADEM,[12] giving an estimated rate of 5 cases per million vaccines. Reported rates vary from 1 in 4000 to 1 in 80,000 after primary vaccination, and from 1 in 50,000 to 1 in 450,000 after revaccination.[13]

The reported incidence of ADEM following the Japanese encephalitis vaccination varies significantly depending on the population.[2,14,15] Between 1996 and 1998, the Japanese reported a rate of 0.2 per 100,000 as compared to a more frequent occurrence of 1 in 50,000 to 75,000 in Denmark.[15] Meanwhile there were no such events reported in the USA after administering more than 813,000 doses of Japanese encephalitis vaccine between 1993 and 1998.[15] In contrast, Menge et al. quoted an incidence rate as high as 1 in 600.[2]

The association between the influenza vaccination and ADEM has only come to light in the recent years, and hence there have been no large population studies and no estimated incidence rates. A 14-year-old female developed ADEM 2 weeks after an influenza vaccination,[16] while 2 adult males, aged 62 and 70, were diagnosed with ADEM and transverse myelitis with acute motor axonal neuropathy respectively within 1 week of vaccination.[17] In a review of adverse events associated with intranasal influenza vaccine between 2003 and 2005 in the USA, 10 neurological events were reported of the total 460 adverse events,[18] which included 2 people with Guillain-Barre syndrome, 1 with Bell's palsy, one ADEM, one febrile convulsion, and 3 non-serious reports of dystonic tongue, tongue paraesthesia and ataxia/vertigo. Nakayama and Onoda found 3 cases of ADEM and nine of Guillain-Barre among 38.02 million doses of influenza vaccine administered between 1994 and 2004 by the Kitasato Institute, Japan.[19] Giant cell arteritis has also been associated recently with the influenza vaccination.[15] All the influenza vaccines available in Australia are either split virion or subunit vaccines prepared from purified inactivated influenza virus cultivated in embryonated hens' eggs. The safety and efficacy of split virion and subunit vaccines are generally considered to be equivalent.[20]

Initially, post-vaccination ADEM was thought to be caused by the vaccine's viral component but it was later recognised that it could also be related to contamination with CNS tissue in which the vaccine was propagated.[1,2] For example, the anti-rabies vaccine had been cultured from rabbit, sheep or goat brain, and the Japanese encephalitis vaccine from murine brain.[2,21] This theory has been highlighted by a significant drop in post-vaccination ADEM incidence rates after the development of vaccines based on recombinant proteins rather than from *in-vivo* infected tissue.[1,2] Results from experimental allergic encephalomyelitis (EAE) also support this concept. In EAE, a disease that clinically and pathologically resembles ADEM, inflammation is produced when an experimental animal is inoculated with myelin or myelin antigens.[1] High-affinity antibodies directed against myelin-basic protein (MBP) have been identified in ADEM patients vaccinated with Semple strain rabies, but not MS patients.[2] However, this does not explain the origin and cause of post-vaccination ADEM due to smallpox and several other vaccines that are prepared without the involvement of neural tissues.[1]

The most common vaccinations associated with ADEM are the non-neural measles, mumps and rubella vaccines. The incidence of 1–2 per million for live measles vaccine is less than the reported 1 in 1000 incidence of post-infectious ADEM following infection with the measles virus itself.[1] Arguably, with both the virus and the vaccine as causes, vaccination dramatically reduces the incidence of ADEM.

2.3. Post-infectious encephalomyelitis

Post-infectious ADEM is associated with a preceding or concomitant infection that is most commonly viral. Measles, mumps, rubella, varicella-zoster, Epstein-Barr virus (EBV), cytomegalovirus (CMV), herpes simplex virus, hepatitis A or B, Coxsackie virus, influenza A or B, human immunodeficiency virus (HIV), human T-cell lymphotropic virus-1 (HTLV-1), human herpes virus 6, vaccinia, Rocky Mountain spotted fever, and human corona virus have been implicated as causing post-infectious ADEM.[1,7,22] Bacterial infections are also associated with post-infectious forms of ADEM, most commonly *Mycoplasma pneumoniae*.[7] Other culprit pathogens include *Borrelia*, *Campylobacter*, *Leptospira*, *Chlamydia*, *Legionella*, and group A beta-haemolytic streptococci.[1,7]

2.4. Other causes of ADEM

Reports of ADEM following solid organ transplantation are rare, and include one in which EBV was identified as the pathogen.[22,23] It is unclear, however, whether the overall incidence of ADEM is higher in recipients of organ transplantation than in the general population.

ADEM has also been described as a paraneoplastic disorder in some cases of leukaemia and non-Hodgkin's lymphoma.[22]

2.5. Multiphasic disseminated encephalomyelitis

Recurrent ADEM where episodes differ clinically is termed multiphasic disseminated encephalomyelitis (MDEM). In some cases of ADEM, the premature cessation

or tapering of therapy may lead to symptom recurrence. Hence, the monophasic nature of ADEM is defined as a lack of recurrence (within 3 months) in the absence of treatment or while on appropriate treatment; and relapse that occurs during cessation or tapering of treatment should be considered as belonging to one monophasic episode.[6] The following criteria apply when differentiating MS and MDEM:[24]

a) Altered mental state, relapses <5 months apart, rapidly evolving deficits and swift, complete recovery favour MDEM. Diplopia and asymmetrical deficits mainly resemble MS.
b) The number, morphology and distribution of lesions on MRI, with lesions >1 cm or involving the cortical ribbon or thalamus, or located infratentorially, and the later disappearance of T2 abnormalities, being distinctive of ADEM. The subsequent development of new lesions on MRI is quite typical of MS.
c) Marked cerebrospinal fluid (CSF) pleocytosis and a normal IgG index are typical for ADEM and would be highly unusual in MS.
d) Bilateral prolonged visual evoked potentials (VEPs) with no history of optic neuritis occurs commonly in MS, but rarely in ADEM.

2.6. Pathogenesis of ADEM

2.6.1. Molecular mimicry

Antigenic epitopes, comprising of delicate structural or partial amino-acid sequence homologies, are shared between an inoculated pathogen or vaccine, and a host CNS protein.[2] As a result, the pathogen is not recognised as "foreign" for elimination, nor "self" for immune tolerance. At the inoculation site the pathogen is initially processed by T cell activation and cross activation of antigen-specific B cells. These autoreactive cells can enter the CNS during immune surveillance and by chance, may encounter the homologous myelin protein. The local reactivation by antigen presenting cells subsequently culminates in a destructive autoimmune process in the CNS.[2] Much research has focused on T cell mediated autoimmune response to myelin autoantigens, such as MBP, proteolipid protein and myelin oligodendrocyte glycoprotein, which can induce ADEM.[23] Some studies have suggested a role for B cells and antibodies to gangliosides such as GM1 and GD1a, while others have identified T helper 2 cells reactive to MBP, which were found in the peripheral blood of ADEM patients.[23]

2.6.2. The re-infectious aetiology

The re-infectious aetiology theory postulates that CNS demyelination occurs as a possible result of direct neurotoxicity of a neurotropic virus, and that vaccination with an attenuated virus strain may cause problems only if administered during a preceding infection, in which previously primed virus-specific cytotoxic T cells are reactivated.[2]

2.6.3. The post-infectious aetiology

The disruption of the blood-brain barrier sustained after direct CNS infection with a neurotropic virus may subsequently result in the leakage of CNS autoantigens into the systemic circulation. These autoantigens are then processed in the systemic lymphatic organs leading to a breakdown in tolerance and emergence of a self-reactive and encephalitogenic T cell response.[2] Studies on patients who developed ADEM following anti-rabies vaccination suggest that MBP may be encephalitogenic in this scenario.[7]

2.6.4. Immuno-inflammatory model

The immuno-inflammatory model combines the concept of molecular mimicry with the inflammatory cascade process.[2] A "first hit" is experienced after an antecedent infection with a virus that expresses determinants allowing molecular mimicry. This need not be clinically eventful or significant. A second infection with an unrelated virus results in sufficient reactivation of the primed autoreactive T cells to eventuate in demyelination of the CNS. This constitutes the "second hit".

2.6.5. Genetic predisposition

Berkovic et al. noticed that many of the children who experienced vaccination-induced encephalopathy had a similar clinical course to that seen in severe myoclonic epilepsy in infancy (SMEI), which is an epileptic encephalopathy associated with prolonged febrile seizures, intractable myoclonus and other seizures and psychomotor decline.[25,26] The authors identified mutations in the SCN1A gene in 11 of 14 patients with suspected vaccine encephalopathy. This leads to the question of whether the SCN1A mutation was a predisposing factor waiting to be triggered by fever or other stresses. More than 50% of SMEI patients experienced their first seizure after DPT vaccination.[25] Similarly, patients who have a certain underlying genetic predisposition may be more prone to developing ADEM post-vaccination.

2.7. Pathology

There is a distinct histopathological pattern of ADEM lesions of perivenous inflammation surrounding small vessels in both the CNS grey and white matter.[2] Most of these lesions seem of similar age, and are infiltrated by lymphocytes, macrophages and to a lesser degree, neutrophils. Perivascular oedema, endothelial swelling and vascular endothelial infiltrations are additional findings. Demyelination may not be evident in hyperacute or acute lesions, but may develop later in the lesion evolution in a pathognomonic "sleeve like" fashion where it is confined to the hypercellular areas. There is only a small degree of axonal damage.

2.8. Clinical features

Depending on the inciting agent, the onset of symptoms may vary slightly: from 1 to 14 days with non-neural vaccines, less than 1 week after the appearance of a rash in exanthematous illnesses, and 1 to 3 weeks after rabies vaccination.[1] Symptom onset is usually rapid with progression over hours to a peak in days, and the neurological features vary with both focal and non-focal elements. No pathognomonic clinical features are seen in ADEM, but a combination of altered consciousness or behaviour and multifocal neurological deficits especially if closely timed to an infection or immunisation, should alert the clinician to its possible diagnosis.[2] Certain series show that spinal cord dysfunction is more prevalent in adults than in children.[27] Seizures are seen more in post-infectious disease but are also associated with vaccination.[28] Encephalopathy, fever, seizures, and meningism are very rare in MS, which is one of the most important differential diagnoses.[1,29]

Focal presentation in ADEM is rather heterogeneous and depends upon the location and the degree of demyelination within the CNS. Multifocal presentations may be combinations of pyramidal and cerebellar signs, which are common, as are cranial neuropathies, which include bilateral optic neuropathies (more common in ADEM than in MS).[1] About 25% of patients present with a paraparesis or quadriparesis with urinary dysfunction when myelitis is predominant. Interestingly, in these cases the peripheral nervous system is also involved more frequently in the post-infectious form[30] but it has also been reported after vaccination, especially following rabies vaccination, with radicular complaints being the most frequent manifestation.[1]

2.9. Investigations

In general, the diagnosis of post-vaccination ADEM is made on clinical grounds with the guidance of MRI after the exclusion of an acute infective condition by lumbar puncture and other microbiological and serological tests.

2.9.1. Cerebrospinous fluid

Lumbar puncture typically reveals a lymphocytic pleocytosis and raised protein levels both greater than those seen in MS.[1] A CSF oligoclonal band is less common in ADEM than in MS and when present (in 0–58% of cases reported), is usually transient, indicating that a disease-causing antigen is only transiently expressed within or outside the CNS.[2]

2.9.2. Radiology

CT scans of the brain in ADEM can be normal but when abnormal, usually shows non-specific, low attenuation subcortical white matter lesions that may or may not enhance. In cases of acute haemorrhagic encephalomyelitis, CT scans may reveal haemorrhage and oedema associated structural changes.[1]

MRI is considered the imaging modality of choice. It can be normal at initial presentation and delays between 5 and 14 days from symptom onset to MRI abnormalities may occur.[1,2] Cerebral lesions are usually disseminated but solitary lesions occur in about 10% to 30% of cases.[31] Lesion patterns often seen in ADEM include widespread, multifocal or extensive white matter lesions and lesions in the deep grey matter (the thalamus and basal ganglia) with the lesion load greater than 50% of the total white matter volume.[2] Although there is no pathognomic MRI appearance for ADEM, in one study cortical involvement or lesions in the basal ganglia were present exclusively in patients with ADEM as compared to MS.[29] Certain authors suggested that the diagnostic hallmark of ADEM is the demonstration of scattered, focal or multifocal (disseminated) areas of inflammation and demyelination within cerebral subcortical and deep cortical white matter, while grey matter involvement is also seen (particularly in the thalamus).[6] It was previously claimed that, all lesions should enhance equally following gadolinium contrast since all lesions should be active and of the same age. Newer studies, however, shown that lesions in ADEM may evolve over several weeks and consequently only some lesions may be enhanced, or there may also be no enhancement.[29] Follow-up MRI scans after a minimum interval of 6 months is recommended to establish or confirm the diagnosis of ADEM at which time there should be a resolution, partial or complete, of old lesions and no new lesions.[1,2] The appearance of new lesions is strongly suggestive of MS.

2.10. Treatment

There is a lack of controlled clinical trials and no proven standard treatment for post-vaccination and other causes of ADEM. Most treatment options are based on empirical and observational evidence. Once ADEM is diagnosed and an acute infectious aetiology excluded, treatment should be instituted as soon as possible. Present treatments are centred on immunosuppression and immunomodulation. The options include corticosteroids, plasma exchange, and intravenous immunoglobulin (IVIg).

2.10.1. Corticosteroids

Corticosteroid therapy is widely accepted as first line therapy for ADEM. The recommended treatment regime is intravenous methylprednisolone 1g daily with a cumulative dose of 3g to 5g followed by a 1 month to 2 month oral prednisolone taper. If there are relapses (as seen in MDEM), they frequently occur shortly after ADEM.[1,2]

2.10.2. Plasma exchange

Plasma exchange is recommended in patients who respond poorly to intravenous corticosteroids. The usual course involves 7 exchanges over 14 days with improvements frequently seen after the first plasma exchange.[1] Plasma exchange is used because serum antibodies directed

against MBP and galactocerebroside are found in patients with post-rabies inoculation ADEM, as well as intrathecal synthesis of these antibodies.[1]

2.10.3. Intravenous immunoglobulin

IVIg is reserved for ADEM that fails to respond to corticosteroid treatment and where plasma exchange is contraindicated or difficult to access. IVIg may be preferred to plasma exchange in cases of post-vaccination encephalomyelitis.[1] The use of IVIg has proven effective with particular subgroups of patients showing both CNS and peripheral nervous system (PNS) involvement and some authors have proposed that in patients with evidence of polyradiculopathy, IVIg should be considered as first line therapy.[27]

2.10.4. Others

With failure of the above treatment modalities, several other therapies have been tried and used with anecdotal success. These include intravenous cyclophosphamide and mitoxantrone.[1,2] Miravalle and Roos discussed the administration of antivaccinia gamma globulin at the time of smallpox vaccination to prevent the complication of post-vaccination ADEM, but it was not effective once the complication had already occurred.[32] It is also advisable to avoid immunisation for at least 6 months after the diagnosis of ADEM as relapse into MDEM has occurred following routine vaccinations.[1]

2.11. Prognosis

Full recovery, and in some cases spontaneous improvement, is the expected outcome in most cases and is seen in about 50% to 75% with a higher proportion (between 70% and 90%) if minor residual deficits are considered.[1,2] The average time to recovery ranges from 1 to 6 months.[1] Suggested predictors of poor outcome include older age, female gender, degree of functional impairment at clinical onset, CSF protein level, spinal cord involvement, PNS damage, and poor response to corticosteroids.[2,27] Functional impairment following ADEM may be assessed by the Scripps neurological rating scale (SNRS), which uses a standardised neurological examination of mentation and mood, visual acuity, eye movements and lower cranial nerves, motor and sensory function, reflexes and Babinski sign, gait and cerebellar function and bladder and bowel function (SNRS score range –10 to 100; the higher the score the better the level of function).[33] In a study of 60 patients with post-infectious inflammatory disorders, Marchioni et al.[27] showed that a mean onset SNRS of 60.3 was associated with a favourable prognosis while a mean SNS of 37.4 was associated with a poorer outcome. The same study also demonstrated that lower mean CSF albumin and IgG concentrations of 68.3 mg/dL and 8.1 mg/dL, respectively, were associated with a favourable prognosis whereas mean concentrations of 106.5 mg/dL and 19.1 mg/dL, respectively, were associated with a poor outcome. In a population of 40 adult ADEM patients studied by

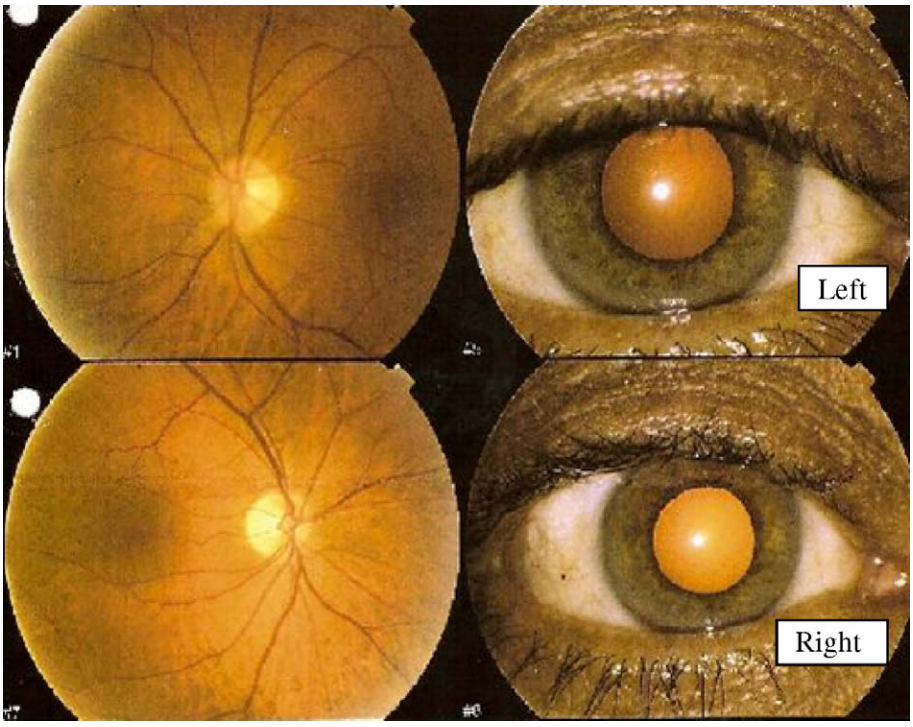

Fig. 1. Images of the patient's optic nerves (see Illustrative case report) taken about 4 weeks after his influenza vaccination showing bilateral optic atrophy, more marked in the right eye (lower image).

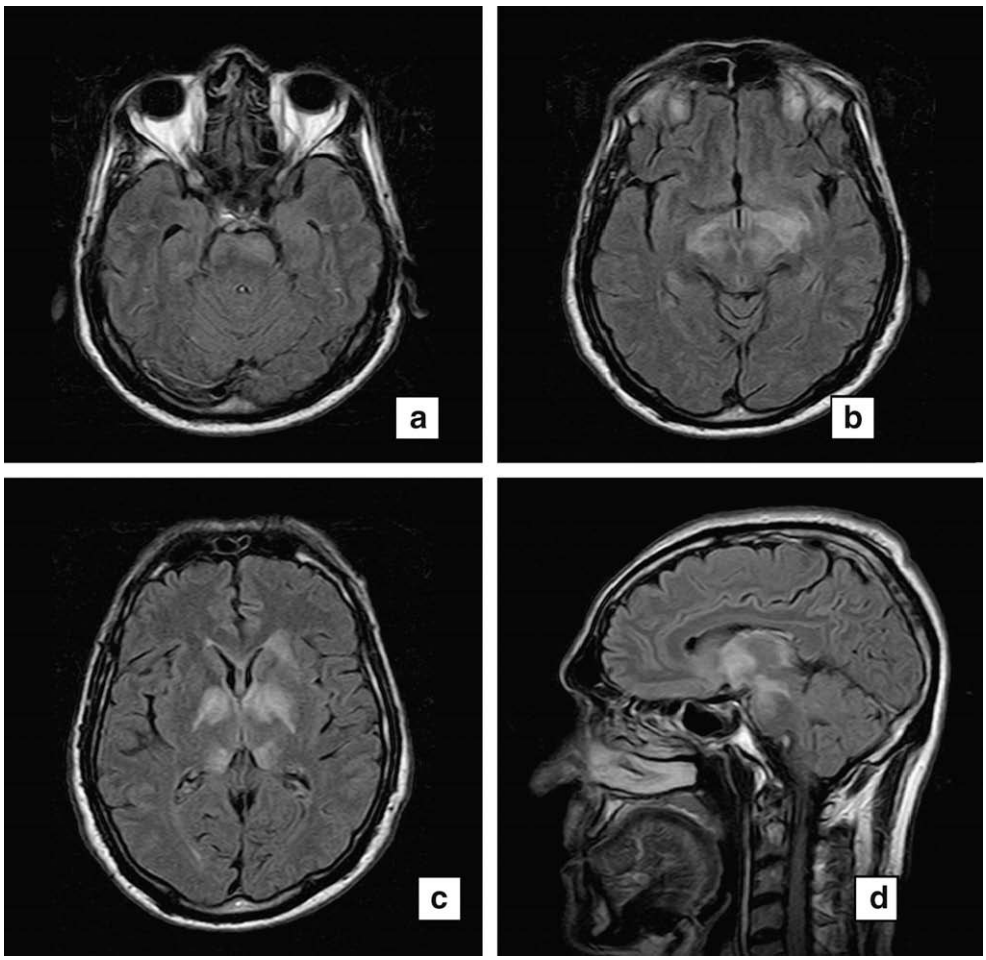

Fig. 2. MRI (a–c) Axial fluid attenuated inversion recovery (FLAIR) images and (d) sagittal FLAIR images performed in October 2005 about 3 months post-influenza vaccination showing involvement of central grey matter including (a) the left pons, (b) centromedial thalami, and (c) right and left globus pallidus.

Schwarz et al., 35% developed clinically definite MS over a mean observational period of 38 months.[29] However, preceding infection or vaccination was not a prerequisite in their initial diagnoses of ADEM.

3. Illustrative case report

A 61-year-old male presented in early July 2005 with a 3-week history of bilateral visual blurring, worse in the right eye, and bilateral pain on eye movement. He had received the inactivated influenza vaccine (Fluvax) 3 weeks prior to symptom onset. His past medical history was unremarkable and he was not receiving regular medication. On examination, his visual acuity was 6/15 in the right eye and 6/6 in the left eye. He demonstrated an afferent pupillary defect on the right. Fundoscopy revealed pallor of both optic discs, more so on the right (Fig. 1). The remainder of his neurological and general medical examination was otherwise unremarkable.

He underwent an initial brain MRI, which was within normal limits. His visual evoked potentials were significantly prolonged at 140 msec bilaterally ($N < 110$ msec). A clinical diagnosis of bilateral optic neuropathies complicating influenza vaccination was made. No specific therapy was instituted. Over the next 2 months, his visual symptoms persisted although he was still able to drive and attend work.

In late September 2005, he presented with a 1 week history of increasing daytime somnolence, fluctuating alertness and orientation consistent with delirium. His clinical examination demonstrated hypersomnolence and mild disorientation to time and place. His visual acuity was 6/18 in both eyes. The rest of his neurological examination was normal. Investigations revealed normal full blood count (FBC), erythrocyte sedimentation rate (ESR) and biochemistry. A CT brain scan was normal. An initial lumbar puncture found a CSF protein concentration of 0.71 g/L (Normal < 0.40) and glucose of 3.1 mmol/L (Normal = 2.4–5.4) with 24×10^6 white blood cells per μL (87% mononuclears). CSF cell surface markers were negative for clonal T cells. Herpes simplex and tuberculosis polymerase chain reaction (PCR) and cryptococcal antigen were negative.

An MRI brain scan revealed fairly symmetric signal abnormality involving the central grey matter predominantly

(Fig. 2a–d). Signal change extended rostrally to the frontal periventricular white matter and caudally to the left pons. There was no gadolinium contrast enhancement and fat-saturated T1-weighted sequences of the orbits showed no abnormal enhancement. Further investigations including vitamin B12 and folate levels, antinuclear antibodies, antineutrophil cytoplasmic antibodies (ANCA), serum angiotensin-converting enzyme, serum electrophoresis, HIV serology, IgM serology for EBV, CMV and Australian encephalitis, mycoplasma serology, Venereal Disease Research Laboratory test (VDRL), antineuronal antibodies and CT scans of the chest, abdomen and pelvis were normal. The patient was diagnosed with ADEM and treated with high dose intravenous steroids consisting of 1g methylprednisolone daily for 5 days followed by oral tapering steroids. His orientation and alertness returned to normal within 2 weeks. On follow-up review 2 months later, he had fully recovered apart from visual acuity of 6/12 bilaterally and bilateral optic disc pallor. A repeat MRI of the brain was significantly improved. He remained clinically well at his last visit in March 2006.

The patient's clinical presentation was most likely due to post-influenza vaccination optic neuritis and encephalomyelitis. A patient with a similar biphasic presentation followed an anti-rabies vaccination[34] has been reported in a 45-year-old male who presented with transverse myelitis 14 days after anti-rabies vaccination and developed bilateral optic neuritis 1 month later.

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EXHIBIT 166

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Review

The spectrum of post-vaccination inflammatory CNS demyelinating syndromes

Dimitrios Karussis *, Panayiota Petrou

Department of Neurology, Multiple Sclerosis Center, Laboratory of Neuroimmunology, The Agnes-Ginges Center for Neurogenetics, Hadassah University Hospital, Jerusalem, Ein-Kerem, Israel

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ABSTRACT

A wide variety of inflammatory diseases temporally associated with the administration of various vaccines, has been reported in the literature. A PubMed search from 1979 to 2013 revealed seventy one (71) documented cases. The most commonly reported vaccinations that were associated with CNS demyelinating diseases included influenza (21 cases), human papilloma virus (HPV) (9 cases), hepatitis A or B (8 cases), rabies (5 cases), measles (5 cases), rubella (5 cases), yellow fever (3 cases), anthrax (2 cases), meningococcus (2 cases) and tetanus (2 cases). The vast majority of post-vaccination CNS demyelinating syndromes, are related to influenza vaccination and this could be attributed to the high percentage of the population that received the vaccine during the HI1N1 epidemia from 2009 to 2012. Usually the symptoms of the CNS demyelinating syndrome appear few days following the immunization (mean: 14.2 days) but there are cases where the clinical presentation was delayed (more than 3 weeks or even up to 5 months post-vaccination) (approximately a third of all the reported cases).
In terms of the clinical presentation and the affected CNS areas, there is a great diversity among the reported cases of post-vaccination acute demyelinating syndromes. Optic neuritis was the prominent clinical presentation in 38 cases, multifocal disseminated demyelination in 30, myelitis in 24 and encephalitis in 17. Interestingly in a rather high proportion of the patients (and especially following influenza and human papiloma virus vaccination-HPV) the dominant localizations of demyelination were the optic nerves and the myelon, presenting as optic neuritis and myelitis (with or without additional manifestations of ADEM), reminiscent to neuromyelitic optica (or, more generally, the NMO-spectrum of diseases). Seven patients suffered an NMO-like disease following HPV and we had two similar cases in our Center. One patient with post-vaccination ADEM, subsequently developed NMO.
Overal, the risk of a demyelinating CNS disease following vaccination, although non-negligible, is relatively low. The risk of onset or relapse of CNS demyelination following infections against which the vaccines are aimed to protect, is substantially higher and the benefits of vaccinations surpass the potential risks of CNS inflammation. This does not in any way exempt us from "learning" the lessons taught by the reported cases and searching new and safer ways to improve vaccination techniques and increase their safety profile.

Contents

* Corresponding author. Tel.: +972 2 6776939.
E-mail address: karus@cc.huji.ac.il (D. Karussis).

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Exhibit 166

1. Introduction

1.1. Vaccinations and autoimmunity

The prevalence of autoimmunity has been continuously rising during the last decades, mainly in the "Western" world. Immune mediated diseases became nowadays one of the leading causes of morbidity and mortality worldwide [1–3]. A complex of genetic and environmental factors have been suggested as responsible [4,5], including various infections, toxins, and drugs that have been all shown to be linked with the onset or exacerbation of autoimmune conditions [6,7].

The more prominent increase of the prevalence of autoimmune diseases in the "Western" world may be also related to additional factors, such as pollution/accumulation of toxic substances, extensive usage of wireless networks, psychological stress and exaggerated use of antibiotics which cause an over-sterilized immunological milieu [8]. All of the above may contribute to an immune-dysregulation and disruption of the delicate networks/mechanisms that maintain self-tolerance, in genetically susceptible individuals, or may act as co-players in a complex interaction with various additional risk-factors.

Other environmental factors that may induce an immune "adjuvant" effect (boosting the immune response) include infectious agents and chemical substances such as silicone, alum and pristane, which are by themselves capable to induce autoimmunity in animal models [9–11].

Vaccines, which contain both attenuated infectious agents or their main immunogenic proteins and chemical adjuvants, represent one of most debatable and characteristic/unique paradigms of "environmentally"-induced trigger of autoimmunity. In this review we will summarize the worldwide experience, based on the published cases during the last 25 years, of central nervous system demyelinating diseases (acute disseminating encephalomyelitis—ADEM, multiple sclerosis—MS, myelitis, neuromyelitis optica—NMO and optic neuritis) associated with various vaccinations. The immunopathogenetic mechanisms involved will be discussed.

2. Vaccinations and CNS inflammatory diseases

A wide variety of inflammatory diseases temporally associated with the administration of various vaccines has been reported in the literature (Table 1). A PubMed search from 1979 to 2013 using the terms "vaccination/encephalitis", "vaccination/encephalomyelitis", "vaccination/ADEM", and "optic neuritis/neuropathy/vaccination" revealed seventy one [71] cases within the above criteria (Table 2). The most commonly reported vaccinations that were associated with CNS demyelinating diseases included influenza (21 cases), human papilloma virus (HPV) (9 cases), Hepatitis A or B (8 cases), rabies (5 cases), measles (5 cases), rubella (5 cases), yellow fever (3 cases), anthrax (2 cases), meningococcus (2 cases) and tetanus (2 cases). As can be seen from Table 2, the vast majority of post-vaccination CNS demyelinating syndromes are related to influenza vaccination and this could be attributed to the high percentage of population that received the vaccine during the H1N1 epidemia from 2009 to 2012.

In terms of the clinical presentation and the affected CNS areas, there is a great diversity among the reported cases of post-vaccination acute demyelinating syndromes (Table 2). Optic neuritis was the prominent clinical presentation in 38 cases, multifocal disseminated demyelination in 30, myelitis in 24 and encephalitis in 17. Interestingly in a very high proportion of the patients (and especially following influenza vaccination) the dominant localizations of demyelination were the optic nerves and the myelon, presenting as optic neuritis and myelitis (with or without additional manifestations of ADEM). This predisposition to the spinal cord and the optic nerves is reminiscent to neuromyelitic optica (or, more generally, the NMO-spectrum of diseases) that are highly associated with anti-aquaporin-4 antibodies. Indeed, as seen in Table 2, seven patients suffered an NMO-like disease following various vaccinations, especially HPV [12–14]. This raises the possibility of cross-reactivities between aquaporin epitopes and certain viral proteins and possibly, a link between ADEM and NMO. The latter is supported by case reports, such one patient with post-vaccination ADEM who subsequently developed NMO [15]. However, the incidence of anti-aquaporin antibodies in ADEM was low, as compared to anti-MOG antibodies [16].

Usually the symptoms of CNS demyelination appear few days following the immunization (mean: 14.2 days—Table 2) but there are cases in which the clinical presentation was delayed (more than 3 weeks or even up to 5 months post-vaccination) (approximately a third of all the reported cases—Table 2).

3. The spectrum of post-vaccination CNS demyelinating syndromes

3.1. Acute disseminated encephalomyelitis (ADEM)

Acute disseminated encephalomyelitis (ADEM) is an inflammatory demyelinating disease of the central nervous system (CNS) [17]. It is a rather rare disease with an incidence of 0.6 to 0.8 per 100,000 per year [18–20]. ADEM can occur in any age but is mainly a disease of children and young adults with a mean age of onset of 5–6 years [21–23] and a higher incidence in males [19]. The clinical presentation of ADEM is widely variable, depending on the distribution of lesions in the CNS. Encephalopathy, occurring in up to 74% of patients [24], is considered mandatory for definite diagnosis. Other neurological signs include pyramidal syndrome, cranial nerve palsies, ataxia, seizures, optic neuritis and speech impairment. ADEM is defined (according to the criteria proposed from the International MS Group [17]) as a first ever clinical event with presumed inflammatory or demyelinating cause, with an acute or subacute onset, that affects multifocal areas of the CNS and is usually polysymptomatic and includes encephalopathy (i.e., behavioral change or altered level of consciousness). Additional criteria include: the presence of focal/multifocal lesion(s) predominantly affecting the white (but also the gray) matter without evidence of previous destructive white matter changes, the occurrence of clinical/radiologic improvement (although there may be residual deficits), and the absence of other etiology that could explain the event.

ADEM has a monophasic course in the majority of patients; if relapse occurs, it usually happens within 3 months from its onset. However, cases with relapse with symptoms different than the original ones have been reported and are defined as recurrent (RADEM) or multiphasic disseminated encephalomyelitis (MDEM) [23,25–33]. The existence of such forms of ADEM remains controversial.

Differentiation between ADEM, multiple sclerosis (MS), is still a challenge [21], especially in the case of relapsing ADEM, where the border between ADEM and MS is more obscure. The lack of oligoclonal antibodies and the high cellularity of the CSF, the involvement of CNS gray matter, and the presence of fever, confusion and headache are some of the main differentiating features between ADEM and MS [18,24,34]. Histopathologically, focal, perivenous and subependymal

Table 1
The spectrum of post-vaccination CNS demyelinating diseases.

ADEM	References: [18,52,135–137,139,141,142,145–148,150–155,158,160,163–166,169–173]
Encephalitis with white matter involvement	References: [135,137,139,140,143,146,147,150,151,153,157,158,160,161,165,171,172]
Myelitis	References: [12–14,52,134,137–139,141,142,144,148,159,161,162,167–169,172]
Optic neuritis	References: [12–14,18,62–77,79,139,145,148,149,151,153,154,156,168,170,173]
NMO-spectrum	References: [12–14,52,148,162,168]

changes (at the early period, mainly T cell infiltrates, accompanied by few plasma cells and later microglial infiltrates) dominate the histopathological pattern of ADEM, leading to the formation of disseminated masses or conglomerates. Despite the predisposition for the white matter, the cortical and deep gray matter (frequently the thalamus) is affected and this further differentiates ADEM from MS. In the long term, only a sparse gliosis can be detected without significant myelin loss in ADEM [18].

The current pathogenetic hypothesis in post-vaccination ADEM is that antigens of viral origin cross-react with myelin components (molecular mimicry) and in a secondary manner induce a hyperergic reaction, that leads to the development of disseminated demyelination. Myelin proteins have shown resemblance to several viral sequences and anti-MBP antibodies have indeed been detected following vaccination with Semple rabies vaccine [35,36]. Another hypothesis is that vaccination may activate in a non-specific way distinct clones of anti-myelin T-cells and that suppressor or regulatory cells that are aimed to control this abnormal reactivity are compromised or malfunctioning.

Post-vaccination ADEM accounts for 5–10% of all cases [18,34]. However, despite a close temporal relation to vaccinations, there is no concrete evidence of a clear pathogenetic correlation. The incidence of post-vaccination ADEM has largely fluctuated over the last decades with a peak occurring between 1927 and 1929 and also –seemingly during the last years. This can be probably related to the methods used for vaccine production, the amount of myelin antigens included and –most importantly– the type of the used adjuvant. The overall incidence of post-vaccination ADEM is estimated to 0.1–0.2 per 100,000 and the higher risk has been reported following immunization against measles. Other common causes of post-vaccination ADEM include vaccines against the varicella zoster, the rubella, the smallpox and the influenza viruses [18]. On the other hand, surprisingly, certain vaccines such as anti-tetanus vaccination were shown to have a negative correlation with ADEM (statistically significant decreased risk) [37].

Vaccination against Hepatitis B is one of the most controversial possible causes of demyelinating disease. An increased risk for ADEM was suggested by Touze et al. [38,39] and Mikaeloff et al. reported a slight increase of the incidence of CNS demyelinating diseases specifically following Engerix B vaccine [40]. For the present, there is no convincing proof of a causative correlation between HBV and ADEM/MS or other acute demyelinating diseases [41–45]. Analysis of the existing data argues against such causal relationship and indicates that the benefits of the vaccine clearly surpass the potential risks of CNS inflammation [46].

Gardasil vaccination is a novel type of vaccine targeting the human papilloma virus that has been shown to be efficient for the prevention of cervical, vulval and vaginal dysplasia and cervical cancer [47–49]. Only few cases of post-vaccination ADEM have been reported in the literature (Table 2).

Post-vaccination ADEM is usually observed after primary vaccination and much less following revaccination [18], but there are reports where a relapse, or a second neurological event was observed after repeated immunizations with the same virus [12–14]. Noteworthy, there is a substantial percentage of the reported post-vaccination cases that subsequently developed MS (Table 2) [46,50–52].

3.2. Isolated optic neuritis

Optic neuritis (ON) is an inflammatory demyelinating condition of the optic nerve. Most cases are idiopathic or associated with ADEM or MS. ON is the most common acute optic neuropathy in young adults with an incidence of 1–3 per 100,000 population per year [53–59].

Acute ON usually presents as an isolated clinical event without additional neurological involvement (monosymptomatic ON) [60]. Clinical features include periocular pain, abnormal visual acuity and visual field defects, reduced color vision, a relative afferent pupillary defect, and abnormal visual evoked potentials. The fundus appears normal but occasionally edema of the optic nerve head (papillitis) is observed [60]. MRI white matter abnormalities identical to those seen in MS can be found in half of the monosymptomatic ON cases [61]. The visual deficits peak over 1 to 2 weeks and usually substantially improve over the following month. However, many patients continue to have residual visual dysfunction, even when visual acuity improves ad integrum.

Optic neuritis represents a unique paradigm of an association of vaccines with demyelination. There are numerous reports of isolated (either unilateral or bilateral) optic neuritis following various types of vaccinations against infectious agents [62], including measles [63–65], anthrax [66], rubella [63–65], Hepatitis A and B [67–69], influenza [70–75], pneumonococcal vaccine [12], meningococcal vaccine [76], rabies [77,78] and BCG [79]. As shown in Table 2, the most often demyelinating clinical presentation associated with vaccinations is optic neuritis, accounting for more than half of the reported cases in the literature.

3.3. Multiple sclerosis

Multiple sclerosis (MS) is a chronic disease of the central nervous system (CNS) that is characterized by loss of motor and sensory function, caused by immune-mediated inflammation, demyelination and subsequent axonal damage [80–82]. MS could be considered as the chronic form of ADEM and ADEM can sometimes develop to MS, as mentioned above.

Clinically, most MS patients experience recurrent episodes (relapses) of neurological impairment but eventually in most of the cases the course of the disease becomes chronic and progressive with time, leading to accumulating motor disability, and cognitive deficits.

Histologically, perivenular inflammatory lesions (consisting of mononuclear infiltrations) are evident in the earlier phases of the disease, resulting in demyelinating plaques, the pathological hallmark of MS [81]. Inflammation leads to damage of oligodendrocytes and demyelination causes disruption of the conduction of neuronal signals in the affected regions. As the disease progresses disability and neuronal damage [83] become permanent and irreversible.

The inflammatory process in MS is propagated by an autoimmune cascade, involving mainly T-cells that target myelin self antigens [84,85], possibly mediated by mechanisms of molecular mimicry (cross-reactive antigens expressed by viruses or other microorganisms and myelin components) [86]. An alternative hypothesis is that "naturally" existing myelin-specific T-cells, especially of the Th17 phenotype, may expand to critical pathogenic quantities [87] due to malfunctioning immunoregulatory mechanisms (such as those involving the Th2, Th3, Tr1, Treg, and CD8 + T-cells).

Additionally, environmental, genetic and infectious factors seem to play important roles in MS pathogenesis, similarly to autoimmunity in general. MS is not a homogenous disease and several distinct immunopathological profiles of the disease exist, including forms in which humoral immune mechanisms are prominent [88]. Indeed, increased B-cell numbers, mainly memory cells and short-lived plasmablasts can be detected in the CNS of MS patients [89]. Plasmablasts persist in the cerebrospinal fluid (CSF) and their number correlates with the intrathecal IgG synthesis (evidenced by the presence of oligoclonal antibodies in the CSF, one of the hallmarks of MS diagnosis) [89,90]. Moreover, B cells, plasma cells, autoantibodies and complement have been detected in MS lesions [91,92]. Recently, ectopic lymphoid follicles have been found in the CNS of patients with MS [93,94], especially in those with progressive disease.

Numerous studies have shown that the risk of MS relapse is increased by infections (approximately twofold [95]), accompanied by enhanced lesion activity in the MRI [96]. Moreover, relapses associated with an infection seem to cause more neurological dysfunction [95].

Vaccinations have been also incriminated/implicated as triggers of the onset of MS in susceptible individuals [97]. Some studies [28,37,98] indicated a significant risk for CIS or conversion to clinically

Table 2
A list of case reports with various post-vaccination CNS demyelinating syndromes, published in the literature.

	Type of vaccine	Age/ gender	Onset time post-vaccination	Clinical syndrome					Response to treatment and outcome	Reference
				ADEM	Encephalitis	Myelitis	Optic neuritis	NMO		
1	Influenza	70/M	7 days			+			Partial recovery after steroids + PE	Nakamura et al. [134]
2	Influenza	62/M	5 days			+			Partial recovery after steroids + PE in the second	Nakamura et al. [134]
3	Influenza	75/F	3 weeks	+	+				No response to PE and steroids; death	Shoamanesh and Traboulsee [135]
4	Influenza	61/M	3 weeks, 3 months	+			+		IVMP: full recovery except 50% reduction of visual acuity	Huynh et al. [18]
5	Influenza	6/M	16 days	+					Steroids treatment/resolved	Iyoda et al. [136]
6	Influenza	83/F	8 days	+	+	+			Dramatic response to PE; died later of pneumonia	Machicado et al. [137]
7	Influenza	61/M	2 weeks			+			I.v. steroids and IVIG; full recovery	Ravaglia et al. [138]
8	Influenza	60/F	10 days			+			I.v. steroids and IVIG; full recovery	Ravaglia et al. [138]
9	Influenza	NA	NA	+	+	+	+		Recovery after i.v. steroids	Vilain et al. [139]
10	Influenza	59/F	2 weeks				+			Hull and Bates [71]
11	Influenza	61/F	NA				+		Good recovery following steroids	Ray and Dreizin [74]
12	Influenza	NA	NA				+		Recovery after steroids	Perry et al. [73]
13	Influenza	62/F	15 days				+		Improvement with steroids	Laffon-Pioger et al. [72]
14	Influenza type A + B	18/M	2 weeks				+		Recovery after steroids	Rubinov et al. [75]
15	Influenza (LAIV) as nasal flu vaccine	13/M	NA				+		Recovery after steroids	Crawford et al. [70]
16	H1N1 influenza	2/M	25 days		+				Full recovery after steroids treatment	Fujii et al. [140]
17	H1N1 influenza	33/F	15 days	(+)		+			Improvement with steroids	Maeda and Idehara [141]
18	H1N1 influenza	36/M	10 days	+		(+)			Marked improvement with steroids	Hoshino et al. [142]
19	H1N1 influenza	34/M months	5 days		+				Complete recovery with steroids	Lee et al. [143]
20	H1N1 influenza	NA/F	4 days			+			Improved without treatment	Arcondo et al. [144]
21	H1N1 influenza	2/M	4 days; 6 days						Full recovery after steroids	Lapphra et al. [145]
22	Rabies	31/M	5 months	+	(+)				Paresis resolved; persisting neurological signs: seizures, behavioral changes	Gamboa et al. [146]
23	Rabies	24/M	1 week	+	+				Died after 37 years of encephalopathy; demyelinating lesions in pathology	Iizuka et al. [147]
24	Rabies	45/M	14 days	+		+	+	+	Improvement with steroids	Kulkarni, et al. [148]
25	Rabies	15/M	25 days				+			Gupta et al. [149]
26	Rabies	NA	NA				+			Dadeya et al. [77]
27	Polyvaccination	27/M	10 days	+	+				Death (day 21)	Labauge et al. [150]
28	Early summer encephalitis	36/M	After repeated immunization	+	+		+		Partial improvement	Schattenfroh [151]
29	Group A + C meningococcal vaccine	25/F	NA	+					Fast disappearance of symptoms and gradual resolution of lesions in MRI after i.v. MP	Py and Andre [152]
30	Meningococcal C	13/M	NA				+			Laria et al. [76]
31	Pneumococcal polysaccharide	87/M	Few days			+	+	+	Improvement of vision but not of quadriparesis with steroids	Kitazawa et al., Intern Med, 2012 [12]
32	Hepatitis B	40/M	6 weeks	+	+		+		Partially resolved after steroids	Cabrera-Gomez et al. [153]
33	Hepatitis B	39/F	4 weeks after the 2nd dose	+			+		Craniotomy and dexamethasone; residual dyslexia hemianopsia; resolution of the MRI lesions, except a poroencephalic cyst	Konstantinou et al. [154]
34	Hepatitis A	23/F	3–7 days	+					Improvement of CNS signs with steroids but not of axonal neuropathy	Huber et al. [155]
35	Hepatitis A	39/M	6 days				+		Partial recovery after steroids	Huang et al. [69]

36	Hepatitis B	28/M	10 days				+			Albitar et al. [67]
37	Hepatitis B	9/F	7 days				+		Recovery after steroids	Erguven et al. [68]
38	Hepatitis and rabies	40/M	NA				+		Improvement with steroids	van de Geijn et al. [62]
39	Hepatitis B and polio	NA	NA				+			Stewart et al. [156]
40	Lyssa	NA	10 days		+				Died 3 days later; demyelinating lesions in CNS and optic nerves	Varga et al. [157]
41	Measles	7/M	3 days	+	+				Complete resolution of lesions within 2 weeks	Shu et al. [158]
42	Measles	19/F	4 days			+			Recovery after pulses of steroids	Hamano et al. [159]
43	Measles	13/M	3 weeks				+			Stevenson et al. [65]
44	Measles	15/M	Few hours				+		Good improvement with steroids	Moradian and Ahmadieh [63]
45	Measles, rubella	16/M	Few hours				+			Arshi et al. [64]
46	Diphtheria, tetanus toxoid, whole cell pertussis	6/M months	6 days	+	+				Recovery without treatment	Aydin et al. [160]
47	Yellow fever	53/M	NA		+	+			Death	Kengsakul et al. [161]
48	Yellow fever	56/M	45 days			+		+	Symptoms improved 5 days later	Chaves et al. [162]
49	Yellow fever	NA		+					NA	Miravalle et al. [163]
50	Smallpox, typhoid, polio, anthrax doses 1–2	19/M	12 days, 17 days, 17 and 5 days	+					Full recovery with IVIG, steroids and vaccinia immunoglobulin	Van Dam et al. [164]
51	Smallpox	30/M	10 days	+	+				Neurologic sequelae still present 1 year after	Sejvar, et al. [165]
52	Tetanus	NA	NA	+					Recovery without steroids	Hamidon and Raymond [166]
53	Tetanus	28/F	15 days			+			Steroids; partial resolvement	Cisse et al. [167]
54	Rubella	31/F	5 days			+	+	+	Partial recovery with steroids	Kline et al. [168]
55	Rubella	14/M	16 days	+		+			Rapid improvement with steroids	Tsuru et al. [169]
56	Rubella	23/F	13 days				+			Stevenson et al. [65]
57	Rubella	17/M	Few hours				+		Recovery	Moradian and Ahmadieh [63]
58	Japanese encephalitis	15/M	3 weeks			+	+	+	Recovery after steroids	Furukawa et al. [13]
59	Diphtheria/tetanus/poliomyelitis	7/M	NA	+			+		Full recovery	Mancini et al. [170]
60	Anthrax	39/M	1 month				+		Excellent recovery	Kerrison et al. [66]
61	Anthrax	23/M	2 weeks				+		Improvement with immunosuppression	Kerrison et al. [66]
62	BCG	12/F	5 days				+		Recovery	Yen and Liu [79]
63	HPV	20/F	28 days after second immunization	+	+				Relapsed twice after steroids cessation, required immunosuppression	Wildemann et al. [171]
64	HPV	15/F	23 days after second immunization	+	+	+			Neurological recovery and resolution of lesions after steroid treatment	Schaffer et al. [172]
65	HPV	17/F	4 months after the 3rd dose			+	+	+	Partial improvement with steroids, PE and Rituximab. At 5 months: still paraparesis and ON.	Menge et al. [14]
66	HPV	14/F	4 months post-3rd dose			+	+	+	Steroids, Rituximab, mycophenolate; stopped recurrences	Menge et al. [14]
67	HPV	13/F	Post-2nd immunization, time unknown			+	+		No response to steroids and PE; Rituximab started	Menge et al. [14]
68	HPV	18/F	5 months after 2nd immunization			+	+		NA	Menge et al. [14]
69	HPV	16/F	10 days post-2nd immunization	+			+		No further demyelinating events within 18 months follow-up. Patient remained with severe visual loss	DiMario et al. [173]
70	HPV	16/F	21 days post-3rd immunization	+		+		+	NA	Sutton et al. [52]
71	HPV	25/F	16 days post-2nd immunization	+					4 months later, the patient suffered from a second event and MRI showed a new lesion (diagnosed then as CDMS)	Sutton et al. [52]

definite MS (CDMS) following Hepatitis B (HBV) immunization, whereas other investigators did not confirm this observation [99]. A recent extensive review from the US Institute of Medicine did not find sufficient evidence to support a causal relationship between the onset of MS and various common vaccinations (measles, mumps and rubella, influenza, Hepatitis A, Hepatitis B, human papilloma virus (HPV), diphtheria, tetanus, acellular pertussis, or meningococcus) [100]. Furthermore, pooled analyses found no evidence that vaccination against tuberculosis (BCG), or against Hepatitis B, influenza, measles, typhoid fever, diphtheria or tetanus, was associated with an increased risk of developing MS [101].

Vaccinations have been also linked to the occurrence of relapses of MS [50]. Deterioration or exacerbation of MS has been described in association with several vaccines [summarized by Loebermann et al. [51]] including lately human papilloma virus vaccination for protection against gynecological cancers [46,52]. In some studies, this increase was impressive; for instance a ten-fold increase of relapse risk and higher MRI activity were reported in the 3 months following vaccination for yellow fever [102].

However, Confavreux et al. [103] in a European database study that evaluated a total of 653 MS relapses showed that of all the patients with a relapse, only 2.3% had been vaccinated during the preceding two-month risk period as compared with 2.8 to 4.0% during the control periods. The relative risk of relapse associated with exposure to any vaccination during the previous two months was 0.71. In any case, the risk of a relapse following the infection itself seems to be much higher than the risk imposed in vaccination. De Keyser et al. reported an overall risk of relapse of 30% in patients suffering an influenza infection as compared to only 5% following vaccination against influenza [104].

3.4. Myelitis

Inflammatory diseases of the spinal cord are collectively described as myelitis. Myelitis can be either infectious or immune mediated (autoimmune). It can be classified according to the areas of the spinal cord affected as [105,106]:

i. *transverse myelitis* (affecting one or two segments and predominantly the white matter of the cord—it can involve the whole width of the cord or half of it),
ii. *long extensive myelitis* (*LETM*), which affects most of the width of the cord at more than three consequent segments
iii. *poliomyelitis*, that affects multiple areas of the gray matter of the cord (the anterior horns),
iv. *myeloradiculitis*, that affects both the white matter of the cord and the roots.

In terms of the causative factors, the most common type of myelitis worldwide is infectious myelitis, either caused by viruses (polio virus, HTLV-1, EBV, CMV, HSV), by bacteria (such as, *Borrelia burgdorferi* – Lyme's disease–, in Europe and North America and Brucella, especially in less developed areas of the world where milk is not pasteurized), by mycoplasma infection and by parasites such as schistosoma. During the last decade, HIV-associated myelitis has emerged, especially in the HIV-endemic areas in Africa.

Immune mediated myelitis propagated by autoimmune mechanisms attacking mainly the white matter of the spinal cord is usually presented as *acute transverse myelitis* (*TM*) [105,106], which may be idiopathic (isolated or as a part of ADEM/MS), post-infectious or post-vaccination. TM is characterized by inflammation and demyelination across both sides of one level, or segment, of the spinal cord resulting in symptoms of neurological disconnection and dysfunction below the level of the demyelinating area [106]. Myelitis can be also one of the hallmarks of neuromyelitis optica (NMO) (see next section).

As shown in Table 2, in 24 out of the 71 reported cases in our review, one of the major presentations of the CNS demyelinating syndrome was myelitis, including 7 cases that met the criteria of NMO-spectrum of diseases.

3.5. Neuromyelitis optica

Neuromyelitis optica (NMO), also known as Devic's disease, is an idiopathic, severe, demyelinating disease of the central nervous system that preferentially affects the optic nerve and spinal cord. NMO has long been thought to be a variant of multiple sclerosis (MS) but it can be clearly distinguished from MS by clinical, neuroradiological, and pathological criteria and the presence of the highly specific serum autoantibodies, against the water channel aquaporin-4 [107–109] in ~75% of NMO patients [107–112]. However, the exact role of antibodies against AQP4 in the pathogenesis of the disease is not clear.

Among all the reported cases with post-vaccination CNS demyelinating syndromes, there were 7 cases with NMO spectrum of diseases (Table 2). Interestingly, in most of these cases the vaccine involved was Gardasil against human papilloma virus, raising the possibility of cross-reactivity between the used viral antigens and aquaporin-4. As expected, the dominant presentation included optic neuritis and/or myelitis with longitudinal spinal lesions [14]. Interestingly, in most of these cases, there was a high incidence of recurrence (second event of demyelination/neurological signs few days up to months following the vaccination).

4. Discussion

Vaccines are one of the greatest achievements of modern medicine and are commonly and safely administered to humans worldwide. However, in rare occasions, vaccines can give rise to enigmatic inflammatory conditions [40,113] and even cause overt autoimmune diseases, by inducing the production of autoantibodies [113], or by breaking the mechanisms of self-tolerance. These rare events are usually documented within weeks following vaccination [40,114] (Table 2), making difficult if not impossible to delineate a causal relationship between vaccination and autoimmune disease. Nevertheless, for some vaccines such a causal link seems very logical. In 1976 an outbreak of Guillain–Barré syndrome (GBS) followed immunization with the “swine flu” vaccine [115,116]. Similar causal relationship has been shown in transverse myelitis (after oral polio vaccine), in arthritis (following diphtheria–tetanus–pertussis and measles–mumps–rubella vaccine) and in autoimmune thrombocytopenia (after measles–mumps–rubella vaccine) [113].

In addition, a number of animal models enabled a better way of studying the link between vaccines and autoimmunity. Immunization of dogs induced the production of 9 different autoantibodies including lupus-associated ones [117] and vaccination of diabetic prone newborn animals was associated with an increased occurrence of diabetes mellitus [118]. Intra-peritoneal immunization of salmon fish with vaccines embedded in oil-adjuvants also induced autoantibodies and the outbreak of granulomatous disease of the liver and peritoneum and immune mediated glomerulonephritis [119].

Specifically, regarding vaccination-induced autoimmunity of the central nervous system (CNS), application of unpurified rubies vaccine (which contained fragments of myelin with antigenic properties) [18] was shown to induce encephalomyelitis/ADEM, resembling the induction methods of experimental autoimmune encephalomyelitis (EAE) in animals, through immunization with myelin antigens in adjuvant [120–122].

A striking clinical example of post-vaccination CNS inflammatory disease is that of development of ADEM in Alzheimer patients, following administration of an experimental vaccine that contained aggregates of synthetic Aβ42 fragments of amyloid precursor protein [123,124]. In experimental animals, a similar EAE/ADEM disease was induced with Aβ42 vaccination, but only when the vaccine included complete Freund's adjuvant. The latter observation underlines the importance and central

role of adjuvants in induction of ADEM and of autoimmunity in general [125,126].

The pathogenic role of adjuvants in the induction of autoimmune syndromes has been highlighted by Yehuda Shoenfeld who introduced the term ASIA syndrome (autoimmune syndromes in association with adjuvants) [125,126]. In general, immunologic adjuvants are substances that enhance the antigen-specific immune responses [127] and are therefore commonly used in vaccines. Eventually, the efficacy of most vaccines depends on the presence of an adjuvant in conjunction with the infectious antigen [128].

The effects of the adjuvants are accomplished via several mechanisms that affect both the innate and the adaptive immune systems. Adjuvants mimic evolutionarily conserved molecules (e.g. bacterial cell walls, LPS, unmethylated CpG-DNA) and bind to Toll-like receptors (TLRs). They activate dendritic cells (DCs), lymphocytes and macrophages, increasing subsequently the release of chemokines and cytokines from T-helper and mast cells [9,129–131].

Currently the most widely used adjuvant in medicine is aluminium. Aluminium interferes with lysosomal functions and stimulates the production and secretion of cytokines such as IL-1b; IL-18 and IL-33 [128,131]. Adjuvants also provide physical protection to antigens, enabling thus a longer exposure of the immune system to the antigen, and a more robust (B-cell and T-cell) response. Adjuvants were thought to carry little risks, but several animal and human studies have demonstrated the ability of some of them to inflict an autoimmune process, such as in the case of Tetramethylpentadecane (TMPD—pristine), which was shown to induce a lupus-like disease in mice [132,133].

Adjuvants present in the vaccines can induce a non-specific activation of the immune system with a subsequent expansion of autoreactive (in our case, myelin specific) lymphocytes that may be further accelerated by defective regulatory cells/circuits, in genetically susceptible individuals.

Molecular mimicry i.e. the molecular similarity between the proteins of the viruses used for the vaccination and self antigens (for instance, CNS myelin components) also represents one of the main immunopathogenetic mechanisms in post-vaccination CNS demyelination [125–127].

In addition to the central role of adjuvants and molecular mimicry in the pathogenesis of post-vaccination systemic or CNS autoimmunity, other vaccine-related factors may also significantly contribute to the outbreak of an autoimmune response, including the type and dose of the infectious agent, the degree of its attenuation (live attenuated or dead) and the way of administration. It is theoretically possible that vaccines administered parenterally and not via the "natural" way of infection could carry a greater risk for induction of an autoimmune reaction, bypassing the control mechanisms of self tolerance.

In conclusion, the risk of a demyelinating CNS disease following vaccination, although non-negligible, is relatively low. From the existing data it seems that the risk of onset or relapse of CNS demyelination following infections against which the vaccines are aimed to protect is substantially higher.

Analysis of the existing data from epidemiological studies argues against a clear causal relationship between vaccines in general, and MS or other demyelinating diseases and advocates in favor of the benefits of vaccinations versus the potential risks of CNS inflammation/demyelination. This does not in any way exempt us from "learning" the lessons taught by the reported cases and searching new and safer ways to improve vaccination techniques and increase their safety profile.

Take-home messages

- Central nervous system (CNS) myelin can be often the target of an autoimmune process that follows various vaccinations and leads to a wide spectrum of immune mediated demyelinating syndromes.
- ADEM is the prototype (and most extensively described) white matter disease associated with vaccines. Other common syndromes include acute optic neuritis (the commonest post-vaccination isolated CNS syndrome reported in the literature) and acute transverse myelitis, but also the onset and/or exacerbation of a chronic disease such as multiple sclerosis (MS) or neuromyelitis optica (NMO) has been frequently reported, following various vaccinations.
- The most common vaccines that are reportedly associated with CNS demyelinating diseases in the literature are influenza vaccines (by far the highest number of reported cases) and human papilloma virus (HPV) vaccination. Other vaccines that have been associated with a spectrum of CNS demyelinating diseases include Hepatitis A or B, rabies, measles, rubella, yellow fever, anthrax, meningococcus and tetanus vaccines. The high number of reported post-vaccination CNS demyelinating syndromes related to influenza vaccination could be attributed to the high percentage of population that received the vaccine during the H1N1 epidemia from 2009 to 2012.
- There is no absolute way to definitely link the onset or exacerbation of demyelination with the vaccine, but the close temporal association with the time of vaccination strongly argues in favor of such pathogenetic correlation.
- Usually the CNS demyelinating syndrome appears shortly (during the 3–4 weeks following the vaccination) but there are reports of longer time intervals of up to 6 months.
- Immune adjuvants that are included in the vaccine preparations and aim to enhance the immune responses have been incriminated as one of the main mechanisms responsible for the immunopathogenesis of these syndromes ("ASIA" spectrum of diseases).
- Molecular mimicry i.e. the molecular similarity between the proteins of the viruses used for the vaccination and self antigens (CNS myelin components) represents the second main immunopathogenetic mechanisms in post-vaccination CNS demyelination.
- Other vaccine-related factors that may also significantly contribute to the outbreak of an autoimmune response include the type and dose of the infectious agent, the degree of its attenuation (live attenuated or dead) and the way of administration.
- Environmental and host genetic factors seem to play an important role.
- The overall risk of a demyelinating CNS disease following vaccination, although non-negligible, is relatively low (estimated to around 0.1%) and the risk of onset or relapse of CNS demyelination following infections against which the vaccines are aimed to protect is substantially higher.
- The existing epidemiological data indicate that the benefits of vaccinations clearly prevail over the potential risks of CNS inflammation/demyelination.
- This, of course, does not in any way exempt us from "learning" the lessons taught by the reported cases and searching new and safer ways to improve vaccination techniques and increase their safety profile.

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EXHIBIT 167

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Myasthenia Gravis Fact Sheet

Myasthenia Gravis Fact Sheet

What is myasthenia gravis?

Myasthenia gravis is a chronic autoimmune, neuromuscular disease that causes weakness in the skeletal muscles that worsens after periods of activity and improves after periods of rest. These muscles are responsible for functions involving breathing and moving parts of the body, including the arms and legs.

The name myasthenia gravis, which is Latin and Greek in origin, means "grave, or serious, muscle weakness." There is no known cure, but with current therapies, most cases of myasthenia gravis are not as "grave" as the name implies. Available treatments can control symptoms and often allow people to have a relatively high quality of life. Most individuals with the condition have a normal life expectancy.

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What are the symptoms of myasthenia gravis?

The hallmark of myasthenia gravis is muscle weakness that worsens after periods of activity and improves after periods of rest. Certain muscles such as those that control eye and eyelid movement, facial expression, chewing, talking, and swallowing are often (but not always) involved in the disorder.

The onset of the disorder may be sudden, and symptoms often are not immediately recognized as myasthenia gravis. The degree of muscle weakness involved in myasthenia gravis varies greatly among individuals.

People with myasthenia gravis may experience the following symptoms:

- weakness of the eye muscles (called ocular myasthenia)
- drooping of one or both eyelids (ptosis)
- blurred or double vision (diplopia)
- a change in facial expression
- difficulty swallowing
- shortness of breath
- impaired speech (dysarthria)
- weakness in the arms, hands, fingers, legs, and neck.

Sometimes the severe weakness of myasthenia gravis may cause respiratory failure, which requires immediate emergency medical care.

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What is a myasthenic crisis?

A myasthenic crisis is a medical emergency that occurs when the muscles that control breathing weaken to the point where individuals require a ventilator to help them breathe. It may be triggered by infection, stress, surgery, or an adverse reaction to medication. Approximately 15 to 20 percent of people with myasthenia gravis experience at least one myasthenic crisis. However, up to one-half of people may have no obvious cause for their myasthenic crisis. Certain medications have been shown to cause myasthenia gravis. However, sometimes these medications may still be used if it is more important to treat an underlying condition.

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What causes myasthenia gravis?

Antibodies

Myasthenia gravis is an autoimmune disease, which means the immune system—which normally protects the body from foreign organisms—mistakenly attacks itself.

Myasthenia gravis is caused by an error in the transmission of nerve impulses to muscles. It occurs when normal communication between the nerve and muscle is interrupted at the neuromuscular junction—the place where nerve cells connect with the muscles they control.

Neurotransmitters are chemicals that neurons, or brain cells, use to communicate information. Normally when electrical signals or impulses travel down a motor nerve, the nerve endings release a neurotransmitter called acetylcholine that binds to sites called acetylcholine receptors on the muscle. The binding of acetylcholine to its receptor activates the muscle and causes a muscle contraction.

In myasthenia gravis, antibodies (immune proteins produced by the body's immune system) block, alter, or destroy the receptors for acetylcholine at the neuromuscular junction, which prevents the muscle from contracting. This is most often caused by antibodies to the acetylcholine receptor itself, but antibodies to other proteins, such as MuSK (Muscle-Specific Kinase) protein, also can impair transmission at the neuromuscular junction.

The thymus gland

The thymus gland controls immune function and may be associated with myasthenia gravis. It grows gradually until puberty, and then gets smaller and is replaced by fat. Throughout childhood, the thymus plays an important role in the development of the immune system because it is responsible for producing T-lymphocytes or T cells, a specific type of white blood cell that protects the body from viruses and infections.

In many adults with myasthenia gravis, the thymus gland remains large. People with the disease typically have clusters of immune cells in their thymus gland and may develop thymomas (tumors of the thymus gland). Thymomas are most often harmless, but they can become cancerous. Scientists believe the thymus gland may give incorrect instructions to developing immune cells, ultimately causing the immune system to attack its own cells and tissues and produce acetylcholine receptor antibodies—setting the stage for the attack on neuromuscular transmission.

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Who gets myasthenia gravis?

Myasthenia gravis affects both men and women and occurs across all racial and ethnic groups. It most commonly impacts young adult women (under 40) and older men (over 60), but it can occur at any age, including childhood. Myasthenia gravis is not inherited nor is it contagious. Occasionally, the disease may occur in more than one member of the same family..

Although myasthenia gravis is rarely seen in infants, the fetus may acquire antibodies from a mother affected with myasthenia gravis—a condition called neonatal myasthenia. Neonatal myasthenia gravis is generally temporary, and the child's symptoms usually disappear within two to three months after birth. Rarely, children of a healthy mother may develop congenital myasthenia. This is not an autoimmune disorder but is caused by defective genes that produce abnormal proteins in the neuromuscular junction and can cause similar symptoms to myasthenia gravis.

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How is myasthenia gravis diagnosed?

A doctor may perform or order several tests to confirm the diagnosis of myasthenia gravis:

- **A physical and neurological examination**. A physician will first review an individual's medical history and conduct a physical examination. In a neurological examination, the physician will check muscle strength and tone, coordination, sense of touch, and look for impairment of eye movements.
- **An edrophonium test**. This test uses injections of edrophonium chloride to briefly relieve weakness in people with myasthenia gravis. The drug blocks the breakdown of acetylcholine and temporarily increases the levels of acetylcholine at the neuromuscular junction. It is usually used to test ocular muscle weakness.
- **A blood test**. Most individuals with myasthenia gravis have abnormally elevated levels of acetylcholine receptor antibodies. A second antibody—called the anti-MuSK antibody—has been found in about half of individuals with myasthenia gravis who do not have acetylcholine receptor antibodies. A blood test can also detect this antibody. However, in some individuals with myasthenia gravis, neither of these antibodies is present. These individuals are said to have seronegative (negative antibody) myasthenia.
- **Electrodiagnostics**. Diagnostic tests include repetitive nerve stimulation, which repeatedly stimulates a

person's nerves with small pulses of electricity to tire specific muscles. Muscle fibers in myasthenia gravis, as well as other neuromuscular disorders, do not respond as well to repeated electrical stimulation compared to muscles from normal individuals. Single fiber electromyography (EMG), considered the most sensitive test for myasthenia gravis, detects impaired nerve-to-muscle transmission. EMG can be very helpful in diagnosing mild cases of myasthenia gravis when other tests fail to demonstrate abnormalities.

- **Diagnostic imaging**. Diagnostic imaging of the chest using computed tomography (CT) or magnetic resonance imaging (MRI) may identify the presence of a thymoma.
- **Pulmonary function testing**. Measuring breathing strength can help predict if respiration may fail and lead to a myasthenic crisis.

Because weakness is a common symptom of many other disorders, the diagnosis of myasthenia gravis is often missed or delayed (sometimes up to two years) in people who experience mild weakness or in those individuals whose weakness is restricted to only a few muscles.

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How is myasthenia gravis treated?

Today, myasthenia gravis can generally be controlled. There are several therapies available to help reduce and improve muscle weakness.

- **Thymectomy.** This operation to remove the thymus gland (which often is abnormal in individuals with myasthenia gravis) can reduce symptoms and may cure some people, possibly by rebalancing the immune system. A NINDS-funded study found that thymectomy is helpful both for people with thymoma and those with no evidence of the tumors. The clinical trial followed 126 people with myasthenia gravis and no visible thymoma and found that the surgery reduced muscle weakness and the need for immunosuppressive drugs.
- **Monoclonal antibody.** This treatment targets the process by which acetylcholine antibodies injure the neuromuscular junction. In 2017, the U.S. Food and Drug Administration approved the use of eculizumab for the treatment of generalized myasthenia gravis in adults who test positive for the antiacetylcholine receptor (AchR) antibody.
- **Anticholinesterase medications.** Medications to treat the disorder include anticholinesterase agents such as mestinon or pyridostigmine, which slow the breakdown of acetylcholine at the neuromuscular junction and thereby improve neuromuscular transmission and increase muscle strength.
- **Immunosuppressive drugs.** These drugs improve muscle strength by suppressing the production of abnormal antibodies. They include prednisone, azathioprine, mycophenolate mofetil, and tacrolimus. The drugs can cause significant side effects and must be carefully monitored by a physician.
- **Plasmapheresis and intravenous immunoglobulin**. These therapies may be options in severe cases of myasthenia gravis. Individuals can have antibodies in their plasma (a liquid component in blood) that attack the neuromuscular junction. These treatments remove the destructive antibodies, although their effectiveness usually only lasts for a few weeks to months.
  - **Plasmapheresis** is a procedure using a machine to remove harmful antibodies in plasma and replace them with good plasma or a plasma substitute.
  - **Intravenous immunoglobulin** is a highly concentrated injection of antibodies pooled from many healthy donors that temporarily changes the way the immune system operates. It works by binding to the antibodies that cause myasthenia gravis and removing them from circulation.

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What is the prognosis?

With treatment, most individuals with myasthenia can significantly improve their muscle weakness and lead normal or nearly normal lives.

Some cases of myasthenia gravis may go into remission—either temporarily or permanently— and muscle weakness may disappear completely so that medications can be discontinued. Stable, long-lasting complete remissions are the goal of thymectomy and may occur in about 50 percent of individuals who undergo this procedure.

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What research is being done?

The mission of the National Institute of Neurological Disorders and Stroke (NINDS) is to seek fundamental knowledge about the brain and nervous system and to use that knowledge to reduce the burden of neurological disease. The NINDS is a component of the National Institutes of Health (NIH), the leading supporter of biomedical

research in the world.

Although there is no cure for myasthenia gravis, management of the disorder has improved over the past 30 years. There is a greater understanding about the causes, structure and function of the neuromuscular junction, the fundamental aspects of the thymus gland and of autoimmunity. Technological advances have led to more timely and accurate diagnosis of myasthenia gravis and new and enhanced therapies have improved treatment options. Researchers are working to develop better medications, identify new ways to diagnose and treat individuals, and improve treatment options.

Medication

Some people with myasthenia gravis do not respond favorably to available treatment options, which usually include long-term suppression of the immune system. New drugs are being tested, either alone or in combination with existing drug therapies, to see if they are more effective in targeting the causes of the disease.

Diagnostics and biomarkers

In addition to developing new medications, researchers are trying to find better ways to diagnose and treat this disorder. For example, NINDS-funded researchers are exploring the assembly and function of connections between nerves and muscle fibers to understand the fundamental processes in neuromuscular development. This research could reveal new therapies for neuromuscular diseases like myasthenia gravis.

Researchers are also exploring better ways to treat myasthenia gravis by developing new tools to diagnose people with undetectable antibodies and identify potential biomarkers (signs that can help diagnose or measure the progression of a disease) to predict an individual's response to immunosuppressive drugs.

New treatment options
Findings from a recent NINDS-supported study yielded conclusive evidence about the benefits of surgery for individuals without thymoma, a subject that had been debated for decades. Researchers hope that this trial will become a model for rigorously testing other treatment options, and that other studies will continue to examine different therapies to see if they are superior to standard care options.

Assistive technologies, such as magnetic devices, may also help people with myasthenia gravis to control some symptoms of the disorder.

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Where can I get more information?

For more information on neurological disorders or research programs funded by the National Institute of Neurological Disorders and Stroke, contact the Institute's Brain Resources and Information Network (BRAIN) at:

BRAIN
P.O. Box 5801
Bethesda, MD 20824
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More information about research on myasthenia gravis supported by NINDS and other NIH Institutes and Centers can be found using NIH RePORTER (*projectreporter.nih.gov*), a searchable database of current and past research projects supported by NIH and other federal agencies. RePORTER also includes links to publications and resources from these projects.

Information is also available from the following organizations:

Myasthenia Gravis Foundation of America, Inc.
355 Lexington Avenue, 15th Floor
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www.myasthenia.org

American Autoimmune Related Diseases Association
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CASE REPORT Open Access

Myasthenia gravis following human papillomavirus vaccination: a case report

Ji Yeon Chung[1†], Seung Jae Lee[2†], Byoung-Soo Shin[3,4] and Hyun Goo Kang[3,4*]

Abstract

Background: Myasthenia gravis (MG), an autoimmune neuromuscular disorder, occurs owing to autoantibodies against acetylcholine receptors. MG symptoms can be triggered by various vaccines. Many studies have evaluated the safety and adverse events of the human papillomavirus (HPV) vaccine. Here, we present a life-threatening case of ocular and bulbar MG symptoms after HPV vaccination and a brief literature review.

Case presentation: A 23-year-old woman presented with binocular diplopia, ptosis, dysarthria, and dysphagia, which occurred on the 3rd day after the second HPV vaccine administration. She was diagnosed with MG based on history, clinical features, and test results. Her symptoms deteriorated on the 3rd day after admission, and she was transferred to the intensive care unit with mechanical ventilation. On the 7th day after admission, due to discomfort in the right chest, pulmonary embolism was suspected. A tracheostomy was performed on the 14th day of mechanical ventilation. In the 4th week, the tracheostomy tube was removed; all symptoms had completely resolved at discharge. She was followed up for 5 months without recurrence or further treatment.

Conclusion: HPV vaccination may cause MG owing to unexpected abnormal autoimmune responses. Additional studies are needed to clarify the possible causal relationship between the HPV vaccine and neurological complications and to evaluate the safety of the vaccine.

Keywords: Adverse event, Human papillomavirus vaccine, Myasthenia gravis, Myasthenia gravis crisis, Vaccination

Background

Myasthenia gravis (MG) is an autoimmune disease that causes a neuromuscular junction disorder owing to blockage of the nicotinic acetylcholine receptor (AChR). MG may be associated with autoimmune reactions, such as autoantibodies and autoimmune responses against AChR [1]. Furthermore, MG is related to thymus disorders and other autoimmune diseases [2]. The human papillomavirus (HPV) vaccination was developed to prevent cervical cancer and is recommended for female individuals aged 9–26 years. In South Korea, bi- and quad-rivalent vaccines have been used; however, since 2016, the nine-valent vaccine has been used, with no serious adverse effects reported to date. Here, we present a case of MG after HPV nine-valent vaccination in a patient whose condition rapidly progressed to MG.

Case presentation

A 23-year-old woman with binocular vertical diplopia, bilateral ptosis (which worsened with left and down gazing), dysarthria, and dysphagia visited the outpatient department. She had received a primary HPV nine-valent vaccination 2 months prior and a second vaccination 5 days before the visit. The symptoms occurred on the 3rd day after the second vaccination. The muscular strength of her upper and lower extremities was normal, and the deep tendon reflex of both sides was normal. Her ptosis and diplopia temporarily improved with an ice pack and pyridostigmine test. The repetitive nerve stimulation (RNS) did not reveal a significant decrement in deltoid, abductor digiti minimi, flexor carpi, and orbicularis oculi muscles. The serum AChR antibody titer was 1.66 nmol/L. Other autoimmune disease tests, including rheumatoid factor and antinuclear antibody, were negative. A thyroid

* Correspondence: hgkang@jbnu.ac.kr
†Ji Yeon Chung and Seung Jae Lee contributed equally to this work.
[3]Department of Neurology, Chonbuk National University School of Medicine, Jeonju 54896, Republic of Korea
[4]Department of Neurology & Research Institute of Clinical Medicine of Chonbuk National University - Biomedical Research Institute of Chonbuk National University Hospital, 20 Geonji-ro, Deokjin-gu, Jeonju-si, Jeonbuk-do 54907, South Korea
Full list of author information is available at the end of the article

function test was normal, and no thymus abnormality was observed on chest computed tomography (CT).

She was diagnosed with MG, and pyridostigmine oral administration and high-dose intravenous steroid therapy were initiated. Her dyspnea became more severe on the 2nd day after admission, and oxygen saturation decreased; therefore, she received intravenous immunoglobulin therapy. Afterward, spontaneous breathing became more difficult, and the dysphagia and bilateral ptosis worsened. These symptoms were determined to demonstrate an MG crisis, and mechanical ventilation was initiated after endotracheal intubation (Fig. 1). Although muscle strength was normal at admission, the extension power of the distal fingers decreased.

On the 7th day after admission, sudden tachycardia was observed, the patient experienced persistent pressure in the right chest, and oxygen saturation decreased during mechanical ventilation. Chest CT revealed a low-density-filling defect of the pulmonary artery in the right lower lobe. Ischemic changes in the lung parenchyma and an increased D-dimer level (2199 ng/mL) were also observed. Therefore, heparin treatment was initiated owing to the possibility of pulmonary embolism. The D-dimer level decreased to within the normal range (99 ng/mL) in the 2nd week after the increase.

In the 4th week of hospitalization, the symptoms further improved such that she could walk and the AChR antibody titer decreased (0.99 nmol/L). However, right eye ptosis and binocular vertical diplopia persisted. She was discharged after the dysphagia had completely resolved. The AchR antibody titer was normal (0.05 nmol/L) at discharge. The patient has returned to daily life without symptom recurrence or further treatment.

Discussion

The HPV vaccination was first approved in 2006 for preventing cervical cancer. However, in Japan, serious adverse events, such as Guillain-Barré syndrome, acute disseminated encephalomyelitis, postural orthostatic tachycardia syndrome, and complex regional pain syndrome have been reported in patients receiving the HPV vaccination, which were suspected to be associated with the HPV vaccination [3]. The causal relationship between these adverse events and HPV vaccination has not yet been elucidated, and the underlying pathogenesis remains unclear. Studies in Japan have hypothesized that an antibody that cross-reacts with autonomic ganglia, neurons, and cardiac proteins or ß1/2-adrenergic and M2/3 muscarinic receptors could be synthesized owing to the epitope of the HPV vaccination [4], and that cytotoxic T cells could be

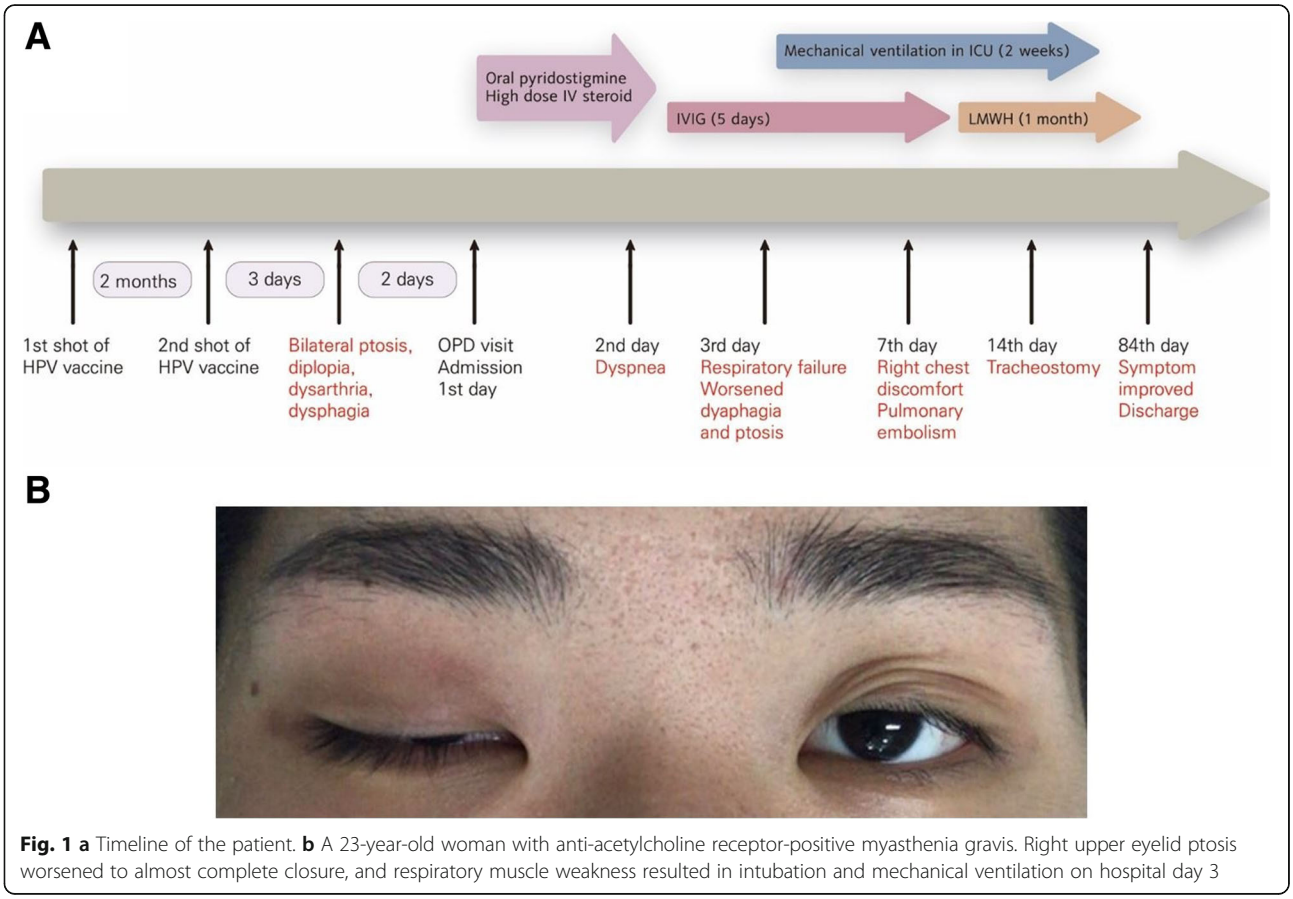

Fig. 1 **a** Timeline of the patient. **b** A 23-year-old woman with anti-acetylcholine receptor-positive myasthenia gravis. Right upper eyelid ptosis worsened to almost complete closure, and respiratory muscle weakness resulted in intubation and mechanical ventilation on hospital day 3

Table 1 Case reports of new-onset myasthenia gravis after vaccination

Author	Age/Sex	Vaccine Type	Time to onset	Initial symptoms	Treatment	Prognosis (Time to recovery)	Thymoma	MGFA Class
Biron [9]	48/M	HBV	1 mo after 2nd shot	Ocular	Edrophonium,, plasma exchange, cyclophosphamide, steroid	Improved. (After 30 PE)	+	I
Bahri [10]	46/F	HBV	1mo after 2nd shot	Ocular, bulbar	Pyridostigmine, steroid	Improved (Not mentioned)	+	IIb
Takizawa [8]	69/M	BCG	6 wks	Ocular	Pyridostigmine	Improved (70 days)	–	IIa
Our case	23/F	HPV	3 d after 2nd shot	Ocular, bulbar	Pyridostigmine, steroid, IVIG	Improved (84 days)	–	V

Mo months, *d* days, *HBV* hepatitis B vaccine, *BCG* Bacillus Calmette-Guerin, *HPV* human papillomavirus, *IVIG* intravenous immunoglobulin, *MGFA* myasthenia gravis foundation of America clinical classification

activated by stimulating the antibody production by binding to acetylcholine receptors [5]. A recent study reported a complex involving HPV and p53 pro-apoptotic tumor suppressor, and the inhibitory enzyme is degraded upon complex formation [6]. Viral oncoprotein E6 can recognize a short leucine-rich consensus sequence within ligase E6AP, and this complex finally degrades p53. The E6 domain has two zinc ions, which maintain structural features for the interactions. Non-specific interactions of HPV with AChR may result in the complex formation and unexpected side effects of HPV, which need to be investigated.

Our patient had no problems with the primary HPV vaccination but exhibited acute bilateral ptosis, dysarthria, and dysphagia on the 3rd day after the second vaccination. She also experienced acute respiratory failure and pulmonary embolism. In our case, blood stasis owing to immobilization while in the intensive care unit could be a risk factor for pulmonary embolism. However, because venous thromboembolism was reported as an adverse event of the HPV vaccination [7], pulmonary embolism could occur owing to the HPV vaccination.

In our patient, MG may have been induced by the HPV vaccination as an adverse event or incidentally without an association between the two factors. Therefore, it may be difficult to suggest a strong relationship between HPV vaccination and MG outbreak. However, previous studies have reported MG occurrence after inoculation with other vaccines [8–10], suggesting an association between symptoms and changes in immune responses in the body following vaccination. This study summarized the studies that reported the first occurrence of MG after vaccinations based on the available literature (Table 1). Additionally, there is no absolute contraindication to the use of the HPV vaccination. Our patient developed MG after receiving the nine-valent vaccine, and the relative risk of the nine-valent vaccine is unclear.

This case report implies that the HPV vaccination may cause MG. Other neurological manifestations may occur owing to unexpected abnormal autoimmune responses such as autonomic dysfunction and pain. It is important to inform patients prior to inoculation and observe the occurrence of abnormal symptoms. Moreover, it is critical to intervene promptly and treat the patient when fatal deterioration is observed. We believe that additional studies are needed to assess the possible causal relationship between the HPV vaccine and neurological complications and to evaluate the safety of the vaccine.

Abbreviations
AChR: Acetylcholine receptor; CT: Computed tomography; HPV: Human papillomavirus; MG: Myasthenia gravis

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Availability of data and materials
All data and material supporting our findings are contained within the manuscript.

Authors' contributions
JYC and HGK participated the design of this research. JYC, SJL, BSS and HGK collected and analyzed the raw clinical data. BSS, SJL, and HGK carried out computational studies and wrote the manuscript. JYC and SJL contributed equally to this work as co-first authors. All authors have read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Written informed consent was obtained from the patient for publication of this case report and any accompanying images. A copy of the written consent is available for review by the editor of this journal.

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Author details

[1]Department of Neurology, Chosun University School of Medicine, Gwangju 61453, Republic of Korea. [2]Institute for Molecular Biology and Genetics and Department of Chemistry, Chonbuk National University, Jeonju 54896, Republic of Korea. [3]Department of Neurology, Chonbuk National University School of Medicine, Jeonju 54896, Republic of Korea. [4]Department of Neurology & Research Institute of Clinical Medicine of Chonbuk National University - Biomedical Research Institute of Chonbuk National University Hospital, 20 Geonji-ro, Deokjin-gu, Jeonju-si, Jeonbuk-do 54907, South Korea.

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Review

Neuromuscular disorders associated with Hepatitis B vaccination

Joerg-Patrick Stübgen *

Department of Neurology and Neuroscience, Weill Cornell Medical College/New York Presbyterian Hospital, 525 East 68th Street, New York, NY 10065-4885, USA

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ABSTRACT

The hepatitis B virus (HBV) is an important infectious cause of acute and chronic liver disease throughout the world. Recombinant hepatitis B vaccines have been developed to combat morbidity and mortality associated with HBV infection. These vaccines have been associated with autoimmune diseases mostly among adult vaccine recipients. Epidemiological surveys have not established unequivocal causality between the hepatitis B vaccine and the development of various autoimmune neuromuscular disorders. However, case histories and series hint at a temporal association between hepatitis B vaccines and the development of various neuropathy syndromes, polyarteritis nodosa complicated by vasculitic neuropathy, myasthenia gravis and dermatomyositis. Conceivably, the hepatitis B vaccines have a potential to occasionally trigger the onset of immune diseases in individuals with an underlying genetic or immunological susceptibility.

Contents

1. Introduction

The hepatitis B virus (HBV), a small DNA virus of the *Hepadnaviridae* family, is one of the most important infectious causes of acute and chronic liver disease throughout the world [1]. Before the introduction of universal childhood vaccination in the US, about 300,000 people in the US acquired new HBV infection every year [2]. Up to 30,000 people became HBV carriers, adding to the population at risk for chronic liver disease, such as chronic active hepatitis, cirrhosis and hepatocellular carcinoma. In the US, 97% of reported disease cases and about 75% of new HBV carriers result from infection of adults [3]. HBV is transmitted by percutaneous or permucosal exposure to infected blood via sexual contact, contaminated needles or blood products [4].

In an attempt to combat the potential life-threatening effects of HBV, recombinant hepatitis B (HB) vaccines have been developed. The medical and scientific communities consider the recombinant HB vaccines safe, but components of the vaccines (yeast, aluminum, thimerosal and hepatitis B surface antigen epitopes) have infrequently been associated with the development of a variety of autoimmune diseases usually among susceptible adult vaccine recipients [5]. In the US, licensed HB vaccines are recommended for use in the childhood vaccination schedule and among the high-risk adult population to be given as a 3-dose series [1]. HB vaccine is the second most frequently dispensed vaccine in the US, and there seems little doubt that the benefits of administration overall far outweigh its risks [5,6].

The purpose of this article is to review in a single text the occurrence and significance of various presumed immune-mediated neuromuscular disorders associated with the administration of HB vaccines. A Medline search was conducted of all relevant publications from 1966 through December 2009 with specific emphasis on search

* Tel.: +1 212 746 2334; fax: +1 212 746 8742.
E-mail address: pstuebge@med.cornell.edu.

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Exhibit 169

of epidemiological studies, but also case reports and series, that reported on neuropathy, myopathy or neuromuscular junction disorders following HB vaccination. All relevant publications were retrieved and critically analyzed.

2. Hepatitis B vaccination and autoimmunity

The pathogenesis of autoimmune phenomena after vaccinations may be similar to the mechanism following natural infections [7]. Several mechanisms have been proposed to explain how infectious agents may induce autoimmune diseases: (a) molecular mimicry (implies a structural homology between an infectious and host antigenic components); (b) epitope spreading (or the appearance of a new antibody or the T cell response, to different epitopes on the same or on another antigen); (c) direct bystander activation (a mechanism whereby priming of microbial antigen-specific T cell takes place under the immune milieu); (d) release of cryptic epitopes; (e) reactivation of memory T cells; (f) superantigenic T cell activation (infectious products may cross-link the T cell receptor and MHC molecule independent of specific antigen recognition), and (g) priming of reactive T cells (infectious agents may cause tissue injury, thereby releasing autoantigens, which are processed and presented by antigen-presenting cells, which leads to priming of autoreactive T cells [5,8–10]. Similar mechanisms may play a role when an antigen of a recombinant vaccine or a live attenuated virus triggers an autoimmune reaction. The exact mechanism involved likely depends on the presumed pathogenesis of any particular immune disorder. Factors that may account for the development of autoimmunity after vaccination include: (a) patient genetic predisposition for autoimmunity; (b) complex vaccines with an array of varied antigens, and (c) the presence of an adjuvant, which potently enhances the immune system [5,11].

The autoimmune response to vaccine particles may be enhanced by the presence of adjuvants, which are potent stimulators of the immune system [12]. Rarely, adjuvants can provoke autoimmune phenomena without an accompanying antigen [13]. Various adjuvants likely exert their immune-enhancing effects via any one or more mechanisms: (a) translocation of antigens to lymph nodes so that antigens can be recognized by, and stimulate, T cells; (b) protection of antigens so that prolonged delivery and exposure to the immune system up-regulates the production of B and T cells; (c) increase in local injection site reaction to facilitate release of stimulatory chemokines by T cells and mast cells; (d) induction of the release of inflammatory cytokines thereby recruiting B and T cells at sites of infection and increasing translational events, and (e) interaction with pattern recognition receptors on leukocyte (accessory cell) membranes to increase the innate immune response to antigen [14–16].

The recombinant HB vaccines are highly purified, genetically engineered, single antigen vaccines [5]. The Center for Disease Control and Prevention (CDC) states that there is no confirmed scientific evidence that HB vaccine causes chronic illness, including multiple sclerosis, chronic fatigue syndrome, rheumatoid arthritis or other autoimmune diseases. Several independent review committees such as that of the US Institute of Medicine (IOM) and the World health Organization's (WHO) Global Advisory committee on Vaccine Safety (GACVS) found no evidence of an unequivocal link between the HB vaccine and chronic illness, so that vaccination is considered safe [17]. However, there exist long lists of diverse autoimmune phenomena that developed in patients vaccinated against HBV; the relatively small number of reports is notable compared to the many millions of HB vaccinations administered [18]. No certain causality has been proven between the HB vaccine and autoimmunity [19], but evidence in the form of a case reports, epidemiological statistics and biological mechanisms hint at an association between serious acute and chronic adverse reactions and HB vaccination [5].

Conceivably, HB vaccine rarely exacerbates or triggers the onset of immune disease presumably in individuals with an underlying genetic and immunological susceptibility [20].

3. Definition of a drug-induced illness

Miller et al. proposed a framework to evaluate case reports as the first stage in identifying environmentally associated rheumatic disorders [21]. An illness (e.g. neuropathy or myopathy) may be considered drug (read vaccine)-related when at least 4 of 8 attribution elements have been satisfied: (1) temporal association; (2) lack of likely alternative explanations; (3) stabilization/improvement of the disorder by interrupting exposure to presumed inciting agent; (4) re-challenge; (5) biologic plausibility; (6) analogy; (7) dose responsiveness, and (8) specificity, i.e. the manifestation of an illness is similar to previous cases exposed to the same drug or agent.

4. Hepatitis B vaccination and neuropathy

Various peripheral nerve syndromes have been described in association with the HB vaccine. There exist isolated literature reports on an association between recombinant [22–24] or plasma-derived [25–29] HB vaccination and GBS. Patients had received 1 to 4 vaccine injections; symptoms of neuropathy started between 1 day and 8 weeks after administration. Other case reports include sensory neuropathy involving also the mental nerve [30], vestibulocochlear neuropathy [31], and precipitation of hereditary motor-sensory neuropathy [32]. A cause-effect relationship between these neuropathy syndromes and immunization was not absolutely demonstrated in any patient. Limited support for HB vaccine-related neuropathies was based only on the temporal sequence of events and biological plausibility. Moreover, there exist no pathological studies that prove a direct link between the vaccine antigen and neuropathies.

A post-marketing surveillance (1982–1985) for neurological events after plasma-derived HB vaccination included case reports of Bell's palsy (10 cases), 9 patients with Guillain-Barré syndrome (GBS), lumbar radiculopathy (5 patients), brachial plexopathy (3 cases) [33]. Half the cases occurred after the first of 3 required vaccine doses. Calculations of the relative risks of these illnesses after vaccination was highly dependent on diagnostic classification of the cases, estimates of the size of the vaccinated population, background incidence of the diseases, and the length and distribution of the hypothetical at risk interval used in the analysis. However, based on this retrospective epidemiological review, no conclusive association was proved between the HB vaccine and any neurological adverse events.

A comprehensive search (Vaccine Adverse Event Reporting System [VAERS] and published literature [PubMed from 1966–2003]) examined adverse events and positive re-challenge of symptoms following HB vaccination [34]. VAERS, a cooperative program instituted by the Center for Disease Control and Prevention and the US Food and Drug Association, collects information about adverse events that occur after the administration of US-licensed vaccines. HB vaccine was associated with a number of serious conditions and positive re-challenge or significant exacerbation of symptoms following immunization: 101 cases of GBS were documented. Based on evidence (biological plausibility, case reports or series, epidemiological data, positive re-challenge and exacerbation of symptoms, and events in identical twins), the authors felt obligated to at least consider a causal relationship between the HB vaccine and serious autoimmune disorders among certain susceptible vaccine recipients in a defined temporal period following immunization.

A recent review assessed the rate and characteristics of GBS after the administration of various vaccines in the United States by reviewing data from the VAERS for period 1990 to 2005 [35]. After the influenza vaccine the HB vaccine was most frequently linked to post-vaccination GBS (94 cases). The incidence of HB vaccine-related

GBS was calculated at about 2.4 cases per million vaccinations, a figure that is substantially lower than the incidence of GBS in the general population (about 2 cases per 100 000 per year) [36]. GBS developed within 6 weeks (59%) or after 6 weeks (15%) after vaccination, or was unreported (27%); the peak time of GBS onset was 2 weeks after vaccination. Disability (22%) and mortality (1%) rates after HB vaccination were comparable to those reported for GBS in the general population. The majority of cases (80%) occurred in patients under 65 years old, and likely reflected vaccination campaigns amongst children and young adults. Any possibility of an association between GBS and the HB vaccine was based on the temporal sequence of events because the probability of observing this degree of imbalance (i.e., events within 6 weeks of injection) by chance alone was considered to be low [37,38]. However, these analyzed data suffered from the inherent limitations of a passive surveillance methods such as VAERS, e.g. underreporting, differential reporting, ascertainment bias, and variability in report quality and completeness [37,39]. It is important to recognize that the data reported to VAERS consist of a series of single case reports, and that without a case cohort control group, any meaningful conclusions are near impossible. Therefore, prospective controlled studies are needed to clarify any association between GBS and HB vaccines.

5. Hepatitis B vaccination and polyarteritis nodosa

Multiple case reports hint at a temporal association between the HB vaccine and PAN [40–46], as well as leukocytoclastic/hypersensitivity vasculitis [47–51]. Post-vaccination PAN also affected children [41,43,46]. PAN occurred after the first injection [40], as well as after booster doses years after initial vaccination [42]. The interval to onset of symptoms varied from 1 week [46] to two months [41]. PAN manifestations were mostly cutaneous. The peripheral nervous system was affected in 2 additional patients characterized as an axonal peripheral polyneuropathy [41], and a delayed-onset mononeuropathy simplex [44]. Patients responded favorably to various immunomodulatory drugs, including hydroxychloroquine and colchicine [40], azathioprine [41,46], cyclophosphamide [41,45], and/or oral steroids.

The immune mechanisms of any proposed association between the HB vaccine and PAN have not been established. In immunohistochemical studies, no virus B antigen could be detected in the vessel walls [40]. Perhaps the HBsAg behaved like a classical heterologous protein that induced the formation and vascular deposition of circulating immune complexes, thereby activating the complement system and initiating disease [52]. As the complete HBV is a recognized trigger of PAN [53,54], induction of this complication by immunization with specific HB-related antigens may not be totally unexpected. Admittedly, case series analyses are not appropriate for hypothesis testing.

A retrospective study investigated the hypothesis that HB vaccination can rarely cause PAN [55]. From 1990 through 2001, the VAERS received 25 reports of PAN following HB vaccination. When a strict causal inference framework was applied to these adverse events [21], only 10 patients met case definition criteria of HB vaccine-associated PAN. The modal peak time to symptom onset after vaccination was 2 weeks. Only a single patient suffered “disseminated neuritis” manifesting as focal weakness and paresthesiae. Many reports were marred by lack of documentation to conclusively rule out established provoking infections, and there was evidence of reporting bias. While the authors identified some supportive evidence, overall, the existing adverse event reports and a review of the published literature did not support a causal link between the HB vaccine and PAN. To help establish a relation of cause and effect, appropriate prospective evaluation of future post-vaccination PAN cases should entail rigorous confirmation of diagnosis, appropriate testing for possible provoking infections, and attempts to relate vaccine antigen to vessel pathology.

6. Hepatitis B vaccination and myasthenia gravis

The onset or exacerbation of myasthenia gravis (MG) was very rarely associated with the HB vaccine [18]. Three atopic patients suffering asthma developed manifestations of MG 1 to 4 weeks after HB vaccination [56–58]. Both plasma-derived and recombinant HB vaccines were invoked. Anti-acetylcholine receptor (AChR) antibodies were positive [57], negative [58], or not measured [56]. Thymus pathology [56,58] and computerized tomography imaging study [57] were normal. Stabilization of MG entailed plasma exchanges [56], and immunosuppressive drugs (cyclophosphamide, cyclosporine and/or oral steroids). One patient developed a myasthenic crisis 4 years after onset, and required ventilator care and IVIG [58]. Any association appeared temporal only; a molecular mimicry relationship seemed unlikely because the HBsAg is structurally unrelated to the nicotinic AChR [56]. Conceivably, vaccine provoked symptomatic disease in previously unrecognized, asymptomatic MG. Perhaps a background history of atopic illness increased patient risk to develop vaccine-related autoimmunity.

A fourth patient with stabilized, general MG (post-thymectomy) worsened dramatically in the month following the second injection of HB vaccine [57]. Delayed treatment with IVIG and oral steroids resulted only in partial improvement of strength. From this single report it appears that no special precautions are necessary when offering the HB vaccine to patients with MG.

7. Hepatitis B vaccination and myopathy

Isolated case histories report on a temporal association between HB vaccination and the development of juvenile DM [8,59,60]. Patients ranged in age from 6 to 17 years. Weakness developed after the first dose of vaccine, and recurred in 1 patient after a second dose [60]. The interval between injection and onset of symptoms ranged between 1 and 3 weeks. Muscle biopsy confirmed the inflammatory nature of muscle disease (endomysial or perimysial perivascular inflammatory infiltrates with/without muscle fiber necrosis) in 2 patients [8,59]. Weakness resolved spontaneously in 1 month [56], or after oral steroid treatment [59], or was not reported [8]. Genetic susceptibility (i.e., HLA-DR3) possibly predisposed 1 patient to develop DM after vaccination [59].

Clues to a possible mechanism of HB vaccine-associated inflammatory muscle disease come from the only detailed study (using indirect immunofluorescence and immunoelectron-microscopy) on HBV-related polymyositis [61]. HBV antigens (HBsAg and HBcAg) were detected within intact muscle fibers. Major histocompatibility complex (MHC) class 1 antigens were co-expressed with viral antigens. An *in situ* PCR study revealed positive signals within muscle fibers. No viral particles were found so that the infection appeared non-replicative. It seemed likely that HBV infection induced MHC-1 expression, so that viral antigens co-expressed with MHC-1 made infected fibers the target of an immune mediated response. A direct virus infection of muscle fibers has never been demonstrated.

Only limited data are available from other studies. A retrospective study from 9 French hospitals, detected no cases of inflammatory myopathy amongst 22 patients with HB-related rheumatic/ connective tissue disorders [62]. A prospective, case-control epidemiological study was conducted to evaluate serious autoimmune adverse events reported to the VAERS database following HB vaccinations, compared to an age, sex, and vaccine year-matched unexposed tetanus-containing vaccine group for autoimmune conditions that had previously been identified from case reports [6]. HB vaccination of adults was associated with an increased odds risk to develop a variety of serious autoimmune adverse events. Unfortunately, the risk for inflammatory myopathies was not analyzed, as myositis had not previously been identified as a HB vaccine-associated at-risk disease based *a priori* from case reports to VAERS. From available published information based

only on rare case reports, there is insufficient evidence of a causal link between HB vaccination and inflammatory myopathies.

8. Conclusion

Epidemiological surveys and isolated retrospective studies do not support the unequivocal causality between the recombinant HB vaccines and the development of various presumed autoimmune neuromuscular diseases. Furthermore, weak case report evidence often cited in obscure journals points almost exclusively to a mere temporal association between such events, and offers no proof of a cause-effect relationship. At present, it is not possible to identify individuals most prone to develop such unforeseen complications after immunization. Thus, there seems little doubt that the overall benefits of vaccine administration far outweigh its risks particularly in adults with lifestyles or occupations that increase the hazard of HBV exposure or infection.

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EXHIBIT 170

Narcolepsy Fact Sheet

Narcolepsy Fact Sheet

What is narcolepsy?

Narcolepsy is a chronic neurological disorder that affects the brain's ability to control sleep-wake cycles. People with narcolepsy usually feel rested after waking, but then feel very sleepy throughout much of the day. Many individuals with narcolepsy also experience uneven and interrupted sleep that can involve waking up frequently during the night.

Narcolepsy can greatly affect daily activities. People may unwillingly fall asleep even if they are in the middle of an activity like driving, eating, or talking. Other symptoms may include sudden muscle weakness while awake that makes a person go limp or unable to move (cataplexy), vivid dream-like images or hallucinations, and total paralysis just before falling asleep or just after waking up (sleep paralysis).

In a normal sleep cycle, a person enters rapid eye movement (REM) sleep after about 60 to 90 minutes. Dreams occur during REM sleep, and the brain keeps muscles limp during this sleep stage, which prevents people from acting out their dreams. People with narcolepsy frequently enter REM sleep rapidly, within 15 minutes of falling asleep. Also, the muscle weakness or dream activity of REM sleep can occur during wakefulness or be absent during sleep. This helps explain some symptoms of narcolepsy.

If left undiagnosed or untreated, narcolepsy can interfere with psychological, social, and cognitive function and development and can inhibit academic, work, and social activities.

Who gets narcolepsy?

Narcolepsy affects both males and females equally. Symptoms often start in childhood, adolescence, or young adulthood (ages 7 to 25), but can occur at any time in life. It is estimated that anywhere from 135,000 to 200,000 people in the United States have narcolepsy. However, since this condition often goes undiagnosed, the number may be higher. Since people with narcolepsy are often misdiagnosed with other conditions, such as psychiatric disorders or emotional problems, it can take years for someone to get the proper diagnosis.

What are the symptoms?

Narcolepsy is a lifelong problem, but it does not usually worsen as the person ages. Symptoms can partially improve over time, but they will never disappear completely. The most typical symptoms are excessive daytime sleepiness, cataplexy, sleep paralysis, and hallucinations. Though all have excessive daytime sleepiness, only 10 to 25 percent of affected individuals will experience all of the other symptoms during the course of their illness.

- **Excessive daytime sleepiness (EDS).** All individuals with narcolepsy have EDS, and it is often the most obvious symptom. EDS is characterized by persistent sleepiness, regardless of how much sleep an individual gets at night. However, sleepiness in narcolepsy is more like a "sleep attack", where an overwhelming sense of sleepiness comes on quickly. In between sleep attacks, individuals have normal levels of alertness, particularly if doing activities that keep their attention.
  - **Cataplexy**. This sudden loss of muscle tone while a person is awake leads to weakness and a loss of voluntary muscle control. It is often triggered by sudden, strong emotions such as laughter, fear, anger, stress, or excitement. The symptoms of cataplexy may appear weeks or even years after the onset of EDS. Some people may only have one or two attacks in a lifetime, while others may experience many attacks a day. In about 10 percent of cases of narcolepsy, cataplexy is the first symptom to appear and can be misdiagnosed as a seizure disorder. Attacks may be mild and involve only a momentary sense of minor weakness in a limited number of muscles, such as a slight drooping of the eyelids. The most severe attacks result in a total body collapse during which individuals are unable to move, speak, or keep their eyes open. But even during the most severe episodes, people remain fully conscious, a characteristic that distinguishes

cataplexy from fainting or seizure disorders. The loss of muscle tone during cataplexy resembles paralysis of muscle activity that naturally occurs during REM sleep. Episodes last a few minutes at most and resolve almost instantly on their own. While scary, the episodes are not dangerous as long as the individual finds a safe place in which to collapse.

- **Sleep paralysis**. The temporary inability to move or speak while falling asleep or waking up usually lasts only a few seconds or minutes and is similar to REM-induced inhibitions of voluntary muscle activity. Sleep paralysis resembles cataplexy except it occurs at the edges of sleep. As with cataplexy, people remain fully conscious. Even when severe, cataplexy and sleep paralysis do not result in permanent dysfunction—after episodes end, people rapidly recover their full capacity to move and speak.
- **Hallucinations**. Very vivid and sometimes frightening images can accompany sleep paralysis and usually occur when people are falling asleep or waking up. Most often the content is primarily visual, but any of the other senses can be involved.

Additional symptoms of narcolepsy include:

- **Fragmented sleep and insomnia**. While individuals with narcolepsy are very sleepy during the day, they usually also experience difficulties staying asleep at night. Sleep may be disrupted by insomnia, vivid dreaming, sleep apnea, acting out while dreaming, and periodic leg movements.
- **Automatic behaviors**. Individuals with narcolepsy may experience temporary sleep episodes that can be very brief, lasting no more than seconds at a time. A person falls asleep during an activity (e.g., eating, talking) and automatically continues the activity for a few seconds or minutes without conscious awareness of what they are doing. This happens most often while people are engaged in habitual activities such as typing or driving. They cannot recall their actions, and their performance is almost always impaired. Their handwriting may, for example, degenerate into an illegible scrawl, or they may store items in bizarre locations and then forget where they placed them. If an episode occurs while driving, individuals may get lost or have an accident. People tend to awaken from these episodes feeling refreshed, finding that their drowsiness and fatigue has temporarily subsided.

What are the types of narcolepsy?

There are two major types of narcolepsy:

- **Type 1 narcolepsy** (previously termed narcolepsy with cataplexy). This diagnosis is based on the individual either having low levels of a brain hormone (hypocretin) or reporting cataplexy and having excessive daytime sleepiness on a special nap test.
- **Type 2 narcolepsy** (previously termed narcolepsy without cataplexy). People with this condition experience excessive daytime sleepiness but usually do not have muscle weakness triggered by emotions. They usually also have less severe symptoms and have normal levels of the brain hormone hypocretin.
  A condition known as **secondary narcolepsy** can result from an injury to the hypothalamus, a region deep in the brain that helps regulate sleep. In addition to experiencing the typical symptoms of narcolepsy, individuals may also have severe neurological problems and sleep for long periods (more than 10 hours) each night.

What causes narcolepsy?

Narcolepsy may have several causes. Nearly all people with narcolepsy who have cataplexy have extremely low levels of the naturally occurring chemical hypocretin, which promotes wakefulness and regulates REM sleep. Hypocretin levels are usually normal in people who have narcolepsy without cataplexy.

Although the cause of narcolepsy is not completely understood, current research suggests that narcolepsy may be the result of a combination of factors working together to cause a lack of hypocretin. These factors include:

- **Autoimmune disorders**. When cataplexy is present, the cause is most often the loss of brain cells that produce hypocretin. Although the reason for this cell loss is unknown, it appears to be linked to abnormalities in the immune system. Autoimmune disorders occur when the body's immune system turns against itself and mistakenly attacks healthy cells or tissue. Researchers believe that in individuals with narcolepsy, the body's immune system selectively attacks the hypocretin-containing brain cells because of a combination of genetic and environmental factors.
- **Family history**. Most cases of narcolepsy are sporadic, meaning the disorder occurs in individuals with no known family history. However, clusters in families sometimes occur—up to 10 percent of individuals diagnosed with narcolepsy with cataplexy report having a close relative with similar symptoms.
- **Brain injuries**. Rarely, narcolepsy results from traumatic injury to parts of the brain that regulate

wakefulness and REM sleep or from tumors and other diseases in the same regions.

How is narcolepsy diagnosed?

A clinical examination and detailed medical history are essential for diagnosis and treatment of narcolepsy. Individuals may be asked by their doctor to keep a sleep journal noting the times of sleep and symptoms over a one- to two-week period. Although none of the major symptoms are exclusive to narcolepsy, cataplexy is the most specific symptom and occurs in almost no other diseases.

A physical exam can rule out or identify other neurological conditions that may be causing the symptoms. Two specialized tests, which can be performed in a sleep disorders clinic, are required to establish a diagnosis of narcolepsy:

- **Polysomnogram (PSG or sleep study)**. The PSG is an overnight recording of brain and muscle activity, breathing, and eye movements. A PSG can help reveal whether REM sleep occurs early in the sleep cycle and if an individual's symptoms result from another condition such as sleep apnea.
- **Multiple sleep latency test (MSLT).** The MSLT assesses daytime sleepiness by measuring how quickly a person falls asleep and whether they enter REM sleep. On the day after the PSG, an individual is asked to take five short naps separated by two hours over the course of a day. If an individual falls asleep in less than 8 minutes on average over the five naps, this indicates excessive daytime sleepiness. However, individuals with narcolepsy also have REM sleep start abnormally quickly. If REM sleep happens within 15 minutes at least two times out of the five naps and the sleep study the night before, this is likely an abnormality caused by narcolepsy.
  Occasionally, it may be helpful to measure the level of hypocretin in the fluid that surrounds the brain and spinal cord. To perform this test, a doctor will withdraw a sample of the cerebrospinal fluid using a lumbar puncture (also called a spinal tap) and measure the level of hypocretin-1. In the absence of other serious medical conditions, low hypocretin-1 levels almost certainly indicate type 1 narcolepsy.

What treatments are available?

Although there is no cure for narcolepsy, some of the symptoms can be treated with medicines and lifestyle changes. When cataplexy is present, the loss of hypocretin is believed to be irreversible and lifelong. Excessive daytime sleepiness and cataplexy can be controlled in most individuals with medications.

Medications

- **Modafinil**. The initial line of treatment is usually a central nervous system stimulant such as modafinil. Modafinil is usually prescribed first because it is less addictive and has fewer side effects than older stimulants. For most people these drugs are generally effective at reducing daytime drowsiness and improving alertness.
- **Amphetamine-like stimulants**. In cases where modafinil is not effective, doctors may prescribe amphetamine-like stimulants such as methylphenidate to alleviate EDS. However, these medications must be carefully monitored because they can have such side effects as irritability and nervousness, shakiness, disturbances in heart rhythm, and nighttime sleep disruption. In addition, health care professionals should be careful when prescribing these drugs and people should be careful using them because the potential for abuse is high with any amphetamine.
- **Antidepressants**. Two classes of antidepressant drugs have proven effective in controlling cataplexy in many individuals: tricyclics (including imipramine, desipramine, clomipramine, and protriptyline) and selective serotonin and noradrenergic reuptake inhibitors (including venlafaxine, fluoxetine, and atomoxetine). In general, antidepressants produce fewer adverse effects than amphetamines. However, troublesome side effects still occur in some individuals, including impotence, high blood pressure, and heart rhythm irregularities.
- **Sodium oxybate**. Sodium oxybate (also known as gamma hydroxybutyrate or GHB) has been approved by the U.S. Food and Drug Administration to treat cataplexy and excessive daytime sleepiness in individuals with narcolepsy. It is a strong sedative that must be taken twice a night. Due to safety concerns associated with the use of this drug, the distribution of sodium oxybate is tightly restricted.
  Lifestyle changes

  Not everyone with narcolepsy can consistently maintain a fully normal state of alertness using currently available medications. Drug therapy should accompany various lifestyle changes. The following strategies may be helpful:

- **Take short naps**. Many individuals take short, regularly scheduled naps at times when they tend to feel

sleepiest.

- **Maintain a regular sleep schedule**. Going to bed and waking up at the same time every day, even on the weekends, can help people sleep better.
- **Avoid caffeine or alcohol before bed**. Individuals should avoid alcohol and caffeine for several hours before bedtime.
- **Avoid smoking**, especially at night.
- **Exercise daily**. Exercising for at least 20 minutes per day at least 4 or 5 hours before bedtime also improves sleep quality and can help people with narcolepsy avoid gaining excess weight.
- **Avoid large, heavy meals right before bedtime**. Eating very close to bedtime can make it harder to sleep.
- **Relax before bed**. Relaxing activities such as a warm bath before bedtime can help promote sleepiness. Also make sure the sleep space is cool and comfortable.
-
- Safety precautions, particularly when driving, are important for everyone with narcolepsy. People with untreated symptoms are more likely to be involved in automobile accidents although the risk is lower among individuals who are taking appropriate medication. EDS and cataplexy can lead to serious injury or death if left uncontrolled. Suddenly falling asleep or losing muscle control can transform actions that are ordinarily safe, such as walking down a long flight of stairs, into hazards.
- The Americans with Disabilities Act requires employers to provide reasonable accommodations for all employees with disabilities. Adults with narcolepsy can often negotiate with employers to modify their work schedules so they can take naps when necessary and perform their most demanding tasks when they are most alert. Similarly, children and adolescents with narcolepsy may be able to work with school administrators to accommodate special needs, like taking medications during the school day, modifying class schedules to fit in a nap, and other strategies.

  Additionally, support groups can be extremely beneficial for people with narcolepsy who want to develop better coping strategies or feel socially isolated due to embarrassment about their symptoms. Support groups also provide individuals with a network of social contacts who can offer practical help and emotional support.

What is the state of the science involving narcolepsy?

In the past few decades, scientists have made considerable progress in understanding narcolepsy and identifying genes strongly associated with the disorder.

Groups of neurons in several parts of the brain interact to control sleep, and the activity of these neurons is controlled by a large number of genes. The loss of hypocretin-producing neurons in the hypothalamus is the primary cause of type 1 narcolepsy. These neurons are important for stabilizing sleep and wake states. When these neurons are gone, changes between wake, REM sleep, and non-REM sleep can happen spontaneously. This results in the sleep fragmentation and daytime symptoms that people with narcolepsy experience.

It remains unclear exactly why hypocretin neurons die. However, research increasingly points to immune system abnormalities. HLA—human leukocyte antigen—genes play an important role in regulating the immune system. This gene family provides instructions for making a group of related proteins called the HLA complex, which helps the immune system distinguish between good proteins from an individual's own body and bad ones made by foreign invaders like viruses and bacteria. One of the genes in this family is *HLA-DQB1*. A variation in this gene, called *HLA-DQB1*06:02*, increases the chance of developing narcolepsy, particularly the type of narcolepsy with cataplexy and a loss of hypocretins (also known as orexins). *HLA-DQB1*06:02* and other HLA gene variations may increase susceptibility to an immune attack on hypocretin neurons, causing these cells to die. Most people with narcolepsy have this gene variation and may also have specific versions of closely related HLA genes.

However, it is important to note that these gene variations are common in the general population and only a small portion of the people with the *HLA-DQB1*06:02* variation will develop narcolepsy. This indicates that other genetic and environmental factors are important in determining if an individual will develop the disorder.

Narcolepsy follows a seasonal pattern and is more likely to develop in the spring and early summer after the winter season, a time when people are more likely to get sick. By studying people soon after they develop the disorder, scientists have discovered that individuals with narcolepsy have high levels of anti-streptolysin O antibodies, indicating an immune response to a recent bacterial infection such as strep throat. Also, the H1N1 influenza epidemic in 2009 resulted in a large increase in the number of new cases of narcolepsy. Together, this suggests that individuals with the *HLA-DQB1*06:02* variation are at risk for developing narcolepsy after they are exposed to a specific trigger, like certain infections that trick the immune system to attack the body.

What research is being done?

The mission of the National Institute of Neurological Disorders and Stroke (NINDS) is to seek fundamental knowledge about the brain and nervous system and to use that knowledge to reduce the burden of neurological disease. The NINDS is a component of the National Institutes of Health (NIH), the leading supporter of biomedical research in the world.

The NINDS, along with several other NIH Institutes and Centers, supports research on narcolepsy and other sleep disorders through grants to medical institutions across the country. Additionally, the NIH's National Heart, Lung, and Blood Institute manages the National Center on Sleep Disorders Research (NCSDR), which coordinates Federal government sleep research activities, promotes doctoral and postdoctoral training programs, and educates the public and health care professionals about sleep disorders. For more information, visit the NCSDR website at **www.nhlbi.nih.gov/about/ncsdr**.

Genetics and biochemicals

NINDS-sponsored researchers are conducting studies devoted to further clarifying the wide range of genetic —both HLA genes and non-HLA genes—and environmental factors that may cause narcolepsy. Other investigators are using animal models to better understand hypocretin and other chemicals such as glutamate that may play a key role in regulating sleep and wakefulness. Researchers are also investigating wake-promoting compounds to widen the range of available therapeutic options and create treatment options that reduce undesired side effects and decrease the potential for abuse. A greater understanding of the complex genetic and biochemical bases of narcolepsy will eventually lead to new therapies to control symptoms and may lead to a cure.

Immune system

Abnormalities in the immune system may play an important role in the development of narcolepsy. NINDS-sponsored scientists have demonstrated the presence of unusual immune system activity in people with narcolepsy. Further, strep throat and certain varieties of influenza are now thought to be triggers in some at-risk individuals. Other NINDS researchers are also working to understand why the immune system destroys hypocretin neurons in narcolepsy in the hopes of finding a way to prevent or cure the disorder.

Sleep biology

The NINDS continues to support investigations into the basic biology of sleep, such as examining the brain mechanisms involved in generating and regulating REM sleep and other sleep behaviors. Since sleep and circadian rhythms are controlled by networks of neurons in the brain, NINDS researchers are also examining how neuronal circuits function in the body and contribute to sleep disorders like narcolepsy. A more comprehensive understanding of the complex biology of sleep will give scientists a better understanding of the processes that underlie narcolepsy and other sleep disorders.

How can I help research?

The NINDS supports the **NIH NeuroBioBank**, a national resource for investigators using human post-mortem brain tissue and related biospecimens for their research to understand conditions of the nervous system. The NeuroBioBank serves as a central point of access to collections that span neurological, neuropsychiatric, and neurodevelopmental diseases and disorders. Tissue from individuals with narcolepsy is needed to enable scientists to study this disorder more intensely. Participating groups include brain and tissue repositories, researchers, NIH program staff, information technology experts, disease advocacy groups, and, most importantly, individuals seeking information about opportunities to donate. More information about NeuroBioBank and opportunities to donate tissue is available at ***https://neurobiobank.nih.gov/***.

Additionally, the NINDS supports genetic and immunological research in narcolepsy at Stanford University. Blood samples from individuals with narcolepsy can be sent by mail and are needed to enable scientists to study this disorder more intensely. Prospective donors may contact:

Stanford University Center for Narcolepsy
450 Broadway Street
M/C 5704
Redwood City, CA 94063
650-721-7574
https://med.stanford.edu/narcolepsy.html

Where can I get more information?

For more information on neurological disorders or research programs funded by the National Institute of Neurological Disorders and Stroke, contact the Institute's Brain Resources and Information Network (BRAIN) at:

BRAIN
P.O. Box 5801
Bethesda, MD 20824
800-352-9424

Information is also available from the following organizations:

Narcolepsy Network
46 Union Drive #A212
North Kingstown, RI 02852
401-667-2523
888-292-6522

National Sleep Foundation
1010 N. Glebe Road, Suite 420
Arlington, VA 22201
703-243-1697

Wake Up Narcolepsy
P.O. Box 60293
Worcester, MA 01606
978-751-3693

National Heart, Lung, and Blood Institute (NHLBI) Health Information Center
National Institutes of Health/DHHS
P.O. Box 30105
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Prepared by:
Office of Communications and Public Liaison
National Institute of Neurological Disorders and Stroke
National Institutes of Health
Bethesda, MD 20892

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EXHIBIT 171

AS03 Adjuvanted AH1N1 Vaccine Associated with an Abrupt Increase in the Incidence of Childhood Narcolepsy in Finland

Hanna Nohynek[1]*, Jukka Jokinen[1], Markku Partinen[2], Outi Vaarala[1], Turkka Kirjavainen[3], Jonas Sundman[1], Sari-Leena Himanen[4], Christer Hublin[5], Ilkka Julkunen[6], Päivi Olsén[7], Outi Saarenpää-Heikkilä[8], Terhi Kilpi[1]

1 Department of Vaccines and Immune Protection, National Institute for Health and Welfare, Helsinki, Finland, **2** Helsinki Sleep Clinic, Vitalmed Research Centre, Helsinki, Finland, **3** Department of Pediatrics, Children's Hospital, Helsinki University Hospital, Helsinki, Finland, **4** Department of Clinical Neurophysiology, Tampere University Hospital, Tampere, Finland, **5** Finnish Institute of Occupational Health, Helsinki, Finland, **6** Department of Infectious Disease Surveillance and Control, National Institute for Health and Welfare, Helsinki, Finland, **7** Department of Child Neurology, Oulu University Hospital, Oulu, Finland, **8** Department of Pediatrics, Tampere University Hospital, Tampere, Finland

Abstract

Background: Narcolepsy is a chronic sleep disorder with strong genetic predisposition causing excessive daytime sleepiness and cataplexy. A sudden increase in childhood narcolepsy was observed in Finland soon after pandemic influenza epidemic and vaccination with ASO3-adjuvanted Pandemrix. No increase was observed in other age groups.

Methods: Retrospective cohort study. From January 1, 2009 to December 31, 2010 we retrospectively followed the cohort of all children living in Finland and born from January 1991 through December 2005. Vaccination data of the whole population was obtained from primary health care databases. All new cases with assigned ICD-10 code of narcolepsy were identified and the medical records reviewed by two experts to classify the diagnosis of narcolepsy according to the Brighton collaboration criteria. Onset of narcolepsy was defined as the first documented contact to health care because of excessive daytime sleepiness. The primary follow-up period was restricted to August 15, 2010, the day before media attention on post-vaccination narcolepsy started.

Findings: Vaccination coverage in the cohort was 75%. Of the 67 confirmed cases of narcolepsy, 46 vaccinated and 7 unvaccinated were included in the primary analysis. The incidence of narcolepsy was 9.0 in the vaccinated as compared to 0.7/100,000 person years in the unvaccinated individuals, the rate ratio being 12.7 (95% confidence interval 6.1–30.8). The vaccine-attributable risk of developing narcolepsy was 1:16,000 vaccinated 4 to 19-year-olds (95% confidence interval 1:13,000–1:21,000).

Conclusions: Pandemrix vaccine contributed to the onset of narcolepsy among those 4 to 19 years old during the pandemic influenza in 2009–2010 in Finland. Further studies are needed to determine whether this observation exists in other populations and to elucidate potential underlying immunological mechanism. The role of the adjuvant in particular warrants further research before drawing conclusions about the use of adjuvanted pandemic vaccines in the future.

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Competing Interests: HN received honoraria for technical consultancy from GlaxoSmithKline (GSK), and Pfizer for development of pneumococcal conjugate vaccines. JJ is co-investigator of a nationwide effectiveness study of the ten-valent pneumococcal conjugate vaccine mainly funded by GlaxoSmithKline. MP has been consultant for Bioprojet and UCB Pharma and received funding support and travel grants from Boehringer-Ingelheim, Bioprojet, GSK, Cephalin, MSD, Leiras and Servier. T. Kilpi is principal investigator of a nationwide effectiveness study of the ten-valent pneumococcal conjugate vaccine mainly funded by GlaxoSmithKline, and her unit received funding for a clinical trial on the safety and immunogenicity of a prototype pandemic influenza vaccine from Solvay Pharmaceuticals. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials. All other authors confirm they have no conflicts of interest.

* E-mail: hanna.nohynek@thl.fi

Introduction

To protect the population from death and serious forms of disease caused by the pandemic AH1N1 infection, the ASO3 adjuvanted vaccine Pandemrix was introduced nation-wide in Finland from October 2009 onwards according to the strategic prioritization order (Table 1) [1]. No other pandemic vaccines were available in the country. Vaccination was carried out as soon as the vaccines arrived in the country, starting 12th October 2009. Following recommendation of the European Medicines Agency (EMA), enhanced passive surveillance of vaccine related adverse events was initiated. Excess number of narcolepsy-cataplexy

Table 1. The prioritization order of the pandemic influenza vaccinations in Finland during the A(H1N1) pandemic recommended by the National Advisory Committee on Vaccinations.

1.	Social and health care professionals who work with A(H1N1) infected patients or patients presumably exposed to the infection, as well as ambulance personnel, and pharmacists who work in customer service
2.	Pregnant women
3.	People aged 6 months to 64 years at high risk due to their underlying illness. This category includes persons who require regular medication for heart or lung disease, metabolic disease, chronic liver or kidney disease, immune deficiency because of an underlying condition or treatment, chronic neurological disease or neuromuscular disease
4.	Healthy children from 6 to 35 months of age
5.	Healthy children and adolescents from 3 to 24 years of age as well as army conscripts
6.	People aged 65 years and above who belong to high risk group due to an underlying illness. After this
7.	The rest of the population

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among children and adolescents was observed a few months after the A(H1N1) epidemic and pandemic vaccination [2]. Narcolepsy was not among the sentinel events EMA encouraged to be followed.

Narcolepsy, a rare neurological sleep disorder characterized by excessive daytime sleepiness (EDS) and cataplexy, has never before the A(H1N1) pandemic been reported in association with vaccination [3,4]. The cause of narcolepsy is unknown. Immunological mechanisms are considered instrumental to the onset of narcolepsy in genetically susceptible persons [5–7]. In addition, environmental factors capable of modulating immune system, e.g. streptococcal A and viral infections, have been suggested to trigger or accelerate disease development [6,8–14].

To evaluate the observed safety signal suggesting association between Pandemrix vaccination and abrupt manifestation of narcolepsy in childhood and adolescence [1,2], we first estimated the incidence of narcolepsy from register data and then performed a population based retrospective cohort study to verify the signal and to characterize its association with the pandemic vaccination.

Methods

The study was done in Finland, a Northern European country with a population of 5.3 million and an annual birth cohort of approximately 60,000.

Study population

The Finnish Population Information System, a computerised national register, allowed us to scrutinize the entire population. Personal data including name, gender, personal identity code, address, date of birth and death of all residents are recorded in this register. The personal identity code remains unchanged throughout a person's lifetime.

Exposure to Pandemrix vaccination

Finnish municipalities (local governments) are responsible for the primary health care and subsequently the administration of the vaccines for the citizens in their region. Vaccinations with Pandemrix of those 19 years and below almost exclusively took place between weeks 44–52, 2009 (Figure 1), and were recorded in the electronic primary health care databases, which are linked to the Population Information System. Personal identity codes of the vaccinees and dates of vaccinations administered up till September 2010 were retrieved from these databases. The completeness of the exposure data was investigated by reviewing vaccination records of 1000 individuals that were randomly selected from the Population Information System.

Screening of narcolepsy

Information on visits and hospitalizations assigned ICD-10 code G47.4 was obtained from the national care register covering all care provided in the Finnish hospitals for the years 1999–2009 and from the local hospital care registers for the year 2010. The same search was done in registers of the three specialized health care centers known to have the capacity of making the diagnosis of narcolepsy. The first recorded date was regarded as the date of diagnosis for that particular individual with narcolepsy. Incident cases of narcolepsy were calculated for the years 2009–2010 by using hereby determined dates of diagnosis assuming that if G47.4 was recorded for the first time in 2009 or later in the data representing years 1999–2010, it truly was the time when the diagnosis was set.

Retrospective cohort study in the subgroup with increased incidence of narcolepsy

Having established that the increase in the incidence of narcolepsy occurred solely in the age group between 4–19 years [1,2], we designed a retrospective cohort study of all children born during the period from January 1, 1991, to December 31, 2005 and living in Finland at any time during the years 2009–10. The primary follow-up period for this cohort started on January 1, 2009 and ended on August 15, 2010, the day before media attention on post-vaccination narcolepsy started in Finland.

Special attention was paid to case ascertainment and determining disease onset. All the relevant records of the ICD-10 G47.4-coded new patients belonging to the cohort and diagnosed during 2009–10 were reviewed [2]. Two narcolepsy experts (MP, TKir) independently reviewed the patient records and classified the cases according to the Brighton Collaboration criteria for diagnostic accuracy (Level 1, Level 2, Level 3, Unknown, or Not a case; work in progress www.brightoncollaboration.org, Table 2), The criteria are an extension of the American Academy of Sleep Medicine criteria for narcolepsy with added estimation of the reliability of the diagnosis. In the discrepant cases, the final level of diagnosis was set by a panel of three other narcolepsy experts (SLH, PO alternating with CH, OSH). A case was considered narcoleptic in the primary analysis, if it was classified as Level 1–3.

In the primary analysis, the onset of narcolepsy was defined as the day when for the first time a school nurse, medical practitioner or other health care professional attended the patient because of the parental or own complaint of EDS, and recorded the observation in the patient records. This was considered the earliest objective time point available to define the onset time, and unlike the other time points available, less susceptible to the impact

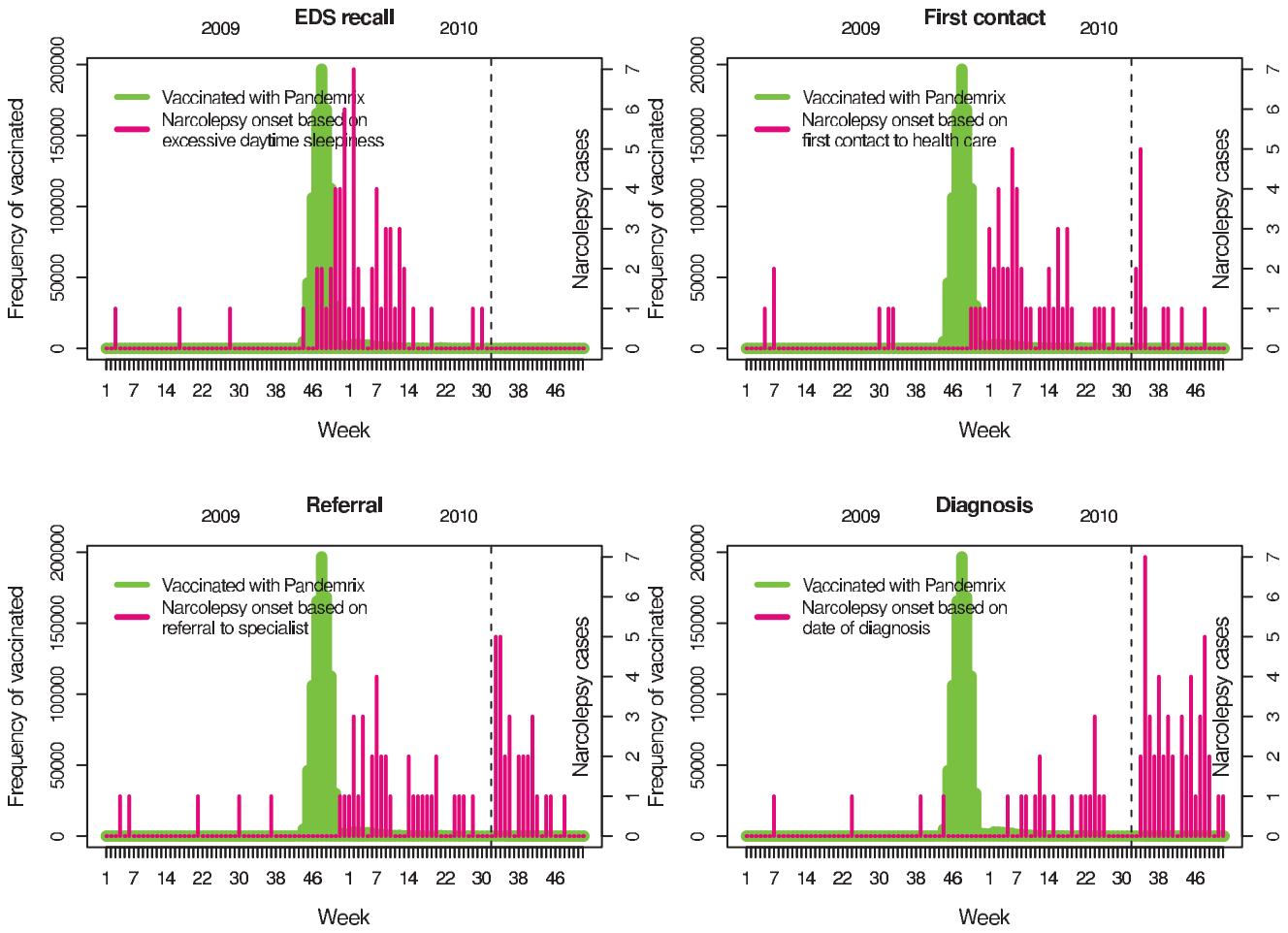

Figure 1. The temporal associations of pandemic vaccination, onset of narcolepsy (with four different definitions), and August 16, 2010, i.e. the date when the Swedish Medical Agency published the press release on the observation on the association between narcolepsy and Pandemrix vaccination (vertical dotted line). Panel top left is Recall = Parental/Patient recall when excessive daytime sleepiness (EDS) started; Panel top right is First contact = first contact to health care because of EDS; Panel bottom left is Referral = referral to specialist (paediatrician, neurologist); and Panel bottom right is Diagnosis = when diagnosis of narcolepsy was set.
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of media attention, which was initiated on August 16, 2010 by the press release on narcolepsy after Pandemrix vaccination given out by the Swedish Medical Authorities.

In the sensitivity analyses, three additional onset times were used, also to allow for comparison with earlier register data and with other studies.

Patient or parental recall. The two reviewers (MP, TKir) gave independent estimates of the onset time of symptoms (EDS and/or cataplexy) by reviewing the patient records. The patient or parental report of the time of onset usually had been recorded at the time of diagnostic workup. The mean date of these two estimates was used in the analysis.

Referral. The date of referral to a pediatrician or pediatric neurologist was the day when the attending clinician wrote a request of referral to a specialist.

Diagnosis. The date of diagnosis was defined as the date when the ICD code G47.4 was for the first time noted in the patient records.

Statistical methods

The incidence of narcolepsy after exposure to H1N1 vaccination was compared to the incidence of narcolepsy without exposure to H1N1 vaccination using Poisson regression. Pandemic vaccination was treated as a time-dependent covariate meaning that subjects moved over from the unexposed state to the exposed state at the time of vaccination. Narcolepsy cases were grouped by vaccination status at the time of disease onset and the person times of the cohort in the vaccinated and unvaccinated states were used as weights in the analysis. The total person time in the cohort was calculated based on aggregate numbers of individuals by sex, year of birth, and region at the turn of 2009/2010 (immigration and emigration in the age group 4 to 19-year-olds in Finland is less than 0.3%). Person time in the vaccinated state was calculated based on weekly cumulative aggregates of the vaccinated during the follow-up. The results are expressed as the rate ratio with 95% confidence intervals based on profile likelihood. The relative rate was calculated by comparing incidences in the vaccinated and unvaccinated states during the follow-up in question. Absolute incidences were calculated by number of narcolepsy cases divided by the person times in the population in the respective states (vaccinated/unvaccinated). The vaccine attributable risk was calculated as the cumulative incidence in the vaccinated minus the expected cumulative incidence without vaccination during the same follow-up time.

Table 2. Brighton collaboration criteria for diagnostic accuracy of narcolepsy.

Level	The Brighton collaboration criteria
Level 1	
In the *presence* of	
criterion 1	Excessive daytime sleepiness and/or definite cataplexy, AND
criterion 2	CSF hypocretin-1 deficiency
Level 2	
In the *presence* of	
criterion 1	Excessive daytime sleepiness, AND
criterion 2	Definite cataplexy, AND
criterion 3	Level 1 or 2 Multiple Sleep Test (MSLT) abormalities
Level 3	
In the *presence* of	
criterion 1	Excessive daytime sleepiness, AND
criterion 2	Level 1 MSLT abnormalities
In the *absence* of	Other mimicking disorders

doi:10.1371/journal.pone.0033536.t002

In the primary analysis, the date of first contact to health care was used to pinpoint disease onset, and the follow-up time was from January 1st 2009 until August 15th 2010. The follow-up in the primary analysis started 10 months prior to the vaccination campaign. This was done in order to obtain information about the baseline incidence and to aqcuire more power to estimate the risk in the unvaccinated. Several sensitivity analyses using different onset definitions and follow-up times were conducted to investigate changes in the risk of the unvaccinated in calendar time, and bias potentially introduced by the increasing awareness among the health care workers and the public of the suspicion that there was a link between Pandemrix and narcolepsy. To minimize potential detection bias, follow-up periods ending as early as February 22, 2010 were also tested. This was the date when one of the authors (MP) for the first time raised the question of the association of one of the cases and H1N1 infection in a discussion between colleagues.

Ethics statement

The study protocol was reviewed and approved by the Institutional Review Board of the National Institute for Health and Welfare (THL), Finland.

Results

Vaccination coverage in the population

In total, 2,76 million Pandemrix vaccine doses were given between October 2009 and August 2010. Vaccination coverage across the country was 52%, but varied from 32 to 82% in the different age groups (Table 3). In contrast, the geographical variability measured as variability across the 21 hospital districts of the country was low, particularly in children and adolescents ranging from 64 to 81%. Of the 915,854 individuals born between 1991 and 2005, 688,566 (75%) were vaccinated. All vaccinated individuals had received only one dose as recommended. The review of the vaccination records of the randomly selected 1000 individuals belonging to the study cohort revealed discrepancy between the local health care records and the electronic register

Table 3. The age-specific Pandemic vaccination coverage in Finland during the influenza pandemic season in 2009–10.

Age group	N vaccinated[1]	N total[2]	Percentage
0–4	221,297	298,114	74.2
5–9	232,023	287,786	80.6
10–14	247,720	302,423	81.9
15–19	189,247	334,636	56.6
20–24	104,535	324,472	32.2
25–29	109,387	344,634	31.7
30–34	133,026	337,970	39.4
35–39	130,096	310,768	41.9
40–44	149,077	358,754	41.6
45–49	160,040	378,341	42.3
50–54	168,853	378,037	44.7
55–59	189,854	388,165	48.9
60–64	220,640	396,886	55.6
65–69	149,071	258,319	57.7
70–74	131,876	225,043	58.6
75–79	101,793	179,671	56.7
80-	122,791	247,408	49.6
Total	2,761,326	5,351,427	51.6

Sources:
[1]Electronic patient records in Finnish health care centres.
[2]Population register of Finland;
doi:10.1371/journal.pone.0033536.t003

data in four cases, all of whom had been vaccinated according to record review but not according to the database search. In addition to the sample of 1000, the vaccination records of all newly diagnosed narcolepsy cases born between 1991 and 2009 were also reviewed. No discrepancies were found.

Patients with confirmed diagnosis in the retrospective cohort

Altogether 71 new diagnoses of narcolepsy were set in children and adolescents aged 4 to 19 years of age in 2009–10 according to the G47.4 ICD10 code. Medical records were obtained from all. Based on the expert review of the hospital and primary care records, the diagnosis of narcolepsy was classified as being level 1 in 11 (16%), level 2 in 51 (76%), and level 3 in 5 (8%) of the patients according to the Brighton collaboration definitions. The two reviewers differed in their opinion on level of classification in three cases. In full agreement by the reviewers, four cases were classified as unknown or not a case. Of the 67 confirmed cases, 57 (85%) sought medical care and 61 (91%) received the diagnosis after pandemic vaccination. Thirty-three were female, 34 male. A detailed clinical description of the patients constituting most of the cohort of narcoleptic cases seen in 2010 has been provided elsewhere [2].

Twenty of the first health care contacts were documented in school medical records, 21 in health centres, 8 in private practice, and the rest in hospitals. The time elapsed from vaccination to the onset of disease varied depending on the definition used for onset (Figure 2). Eighteen children were referred to a specialist already after Christmas 2009 and prior to the end of February 2010, 15 children were referred between 1 March 2010 to 15 August 2010, prior to the media attention, and 27 on or shortly after this date (Figure 2). The effect of the media attention shows as a bimodal

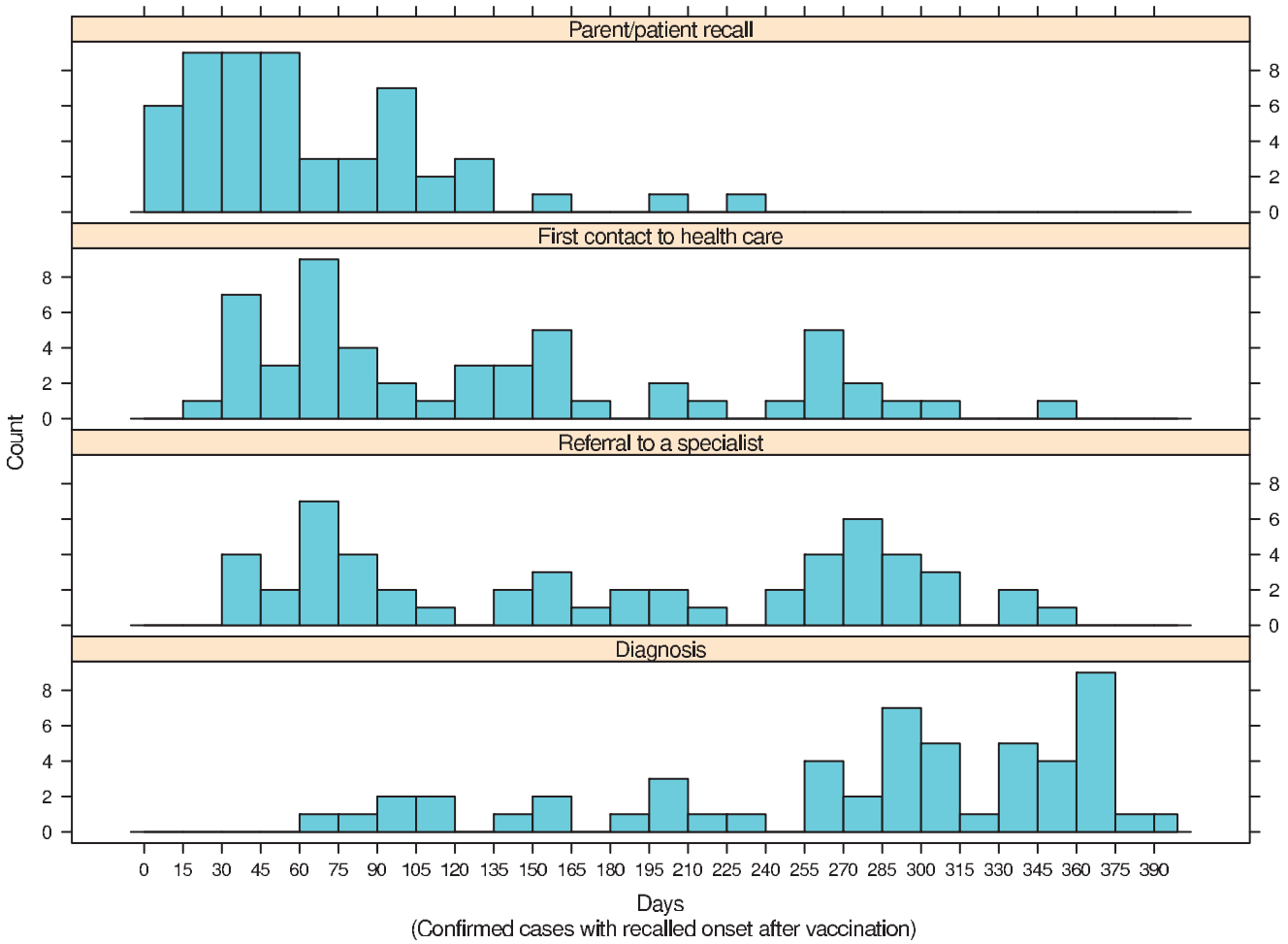

Figure 2. The different time intervals from the vaccination to the onset of narcolepsy depending on the definition of the onset time point, i.e. a) estimated onset time based on the extensive review of the patient records by a sleep and/or narcolepsy specialist, and closest to the parental/patient recall; b) first recorded contact to health care because of excessive sleepiness; c) date of referral to paediatrician or pediatric neurologist; and d) date of setting the diagnosis of narcolepsy, ICD-10 G47.4.
doi:10.1371/journal.pone.0033536.g002

distribution of the date of the first contact and referral (Figure 1, panels top right and bottom left; Figure 2). The delay from referral to the diagnosis was generally shorter for those referred on or after August 16, 2010 than before (mean delay 42 vs.122 days). The vaccinated patients were younger than those unvaccinated (Figure 3). Geographically, cases occurred in 16/21 Finnish hospital districts. This is in accordance with the underlying population size.

In the primary analysis, the incidence of narcolepsy was 9.0 in the vaccinated as compared to 0.7/100,000 person years in the unvaccinated children and adolescents, translating into a rate ratio of 12.7 (95% confidence interval 6.1–30.8) (Table 4). The lower limit of the 95% confidence level of the rate ratio was well above one in all sensitivity analyses using different follow-up periods and onset time definitions, except for the date of diagnosis as onset definition and follow-up period ending February 22, 2010 (Figure 4).

Six cases of narcolepsy had their first health care contact prior to the first H1N1 epidemic and the vaccination campaign. During the prepandemic and prevaccination follow-up period from January to October 2009, the baseline incidence of narcolepsy in the age-group of 4–19-year-olds was estimated as 0.79/100,000 person-years. No obvious change in the rate of unvaccinated was observed after the start of the campaign: By the time of media attention in August 2010, one case was recorded in the 227,288 unvaccinated, compared to an expected of 1.8 cases. With the estimated incidence in the vaccinated (9.0/100,000 person-years), one would have expected 20.6 unvaccinated cases.

Based on the primary analysis, the vaccine attributable risk of developing narcolepsy within approximately 8 months after Pandemrix vaccination was estimated to be 1 in 16,000, with 95% confidence interval from 1 in 13,000 to 1 in 21,000 vaccinated.

Discussion

We found a 12.7-fold risk of narcolepsy in 4–19-year-old individuals within approximately 8 months after Pandemrix vaccination as compared to unvaccinated individuals in the same age group. This translates into a vaccine attributable risk of 1:16,000.

Our study covers the entire population of Finland and is based on comprehensive data on individual Pandemrix vaccinations, diagnoses of narcolepsy and linkage of the two using unique

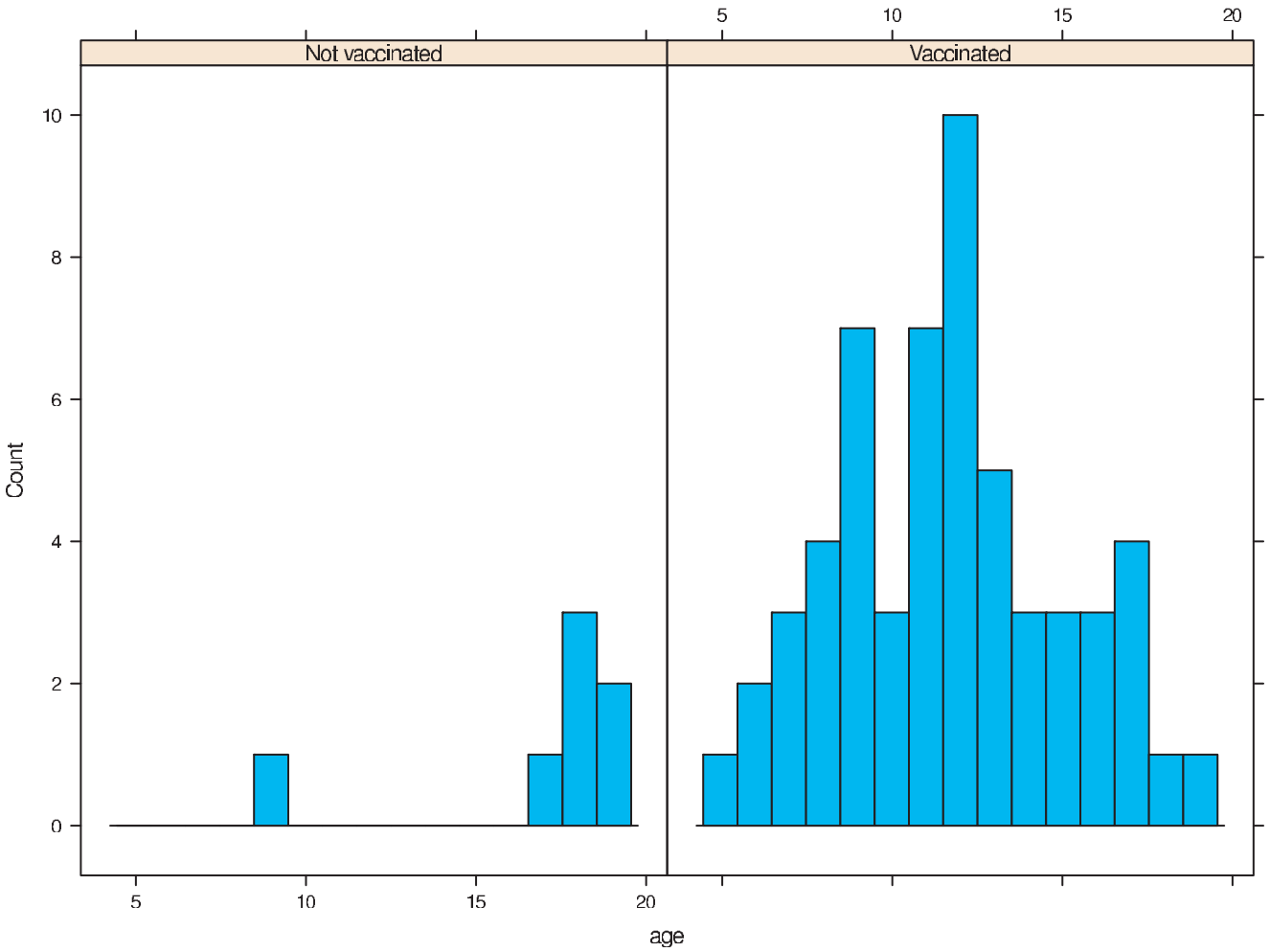

Figure 3. The age distribution of the new narcoleptic cases among the Pandemrix vaccinated and unvaccinated children and adolescents. Age presented in years.
doi:10.1371/journal.pone.0033536.g003

personal identification codes assigned to all residents in Finland. Vaccination records were retrieved from primary health care databases. The high accuracy of the exposure data was confirmed through a validation check on a random sample. Newly diagnosed cases of narcolepsy were identified via a systematic nationwide search from the hospital registers, and the diagnoses were verified through a systematic stepwise expert review procedure.

Some parents may have been tempted to recall the onset of symptoms as occurring after, rather than before their child received the pandemic vaccine. Therefore, we used different definitions for disease onset to evaluate the significance of the timing of onset on the observed association. In the primary analysis, the earliest note of EDS in the patient's medical records was used to limit recall bias.

A particular concern is that the observed association is a result of increased detection of narcolepsy among vaccinated children. According to such a view, a similar increase in narcolepsy among unvaccinated children has occurred but is yet to be observed. This argument, however, is not supported by the factual circumstances. In early 2010, narcolepsy was a rare disease unknown to most parents. Also, very few primary care physicians had seen a narcoleptic child, and no beliefs, even less conviction associated narcolepsy with the pandemic vaccine. Yet considerable numbers of Pandemrix vaccinated children were already referred to specialist before the end of February 2010 and later diagnosed with narcolepsy. The sudden surge of referrals during the first months of 2010 can hardly be explained by increased awareness and changes in diagnostic practices alone. Awareness was aroused and referrals to specialist and diagnostic workup expedited only after the media attention from Sweden broke out in August 2010.

Should a confounding factor instead of vaccination be the true cause of the association, it would have to be even more strongly associated with narcolepsy than the pandemic vaccination as we now report. In addition, such a risk factor should have a strong and time dependent positive correlation with the vaccination itself. A recent study in China found a 3–4-fold greater than predicted occurrence of narcolepsy onset following the 2009–10 H1N1 pandemic season, which was independent of vaccination [14]. In our study, there was no evidence of change in the incidence among the unvaccinated 4–19-year-olds after the first H1N1 epidemic in Finland, whereas a considerably increased risk was associated with vaccination. As H1N1 infection was hardly more common in the vaccinated than in the unvaccinated population, our findings

Table 4. Main results of the cohort analysis using two follow-up periods among those born at or after 1 January 1991.

Incidence in confirmed narcolepsy cases

Follow-up period	Narcolepsy cases		Follow-up years		Relative Risk		
	Not vaccinated	Vaccinated	Not vaccinated	Vaccinated	Risk ratio	95%LCL	95%UCL
First contact: 2009-01-01 to 2010-12-31	7	57	1,069,247	762,461	11.4	5.6	27.5
First contact: 2009-01-01 to 2010-08-16[1]	7	46	986,195	510,874	12.7	6.1	30.8

[1]The date when the news on the possible association between narcolepsy and Pandemrix vaccination observed in Sweden was published in the national media in Finland.
LCL = Lower confidence limit, UCL = Upper confidence limit.
doi:10.1371/journal.pone.0033536.t004

contradict the Chinese observation. We can think of several infectious, environmental, social or psychological factors that could modify the strength of the association seen in this study but none that could completely undo an association of this magnitude.

Our finding is supported by the recent results from Sweden, where a cohort study covering the entire population reported an almost 7-fold incidence of narcolepsy with cataplexy in children vaccinated with Pandemrix compared to those in the same age

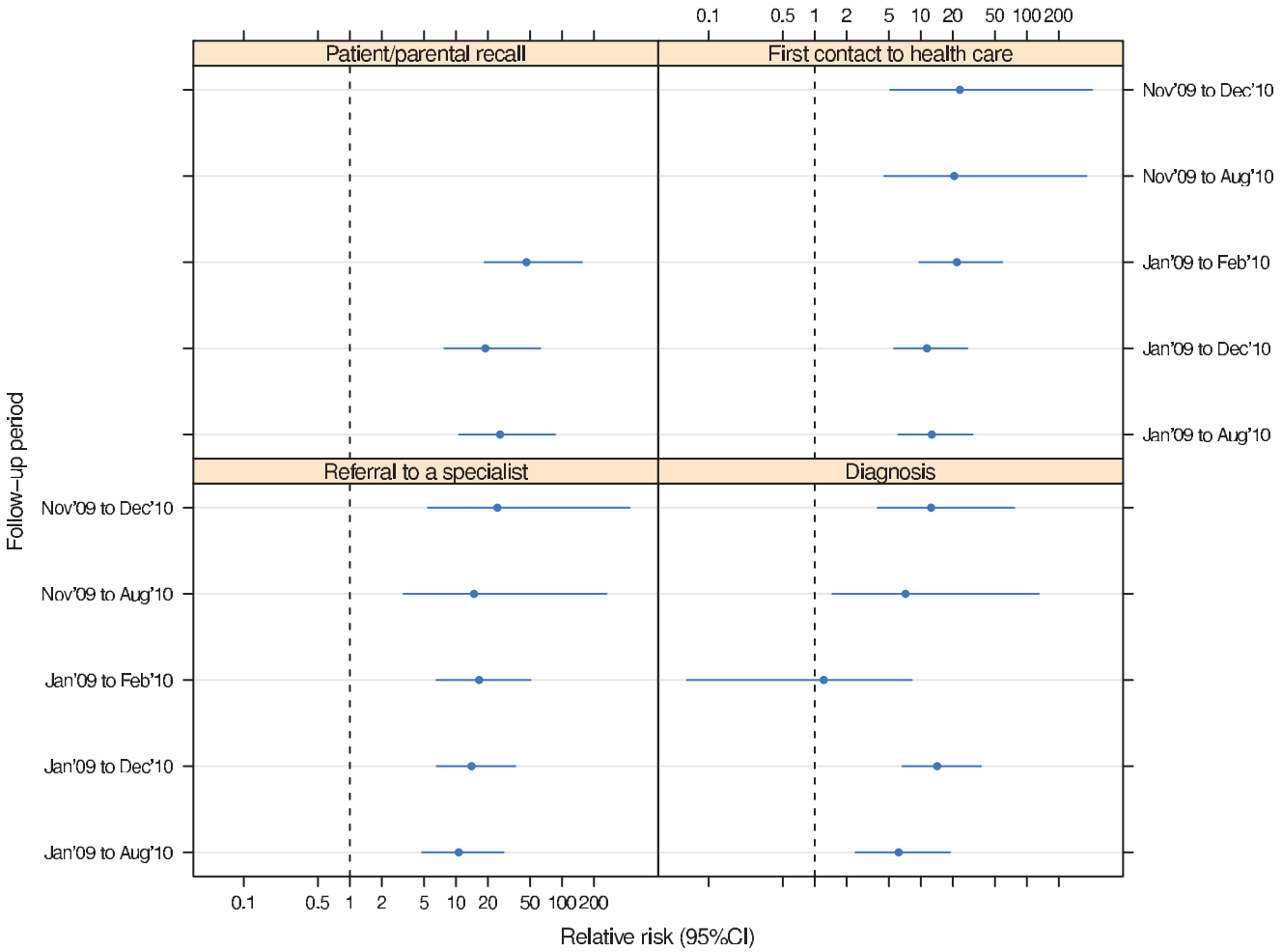

Figure 4. Sensitivity analyses of the risk ratio of Pandemrix vaccination and narcolepsy using different definitions of the onset dates of narcolepsy and follow-up time periods. The two intervals in the top left panel are missing because of infinite estimates (i.e. no cases among unvaccinated).
doi:10.1371/journal.pone.0033536.g004

group who were not vaccinated [4]. The incidence in the unvaccinated (0.64/100,000 person-years) compares well to that seen in our study. Preliminary passive reporting system data from France, Norway and Ireland also indicate higher than expected number of cases in children and adolescents after Pandemrix vaccination [15–17]. On the other hand, it is perplexing that both Canada and the United Kingdom lack the signal. In these two countries, genetic susceptibility to narcolepsy is as common as in the Nordic countries. This suggests multifactorial nature of the observed phenomenon.

The biological plausibility for a vaccine contributing to the increased risk of narcolepsy particularly in the signal-generating age group is based firstly on the immunomodulatory effects of vaccination and secondly on the fact that narcolepsy is strongly linked to the HLA DQB1*0602 allele [18]. An analogous example of a similar disease process affecting children and adolescents in particular is provided by type 1 diabetes, in which insulin-producing beta-cells are destroyed by immunological mechanisms in genetically predisposed individuals with HLA DQB1*0302 and 02 alleles [19–21]. Neither an increase nor an imbalance between the vaccinated and unvaccinated in the incidence of narcolepsy was seen in the population older than 19 years [2]. It is noteworthy that the HLA DQB1*0602 allele is approximately twice as common in northern than in southern Europe [22] and that apart from the Nordic countries, Ireland and Canada, the AS03 adjuvanted vaccine was not widely used in the age group from 4 to 19 years. It should therefore not be surprising that the signal was detected in Sweden and Finland.

Vaccinations may induce bystander activation of immunological responses especially due to function of adjuvants. The age-related differences in the immune responsiveness to Pandemrix vaccination may be of importance in the induction of the bystander activation of immune system [23]. Pandemrix vaccination could have accelerated an on-going disease process rather than triggered narcolepsy associated autoimmunity. As computer search for peptide homologies between H1N1 virus and neuron-specific proteins did not reveal any potential molecular mimicry [7,24–27], it seems unlikely that H1N1 virus infection or vaccination induced cross-reactive autoimmunity against hypocretine-producing neurons.

Our finding raises concerns of lipid containing adjuvants. Animal models have suggested that squalene, although at higher doses than used in human vaccines, is capable of contributing to the development of autoimmunity [28–30]. In humans, the epidemiological data available until now has not supported the induction of autoimmunity by squalene containing adjuvants. Adjuvanted vaccines are much needed to enhance immune responses, especially in immune compromised persons. The large scale use of new adjuvanted vaccines in human populations calls for further research of their association with adverse effects, such as autoimmunity.

Further studies are urgently needed to determine whether the association between adjuvanted pandemic vaccinations and narcolepsy can be demonstrated in other populations. The underlying immunological mechanism also warrants further research.

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The following report in Finnish previously reported some of the results of the study: Kansallisen narkolepsiatyöryhmän loppuraportti 31.8.2011 ISSN 1798-0089 http://www.thl.fi/thl-client/pdfs/c02a3788-a691-47a4-bca8-5161b6cff077 (“The final report of the National Narcolepsy Task Force”).

Author Contributions

Conceived and designed the experiments: JJ T. Kilpi HN. Analyzed the data: JJ T. Kilpi HN. Contributed reagents/materials/analysis tools: JJ JS. Wrote the paper: HN JJ MP OV T. Kirjavainen JS SLH CH IJ PO OSH T. Kilpi. Designed the database: JJ JS. Obtained permissions: JJ HN T. Kilpi. Obtained data: JJ JS HN. Validated cases: MP T. Kirjavainen PO SLH OSH CH.

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EXHIBIT 172

BRAIN
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Increased risk of narcolepsy in children and adults after pandemic H1N1 vaccination in France

Yves Dauvilliers,[1,2] Isabelle Arnulf,[2,3] Michel Lecendreux,[2,4] Christelle Monaca Charley,[5] Patricia Franco,[2,6] Xavier Drouot,[7] Marie-Pia d'Ortho,[8] Sandrine Launois,[9] Séverine Lignot,[10] Patrice Bourgin,[11] Béatrice Nogues,[12] Marc Rey,[13] Sophie Bayard,[1,2] Sabine Scholz,[1,2] Sophie Lavault,[2,3] Pascale Tubert-Bitter,[14] Cristel Saussier[15] and Antoine Pariente[10] on behalf of the Narcoflu-VF study group

1 Sleep Disorder Centre, Neurology Department, Gui de Chauliac hospital, CHU Montpellier, INSERM, U1061, Montpellier, France
2 National Reference Network for Orphan Diseases (Narcolepsy, Idiopathic Hypersomnia and Kleine Levin syndrome), France
3 Sleep Disorder Unit, Hospital la Pitié-Salpêtrière, AP-HP, Paris, France
4 Paediatric Sleep Disorder Centre, CHU Robert-Debré, AP-HP, Paris, France
5 Sleep Unit, Clinical Neurophysiology Department, Université Lille Nord de France; Lille University Hospital, Lille, France
6 INSERM, U1028, Lyon, France; Paediatric Sleep Unit, Hôpital Femme-Mère-Enfant, Lyon, France
7 Sleep Centre Physiology Department, Functional Explorations, Hôpital Henri Mondor, Creteil, France
8 Sleep Centre – Neurophysiology, Department of Physiology, Functional Explorations, Bichat Hospital, AP-HP, Université Denis Diderot Paris 7Paris, France
9 Sleep Laboratory and Physiology Department, Grenoble University Hospital, Inserm, U1042, Grenoble, France
10 Inserm, CIC-P 0005, Pharmacoepidemiology Department, CHU de Bordeaux, France
11 Sleep Disorder Centre, CHU Strasbourg, CNRS UPR 3212, Strasbourg, France
12 Functional Explorations Laboratory, Rare Hypersomnia Competence Centre, Hôpital Laennec, Nantes, France
13 Clinical Neurophysiology Unit, La Timone Hospital, Marseille, France
14 INSERM, U1018, Centre for Research in Epidemiology and Population Health (CESP), Biostatistics Team, Villejuif, France. Univ Paris Sud, UMRS 1018, Villejuif, France
15 Agence Nationale de Sécurité des Médicaments et des Produits de Santé, Saint-Denis, France

Correspondence to: Yves Dauvilliers, MD, PhD.
National Reference Network for Narcolepsy,
Sleep-Disorders Center, Department of Neurology,
Hopital Gui de Chauliac, INSERM U1061, UM1,
Montpellier, France
E-mail: ydauvilliers@yahoo.fr

Correspondence may also be addressed to: Antoine Pariente, MD, PhD. CIC 0005, Pharmacology Department, CHU de Bordeaux, Hopital Pellegrin, Pl Amelie Raba Leon, 33000, Bordeaux, France. E-mail: antoine.pariente@u-bordeaux2.fr

An increased incidence of narcolepsy in children was detected in Scandinavian countries where pandemic H1N1 influenza ASO3-adjuvanted vaccine was used. A campaign of vaccination against pandemic H1N1 influenza was implemented in France using both ASO3-adjuvanted and non-adjuvanted vaccines. As part of a study considering all-type narcolepsy, we investigated the association between H1N1 vaccination and narcolepsy with cataplexy in children and adults compared with matched controls; and compared the phenotype of narcolepsy with cataplexy according to exposure to the H1N1 vaccination. Patients with narcolepsy-cataplexy were included from 14 expert centres in France. Date of diagnosis constituted the index date. Validation of cases was performed by independent experts using the Brighton collaboration criteria. Up to four controls were individually matched to cases according to age, gender and geographic location. A structured telephone interview was

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performed to collect information on medical history, past infections and vaccinations. Eighty-five cases with narcolepsy-cataplexy were included; 23 being further excluded regarding eligibility criteria. Of the 62 eligible cases, 59 (64% males, 57.6% children) could be matched with 135 control subjects. H1N1 vaccination was associated with narcolepsy-cataplexy with an odds ratio of 6.5 (2.1–19.9) in subjects aged <18 years, and 4.7 (1.6–13.9) in those aged 18 and over. Sensitivity analyses considering date of referral for diagnosis or the date of onset of symptoms as the index date gave similar results, as did analyses focusing only on exposure to ASO3-adjuvanted vaccine. Slight differences were found when comparing cases with narcolepsy-cataplexy exposed to H1N1 vaccination ($n = 32$; mostly AS03-adjuvanted vaccine, $n = 28$) to non-exposed cases ($n = 30$), including shorter delay of diagnosis and a higher number of sleep onset rapid eye movement periods for exposed cases. No difference was found regarding history of infections. In this sub-analysis, H1N1 vaccination was strongly associated with an increased risk of narcolepsy-cataplexy in both children and adults in France. Even if, as in every observational study, the possibility that some biases participated in the association cannot be completely ruled out, the associations appeared robust to sensitivity analyses, and a specific analysis focusing on ASO3-adjuvanted vaccine found similar increase.

Keywords: narcolepsy; cataplexy; H1N1; vaccine; infection

Abbreviations: MSLT = Multiple Sleep Latency Test

Introduction

Narcolepsy with cataplexy is a disabling orphan disorder caused by a loss of hypothalamic hypocretin/orexin-producing neurons with the main peak of disease onset at 16 years of age (Dauvilliers *et al*., 2001, 2007). An autoimmune basis for narcolepsy-cataplexy has long been suspected based on the tight association with HLA-DRB1*15:01-DQB1*06:02 haplotype, T cell receptor alpha and purinergic receptor P2RY11 polymorphisms (Mignot *et al*., 2001; Hallmayer *et al*., 2009; Hor *et al*., 2010; Kornum *et al*., 2011*a*), the presence of elevated Tribbles homolog 2 and anti-streptolysin O antibodies (Aran *et al*., 2009; Cvetkovic-Lopes *et al*., 2010), the low vitamin D levels (Carlander *et al*., 2011), and the positive effect of intravenous IgG to normalize CSF hypocretin-1 level in a single patient (Dauvilliers *et al*., 2009). However, the precise aetiology of narcolepsy-cataplexy remains unknown with both genetic and environmental factors playing a major role (Dauvilliers *et al*., 2007; Kornum *et al*., 2011*b*).

In early 2010, an increased incidence of narcolepsy was detected in children in Finland and Sweden where pandemic H1N1 influenza vaccine (Pandemrix® containing adjuvant ASO3, squalene and alphatocopherol) was used (THL, 2010; MPA Sweden, 2011; Nohynek *et al*., 2012; Partinen *et al*., 2012). We reported some cases with narcolepsy-cataplexy from France, Canada and the USA using the ASO3-adjuvanted H1N1 vaccine, some cases using non-adjuvanted vaccine and some cases who developed narcolepsy-cataplexy after H1N1 infection (Dauvilliers *et al*., 2010). Recent studies confirmed the increased childhood/adolescent incidence rate of narcolepsy in western Sweden, England and Ireland after the H1N1 Pandemrix® vaccination campaign (National Narcolepsy Study Steering Committee, 2012; Miller *et al*., 2013; Szakács *et al*., 2013). The majority of these did not consider the adult population.

A large increase of childhood cases with narcolepsy-cataplexy was reported after the winter of 2009–2010 in China, independent of vaccination (Han *et al*., 2011). In South Korea no increase was seen in the incidence rate of narcolepsy after the H1N1 vaccination campaign using non-adjuvanted and MF59-adjuvanted H1N1 vaccines (Choe *et al*., 2012).

Differences exist in the rates of pandemic H1N1 vaccination in the general population across the different countries and according to the age group (children versus adults), the presence of adjuvanted versus non-adjuvanted vaccine, and the adjuvant ASO3 or not, that preclude any definitive conclusion on the real risk of narcolepsy after H1N1 vaccine exposure. In 2010, the European Centre for Disease Controls (ECDC) funded a multinational case-control study in eight European countries coordinated by the Vaccine Adverse Event Surveillance and Communication (VAESCO) consortium (ECDC, 2012) to study the association between all-type narcolepsy and H1N1 vaccination. In this context, the French drug agency (*Agence Nationale de Sécurité du Médicament et des produits de santé*, ANSM) co-funded a study in France, Narcoflu-VF, to contribute both to the VAESCO study and to pursue specific objectives. These specificities in design and objective consisted of a longer period of recruitment (up to April 2011), and in a sub-analysis focusing on the risk of narcolepsy-cataplexy.

From October 2009 to February 2010, a campaign of vaccination against H1N1 influenza targeting all subjects was implemented in France, with swine flu vaccine administered to 5.7 million individuals. Of these, 4.1 million were vaccinated with Pandemrix® and 1.6 million with Panenza® (non-adjuvanted vaccine). Panenza® was indicated for the vaccination of children aged <24 months (then extended to children <9 years), pregnant females and immunocompromised patients. The final population coverage was estimated at 8.8% at the end of the vaccination campaign. Panenza® was used in ~90% of the vaccinated aged <9 years and Pandemrix® in ~89% of the vaccinated aged ⩾9 years (data obtained from ANSM).

The aim of the sub-analysis of the Narcoflu-VF study presented here was (i) to investigate the association between pandemic H1N1 vaccination and narcolepsy-cataplexy in both children and adults compared with gender-, age- and geographic location-matched controls in France; and (ii) to compare the phenotype of cases with narcolepsy-cataplexy according to exposure to H1N1 vaccination.

Materials and methods

The Narcoflu-VF study is a multicentre case-control study performed in the institutions of 14 French expert orphan disease narcolepsy centres being easily identifiable by professionals and patients, which has allowed a specialized homogeneous care for both diagnosis and management of patients with narcolepsy in France. The information provided when the study was proposed to patients specified that the study aimed to investigate narcolepsy risk factors and potential associations with infections, medical drug use, and vaccinations. No specific emphasis was put on H1N1 vaccination, to limit the possibility of a participation bias related to this specific exposure. The protocol was approved by the research scientific committee of the ANSM and the Bordeaux hospital ethics committee. All subjects gave written informed consent to participate.

Recruitment of cases with narcolepsy-cataplexy

Patients with narcolepsy-cataplexy were referred to one of the participating sleep centres to confirm the diagnosis by polysomnography as well as the Multiple Sleep Latency Test (MSLT) between 1 October 2009 and 30 April 2011. Diagnosis of narcolepsy-cataplexy was established using the revised International Classification of Sleep Disorders (ICSD, 2005). All patients presented excessive daytime sleepiness, typical cataplexy, at least two sleep-onset REM periods and a mean sleep latency <8 min during the MSLT. The date retained as the main index date for the analysis was the date of polysomnography-MSLT confirmed diagnosis. Participating centres identified retrospectively from lists of medical records completed by reference centres for orphan diseases as required by the French government and from hospital statistic databases all their patients with narcolepsy-cataplexy potentially matching the eligibility criteria. All potentially eligible cases were asked to participate.

All cases agreeing to participate were validated using the Brighton collaboration criteria by an expert committee to fully confirm the diagnosis of typical narcolepsy-cataplexy (Poli *et al*., 2012). Two experts from the committee were assigned to assess each case, and a third one solicited in case of discrepancy. The investigator who included the case could not be an expert for the validation of the case to maintain blinding and objective assessment. Brighton case definition level 1 included the presence of excessive daytime sleepiness and/or unambiguous cataplexy, and CSF hypocretin-1 deficiency; and level 2 included the presence of excessive daytime sleepiness, unambiguous cataplexy, and abnormal MSLT including either mean sleep latency <8 min (or 12 min for children) or at least two sleep-onset REM periods. Subjects classified with levels 3 and 4 (less degree of reliability of narcolepsy-cataplexy) were excluded from the study.

Only confirmed cases of narcolepsy-cataplexy for whom the date of onset of symptoms—either excessive daytime sleepiness or cataplexy—was after 1 January 2005, and after the date of H1N1 vaccination for exposed cases, were included in the main analysis.

Recruitment of control subjects

Up to four control subjects were individually matched to each case according to sex, age (year of birth $\pm$ 2 years) and geographic location during the same recruitment period. Control subjects were recruited from: (i) patients from the hospitals to which the participating sleep centres belonged; and (ii) healthy volunteers from a national database (Narcobank).

For hospital controls, the reason for healthcare requirement had to be unrelated to narcolepsy or pandemic H1N1 vaccination. The reason for this eligibility criterion was to obtain a population of hospital controls that would not exclusively represent patients with specific indication to H1N1 vaccination or contraindication to it. It was specified not to recruit controls from departments that specialized in the treatment of patients for which H1N1-vaccination was especially recommended (e.g. departments specialized in the management of AIDS, and immunocompromised patients). However, if a control subject admitted for surgery had a history of asthma, she/he was considered eligible. No patient was eliminated on the basis of the presence of a specific historical condition; the recruitment procedure was only thought to avoid constituting a population of controls in which vaccination rate would have been specifically affected by a recommendation targeting a condition that would have been over-represented in the recruitment department (e.g. recruitment of controls in a Chronic Obstructive Pulmonary Disease management department).

Narcobank is a study financed by a national research programme from the French Health Ministry in 2007 with aims to study biomarkers and genetic risk factors of narcolepsy and other rare central hypersomnias. All subjects (patients and healthy controls matched for age and gender) were recruited between 2008 and 2010 from five sleep centres, all participating to the Narcoflu-VF study. The population of Narcobank was recruited without considering exposure to H1N1 vaccination. Some cases with narcolepsy-cataplexy may have participated in both Narcoflu-VF and Narcobank. The recruitment of control subjects from Narcobank mainly concerns these cases.

Data collection

All subjects were recruited by investigators before information on H1N1 vaccination status was collected. They were contacted for a standardized telephone interview to collect data on body mass index, smoking, medical history, history of viral or bacterial infections, and history of vaccinations. Date and type of all vaccines performed between January 2005 and index date were collected including seasonal flu, pandemic H1N1 vaccinations (Pandemrix® or Panenza®), and no-flu vaccinations. Excessive daytime sleepiness was assessed using the Epworth scale for adults (Johns, 1991), and the Paediatric Daytime Sleepiness Scale for children (Drake *et al*., 2003). Patients had to have their medical booklet and vaccination certificate (a further interview was scheduled if the patient did not have them). This ensured complete information for most patients concerning type of vaccine and date of vaccination. However, this could not be ascertained for some participants. For these, the type of vaccine was considered 'undocumented', and the date of vaccination was the reported month and year of vaccination, day being set at the first of the month. A procedure of exception was retained for the H1N1 vaccination campaign in France; purpose vaccination centres were opened, in limited number, to which patients were called-up by mail; being vaccinated implicated an individual voluntary step and prolonged wait. For these reasons, patient knowledge on this specific vaccination appear reliable at least for the fact of being vaccinated and the period of vaccination, even if vaccination type and exact date were considered only when they could be certified according to information from the medical booklet or the additional H1N1 vaccination certificate.

Information on the characteristics of narcolepsy-cataplexy was collected from medical records. It included index date, date of referral for MSLT, symptoms severity and date of onset, polysomnography and

MSLT results, HLA typing, and CSF hypocretin-1 concentration when available.

Date of onset of symptoms was obtained from patients. As it can be difficult to assess, patients were asked to report the period of onset of their first symptoms (month and year of beginning and ending of this period). Cases reporting vaccination were thus classified as follows for the main and sensitivity analysis: (i) first symptoms after vaccination (i.e. left margin of the first symptoms onset period after vaccination date, at least in the following month if day unknown, most cases): cases considered exposed for all analyses; and (ii) first symptoms before vaccination or potentially concomitant (i.e. right margin of the first symptoms onset period before vaccination date, at least in the following month if day unknown, most cases or insufficient precision to exclude anteriority of first symptoms to vaccination): cases excluded from the analyses on date of diagnosis and date of referral for MSLT, and considered as non-exposed for analyses on date of first symptom onset.

Statistical analysis

Characteristics of cases and controls were compared in univariate analyses using Mc-Nemar Chi^2 test for qualitative variables and Student *t*-test for paired series for quantitative variables (or non-parametric Wilcoxon test when Student *t*-test could not be used). The association between H1N1 vaccination and narcolepsy-cataplexy was estimated using conditional logistic regression models; it was expressed using odds ratios and their 95% confidence intervals (CI). Variables were included in the multivariate models if they were associated with narcolepsy-cataplexy with $P < 0.25$ after univariate analyses. They were considered in the final model if they were associated with narcolepsy-cataplexy with $P < 0.2$ or found responsible for confounding or H1N1 vaccination effect modification. Association was estimated for the whole population, by age category (<18 or $\geqslant 18$ years at index date), and by time period of the date of diagnosis. Sensitivity analyses were performed that considered as index date (i) the date of referral for polysomnography-MSLT; and (ii) the date of first symptom onset. Characteristics of exposed and non-exposed cases contributing to any of these analyses were compared using Chi^2 test or exact Fisher test for qualitative variables and Student *t*-test or non-parametric Mann-Whitney Wilcoxon test for quantitative variables.

Additional analyses were performed: (i) an analysis including cases with narcolepsy-cataplexy that were excluded from the main analysis because their date of onset of symptoms was before the date of H1N1 vaccination. In this additional analysis, these were considered as non-exposed; and (ii) an analysis considering only exposure to AS03-adjuvanted vaccine. All cases exposed to other H1N1 vaccines and their matched controls, as well as controls exposed to other or undocumented H1N1 vaccines were excluded from this analysis.

We used SAS 9.3 for Windows (SAS Institute Inc). All reported *P*-values are two-tailed, with a significance level set at 0.05.

Results

Of the 177 cases with narcolepsy (all types considered) initially contacted to participate to the full Narcoflu-VF study, 127 responded and agreed to participate. Of these, 85 were confirmed cases with narcolepsy-cataplexy and were initially included, with 23 patients later excluded from the main analysis based on index date ineligibility or onset of symptoms starting before H1N1 vaccination (Fig. 1). Among the 62 remaining eligible cases, 66.1% were males, median age was 15.3 years (range 5–51 years), and 35 (56.5%) were <18 years of age. CSF hypocretin-1 was available in 24 patients, all with levels <110 pg/ml. All cases were validated using the Brighton classification showing level 1 in 37.1% and level 2 in 62.9%.

Of the 62 eligible cases with narcolepsy-cataplexy, 59 could be matched to 135 control subjects, with regards to sex, year of birth and geographic area (Fig. 1 and Table 1). If body mass index was higher in cases, no significant difference was found between cases and controls for smoking (either personal or in relatives), age at puberty onset, medical history of diabetes, asthma, migraine, head trauma, cancer or familial history of autoimmune diseases.

We studied the history of infections and vaccinations between 1 January 2005 and the index date (Table 2). No between-group differences were found regarding the frequency of history of infectious episodes (Epstein-Barr virus streptococcal, upper respiratory or gastrointestinal tract infections or flu-like episodes), seasonal influenza or non-flu vaccinations. Conversely, pandemic H1N1 vaccination was found in 31 cases (27 Pandemrix® and four Panenza®) and 24 controls (17 Pandemrix®, one Panenza®, and six unknown) (52.5% versus 17.8%, $P < 10^{-4}$).

H1N1 vaccination was associated with narcolepsy-cataplexy with an odds ratio of 5.5 (95% CI 2.5–12.0) when considering the whole population over the complete study period. When considering patients <18 years of age and their matched controls, the odds ratio was estimated at 6.5 (95% CI 2.1–19.9), and 4.7 (95% CI 1.6–13.9) in those aged 18 and over. For cases with a diagnosis date before July 2010 and matched controls, the odds ratio was estimated at 2.8 (95% CI 0.8–10.5); it was estimated at odds ratio 7.6 (95% CI 2.8–20.8) for those with a later index date. In this analysis, after running multivariate models, H1N1 vaccination was the only study variable associated with narcolepsy-cataplexy with $P < 0.2$ or found responsible for confounding or H1N1 vaccination effect modification (Table 3). Sensitivity analyses using different index dates found similar results (Table 3), as well as additional analyses considering cases with narcolepsy-cataplexy with date of first symptoms onset before the date of H1N1 vaccination as non-exposed, and considering only exposition to AS03-adjuvanted vaccine (Table 4).

As some healthcare professionals were included in the volunteer controls ($n = 36$), with theoretically increased risk of being vaccinated, we differentiated the rate of vaccination between healthcare control subjects and other control subjects (35.5% versus 8.7% of corresponding cases, $P < 10^{-3}$). Excluding healthcare professional controls from the analysis did not change the estimates in children but led to the model not converging in adults.

We further compared among the 62 cases with narcolepsy-cataplexy, those exposed to H1N1 vaccination ($n = 32$ including 28 with Pandemrix®) to those unexposed (Table 5). Median delay between vaccination and onset of excessive daytime sleepiness was 2.5 months [interquartile range (IQR) 1.2–4.5], with cataplexy onset 4.5 months (IQR: 1.6–8.1), and with diagnosis of narcolepsy-cataplexy 10.6 months (IQR: 8.5–13.4). The mean delay between narcolepsy-cataplexy onset (either excessive daytime sleepiness or cataplexy) and its diagnosis was shorter in exposed versus non-exposed cases, together with shorter delay between onset of excessive daytime sleepiness and cataplexy. However,

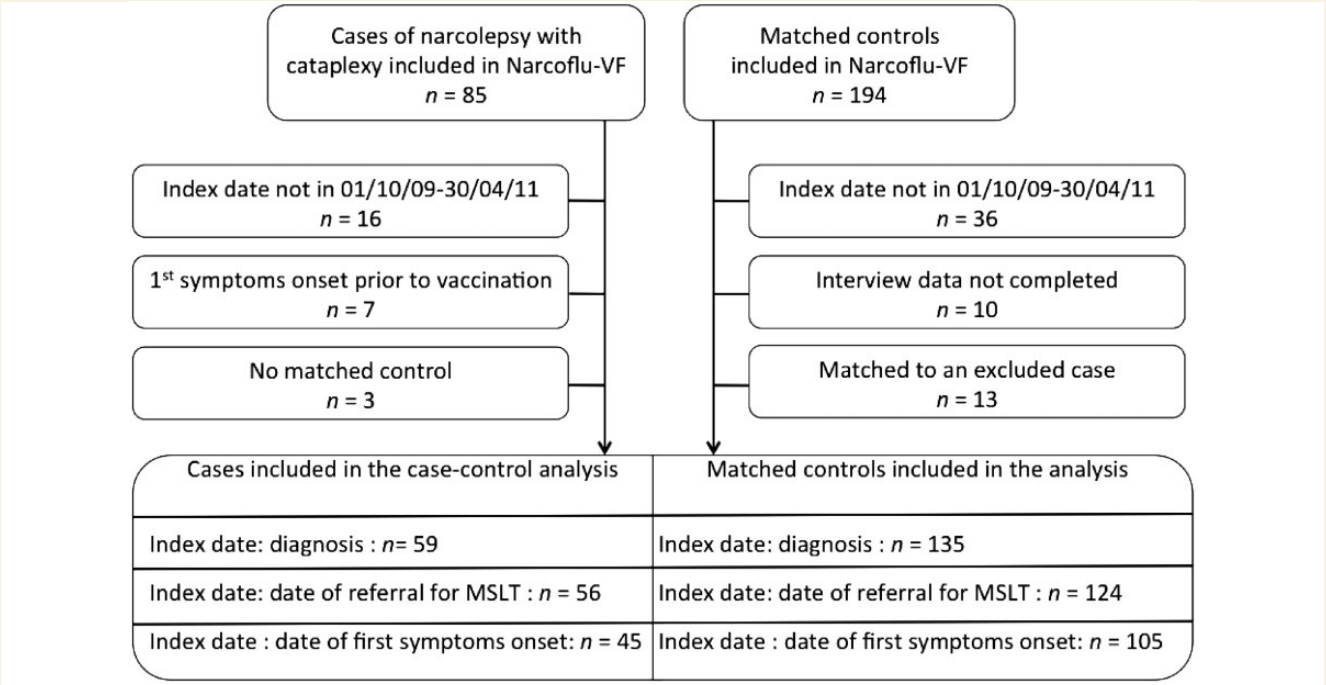

Figure 1 Selection of cases and control subjects for the case-control analysis included in the NarcoFlu-VF study.

Table 1 Comparison of socio-demographic and medical history of patients with narcolepsy-cataplexy and matched control subjects

	Cases *n* = 59	Controls *n* = 135	*P**
Male gender, *n* (%)	38 (64.4)	86 (63.7)	–#
Age at primary index date (i.e. date of referral for MSLT), in years			
Median (IQR)	15.1 (13.0–25.6)	19.4 (13.2–32.2)	–
<18, *n* (%)	34 (57.6)	62 (45.9)	–
Period of recruitment, *n* (%)			
1 October 2009–30 June 2010	16 (27.1)	40 (29.6)	–
1st July 2010–30 April 2011	43 (72.9)	95 (70.4)	–
BMI (kg/m^2), median (IQR)	23.1 (0.3–26.9)	21.3 (19.3–23.5)	<0.0001
Age at onset of puberty, median (IQR)	11 (11–12)	11.5 (10–12)	0.89
Active smoker, *n* (%)	12 (20.3)	21 (15.6)	0.12
Another active smoker at home, *n* (%)	17 (28.8)	41 (30.4)	0.64
Diabetes, *n* (%)	0 (0)	1 (0.7)	0.60
Asthma *n* (%)	7 (11.9)	14 (10.4)	0.94
Migraine, *n* (%)	9 (15.3)	24 (17.8)	0.75
Head trauma, *n* (%)	2 (3.4)	13 (9.6)	0.28
Cancer, *n* (%)	0 (0)	1 (0.7)	0.62
Immunodepression, *n* (%)	1 (1.7)	0 (0.0)	NA
Familial history of autoimmune disease, *n* (%)	7 (11.9)	17 (12.6)	0.4

*P for paired statistical tests; #matching variables: *P* not provided (equal to 1 according to test for paired data).
NA = non-assessable.
BMI = body mass index.

these differences did not remain significant when considering only cases with a similar period of onset of first symptoms (i.e. ⩾1 October 2009) (Table 5). No significant difference was found between exposed and non-exposed cases regarding gender, age at time of diagnosis, positive familial history of narcolepsy-cataplexy, and period of recruitment (before or after 1 July 2010). Severity of narcolepsy-cataplexy assessed clinically through Epworth scale or Paediatric Daytime Sleepiness Scale for excessive daytime sleepiness, frequency of cataplexy at baseline, presence of generalized cataplexy with fall, hypnagogic hallucination or sleep paralysis,

Table 2 Comparison of infectious episodes and vaccination history between patients with narcolepsy-cataplexy and matched control subjects

	Cases $n = 59$	Controls $n = 135$	P*
Infections			
Infectious episodes, between 1 January 2005 and index date, *n* (%)	47 (79.7)	112 (83.0)	0.18
Confirmed Epstein-Barr Virus infection between 1 January 2005 and index date, *n* (%)	2 (4.3)	2 (1.8)	0.37
Confirmed streptococcal infection between 1 January 2005 and index date, *n* (%)	0	4 (3.6)	0.45
Upper respiratory tract infectious episode between 1 January 2005 and index date, *n* (%)	39 (83.0)	99 (88.4)	0.20
Gastrointestinal tract infectious episode between 1 January 2005 and index date, *n* (%)	12 (25.5)	50 (44.6)	0.07
Flu-like episode during the last two flu seasons preceding index date, *n* (%)	13 (22.0)	24 (17.8)	0.20
Vaccinations			
At least one seasonal influenza vaccination between 1 January 2005 and index date, *n* (%)	5 (8.5)	23 (17.0)	0.12
H1N1 influenza vaccination, *n* (%)	31 (52.5)	24 (17.8)	$<10^{-4}$
Pandemrix®	27	17	
Panenza®	4	1	
Undocumented	0	6	
Non-flu vaccinations during the past 2 years, *n* (%)	15 (25.4)	45 (33.3)	0.37
Non-flu vaccinations between 1 January 2005 and index date, *n* (%)			
Diphteria	38 (84.4)	80 (80.8)	0.68
Tetanus	40 (88.9)	83 (83.8)	0.50
Poliovirus	38 (84.4)	80 (80.8)	0.68
Haemophilus influenza type B	7 (15.6)	9 (9.1)	0.39
Pertussis	19 (42.2)	41 (41.4)	0.76
Hepatitis B	6 (13.3)	25 (25.3)	0.79
Human papilloma virus	3 (5.1)	7 (5.2)	0.42

*P for paired for paired statistical tests.

nocturnal agitation or body mass index did not differ between groups. Polysomnography recordings revealed similar total sleep time, REM sleep latency and percentage of patients with apnea/hypopnea index >15/h between groups. We reported a slightly higher number of sleep onset REM periods in exposed patients without any change for the mean sleep latency (Table 5). No between group differences were found for HLA DQB1*06:02 reported in 92% of vaccinated versus 85.2% in non-vaccinated cases. CSF hypocretin-1 levels were assessed more frequently in exposed ($n = 19$) compared to non-exposed ($n = 5$) cases; but all values were <110 pg/ml.

Discussion

We report the first study showing an association between pandemic H1N1 vaccination and narcolepsy-cataplexy by an odds ratio up to 6-fold in children (<18 years) and 5-fold in adults (⩾18 years). Sensitivity analyses taking into account either date of referral for MSLT or date of onset of first symptoms did not change the results. Furthermore, we found almost no difference when comparing the characteristics of cases with narcolepsy-cataplexy exposed to H1N1 vaccination (mostly Pandemrix®) with non-exposed cases, except for a shorter delay of diagnosis and higher number of sleep onset REM periods in exposed cases. No differences were found regarding the frequency of history of infections.

After the 2009–2010 H1N1 flu pandemic and related vaccination campaigns, an increased risk of narcolepsy after vaccination was reported, particularly in Scandinavian children—especially in Finland and Sweden—characterized by a high coverage of Pandemrix® vaccination (THL, 2010; MPA Sweden, 2011); the results from Finland, but also more recently those from western Sweden, England and Ireland, showed a particular increase of narcolepsy-cataplexy in children (National Narcolepsy Study Steering Committee, 2012; Nohynek *et al*., 2012; Partinen *et al*., 2012; Miller *et al*., 2013; Szakács *et al*., 2013). We previously reported the first cases with narcolepsy-cataplexy from France and Canada using the similar adjuvanted H1N1 vaccine (Dauvilliers *et al*., 2010). This study confirms the initial signs from France showing an association between narcolepsy-cataplexy and H1N1 vaccine, mostly represented by Pandemrix®, and shows a similar increased risk in vaccinated children and adults; the increased risk for adults being previously unreported. Our findings also show that narcolepsy-cataplexy post-H1N1 exposure is almost similar to genuine narcolepsy-cataplexy.

Monozygotic twin narcolepsy-cataplexy studies have suggested that environmental factors play a major role in narcolepsy-cataplexy pathophysiology (Dauvilliers *et al*., 2004). Recent data reported a large increase in onset of childhood cases with narcolepsy-cataplexy after the winter of 2009–2010 in China, together with a seasonality of disease onset (Han *et al*., 2011). These findings suggested a role of the influenza pandemic H1N1-infection independently of any vaccination, with data showing a decreased incidence the following year (Han *et al*., 2012).

Table 3 Estimation of the association between H1N1 vaccination and the risk of narcolepsy-cataplexy

Analysis setting	Odds ratio (95% CI)
Main analysis (index date: date of diagnosis)	
Whole population	5.5 (2.5–12.0)
Cases aged <18 years and their controls	6.5 (2.1–19.9)
Cases aged ⩾18 years and their controls	4.7 (1.6–13.9)
Index date before July 2010	2.8 (0.8–10.5)
Index date from July 2010 onwards	7.6 (2.8–20.8)
Sensitivity analysis (index date: date of referral for MSLT)	
Whole population	6.1 (2.4–15.0)*
Cases aged <18 years and their controls	6.1 (2.0–18.9)
Cases aged ⩾18 years and their controls	6.1 (1.3–27.9)*
Index date before July 2010	9.6 (1.6–59.0)
Index date from July 2010 onwards	4.9 (1.7–14.4)*
Sensitivity analysis (index date: date of onset of first symptoms)	
Whole population	24.6 (5.6–108.6)*
Cases aged <18 years and their controls	27.3 (3.6–209.1)
Cases aged ⩾18 years and their controls	16.8 (1.9–149.10)*
Index date before July 2010	40.5 (5.2–317.7)
Index date from July 2010 onwards	9.9 (1.2–85.1)

*Adjusted for smoking.
Results of the main analysis considering date of diagnosis as the index date, and of the sensitivity analyses performed regarding date of referral for MSLT or date of first symptoms onset.

Associations between streptococcus infections and recent onset cases of narcolepsy-cataplexy were also reported with large frequency of high levels of serum antibodies against streptolysin O (65%) within 1 year of onset compared with age-matched controls (26%) (Aran *et al*., 2009). We did not find such associations with flu infection symptoms, streptococcal, upper respiratory or gastrointestinal tract infections, but also not with non-H1N1 flu vaccines in our study.

This study has several strengths. First, each case was validated independently by two experts blind to the vaccination status and who had not been involved in the case recruitment. This validation used the Brighton classification criteria (Poli *et al*., 2012), thus misclassification of cases is unlikely to have occurred. Second, to allow for consideration of cases who, after the exposure period, would have had a longer time to develop symptoms and be diagnosed with narcolepsy-cataplexy, we decided to consider a longer period of recruitment after the vaccination campaign than that retained in the VAESCO protocol (ECDC, 2012). Indeed, the delay between onset of first symptoms and diagnosis can be very long in narcolepsy, usually ~8–10 years (Morrish *et al*., 2004; Dauvilliers *et al*., 2007). However, it is possible that only the abrupt onset and the most severely exposed cases were identified, as indicated by the larger proportion of subjects <18 years of age in the vaccinated cases (65.6% versus 46.7%). In contrast, we could not detect major differences in disease severity between exposed and non-exposed cases with either clinical or polysomnography evaluations but we did detect a higher number of sleep onset REM periods in exposed cases, as reported in early-onset cases with narcolepsy-cataplexy in China (Han *et al*., 2012). To prevent the impact of professional/media attention on the rate of narcolepsy-cataplexy recognition, we analysed the results taking into account the recruitment period of date of diagnosis, date of referral for MSLT and date of onset of symptoms before and after 1 July 2010. Results did not vary significantly between analyses, but were not significant for the period before July 2010 when considering diagnosis date as the index date. Although this could indicate a potential media effect, it may also reflect the fact that time to diagnosis is, by essence, longer than time to first symptoms or to MSLT referral, for which the association remained significant for the same period. Another strength of this study was the recruitment of patients from expert narcolepsy sleep centres specifically created by the national government plan for orphan diseases, to which patients suspected of having narcolepsy are usually referred. In those centres, all eligible patients were identified from medical files and hospital statistic databases, and contacted to participate; thus limiting the possibility of a selection bias. After the initial diagnosis was made using ISCD criteria (2005), all cases were validated by an expert committee using the Brighton collaboration criteria (Poli *et al*., 2012). As narcolepsy-cataplexy and narcolepsy without cataplexy are two individualized diseases with different pathophysiology in ~80% of cases (normal CSF hypocretin-1 levels and no association with HLA DQB1*06:02 in the latter condition) (ICSD, 2005; Andlauer *et al*., 2012), we decided to focus in this analysis on patients with typical cataplexy to constitute a more specific and homogeneous group. However, during the same recruitment period 22 patients with narcolepsy without cataplexy were diagnosed in France and included in the French participation of the VAESCO analysis, three of whom had been vaccinated against H1N1 (all with Pandemrix®) before the first onset of symptoms.

The study has several limitations. First, we had a limited number of cases even with regards the rarity of narcolepsy-cataplexy. This resulted in limiting the power for analyses adjustments and prohibited evaluating the effect of adjuvanted versus non-adjuvanted vaccines. We were unable to compare the population of exposed and non-exposed cases according to the different age groups. Considering only exposure to AS03-adjuvanted vaccine, we found a strong association with narcolepsy-cataplexy in both children and adults. Despite the fact that the apparent relative excess of vaccination with Panenza® in cases compared with control subjects could indicate that the increased risk of narcolepsy-cataplexy also associated with this vaccine, we were unable to investigate this point. To date, no evidence supports this hypothesis; however the possibility that other H1N1 vaccines, adjuvanted or not, could be associated with an increased risk of narcolepsy-cataplexy cannot be ruled out. Second, our population was affected by a selection bias, with an over-representation of healthcare professionals characterized by a higher H1N1 vaccination rate. Even if

Table 4 Estimation of the association between H1N1 vaccination and the risk of narcolepsy-cataplexy

Analysis including cases vaccinated after the date of onset of first symptoms (non-exposed)	Odds ratio (95% CI)
Index date: date of diagnosis (65 cases, 148 controls)	
Whole population	5.2 (2.3–11.6)**
Cases aged <18 years and their controls	6.4 (1.9–21.3)**
Cases aged ⩾18 years and their controls	4.1 (1.4–12.2)**
Index date before July 2010	5.8 (1.1 –29.9)*
Index date from July 2010 onwards	5.2 (2.1–12.9)**
Index date: date of referral for MSLT (64 cases, 142 controls)	
Whole population	3.7 (1.7–8.0)*
Cases aged <18 years and their controls	3.6 (1.4–8.9)
Cases aged ⩾18 years and their controls	4.1 (1.1–15.7)*
Index date before July 2010	4.8 (1.2–19.3)
Index date from July 2010 onwards	2.9 (1.2–7.4)*
Index date: date of onset of first symptoms (45 cases, 105 controls)#	
Whole population	24.6 (5.6–108.6)*
Cases aged <18 years and their controls	29.2 (3.8–223.4)
Cases aged ⩾18 years and their controls	16.8 (1.9–149.1)*
Index date before July 2010	40.5 (5.2–317.7)*
Index date from July 2010 onwards	9.9 (1.2–85.1)*
Analysis considering only exposure to AS03-adjuvanted vaccines (all exposed to other H1N1 vaccine and matched controls excluded)	**Odds ratio (95% CI)**
Index date: date of diagnosis (55 cases, 117 controls)	
Whole population	4.4 (2.0–9.7)**
Cases aged <18 years and their controls	4.1 (1.4–12.2)**
Cases aged ⩾18 years and their controls	4.6 (1.5–14.1)
Index date before July 2010	3.3 (0.9–12.2)
Index date from July 2010 onwards	4.9 (1.9–12.9)**
Index date: date of referral for MSLT (51 cases, 106 controls)	
Whole population	4.0 (1.7–9.1)*
Cases aged <18 years and their controls	3.3 (1.2–8.8)*
Cases aged ⩾18 years and their controls	6.6 (1.7–26.1)*
Index date before July 2010	5.6 (1.4–22.2)*
Index date from July 2010 onwards	2.9 (1.0–8.4)*
Index date: date of onset of first symptoms (41 cases, 89 controls)	
Whole population	20.6 (4.7–90.3)*
Cases aged <18 years and their controls	21.5 (2.8–166.6)
Cases aged ⩾18 years and their controls	17.7 (2.1–149.5)*
Index date before July 2010	36.1 (4.8–273.1)
Index date from July 2010 onwards	6.1 (0.7–56.8)

Results of the additional analyses (i) including and considering as non-exposed cases with date of onset of first symptoms prior to date of H1N1 vaccination; (ii) including and considering only in the exposed cases and controls those vaccinated with AS03-adjuvanted vaccine.
*Adjusted for smoking.
**Adjusted for smoking and family history of excessive daytime sleepiness.
#The results from this analysis are similar to that of the corresponding one performed in the sensitivity analysis as the index date considered is the date of onset of first symptoms. Thus, in the sensitivity analysis, cases vaccinated after first onset of symptoms were already considered as non-exposed.

this is unlikely to have modified the estimates in children, this could have lowered the estimation of the association in adults. Third, the reliability of the information on vaccination obtained from patients' interview can be questioned, and was incomplete in some cases. The previously detailed procedure of exception retained for the French H1N1 vaccination campaign showed that subjects' knowledge on this specific vaccination is reliable, at least for the fact of being vaccinated and the period of vaccination. However, this cannot be fully certified and the occurrence of misclassification for exposure cannot be eliminated. The consistency of the results obtained in including cases with date of onset before vaccination, considering them as non-exposed, or restricting the study to exposure to Pandemrix® suggests that, if such misclassification occurred, they are unlikely to have been responsible for the associations found. Other potential recall biases may exist within this study, including the reliance on patient history for critical narcolepsy onset dates, and the non-responders bias as 28% of eligible patient refused to take part of the study.

Table 5 Clinical, polysomnographical and biological characteristics of cases with narcolepsy-cataplexy according to H1N1 vaccination

	Exposed cases $n = 32$	Non-exposed cases $n = 30$	P
Clinical assessment			
Male gender, *n* (%)	22 (68.8)	19 (63.3)	0.65
Age at date of diagnosis, years, median (IQR)*	14.8 (13.3–24.4)	18.3 (12.6–32.4)	0.60
Age at date of diagnosis, *n* (%)			
<18	21 (65.6)	14 (46.7)	0.31
18–25	3 (9.4)	7 (23.3)	
26–40	6 (18.8)	5 (16.7)	
⩾40	2 (6.3)	4 (13.3)	
Period of date index recruitment			
October 2009 to June 2010	6 (18.8)	11 (36.7)	0.11
July 2010 to April 2011	26 (81.3)	19 (63.3)	
Delay between excessive daytime sleepiness and cataplexy onset, in months (median, IQR)	0.0 (0.0–0.6)	1.0 (0.0–11.8)	0.03
Delay of diagnosis, in months (median, IQR)	6.9 (3.5–9.4)	12.6 (4.8–18.5)	0.006
Family history of narcolepsy-cataplexy, *n* (%)	1 (3.1)	2 (6.7)	0.61
Pathological score on sleep scale, *n* (%)*	23 (82.1)	20 (71.4)	0.67
Generalized cataplexy, *n* (%)	23 (71.9)	22 (73.3)	0.9
Daily number of cataplexy attacks, median (IQR)*	1 (1–3)	1 (1–2)	0.12
Hypnagogic hallucinations, *n* (%)	18 (56.3)	16 (53.3)	0.71
Sleep paralysis, *n* (%)	12 (37.5)	9 (30.0)	0.47
Nocturnal sleep disturbances, *n* (%)	13 (40.6)	16 (53.3)	0.32
BMI > 25, *n* (%)	11 (34.4)	12 (40.0)	0.43
Weight gain at disease onset, *n* (%)	18 (56.3)	15 (50.0)	0.72
Polysomnographic assessment			
Total sleep time, min, median, (IQR)	487 (452–513)	473 (447–510)	0.65
Night REM sleep latency, min, median (IQR)	11 (2–122)	55 (4–100)	0.79
Apnoea-hypopnoea index >15, *n* (%)	1 (3.1)	2 (6.7)	0.61
MSLT			
Sleep onset REM period, *n* (IQR)	4 (3–5)	3 (3–4)	0.04
Mean sleep latency, min, median (IQR)	3.4 (2.5;5.0)	3.6 (2.5;5.6)	0.67
CSF hypocretin-1 measurements, *n* (%)	19 (59.4)	5 (16.7)	0.0004
HLA DQB1 06:02, *n* (%)	23 (92.0)	23 (85.2)	1.00

*Sleep scale: Epworth for adults (pathological score > 10), Paediatric Daytime Sleepiness Scale (>30) for children.
BMI = body mass index; IQR = interquartile range.

Although we developed specific procedures to lower the importance of these potential biases (e.g. no emphasis on H1N1 vaccination in information documents), as for all observational studies, the possibility that some biases participated in the reported association cannot be fully ruled out. However, even if it does not eliminate the possibility of such bias, the results are consistent with previously published studies and the consistency of the performed sensitivity analyses is reassuring.

Our results indicate that H1N1 vaccination, mostly represented by Pandemrix® in our study, could have contributed to narcolepsy-cataplexy in both children and adults. The mechanisms underlying such an association remain unclear but may involve either a specific immune response to H1N1 with potential molecular mimicry or a large non-specific stimulation of the immune system through the adjuvanted ASO3 vaccine with increasing brain inflammation/blood–brain permeability, allowing the autoimmune process to reach hypocretin neurons resulting in narcolepsy-cataplexy (Dauvilliers *et al*., 2010; Kornum *et al*., 2011*b*). We may also speculate that patients with narcolepsy-cataplexy after exposure to H1N1 vaccine would have developed the disease later on, as suggested by a decreased narcolepsy incidence in 2011–2012 in Finland (unpublished data). H1N1 vaccination could have precipitated an ongoing disease process through the activation of pre-existing autoreactive T cell clones rather than causing narcolepsy-cataplexy. Taken together, the majority of the results from the H1N1 vaccine story reinforce the underlying autoimmune hypothesis of narcolepsy-cataplexy. A biobank was constituted for the present study and further analyses will be performed to elucidate the biological mechanism underlying this association.

In conclusion, in this case-control study, we found a strong association between H1N1 vaccination, mostly represented by Pandemrix®, and narcolepsy-cataplexy in both children and

adults. This association appeared robust to sensitivity analyses, and a specific analysis focusing on ASO3-adjuvanted vaccine found a similar increase. However, as in all observational studies, the possibility that some biases participated in this association cannot be completely ruled out.

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RESEARCH

Risk of narcolepsy in children and young people receiving AS03 adjuvanted pandemic A/H1N1 2009 influenza vaccine: retrospective analysis

Elizabeth Miller *consultant epidemiologist*[1], Nick Andrews *senior statistician*[2], Lesley Stellitano *public health researcher*[1 3], Julia Stowe *research fellow*[1 4], Anne Marie Winstone *public health researcher*[1 3], John Shneerson *consultant physician*[5], Christopher Verity *consultant paediatric neurologist*[1 3]

[1]Immunisation, Hepatitis and Blood Safety Department, Health Protection Agency, Colindale, London NW9 5EQ, UK ; [2]Statistics, Modelling, and Economics Department, Health Protection Agency, Colindale, London NW9 5EQ, UK; [3]PIND Research Group, Addenbrooke's Hospital, Cambridge CB2 0QQ, UK; [4]General and Adolescent Paediatric Unit, Institute of Child Health, University College London, London NW1 2PF, UK; [5]Respiratory Support and Sleep Centre, Papworth Hospital, Cambridge CB23 3RE, UK

Abstract

Objective To evaluate the risk of narcolepsy in children and adolescents in England targeted for vaccination with ASO3 adjuvanted pandemic A/H1N1 2009 vaccine (Pandemrix) from October 2009.

Design Retrospective analysis. Clinical information and results of sleep tests were extracted from hospital notes between August 2011 and February 2012 and reviewed by an expert panel to confirm the diagnosis. Vaccination and clinical histories were obtained from general practitioners.

Setting Sleep centres and paediatric neurology centres in England.

Participants Children and young people aged 4-18 with onset of narcolepsy from January 2008.

Main outcome measures The odds of vaccination in those with narcolepsy compared with the age matched English population after adjustment for clinical conditions that were indications for vaccination. The incidence of narcolepsy within six months of vaccination compared with the incidence outside this period measured with the self controlled cases series method.

Results Case notes for 245 children and young people were reviewed; 75 had narcolepsy (56 with cataplexy) and onset after 1 January 2008. Eleven had been vaccinated before onset; seven within six months. In those with a diagnosis by July 2011 the odds ratio was 14.4 (95% confidence interval 4.3 to 48.5) for vaccination at any time before onset and 16.2 (3.1 to 84.5) for vaccination within six months before onset. The relative incidence from the self controlled cases series analysis in those with a diagnosis by July 2011 with onset from October 2008 to December 2010 was 9.9 (2.1 to 47.9). The attributable risk was estimated as between 1 in 57 500 and 1 in 52 000 doses.

Conclusion The increased risk of narcolepsy after vaccination with ASO3 adjuvanted pandemic A/H1N1 2009 vaccine indicates a causal association, consistent with findings from Finland. Because of variable delay in diagnosis, however, the risk might be overestimated by more rapid referral of vaccinated children.

Introduction

Narcolepsy is a chronic disorder presenting with excessive daytime sleepiness, often accompanied by a transient loss of muscle tone triggered by strong emotion (cataplexy). Diagnosis is based on clinical criteria and can be confirmed by polysomnography followed by a multiple sleep latency test.[1] Estimates of prevalence generally range between 25 and 50 per 100 000, though might be less in some populations, possibly because of differences in genetic susceptibility or exposure to aetiological risk factors.[2] Information on incidence is more limited. Onset can occur at any age[2] but is commonest in those aged 10-19, in whom an incidence of 3.84 per 100 000 person years has been reported.[3] The interval between onset and diagnosis can be long, with a median of 10.5 years in one study.[4] Diagnostic delay is less in those with cataplexy and in younger patients.[5] There is a strong association with human leucocyte antigen (HLA) DQB1*0602 and reported associations with environmental factors such as streptococcal infection,[6] seasonal influenza,[7] and more recently pandemic A/H1N1 2009 influenza.[8]

In England, a monovalent pandemic strain vaccine containing the oil-in-water adjuvant AS03 (Pandemrix) was introduced in October 2009 during the second wave of infection, initially for people with high risk clinical conditions[9 10] and then in healthy

Correspondence to: E Miller liz.miller@hpa.org.uk

children aged under 5 from mid-December 2009.[11] By March 2010, around 24% of healthy children aged <5 and 37% aged 2-15 in a risk group had been vaccinated in England.[12] A second pandemic vaccine was used (Celvepan) but accounted for less than 1% of the total.

In August 2010 concerns were raised in Finland and Sweden about a possible association between narcolepsy and Pandemrix.[13] A subsequent cohort study in Finland reported a 13-fold increased risk of narcolepsy after vaccination in children and young people aged 4-19, most of whom had onset within three months after vaccination and almost all within six months.[14] To evaluate the risk of narcolepsy after vaccination in England we identified cases in those aged under 19 with onset since 1 January 2008 and compared the proportion vaccinated with that in the age matched English population after adjusting for clinical conditions that were indications for pandemic vaccination.

Methods

Case ascertainment and validation

Cases in children and young people aged 4-18 at onset of narcolepsy from January 2008 were ascertained from sleep centres and paediatric neurology centres in England. With lists supplied by the British Sleep Society and the British Paediatric Neurology Association we identified 23 centres that saw children. In July 2011 we contacted these 23 centres and 16 replied that they had seen affected children in the relevant time period. To provide an alternative means of case ascertainment we identified all the cases in England recorded in the hospital episode statistics database[15] with the ICD-10 (international classification of diseases, 10th revision) diagnosis code G47.4 (narcolepsy and cataplexy) in the same age group in the same time period. Clinical information including the presence of cataplexy and results of relevant tests including polysomnography, multiple sleep latency test, HLA type, and hypocretin concentrations were extracted from case notes during visits to the 16 study centres from August 2011 to February 2012. Details of the clinical features and test results of cases will be reported elsewhere. Patients' general practitioners were sent a questionnaire to ascertain history of pandemic and seasonal influenza vaccination, date of onset of symptoms, date of first healthcare consultation for a sleep problem, and any underlying clinical condition for which pandemic vaccine was indicated. Information on infections preceding narcolepsy was also sought. These data were reviewed by three narcolepsy experts (blinded to vaccination status) who confirmed the cases in which the diagnosis was definite—that is, narcolepsy with cataplexy or narcolepsy without cataplexy according to international classification of sleep disorders criteria.[1] Cases not meeting these criteria but with a convincing clinical history were classified as probable narcolepsy. The remainder were excluded because of insufficient information and were not included in the analysis.

Index dates—definitions

The date of symptom onset was the earliest date of excessive daytime sleepiness or cataplexy as given by the general practitioner or recorded in the centre notes. When the exact date was not available we used the mid-point of the month.

The date of first known healthcare contact was the earliest recorded consultation for a sleep related problem as reported by the general practitioner or in the centre notes.

The key centre visit was when all cases known at the centre were systematically ascertained; cases identified on an ad hoc basis after this were not included.

The date of diagnosis was the earliest date that identified an affected patient at the key centre visit, either on the basis of a clinical history and sleep study confirming narcolepsy or because there was sufficient clinical information to diagnose probable narcolepsy.

Statistical analysis

We assessed the association between vaccination and narcolepsy using the case coverage method[16]: for each patient with narcolepsy in the study the population coverage was ascertained for children of the same age (in months on 30 September 2009) at the relevant index date (that is, date of symptom onset) and with the same risk group status (in a group or not). The association was calculated as the odds ratio for vaccination in the cases compared with the matched population. This was done with logistic regression with the outcome as vaccinated (yes/no) in the cases and with an offset for the log odds of the matched coverage. As the outcome is rare, odds ratios approximate to relative risks. Vaccine coverage by age in years and risk group status came from weekly electronic reports to the Birmingham research unit of the Royal College of General Practitioners by a representative sample of 98 general practices in England for the period September 2009 to August 2010.[17] We analysed patient level electronic records extracted from the practices to derive coverage data for specific age and risk groups. To obtain coverage within 12 weeks or six months before an index date we matched the coverage at the index date and at the date 12 weeks or six months earlier and calculated the difference in coverage. Cases categorised by the experts as definite and probable narcolepsy were combined for all analyses. The primary analysis used first symptoms as the index date and was restricted to diagnoses by 31 July 2011. We carried out sensitivity analyses including all patients with a diagnosis by the key centre visit, using first healthcare contact or diagnosis as the index date, not matching on risk group status, or increasing population coverage by a relative 20% (for example, 10% increasing to 12%). Analyses were performed based on vaccination within 12 weeks, within six months, and at any time before the index date.

We carried out a separate analysis using the self controlled case series method[18] to estimate the incidence of symptom onset within three and six months after vaccination relative to the incidence outside this period (the baseline). Because pandemic influenza vaccination started in October 2009 the observation period for each individual started on 1 October 2009 and ended on 31 December 2010. In a second analysis we used a start date of October 2008 to allow inclusion of additional unexposed person time in the baseline. Analyses were performed with all those with a diagnosis by the key visit date and also restricted to those with a diagnosis by July 2011. Adjustment for time period was made with calendar month of onset. Adjustment by age was not necessary as this was relatively stable within the study period.

Results

Vaccine coverage

We extracted information on 160 400 individuals aged 2-18 from the Royal College of General Practitioners database. Of these, 14 400 (9.0%) were in a clinical risk group, mainly because of asthma. Table 1⇓ gives the uptake of pandemic vaccine by August 2010 by age and risk group status and the

estimated number of first doses given in England by this date, based on 2009 population estimates.[19] The cumulative vaccine uptake by day, age, and risk status is consistent with the initial targeted vaccination of risk groups followed by all children aged under 5 (fig 1⇓).

Study cases

Review of clinical records

We reviewed the clinical records in 245 cases identified by clinicians and/or from the hospital episode statistics database search at the 16 study centres. Although in all cases the diagnoses or hospital admission dates were after January 2008, we excluded 130 because onset of symptoms was before January 2008 and 23 because the diagnosis had not been confirmed by the sleep centre. This left 92 cases for independent review by the narcolepsy expert panel: in 10 there was insufficient information to assign a diagnosis, in three the date of diagnosis was after the key visit, three patients were outside the 4-18 age range, and in one the onset was before January 2008. Of the 75 remaining cases, 66 were definite according to the international classification of sleep disorders criteria (56 had narcolepsy with cataplexy and 10 had narcolepsy without cataplexy). The nine remaining were considered probable narcolepsy. Table 2 shows the demographic and clinical features in these 75 cases⇓; in 55 cases the patients has received a diagnosis by July 2011.

Cases identified from hospital episode statistics

Of the 162 cases identified via this database in England, 130 were identified from the 16 study centres. Only 35 fitted our case definition and were included in the analysis. In the 95 excluded cases, 62 patients had onset before January 2008, and in 25 the diagnosis in the hospital episode statistics database was not confirmed by the study centre (case notes in eight such cases were not available for review). The remaining 32 cases identified from hospital episode statistics were in centres that had not reported cases or were cases at non-centre hospitals; these 32 cases were distributed as follows: two hospitals had four cases each, two had three cases each, and 18 had single cases.

Vaccination history

We obtained vaccination history and risk group status in all 75 study cases; none of the patients with a diagnosis of probable narcolepsy was vaccinated (table 2). Of the 11 definite cases in which the patient had previously received pandemic vaccine, six had onset within three months, one within three to six months, and four between seven and 14 months after vaccination; all had received Pandemrix and age at vaccination ranged between 3 and 16. Figure 2⇓ shows the 75 cases by month of symptom onset and whether they had previously receieved vaccine, together with vaccine uptake. The vaccinated patient with onset in 2011 received Pandemrix in 2011, when residual stocks were used instead of seasonal vaccine.[20] Two were reported to have an influenza-like illness in the six months before first symptoms, neither of whom was vaccinated.

Case coverage analysis

Table 3 shows the results of the case coverage analysis⇓ for patients who had received a diagnosis by July 2011 and by the key study visit with and without adjustment for risk group status. Odds ratios were significantly increased in all analyses; odds ratios without matching on risk group status were generally higher as were those based on date of onset of symptoms. The odds ratio with symptom onset as the index date and with the assumption that all vaccinated patients were in a risk group was 5.0 (1.3 to 19.3) for vaccination within six months and 3.3 (1.2 to 8.7) for "vaccinated at any time," while increasing coverage by a relative 20% gave a risk group adjusted odds ratio of 13.0 (2.5 to 68.3) for vaccination within six months and 11.5 (3.4 to 39.2) for "vaccinated at any time."

Self controlled case series analysis

Only 18 cases diagnosed by the key visit had onset of symptoms between October 2009 and December 2010, of whom seven were unvaccinated, one was vaccinated after onset, and 10 were vaccinated before onset (five within 84 days, six within 182 days, four more than 182 days before). Restriction of cases to those diagnosed by July 2011 excluded four unvaccinated cases and one case vaccinated more than 182 days before onset. Starting the observation period from October 2008 added another 22 unvaccinated cases and two more cases vaccinated after onset. Relative incidence estimates were only significantly raised when we included the period from October 2008 in the baseline (table 4⇓).

Attributable risk

For calculation of the vaccine attributable risk we used the odds ratio of 14.4 based on symptom onset as the index date, diagnosed by July 2011, and "vaccinated at any time" (table 3). If the odds ratio is used to approximate relative risk (RR), the attributable fraction ((RR−1)/RR)) is 13.4/14.4, which applied to the 10 vaccinated patients in this analysis gives an estimate of 9.3 attributable cases. To estimate the number of doses given to the population the cases came from, we used the number of doses given in England to those aged 3-18 by September 2009 (668 000 from table 1, as the youngest vaccinated patient was aged 3 at vaccination). We then adjusted this number assuming a range of 80% to 100% for the proportion of cases captured, which gives a range of 534 400 to 668 000 doses. The figure of 80% used as a minimum proportion of cases captured was obtained by comparing the number of hospital episode statistics cases coded as G47.4 for the period 1 January 2008 to 20 November 2010 that were from the 16 centres (130 cases) to the total number of G47.4 cases in the hospital episode statistics database in England for this period (162). The estimated attributable risk is therefore between 9.3/534 400 and 9.3/668 000 (1 per 57 500 to 1 per 52 000 doses).

Discussion

Principal findings

This study shows a significantly increased risk of narcolepsy in children who received the AS03 adjuvanted pandemic strain vaccine in England. Our case coverage method gave an odds ratio of 14.4 (4.3 to 48.5) for the primary analysis and is consistent with the relative risk of 13 reported from Finland in a retrospective cohort study.[14] The lack of reported cases in other European states and Canada after the initial case reports from Finland and Sweden in August 2010[13] led to speculation that some unidentified factor was operating in these countries and that the association, if real, might be restricted to these Scandinavian populations.[21] Our study confirms the signal raised from Finland and Sweden[13] and indicates that the association is not restricted to those populations.

The increased risk found in our study and in Finland could be because the vaccine accelerates onset of narcolepsy, which would lead to a consequent deficit in incident cases in subsequent years with no vaccine attributable risk in the longer

term. Evaluation of this would require late follow-up. The effect would be difficult to detect in England given the low vaccine coverage but might be detected in Finland and Ireland,[22] where coverage was substantially higher. A spuriously high risk would also be generated if the clinical features of the vaccine associated cases prompted earlier referral, as suggested by the abrupt onset and unusual severity reported in one small case series.[23] A later follow-up could ascertain relatively more unvaccinated than vaccinated patients with onset in 2010-11 with a consequent reduction in the relative risk. The attributable risk, however, could increase as a result of ascertainment of additional vaccinated patients. Our attributable risk estimate of between one in 57 500 and 52 000 doses was lower than reported from Finland (one in 16 000), despite a similar odds ratio/relative risk and annual incidence before vaccine, which was 0.42 per 100 000 in our study (based on the 29 incident cases in 2008) and 0.31 in Finland between 2002 and 2009.[24] This could be because of differences in population susceptibility or because proportionately more vaccine in Finland was given to adolescents, in whom incidence is highest. The same attributable fraction applied to a higher absolute incidence generates a higher attributable risk.

Strengths and weaknesses of our study

Our aim was to conduct a national study in England, and we therefore contacted all sleep centres that see affected children and in addition approached paediatric neurologists to whom such children might have been initially referred. Based on replies to our initial contact in July 2011, we focused on the 16 sleep/neurology centres in England that reported that they had seen affected children with onset since 2008. We did not visit the seven remaining centres that made a negative return, though it is possible that relevant cases were not identified at the time or were referred to them after July 2011. Cases in the hospital episode statistics database that were not in the 16 sleep/neurology centres together accounted for 20% (32/162) of the G47.4 hospital episode statistics diagnoses in England in the study period. Most of these cases would not have been eligible for inclusion judging by the hospital episode statistics cases reviewed at the 16 study centres (where only 35/120 (29%) with available information were eligible). The G47.4 diagnosis code, however, had low sensitivity (as admission is not a necessary part of case management), and it is possible that eligible cases in England were missed. Under the worst case scenario—that, based on the hospital episode statistics diagnoses, only 80% of eligible cases were captured and that those not captured were all in unvaccinated patients—this would add another four unvaccinated cases to the number with onset after October 2009 diagnosed by July 2011 (increasing the total from 17 to 21 in table 3 among those eligible for vaccination at any time before onset). Adding in four cases (one in a risk group) still results in an increased odds ratio of 9.2 (3.1 to 27.2).

Although the case coverage analysis gave a significantly raised odds ratio, the number of cases in patients with onset in 2010 (n=16) was lower than in 2009 (n=21). This deficit was particularly evident for unvaccinated patients; there were six in 2010 compared with 21 in 2009. While delays to diagnosis might partially explain this, based on the distribution of intervals from onset to diagnosis in previous years we might reasonably have expected about seven more unvaccinated children in 2010 to have received a diagnosis by July 2011. The "missing" cases in unvaccinated patients could be just random variation, but to assess the impact of the dearth of unvaccinated patients in 2010 we added seven cases in 2010 with onset dates across the year, one of which was in a patient in a risk group for vaccination. This had the effect of decreasing the odds ratio for vaccination within six months from 16.2 to 8.3 (95% confidence interval 2.2 to 31.5).

The results of our self controlled case series analysis were less clear. This method requires a prespecified risk period after vaccination in which the incidence relative to the baseline incidence is compared.[25] Based on the onsets in the Finnish cases[14] we defined the risk period as within six months. This resulted in the inclusion in the baseline of four patients with symptoms more than six months after vaccination. When more unexposed time was included in the baseline by starting person time from October 2008 the self controlled case series analysis gave results closer to the case coverage estimates. The finding that four of the 11 cases associated with vaccines in our study were in children with onset longer than six months after vaccination could reflect lack of precision in ascertaining onset date or the fact that in our study were included patients with diagnosis in 2011 whereas in the Finnish study follow-up ended in December 2010. Our longer follow-up period would have allowed patients with a later onset to receive a diagnosis. Another assumption of the self controlled case series method that used person time before vaccination is that the narcolepsy condition should not influence whether or not an individual subsequently gets vaccinated. This seems unlikely but could occur if narcolepsy is regarded by some general practitioners as an indication for influenza vaccination or if the symptoms lead to individuals being more or less likely to visit their general practitioner and be offered vaccination opportunistically.

Apart from the inherent problems in conducting timely studies of the association between narcolepsy and exposure to a vaccine first used in late 2009, our study has other potential limitations. There can be difficulty in accurately defining onset of symptoms, which could result in recall bias. Onset dates, however, were obtained from medical records made before the putative association had generated public interest, and the date of first healthcare contact should be objective. Random inaccuracies in defining onset would reduce the estimate of relative risk rather than generating a falsely high estimate. Our case coverage approach depended on the accuracy and representativeness of the Royal College of General Practitioners' coverage data. The patient level data used for the analysis were extracted by established procedures used for estimates of effectiveness of annual influenza vaccine.[26] The Royal College of General Practitioners' population is closely matched to the national population in terms of age, sex, deprivation index, and prescribing patterns,[27] and our coverage estimates by age and risk group status were similar to those in a national coverage survey that provided aggregate data by broad age groups.[12] The case coverage method also depends on the absence of a confounding variable for which coverage could not be stratified. Apart from age and time period, which were adjusted for in the analysis, we are not aware of any other variable that could generate the size of effect observed. Although there is no reported association between having a co-morbidity for which influenza vaccination is recommended and likelihood of subsequently developing narcolepsy, we adjusted for this variable because of its high correlation with vaccination and hence potential to be a confounder. The reduction in odds ratio seen after this adjustment might reflect a true association or be caused by chance. If the association is real then failure to identify whether a vaccinated patient was in a risk group for vaccination could result in spuriously high odds ratios. Under the extreme assumption that all patients were in a risk group, however, there was still an increased odds ratio of 5.0 (1.3 to 19.3) for vaccination within six months before onset. Finally,

our attempt to investigate an association with pandemic influenza was based on a history of influenza-like illness. As a clinical history is not specific, and some infections are asymptomatic, we cannot exclude H1N1 infection as an aetiological factor in some cases. It seems unlikely, however, that previous infection would be more likely in vaccinated patients.

Strengths and weaknesses in relation to other studies

It is difficult to rapidly test the putative association between vaccination and narcolepsy because of the long and variable interval between onset and diagnosis[4] and the considerable potential for underdiagnosis.[28 29] Pandemic vaccine was first used in October 2009 and many patients with onset in 2010 and 2011 will not be yet have a diagnosis. The potential for an accelerated diagnosis in patients in whom an association with vaccination is suspected vaccine once the signal was raised is considerable. We sought to limit this bias by restricting our primary analysis to patients with a diagnosis by July 2011, when reports from Finland and Sweden had not generated media or public interest in the United Kingdom, the first spike in internet searches for "narcolepsy" being in December 2011.

Others have sought to limit ascertainment bias by restricting cases to those with onset or first healthcare contact before media attention.[14 30] As diagnosis is a necessary condition for case capture, however, ascertainment might still be biased because of preferential inclusion of vaccinated patients with accelerated diagnosis after the generation of public interest. Censoring cases by date of diagnosis and using this as the index date for analysing previous vaccine exposure blurs any temporal relation between vaccination and onset because of variable diagnostic delays, and patients vaccinated after onset but before diagnosis will be categorised as "exposed." In our study, as in Finland, risk estimates were substantially lower when we used diagnosis as the index date. An unpublished case-control study that pooled data from five European countries that used the AS03 adjuvanted or other H1N1 pandemic strain vaccine failed to find an association when the multiple sleep latency test date was used as the index date (odds ratio 1.6, 95% confidence interval 0.5 to 6.1), but when they additionally restricted cases to those with symptom onset between April 2009 and June 2010 the odds ratio increased to 4.6 (1.7 to 13.7).[31] As this additional analysis was one of several sensitivity analyses conducted, however, its relevance was perhaps overlooked, resulting in the conclusion that the signal from Finland and Sweden could not be confirmed.[31] In our study, to minimise ascertainment bias and improve precision in defining the risk period after vaccination, we censored case inclusion by date of diagnosis and used symptom onset as the index date for the primary analysis.

Other epidemiological approaches to assessing the association have been adopted. A study in one Swedish county linking a pandemic vaccination register with a healthcare database, while underpowered to investigate the risk of narcolepsy, reassuringly found little evidence of an association with other neurological or autoimmune disorders.[32] Ecological studies that evaluate changes in population incidence of narcolepsy associated with the use of pandemic vaccine have also been reported.[24 33] Establishing causality through such an approach, however, is problematic as other factors can affect the incidence of patients with the diagnosis. Also, unless vaccine coverage is high, as in Finland,[24] an increase might be difficult to detect at the population level. A recent study derived a pooled incidence estimate from automated healthcare databases in six European countries to monitor changes associated with the use of pandemic vaccines.[33] Estimates of baseline incidence, however, varied widely between countries, probably reflecting differences in case capture between databases, and significant increases and decreases in incidence in individual countries unrelated to vaccine use were observed.

Policy implications and future research

In conclusion, we found evidence of an increased risk of narcolepsy in children who received pandemic A/H1N1 2009 influenza vaccine (Pandemrix) in England. Despite attempts to minimise ascertainment bias, the potential for overestimation of risk remains because of more rapid referral of vaccinated patients. Long term follow-up of the cohorts exposed to the vaccine is needed to properly evaluate the attributable risk.

As a precaution, based on the preliminary reports from Sweden and Finland and pending the outcome of confirmatory studies, in July 2011 the European Medicines Agency changed the indication for use of Pandemrix vaccine in people aged under 20 to those for whom seasonal trivalent vaccine was not available and for whom prevention of A/H1N1 2009 influenza was considered necessary.[34] Its licence, however, remains valid, and the vaccine can still be manufactured and sold in any European Union country. While further use of the AS03 adjuvanted vaccine for prevention of seasonal A/H1N1 2009 seems unlikely, our findings have implications for the future licensure and use of AS03 adjuvanted pandemic vaccines containing different subtypes such H5 or H9. Further studies to assess the risk, if any, associated with the other A/H1N1 2009 vaccines used in the pandemic, including those with and without adjuvants, are also needed to inform the use of such vaccines in the event of a future pandemic.

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Contributors: All the authors were involved in study design. JS, CV, AMW, and LS extracted clinical information from the centre notes. JS extracted the HES cases and conducted the GP follow-up. JSh assisted in recruitment of sleep centres and was a member of the expert panel. NA conducted the statistical analysis, and EM wrote the first draft of the paper. All authors contributed to the final version and had access to the dataset.

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What is already known on this topic

A potential association between ASO3 adjuvanted A/H1N1 2009 pandemic vaccine (Pandemrix) and narcolepsy was first identified in Scandinavian countries after clinicians in sleep centres reported temporal associations

An epidemiological study from Finland reported a 13-fold increased risk in children and young people aged 4-19

There is a need for a robust study to independently test the association in a non-Scandinavian country where no signal has been raised by clinician reports

What this study adds

The increased risk of onset of narcolepsy in children and young people after the AS03 adjuvanted pandemic vaccine is not confined to Scandinavian populations

The magnitude of the increased risk found in English children and young people is similar to that reported from Finland

the previous three years; no other relationships or activities that could appear to have influenced the submitted work.

Ethical approval: HPA has approval for England from the National Information Governance Board for Health and Social Care (NIGB) (PIAG ref: PIAG 03-(c)/2001), which allows us access to patient identifiable information for purposes of monitoring vaccine safety.

Data sharing: No additional data available.

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Tables

Table 1| Coverage of vaccination with ASO3 adjuvanted pandemic A/H1N1 2009 vaccine by August 2010* in England by age and risk group, and total doses based on RCGP age specific coverage estimates

Age in years (September 2009)	% Coverage overall	% Coverage in risk groups	% Coverage in non-risk groups	Population of England	Estimated No of people vaccinated in England
2	31.9	46.0	31.2	639 700	204 086
3	29.2	46.1	28.2	619 800	181 119
4	20.5	40.6	18.9	605 800	124 324
5	4.7	34.0	2.1	597 800	28 277
6	4.1	30.6	1.7	576 700	23 771
7	4.4	30.7	1.5	559 400	24 393
8	4.4	30.3	1.7	557 800	24 277
9	4.4	30.2	1.7	571 500	24 948
10	4.6	31.0	1.9	586 600	26 999
11	4.6	28.5	1.9	596 800	27 747
12	4.7	29.4	2.0	612 300	28 958
13	4.9	29.1	2.1	608 700	29 623
14	4.3	24.6	1.9	612 100	26 476
15	4.5	25.6	2.2	628 700	28 472
16	4.0	22.6	1.9	640 900	25 336
17	3.6	20.8	1.8	666 900	24 093
18	2.9	15.5	1.7	686 400	19 634
Total aged 2-4	27.3	43.9	26.2	1 865 300	509 529
Total aged 5-18	4.3	27.1	1.9	8 502 600	363 004

*About 200 000 doses of pandemic vaccine were given in winter of 2010-11[21] but no age or risk group specific coverage data were available.

Table 2| Demographic and clinical features of 75 patients with narcolepsy in cases included in analysis according to ASO3 adjuvanted pandemic A/H1N1 2009 vaccination

Category	Never vaccinated	Vaccinated after first symptoms	Vaccinated before first symptoms	Total
Age at diagnosis (years):				
4-8	18	3	7	28
9-13	21	1	2	24
14-18	21	0	2	23
Sex:				
Male	33	2	8	43
Female	27	2	3	32
Diagnostic category:				
Narcolepsy and cataplexy	42	4	10	56
Narcolepsy, no cataplexy	9	0	1	10
Probable narcolepsy	9	0	0	9
Risk group for influenza vaccine:				
No	49	2	5	56
Yes	11	2	6	19
2010-11 seasonal vaccine given:				
No	56	3	9	68
Yes (before symptoms)	1	0	0	1
Yes (after symptoms)	3	1	2	6

Table 3| Case coverage analysis in patients with narcolepsy showing odds ratios for receipt of ASO3 adjuvanted pandemic A/H1N1 2009 vaccine before narcolepsy using different index dates, follow-up periods, and risk intervals

Interval before index date	No of patients vaccinated	*Total No of patients eligible for vaccination in interval before index date	Not matching on risk group		Matching on risk group	
			Average coverage	Odds ratio (95% CI)	Average coverage	Odds ratio (95% CI)
Index date: symptom onset						
Censored July 31 2011†:						
12 weeks	5	10	0.060	34.7 (7.4 to 163.7)	0.098	18.4 (3.7 to 91.6)
6 months	6	10	0.072	33.1 (8.1 to 135.7)	0.151	16.2 (3.1 to 84.5)
Any time	10	17	0.089	22.2 (7.9 to 62.1)	0.160	14.4 (4.3 to 48.5)
Censored at key visit†:						
12 weeks	5	12	0.051	30.8 (7.1 to 134.2)	0.082	17.8 (3.7 to 86.3)
6 months	6	13	0.060	23.2 (6.5 to 82.0)	0.119	12.5 (2.9 to 53.1)
Any time	11	26	0.083	11.0 (4.8 to 25.4)	0.132	8.3 (3.1 to 22.3)
Index date: first healthcare contact						
Censored July 31 2011†:						
6 months	7	24	0.049	12.7 (4.6 to 34.8)	0.094	6.7 (2.1 to 21.0)
Any time	10	32	0.067	8.4 (3.7 to 19.1)	0.124	4.7 (1.9 to 11.8)
Censored at key visit†:						
6 months	7	26	0.045	12.5 (4.5 to 34.1)	0.087	6.7 (2.1 to 20.8)
Any time	11	42	0.067	6.3 (3.0 to 13.4)	0.112	4.0 (1.7 to 9.3)
Index date: diagnosis						
Censored July 31 2011†:						
Any time	12	44	0.072	5.9 (2.9 to 12.0)	0.129	3.3 (1.5 to 7.4)
Censored at key visit†:						
Any time	14	55	0.071	5.4 (2.8 to 10.2)	0.122	3.2 (1.6 to 6.8)

*As almost all pandemic vaccine was given by end of April 2010 cases were excluded from within 12 weeks and within 6 month analysis if index date was after August 2010 and November 2010, respectively, leading to exclusion of one case vaccinated in 2011.

†Censoring date for inclusion by diagnosis.

Table 4| **Relative incidence estimates and 95% confidence intervals for onset of narcolepsy in different periods after vaccination with ASO3 adjuvanted pandemic A/H1N1 2009 vaccine using self controlled case series analysis**

Analysis	Period of risk after vaccination (days)	Cases*	Relative incidence (95% CI) adjusted for period
Symptoms Oct 2009 to Dec 2010	0-84	5	2.9 (0.6 to 12.9)
Diagnosed by key visit	0-182	6	1.4 (0.3 to 6.4)
Symptoms Oct 2009 to Dec 2010	0-84	5	2.3 (0.5 to 11.0)
Diagnosed by July 2011	0-182	6	1.4 (0.2 to 7.5)
Symptoms Oct 2008 to Dec 2010	0-84	5	7.1 (1.7 to 29.3)
Diagnosed by key visit	0-182	6	5.2 (1.3 to 20.2)
Symptoms Oct 2008 to Dec 2010	0-84	5	10.1 (2.2 to 46.3)
Diagnosed by July 2011	0-182	6	9.9 (2.1 to 47.9)

*Excludes one vaccinated case with onset within three months who received pandemic vaccine after December 2010 when residual stocks were used in place of seasonal influenza vaccine.

Figures

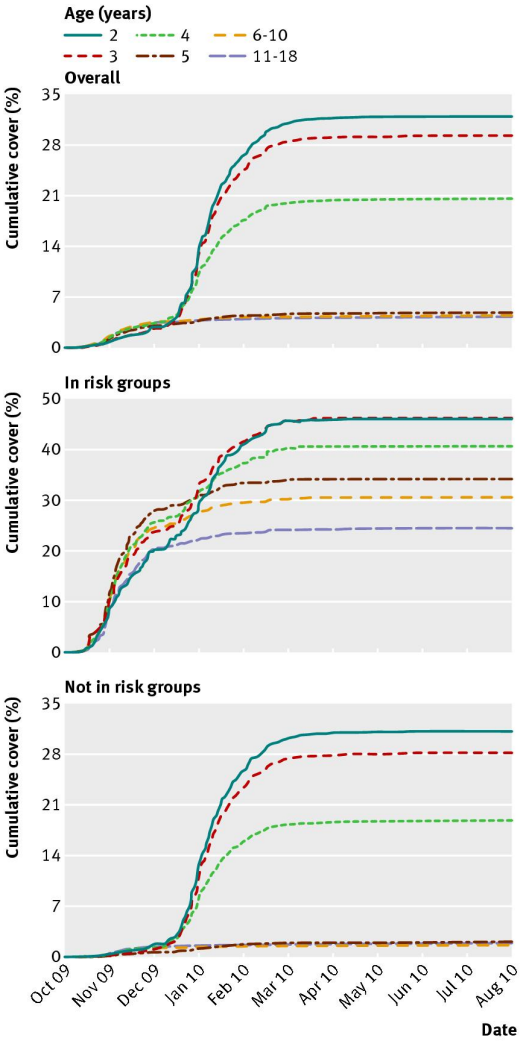

Fig 1 Cumulative population uptake by day of pandemic A/H1N1 2009 influenza vaccine by age at September 2009 and risk group status

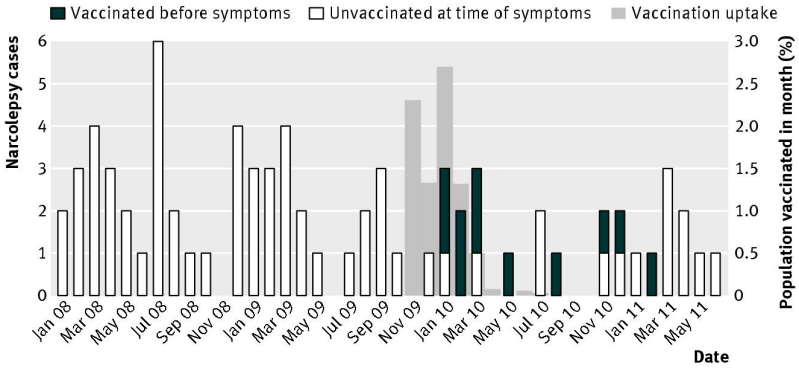

Fig 2 Number of cases of narcolepsy by month and year of onset according to vaccination status at onset. Also shown is population vaccine coverage with pandemic vaccine

EXHIBIT 174

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Risk of Narcolepsy after AS03 Adjuvanted Pandemic A/H1N1 2009 Influenza Vaccine in Adults: A Case-Coverage Study in England

Julia Stowe, BA (Hons)[1]; Nicholas Andrews, PhD[2]; Christopher Kosky, MBBS[3]; Gary Dennis, MD[4]; Sofia Eriksson, PhD[5]; Andrew Hall, MBBChir[6]; Guy Leschziner, PhD[7]; Paul Reading, PhD[8]; John M. Shneerson, DM[9]; Katherine Donegan, PhD[10]; Elizabeth Miller, FRCPath[11]

[1]Research Fellow, Public Health England, London, UK; [2]Senior Statistician, Public Health England, London, UK; [3]Consultant Physician, Guy's and St Thomas' NHS Trust, London, UK; [4]Consultant Neurologist, Royal Hallamshire Hospital, Sheffield, UK; [5]Consultant Neurologist, National Hospital for Neurology and Neurosurgery, London, UK; [6]Consultant in Anaesthesia, Intensive Care and Sleep Disorders Medicine, University Hospitals of Leicester NHS Trust, Leicester, UK; [7]Consultant Neurologist/Clinical Lead – Sleep, Guy's and St Thomas' NHS Trust, London, UK; [8]Consultant Neurologist, South Tees NHS Trust, Middlesborough, UK; [9]Consultant Physician, Papworth Hospital NHS Foundation, Cambridge, UK; [10]Pharmacoepidemiology Research and Intelligence Unit, Medicines and Healthcare Products Regulatory Agency, UK; [11]Consultant Epidemiologist, Public Health England, London, UK

Study Objectives: An increased risk of narcolepsy has been observed in children following ASO3-adjuvanted pandemic A/H1N1 2009 (Pandemrix) vaccine. We investigated whether this risk extends to adults in England.

Methods: Six adult sleep centers in England were visited between November 2012 and February 2014 and vaccination/clinical histories obtained from general practitioners. Suspected narcolepsy cases aged older than 17 y were selected. The risk of narcolepsy following Pandemrix was calculated using cases diagnosed by the time of the center visits and those with a diagnosis by November 30, 2011 after which there was increased awareness of the risk in children. The odds of vaccination in cases and in matched population data were compared using a case-coverage design.

Results: Of 1,446 possible cases identified, most had onset before 2009 or were clearly not narcolepsy. Of the 60 remaining cases, 20 were excluded after expert review, leaving 40 cases with narcolepsy; 5 had received Pandemrix between 3 and 18 mo before onset. All the vaccinated cases had cataplexy, two received a diagnosis by November 2011 and two were aged 40 y or older. The odds ratio for vaccination in cases compared to the population was 4.24 (95% confidence interval 1.45–12.38) using all cases and 9.06 (1.90–43.17) using cases with a diagnosis by November 2011, giving an attributable risk of 0.59 cases per 100,000 doses.

Conclusions: We found a significantly increased risk of narcolepsy in adults following Pandemrix vaccination in England. The risk was lower than that seen in children using a similar study design.

Keywords: adult, case-coverage, narcolepsy, Pandemrix, vaccination

Citation: Stowe J, Andrews N, Kosky C, Dennis G, Eriksson S, Hall A, Leschziner G, Reading P, Shneerson JM, Donegan K, Miller E. Risk of narcolepsy after AS03 adjuvanted pandemic A/H1N1 2009 influenza vaccine in adults: a case-coverage study in England. *SLEEP* 2016;39(5):1051–1057.

Significance

Our study shows that the causal association between narcolepsy and the oil-in-water adjuvanted pandemic H1N1 influenza vaccine is not, as previously thought, confined to children and adolescents and will add further impetus to the research into the etiology of this condition. While possession of the DQB1*06:02 gene is clearly implicated, environmental or other triggers appear to be necessary to instigate the onset in susceptible individuals. Further surveillance of populations who have received pandemic strain vaccines is needed in order to document whether the association is seen with other products and to provide insights into the likely auto-immune pathway by which the oil-in-water adjuvant and/or the viral antigens in the HIN1 pandemic strain trigger the pathological process that results in loss of orexin-producing neurons.

INTRODUCTION

Narcolepsy is a disabling and chronic sleep disorder characterized by excessive daytime sleepiness, hypnagogic hallucinations, sleep paralysis, and cataplexy. Narcolepsy is divided into narcolepsy with cataplexy (type 1) and narcolepsy without cataplexy (type 2).[1] Cataplexy is a unique symptom in which there is transient loss of skeletal muscle tone, with preservation of consciousness that is triggered by emotions such as laughter or anger.

The prevalence of narcolepsy with cataplexy is between 25 and 50 per 100,000 people with an incidence of around 0.74 per 100,000 person-years.[2] Onset usually occurs between 15 and 40 y of age and symptoms develop gradually, so time from onset to diagnosis can be many years. Both environmental and genetic factors play a role in its etiology. There is a strong association with the HLA DQB1*06:02 genotype, but this alone is not sufficient for the disease to develop. Narcolepsy is associated with specific loss of cells producing the neuropeptide hypocretin, resulting in low levels of hypocretin in the cerebrospinal fluid.

An H1N1 ASO3-adjuvanted pandemic vaccine (Pandemrix, GlaxoSmithKline Biologicals, Wavre, Belgium) was used in the United Kingdom (UK) from October 2009, initially for people comprising a seasonal influenza vaccine risk group[3] or health or social care workers, followed by children younger than 5 y from November 2009 onward.[4] Approximately 5.5 million people in the UK were vaccinated with Pandemrix.[5] It was the predominant H1N1 vaccine used within the European Union.[6] In August 2010 concerns were raised in Finland and Sweden about a possible association between narcolepsy and Pandemrix. A cohort study in Finland reported a 13-fold increased risk of narcolepsy following Pandemrix in children aged 4 to 19 y.[7] This was confirmed by a study in sleep centers in England, which identified a 14-fold increased risk in those aged 4–18 y.[8] Other studies subsequently published from Ireland and Norway also indicated an increased risk of narcolepsy in children who received Pandemrix.[9,10]

The initial signal in the Scandinavian countries was in children but more recently adult cases have been reported. A small case-control study in 25 adults in France suggested an elevated risk[11] as did a follow-up study in Finland published as an online report.[12] A record linkage cohort study in Sweden found no overall increased risk in adults, although there was

a marginally elevated risk in those aged 21–30 y.[13] Using the same published methodology as the childhood study in England,[8] we investigated whether there was an increased risk of narcolepsy in adults who received Pandemrix.

METHODS

Case Ascertainment and Validation

The sleep centers in England where the largest numbers of cases of narcolepsy are diagnosed were identified through the Hospital Episode Statistic (HES) database.[14] HES episodes in those age 16 y and older with an ICD 10 code of G474 in any diagnosis field were extracted for the period January 2009 to December 2012. Six sleep centers were identified as being the major centers that together covered 33% of the narcolepsy coded episodes in HES during this period. We estimated that within these centers approximately 30 cases may be seen with onsets from 2010 which should give sufficient power to detect at least a fivefold increased risk (80% power, 5% significance level, 5% vaccine uptake).

The six centers were visited between November 2009 and February 2010 (Table S1, supplemental material) and all those aged 16 y and older at the time of diagnosis were ascertained with the aim to include those aged 18 y and olderon September 1, 2009. These cases were found by searching local databases and electronic clinic letters for the keyword *narco* or searching for multiple sleep latency test (MSLT) reports for a diagnosis of narcolepsy. The cases from HES and those identified from the local searches were then merged and deduplicated using National Health Service (NHS) number or surname and date of birth. These potential cases were reviewed using medical records to establish symptom onset details, clinical history, and sleep study results. If any information was missing from the electronic records, the case notes were reviewed to identify the relevant information.

Details of the anonymized cases collated at center visits were evaluated by a review panel (authors GL, JShn, AH, SE) who were blinded to vaccination status. To expedite the review, cases with a clear history of excessive daytime sleepiness (EDS) and cataplexy or EDS with a positive MSLT or cerebrospinal fluid positive for narcolepsy were not all sent to the panel for review; rather, a few examples of these cases were first shown to the panel for their agreement. The four sleep center consultants on the review panel categorized each case as definite narcolepsy with cataplexy; definite narcolepsy without cataplexy; probable narcolepsy and insufficient evidence to confirm a diagnosis of narcolepsy. The panel based their diagnosis on the International Classification of Sleep Disorders, Second Edition (ICSD-2) criteria.[15] A diagnosis based on the consensus view of three of the four panel members was taken, with remaining cases discussed by teleconference.

Pandemrix vaccination histories for cases with definite or probable narcolepsy were obtained from the patient's general practitioner (GP) who was asked for date and batch number of any pandemic vaccine given, the date of first symptoms and/or first consultation for narcolepsy symptoms, presenting symptoms, history of pandemic influenza illness, and whether the patient was in a clinical risk group for which pandemic strain H1N1 vaccine was recommended.

Index Dates: Definitions

The date of symptom onset was defined as the earliest date of EDS or cataplexy as given by the GP or recorded in the sleep center notes or referral letters. When the exact date was not available we used the midpoint of the month of the approximate date and also approximated an earliest and latest date of onset for sensitivity analysis. The date of first known health care contact was the earliest recorded consultation for a sleep related problem as reported by the GP or in the center notes. The date of diagnosis was the date when there was either a clinical history and sleep study confirming narcolepsy or sufficient clinical information to diagnose probable narcolepsy.

Statistical Analysis

We assessed the association between vaccination and narcolepsy using the case coverage method[16] in which the odds of vaccination in cases is compared to the odds of vaccination in matched population data. The analysis is by logistic regression with the outcome as vaccinated (yes/no) in the cases and with an offset for the log odds of the matched coverage. Population vaccine coverage was calculated from the Clinical Practice Research Datalink (CPRD).[17] We used patient-level data to derive cumulative coverage stratified by exact date (from September 2009 to March 2011), age on January 1, 2010 (categorized as 18, 19, 20–24, 25–29, …, ≥ 80 years) and, when matching by risk group, being in a vaccine target clinical risk group. This was then used to look up the appropriate matched coverage for each narcolepsy case based on their age, risk group status (if matching on risk group) and narcolepsy index date (e.g. date of onset). To determine vaccine coverage within 6 mo of an index date, the coverage 6 mo earlier was subtracted from the matched coverage on the index date. Patients were categorized as being in a risk group if there was any clinical code denoting chronic heart disease, chronic kidney disease, chronic obstructive pulmonary disease, diabetes, chronic liver disease, immunological disorders, multiple sclerosis, or stroke/transient ischemic attack in the 5 y prior to September 2009 for the 2009–2010 vaccination season and September 2010 for the 2010–2011 season. We used similar criteria for allocating narcolepsy cases to a risk group based on the information provided by the GP on clinical conditions considered high risk for influenza.

The primary analysis was restricted to cases diagnosed by November 30, 2011 after which there was increased awareness of the risk seen in children with the potential for accelerated diagnosis in vaccinated cases. It also used first symptoms as the index date and the odds of vaccination at any time before onset. Additional analyses were performed using first health care contact and diagnosis as the index date, all cases diagnosed by the center visit date, not matching coverage by risk group status and calculating the odds of vaccination within 6 mo of the index date. Stratification by age younger than 30 y and age 30 y and older on September 1, 2009 was also done. Sensitivity analyses in which population coverage was increased or decreased by a relative 20% (for example, 10% coverage decreasing to 8% or increasing to 12%) and using the earliest and latest estimated onset dates were also conducted. These analyses were documented in a statistical analysis plan prior to receipt of the data

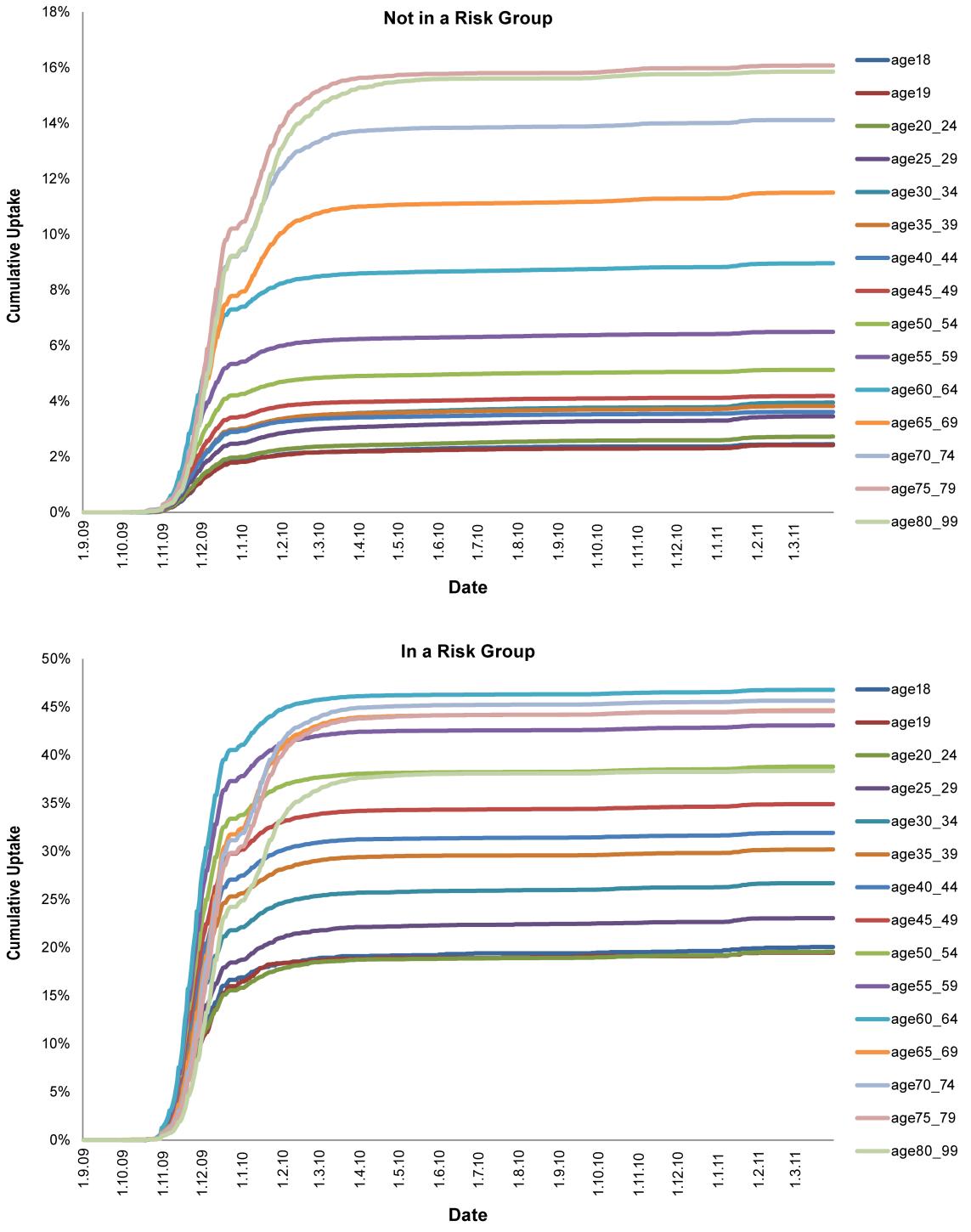

Figure 1—Vaccine uptake by age, risk group, and period from the Clinical Practice Research Datalink (CPRD) by clinical risk group status.

by the statistician (NA) for analysis. Analysis was done using Stata version 13 (StataCorp, College Station, TX).

RESULTS

Vaccine Coverage

Coverage data were obtained from approximately 3.5 million patients aged 18–99 y registered in the CPRD practices on September 1, 2009. Vaccination coverage was low in healthy young adults and increased with age. As expected for those in a risk group, uptake was higher and also increased with age (Figure 1). Most vaccination was during 2009–2010 with only small increases in 2010–2011, which is in agreement with other data.[8]

Study Cases

A total of 2,554 potential patients were identified through the different search strategies and data sources. When cross

Table 1—Demographic features and clinical features of 40 patients with narcolepsy according to ASO3 adjuvanted pandemic A/H1N1 2009 vaccination.

Factor	Level	Unvaccinated	Vaccinated before Onset	Total
Age at September 2009 (years)	18–19	5	1	6
	20–24	7	2	9
	25–29	5	0	5
	30–34	4	0	4
	35–39	3	0	3
	40–44	7	1	8
	45–49	2	0	2
	50–54	1	1	2
	≥ 55	1	0	1
Sex	Male	14	1	15
	Female	21	4	25
Diagnostic category	Narcolepsy with cataplexy	23	5	28
	Narcolepsy without cataplexy	8	0	8
	Probable narcolepsy	4	0	4
HLA DQB1*06:02	Positive	11	2	13
	Negative	3	0	3
	Not known	21	3	24
Comorbidity	No	32	2	34
	Yes	1	3	4
	Not known	2	0	2
Seasonal vaccine before onset (and from 2008/2009)	No	33	2	35
	Yes (before symptoms)	1	2	3
	Not known	1	1	2

HLA, human leukocyte antigen.

referenced and de-duplicated 1,446 patients remained and were taken forward for case note review (Table S1). The majority, 926, had symptom onset before 2009 and 441 clearly did not have narcolepsy when the notes were reviewed; these 1,367 cases were excluded. The case notes of 10 could not be traced and one person was seen in two centres. Of the remaining 68 patients 30 were considered definite cases after reviewing the available information and 38 were sent to the panel for review. The panel members were in initial agreement on 28, with agreement reached after teleconference for the remaining 10. Twenty cases were categorized as not narcolepsy/insufficient evidence and excluded with the remaining 18 cases added to the 30 definite cases. Of the 48 cases, 8 were not included in the final analysis because although age 18 y or older at diagnosis they were younger than 18 y on September 1, 2009. This left a total of 40 adults with narcolepsy of whom 28 were categorized as definite narcolepsy with cataplexy, 8 as definite narcolepsy without cataplexy, and 4 probable narcolepsy.

Four individuals were reported to have an influenza-like illness prior to first symptoms, although only one within 3 mo of symptoms; none of these four cases was vaccinated.

Vaccination History

We obtained vaccination history on all 40 cases and risk group status for 38 (Table 1). Five patients had received Pandemrix prior to first symptoms of whom three were in a clinical risk group recommended for vaccination; all five had cataplexy. One had onset within 3 mo, two within 3 to 6 mo, and two between 7 and 18 mo after vaccination; two had a confirmed human leukocyte antigen (HLA) DQB1*06:02 genotyping, with the other three not tested.

Figure 2 shows the timing of onset for the 40 adult narcolepsy cases by vaccination status and monthly vaccine uptake in the age-matched population. The first vaccinated case had onset in early 2010 and the latest in 2012 after receiving Pandemrix in 2011 when residual stocks were used instead of seasonal vaccine.[18] Mean time from onset to diagnosis using cases with onset in 2009–2011 and diagnosis within 30 mo was 493 days in four vaccinated cases and 434 in 28 nonvaccinated cases (P = 0.69, Kruskal-Wallis test).

Case Coverage Analysis

The primary analysis, which used symptom onset, cases with a diagnosis by November 30, 2011 and matching on risk group, only included two of the five vaccinated cases but showed an elevated odds ratio of 9.06 (1.90–43.17) (Table 2). When including all cases ascertained by the date of the centre visit (five vaccinated cases) the odds ratio was lower but still significant at 4.24 (1.45–12.38). Higher odds ratios (but fewer vaccinated cases) were seen when including only cases with onset within 6 mo of vaccination. When other outcome dates were used such as date of first healthcare contact or date of diagnosis, the odds ratios reduced and some became nonsignificant (Table 2). The sensitivity analyses and age stratification were based on

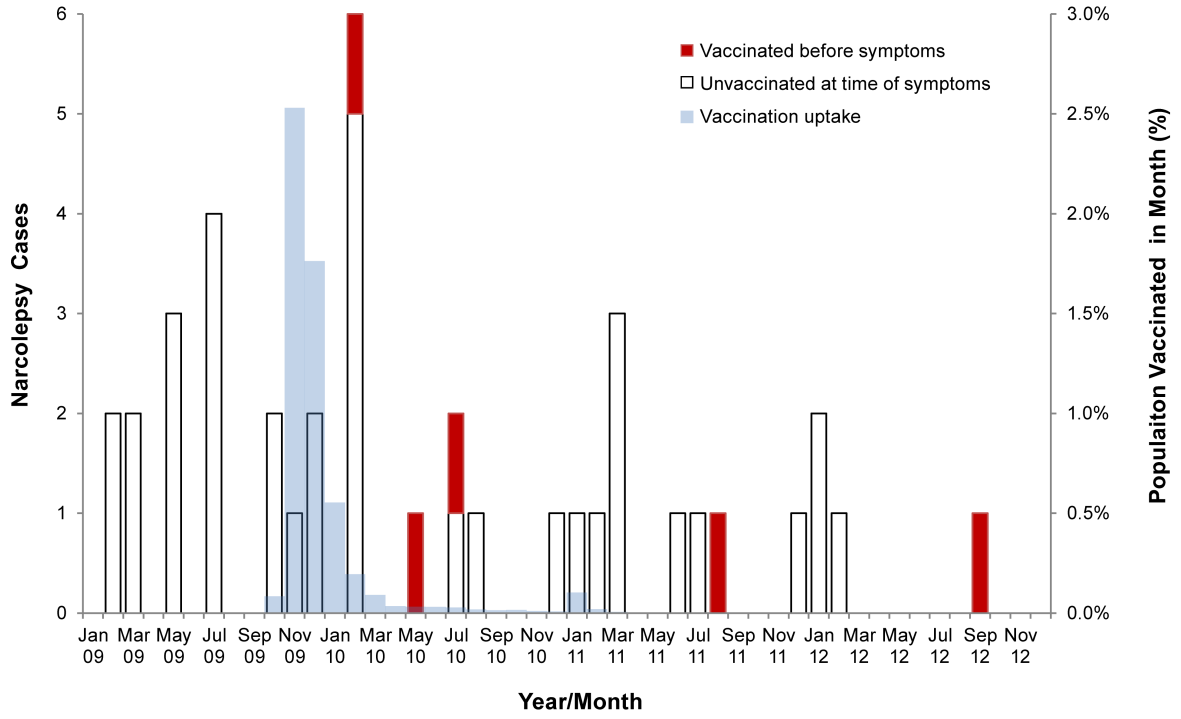

Figure 2—Timing of onset for the 40 adult narcolepsy cases by vaccination status and monthly vaccine uptake in the age matched population.

Table 2—Case coverage analysis in patients with narcolepsy showing odds ratios for receipt of ASO3 adjuvanted pandemic A/H1N1 2009 vaccine before narcolepsy onset using different index dates, follow-up periods, and risk intervals.

Censoring Date for Inclusion by Diagnosis	Interval before Index Date	Number of Patients Vaccinated	Total Patients Eligible for Vaccination in Interval Before Index	Not Matching on Risk Group		Matching on Risk Group	
				Average Coverage	Odds Ratio (95% CI)	Average Coverage	Odds Ratio (95% CI)
USING FIRST SYMPTOMS							
Nov 30, 2011	6 months	2	10	0.026	11.29 (2.05–62.05)	0.016	17.94 (3.34–96.23)
	Any time	2	10	0.043	5.77 (1.02–28.14)	0.027	9.06 (1.90–43.17)
Center visit	6 months	3	22	0.019	9.64 (2.54–36.57)	0.014	12.74 (3.43–47.26)
	Any time	5	27	0.047	4.74 (1.77–12.67)	0.063	4.24 (1.45–12.38)
USING FIRST HEALTH CARE CONTACT							
Nov 30, 2011	6 months	1	12	0.017	6.10 (0.65–57.10)	0.011	9.72 (1.06–88.79)
	Any time	2	13	0.044	4.09 (0.89–18.89)	0.028	6.40 (1.40–29.37)
Center visit	6 months	1	17	0.014	5.16 (0.58–45.73)	0.009	8.05 (0.93–69.76)
	Any time	5	33	0.049	3.54 (1.35–9.27)	0.058	3.37 (1.20–9.48)
USING DATE OF DIAGNOSIS							
Nov 30, 2011	6 months	0	14	0.028	0	0.016	0
	Any time	2	19	0.056	2.03 (0.45–9.14)	0.035	3.32 (0.75–14.66)
Center visit	6 months	0	14	0.028	0	0.016	0
	Any time	5	40	0.054	2.54 (0.98–6.59)	0.057	2.64 (0.97–7.20)

CI, confidence interval.

all cases diagnosed by the center visit date to increase power (Table 3). Results were similar when allowing for uncertainty in the onset date and remained significant when increasing coverage by a relative 20%. Odds ratios were similar for those younger than 30 y and older individuals, but the number of cases in each age group was small.

Table 3—Sensitivity analysis and age stratification using vaccination at any time prior to first symptoms and all cases diagnosed by the center visit date.

Analysis	Number of Patients Vaccinated prior to First Symptoms	Total Patients Eligible for Vaccination prior to First Symptoms	Average Coverage Matching on Risk Group	Odds Ratio (95% CI)
Best estimate of onset date	5	27	0.063	4.24 (1.45–12.38)
Earliest onset date	5	23	0.066	5.25 (1.72–16.02)
Latest onset date	5	28	0.061	4.13 (1.42–12.00)
Coverage reduced by relative 20%	5	27	0.050	5.42 (1.87–15.73)
Coverage increased by relative 20%	5	27	0.075	3.45 (1.18–10.14)
Age 18–29 y on September 1, 2009	3	16	0.059	4.36 (1.11–17.17)
Age 30 y or older on September 1, 2009	2	11	0.067	4.07 (0.73–22.63)

CI, confidence interval.

Attributable Risk

The calculation for the vaccine-attributable risk used the odds ratio of 4.24 based on symptom onset at any time (Table 2). Using the odds ratio to approximate relative risk (RR), the attributable fraction ((RR−1)/RR)) is (3.24/4.24), which applied to the five vaccinated patients in the analysis gives an estimate of 3.82 attributable cases. HES data indicate that the sleep centers visited provided a diagnosis for approximately 33% of the narcolepsy cases in England in the study period, giving an estimated total of 3.82/0.33 = 11.6 attributable cases in England. Counting pandemic vaccine doses administered to those aged 18–59 y gives a total of 1,975,000 based on the final cumulative uptake and the Office for National Statistics population data for England in 2009.[19] The attributable risk is therefore 11.6/1,975,000 = 0.59 per 100,000 doses

DISCUSSION

We found a significantly increased risk of narcolepsy in adults following AS03 adjuvanted pandemic strain vaccine in England. The odds ratio in adults was 9.06 (1.90–43.17) in the primary analysis and 4.24 (1.45–12.38) using all cases with a diagnosis by the date of the sleep centre visit, with an estimated attributable risk 0.59 per 100,000 doses. This risk is lower than we found in children where the comparable odds ratios were 14.4 (4.3 to 48.5) and 8.3 (3.1 to 22.3) respectively, and attributable risk of 1.74 cases per 100,000 doses.[8] As in the Finnish adult study,[12] the risk was highest within 6 mo of vaccination with an odds ratio of 12.74 (3.43–47.26).

The mechanism by which narcolepsy with cataplexy is associated with Pandemrix is not known. HLA DQB1*06:02 is present in 95% of patients with narcolepsy with cataplexy (type 1).[20,21] In this study, all five vaccinated narcolepsy patients developed narcolepsy with cataplexy. The two tested patients were positive for HLA DQB1*06:02. It is possible that Pandemrix provides a second hit in those patients with a genetic vulnerability to the development of narcolepsy with cataplexy. Pandemrix may result in the development or augmentation of autoantibodies to hypocretin-producing cells and the destruction of these cells results in the development of narcolepsy with cataplexy. Others have speculated on autoimmunity as a mechanism to explain the link between narcolepsy and Pandemrix.[21] As with the pediatric study in England,[8] there was no evidence that prior swine influenza infection was a risk factor, with only one study case reporting influenza-like-illness in the 3 mo prior to their narcolepsy symptoms. Recent research, however, suggests that vaccine-induced narcolepsy may be associated with the induction of antibodies to the H1N1 nucleoprotein of the Pandemrix strain that cross-react with hypocretin receptors.[22,23]

Our odds ratio for the primary analysis is lower than found in the French case control study which reported an odds ratio of 16.8 (1.9–149.1) for cases aged 18 years and over using symptom onset as the index date.[11] In that study 28% of eligible cases declined to participate and onset date was based on patient recall, allowing the potential for participation and recall bias which would likely lead to an overestimate of the association. In the Swedish record linkage study, which failed to find an elevated risk in those aged 20 y and older,[13] the narcolepsy diagnosis was not verified and the index date was date of diagnosis, which would likely underestimate the association. In our study, cases were verified by an expert panel according to ICSD-2 diagnostic criteria, and onset date was independently obtained from referral letters, hospital notes, and GP records. Based on this information, we defined the earliest and latest possible date of first symptoms; odds ratios generated with these extreme dates were similar to the odds ratio using the most likely onset date.

To ensure as complete case ascertainment as possible, cases were identified by actively searching local electronic patient records and databases and cross-checking with cases in the national hospital database. This approach should avoid selection bias arising from differential ascertainment of diagnosed cases in vaccinated and unvaccinated individuals, as might occur if reliant on clinician recall. In the primary analysis, data were censored to only include cases diagnosed by November 30, 2011 to limit potential bias from accelerated diagnosis in patients in whom an association with vaccination was suspected once the association had generated media interest in December 2011.[8] We found that the odds ratio using cases diagnosed by the center visit date was lower than that using cases diagnosed by November 30, 2011 rather than higher, which might have occurred if there was a tendency for more rapid diagnosis of vaccinated cases after the association was publicized.

Our case-coverage approach relies on the representativeness of the coverage data used. In this study we used information

from the CPRD, a different GP dataset than we used in the pediatric narcolepsy study.[8] It was reassuring that age- and risk group–specific coverage estimates were similar in both GP datasets (data not shown) and were comparable to national coverage data.[17] The sensitivity analysis showed that even if we have underestimated coverage by as much as a relative 20% (for example, due to vaccination given outside of general practice not getting on the record) the association would still be significant, odds ratio 3.45 (1.18–10.14) for vaccinated at any time before onset.

In conclusion, we found evidence of an increased risk of narcolepsy in adults following AS03 adjuvanted pandemic strain vaccine in England. We were unable to define how the risk varied with age due to the relatively small numbers of cases. However, the data do not suggest a threshold age above which the risk is zero as vaccine-associated cases were identified across the age range studied. Further studies in collaboration with other European countries that used Pandemrix may help to more accurately define the age-specific risk in adults.

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Address correspondence to: Julia Stowe, BA (Hons), Public Health England, 61 Colindale Avenue, London NW9 5EQ; Email: Julia.Stowe@phe.gov.uk

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EXHIBIT 175

Centers for Disease Control and Prevention
CDC 24/7: Saving Lives, Protecting People™

Arthritis

Rheumatoid Arthritis (RA)

What is rheumatoid arthritis (RA)?

Rheumatoid arthritis, or RA, is an autoimmune and inflammatory disease, which means that your immune system attacks healthy cells in your body by mistake, causing inflammation (painful swelling) in the affected parts of the body.

RA mainly attacks the joints, usually many joints at once. RA commonly affects joints in the hands, wrists, and knees. In a joint with RA, the lining of the joint becomes inflamed, causing damage to joint tissue. This tissue damage can cause long-lasting or chronic pain, unsteadiness (lack of balance), and deformity (misshapenness).

RA can also affect other tissues throughout the body and cause problems in organs such as the lungs, heart, and eyes.

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What are the signs and symptoms of RA?

With RA, there are times when symptoms get worse, known as flares, and times when symptoms get better, known as remission.

Signs and symptoms of RA include:

- Pain or aching in more than one joint.
- Stiffness in more than one joint.
- Tenderness and swelling in more than one joint.
- The same symptoms on both sides of the body (such as in both hands or both knees).
- Weight loss.
- Fever.
- Fatigue, or tiredness.
- Weakness.

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What causes RA?

RA is the result of an immune response in which the body's immune system attacks its own healthy cells. The specific causes of RA are unknown, but some factors can increase the risk of developing the disease.

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What are the risk factors for RA?

Researchers have studied a number of genetic and environmental factors to determine if they change person's risk of developing RA.

Characteristics that increase risk

- **Age**. RA can begin at any age, but the likelihood increases with age. The onset of RA is highest among adults in their sixties.
- **Sex.** New cases of RA are typically two-to-three times higher in women than men.
- **Genetics/inherited traits**. People born with specific genes are more likely to develop RA. These genes, called HLA (human leukocyte antigen) class II genotypes, can also make your arthritis worse. The risk of RA may be highest when people with these genes are exposed to environmental factors like smoking or when a person is obese.
- **Smoking**. Multiple studies show that cigarette smoking increases a person's risk of developing RA and can make the disease worse.
- **History of live births.** Women who have never given birth may be at greater risk of developing RA.
- **Early Life Exposures.** Some early life exposures may increase risk of developing RA in adulthood. For example, one study found that children whose mothers smoked had double the risk of developing RA as adults. Children of lower income parents are at increased risk of developing RA as adults.
- **Obesity.** Being obese can increase the risk of developing RA. Studies examining the role of obesity also found that the more overweight a person was, the higher his or her risk of developing RA became.

Characteristics that can decrease risk

Unlike the risk factors above which may increase risk of developing RA, at least one characteristic may decrease risk of developing RA.

- **Breastfeeding.** Women who have breastfed their infants have a *decreased* risk of developing RA.

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How is RA diagnosed?

RA is diagnosed by reviewing symptoms, conducting a physical examination, and doing X-rays and lab tests. It's best to diagnose RA early—within 6 months of the onset of symptoms—so that people with the disease can begin treatment to slow or stop disease progression (for example, damage to joints). Diagnosis and effective treatments, particularly treatment to suppress or control inflammation, can help reduce the damaging effects of RA.

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Who should diagnose and treat RA?

A doctor or a team of doctors who specialize in care of RA patients should diagnose and treat RA. This is especially important because the signs and symptoms of RA are not specific and can look like signs and symptoms of other inflammatory joint diseases. Doctors who specialize in arthritis are called rheumatologists, and they can make the correct diagnosis. To find a provider near you, visit the database of rheumatologists on the American College of Rheumatology (ACR) website.

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How is RA treated?

RA can be effectively treated and managed with medication(s) and self-management strategies. Treatment for RA usually includes the use of medications that slow disease and prevent joint deformity, called disease-modifying antirheumatic drugs (DMARDs); biological response modifiers (biologicals) are medications that are an effective second-line treatment. In addition to medications, people can manage their RA with self-management strategies proven to reduce pain and disability, allowing them to pursue the activities important to them. People with RA can relieve pain and improve joint function by learning to use five simple and effective arthritis management strategies.

For more information about the treatment of RA, review the Clinical Practice Guidelines for the Treatment of Rheumatoid Arthritis from the American College of Rheumatology (ACR) or the ACR's Rheumatoid Arthritis Patient page.

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What are the complications of RA?

Rheumatoid arthritis (RA) has many physical and social consequences and can lower quality of life. It can cause pain, disability, and premature death.

- **Premature heart disease.** People with RA are also at a higher risk for developing other chronic diseases such as heart disease and diabetes. To prevent people with RA from developing heart disease, treatment of RA also focuses on reducing heart disease risk factors. For example, doctors will advise patients with RA to stop smoking and lose weight.
- **Obesity.** People with RA who are obese have an increased risk of developing heart disease risk factors such as high blood pressure and high cholesterol. Being obese also increases risk of developing chronic conditions such as heart disease and diabetes. Finally, people with RA who are obese experience fewer benefits from their medical treatment compared with those with RA who are not obese.
- **Employment.** RA can make work difficult. Adults with RA are less likely to be employed than those who do not have RA. As the disease gets worse, many people with RA find they cannot do as much as they used to. Work loss among people with RA is highest among people whose jobs are physically demanding. Work loss is lower among those in jobs with few physical demands, or in jobs where they have influence over the job pace and activities.

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How can I manage RA and improve my quality of life?

RA affects many aspects of daily living including work, leisure and social activities. Fortunately, there are multiple low-cost strategies in the community that are proven to increase quality of life.

- **Get physically active.** Experts recommend that ideally adults be moderately physically active for 150 minutes per week, like walking, swimming, or biking 30 minutes a day for five days a week. You can break these 30 minutes into three separate ten-minute sessions during the day. Regular physical activity can also reduce the risk of developing other chronic diseases such as heart disease, diabetes, and depression. Learn more about physical activity for arthritis.
- **Go to effective physical activity programs**. If you are worried about making arthritis worse or unsure how to safely exercise, participation in physical activity programs can help reduce pain and disability related to RA and improve mood and the ability to move. Classes take place at local Ys, parks, and community centers. These classes can help people with RA feel better. Learn more about the proven physical activity programs that CDC recommends.
- **Join a self-management education class.** Participants with arthritis and (including RA) gain confidence in learning how to control their symptoms, how to live well with arthritis, and how arthritis affects their lives. Learn more about the proven self-management education programs that CDC recommends.
- **Stop Smoking**. Cigarette smoking makes the disease worse and can cause other medical problems. Smoking can also make it more difficult to stay physically active, which is an important part of managing RA. Get help to stop smoking by visiting I'm Ready to Quit on CDC's Tips From Former Smokers website.
- **Maintain a Healthy Weight.** Obesity can cause numerous problems for people with RA and so it's important to maintain a healthy weight. For more information, visit the CDC Healthy Weight website.

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Learn more about RA

- National Institute of Arthritis and Musculoskeletal and Skin Diseases—Rheumatoid Arthritis
- American College of Rheumatology—Rheumatoid Arthritis

Learn more about arthritis

- Arthritis Types
- Physical Activity for Arthritis
- Frequently Asked Questions (FAQs)
- Arthritis-Related Statistics

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Page last reviewed: January 6, 2020
Content source: Centers for Disease Control and Prevention , National Center for Chronic Disease Prevention and Health Promotion , Division of Population Health

EXHIBIT 176

Centers for Disease Control and Prevention
CDC 24/7: Saving Lives, Protecting People™

Arthritis

Arthritis-Related Statistics

Find basic statistics about arthritis, such as prevalence, disabilities and limitations, quality of life, and costs.

Note: There are different data sources for some of the arthritis related statistics; therefore, case definitions and terminology will also vary. Learn more about **arthritis case definitions**.

Common Types of Arthritis

Osteoarthritis is the most common form of arthritis. Gout, fibromyalgia, and rheumatoid arthritis are other common rheumatic conditions.

Learn more about **specific types of arthritis**.

Prevalence of Arthritis in the United States

National Prevalence

- From 2013–2015, an estimated 54.4 million US adults (22.7%) annually had ever been told by a doctor that they had some form of arthritis, rheumatoid arthritis, gout, lupus, or fibromyalgia.[1] Learn more about **national arthritis statistics**.

Prevalence by State

- The percentage of adults with arthritis varies by state, ranging from 17.2% in Hawaii to 33.6% in West Virginia in 2015. Learn more about **state-level arthritis statistics**.
- To view arthritis prevalence estimates by state, go to the interactive map on the **Chronic Disease Indicators database** and select a state on the map.
- For detailed state-level estimates, see the ***MMWR Surveillance Summary***: "Geographic Variations in Arthritis Prevalence, Health-Related Characteristics, and Management—United States, 2015."

Prevalence by County

- The percentage of adults with arthritis varies considerably by county, ranging from 11.2% to 42.7% in 2015. Learn more about **county-level arthritis statistics**.

Prevalence by Census Track or Largest Cities

- The percentage of adults with arthritis varies by census track or within each of the 500 largest cities in the United States.
- To view arthritis prevalence estimates by census track or large city, go to the interactive map on the **500 Cities Database** and select location type.

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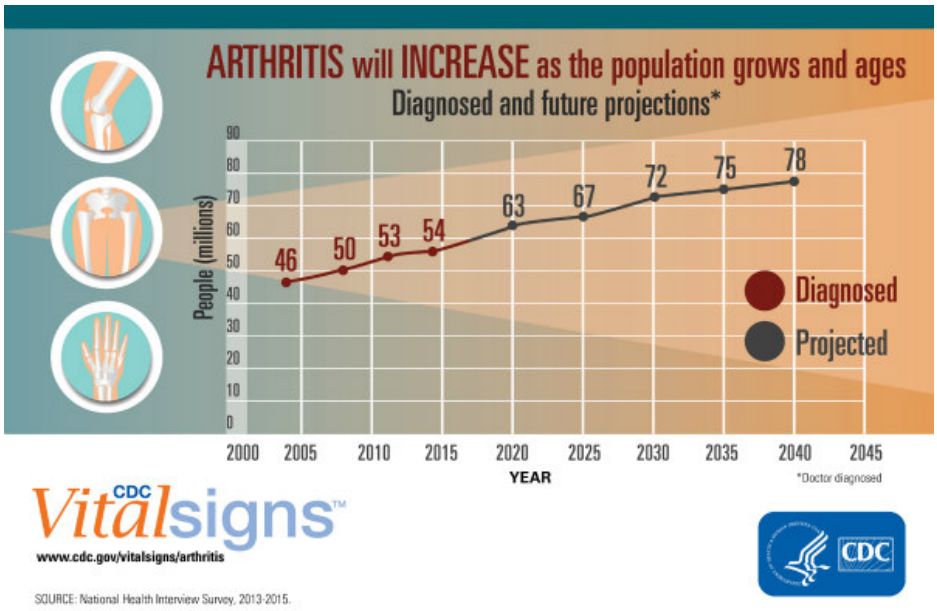
Projected Prevalence of Arthritis in US Adults

By 2040, an estimated 78 million (26%) US adults aged 18 years or older are projected to have doctor-diagnosed arthritis.[2]

Learn more about **future arthritis burden**.



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Prevalence of Arthritis by Age/Race/Gender

Prevalence by Age

- From 2013 to 2015 in the United States
  - Of people aged 18 to 44 years, 7.1% ever reported doctor-diagnosed arthritis.[1]
  - Of people aged 45 to 64 years, 29.3% ever reported doctor-diagnosed arthritis.[1]
  - Of people aged 65 years or older, 49.6% ever reported doctor-diagnosed arthritis.[1]
- The risk of arthritis increases with age and arthritis is more common among women than men.[1]
  - Learn more about **arthritis risk factors**.

Prevalence by Gender

- From 2013 to 2015 in the United States, 26% women and 19.1% men ever reported doctor-diagnosed arthritis.[1]
- To view state-specific prevalence estimates in men and women, go to the interactive map on the **Chronic Disease Indicators Database** and select Gender in the "View by" drop down menu.

Prevalence by Race and Ethnicity

- 4.4 million Hispanic adults ever reported doctor-diagnosed arthritis.[1]
- 41.3 million Non-Hispanic whites ever reported doctor-diagnosed arthritis.[1]
- 6.1 million Non-Hispanic blacks ever reported doctor-diagnosed arthritis.[1]
- 1.5 million Non-Hispanic Asians ever reported doctor-diagnosed arthritis.[1]
- To view state-specific prevalence estimates by race and ethnicity, go to the interactive map on the **Chronic Disease Indicators Database** and select Race/Ethnicity in the "View by" drop down menu.

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Overweight/Obesity and Arthritis

- Adults aged 18 years or older who are overweight or obese report doctor-diagnosed arthritis more often than adults with a lower body mass index (BMI).
  - More than 16% of under/normal weight adults report doctor-diagnosed arthritis.[1]
  - Almost 23% of overweight and 31% of obese US adults report doctor-diagnosed arthritis.[1]
- Learn more about **arthritis comorbidities** like obesity.
- For state-level estimates of arthritis among adults who are obese, see the ***MMWR Surveillance Summary***.
- To view state-specific arthritis prevalence estimates among adults who are obese, go to the interactive **Chronic Disease Indicators Database** and select indicator: Arthritis among adults aged >= 18 years who are obese.

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Severe Joint Pain and Arthritis

- In 2015, 15 million adults reported severe joint pain due to arthritis.
- The percentage of adults with arthritis who have arthritis-attributable severe joint pain varies by state, ranging from 20.3% in Utah to 46% in Mississippi in 2015.
- For state-specific prevalence of severe joint pain, see the ***MMWR Surveillance Summary***.

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Disability/Limitations and Arthritis

Leading Cause of Work Disability

- Arthritis and other rheumatic conditions are a leading cause of work disability among US adults.[3]
- In all US states, 1 in 25 working-age adults aged 18 to 64 years face work limitations they attribute to arthritis; among those with arthritis, at least 1 in 4 have work limitations. The prevalence of work limitations due to arthritis varies by state.
- **View state-specific prevalence data for work limitations.**

Activity Limitation

- Arthritis limits the activities of 23.7 million US adults. Around 44% of adults with doctor-diagnosed arthritis had arthritis-attributable activity limitations in 2013–2015.[1]
- Learn more about **arthritis-related disabilities and limitations**.

Risk of Falls and Fall Injuries

- Adults with arthritis were about 2.5 times more likely to have two or more falls and suffer a fall injury in the past 12 months compared with adults without arthritis.[4]

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Arthritis Costs

- In 2013, the national costs of arthritis were $304 billion overall.
  - Arthritis-attributable medical costs were $140 billion.
  - Arthritis-attributable lost wages were $164 billion.
- Learn more about the **cost of arthritis in US adults.**

More Data and Statistics

- National Statistics
- State Statistics
- Cost Statistics
- Disabilities and Limitations

Related CDC Data Portals

Work directly with the data to create your own filtered datasets, customize visualizations, download data, and more.

- Chronic Disease Indicator (CDI) Data Portal
- 500 Cities Data Portal

More About Arthritis

- Communications Center
- Key Public Health Messages for Arthritis
- About CDC's Arthritis Program
- Tools for Partners
- CDC Publications

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EXHIBIT 177

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Rheumatology INTERNATIONAL

EPIDEMIOLOGY OF RMD

Prevalence of rheumatoid arthritis in the United States adult population in healthcare claims databases, 2004–2014

Theresa M. Hunter[1] · Natalie N. Boytsov[1] · Xiang Zhang[1] · Krista Schroeder[1] · Kaleb Michaud[2] · Andre B. Araujo[1]

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Abstract This study aimed to determine the prevalence of rheumatoid arthritis in the United States (US) adult insured population from 2004 to 2014. This was an observational, retrospective, cross-sectional study based on US administrative health insurance claims databases (Truven Health MarketScan® Research database and IMS PharMetrics Plus database). Trends in RA prevalence focusing on the 10-year period covering January 1, 2004–December 31, 2014 were analyzed using a validated algorithm for the identification of RA. Prevalence rates in the databases were determined and age- and gender-adjusted rates were projected to the US population in 2014. Analysis of data from the two databases indicated that the RA prevalence rate in commercially insured adult US population ranged from 0.41 to 0.54% from 2004 to 2014. The prevalence varied substantially by gender and age in each year and increased gradually across the years for most subgroups. In 2014, out of 31,316,902 adult patients with continuous enrollment in the Truven Health MarketScan® Research database, 157,634 (0.50%) patients met our criteria for RA. Similarly, out of 35,083,356 adult patients in the IMS PharMetrics Plus database, 139,300 (0.50%) patients met our criteria for RA. In 2014, the overall age-adjusted prevalence of RA ranged from 0.53 to 0.55% (0.29–0.31% for males and 0.73–0.78% for females). The prevalence of RA in the US appeared to increase during the period from 2004 to 2014, affecting a conservative estimate of 1.28–1.36 million adults in 2014.

Keywords Rheumatoid arthritis · Prevalence · Claims databases

Introduction

Over the past two decades, there has been much variation between studies that report the prevalence of rheumatoid arthritis (RA) [1]. While there have been several RA prevalence studies conducted in Europe [2–6], limited prevalence studies have been conducted in the United States (US). The most frequently referenced study on RA prevalence in the US assessed data from 1955 to 1985 and found that there was a prevalence of 1073 per 100,000 population in 1985 [7]. This study only analyzed data from Olmsted County, Minnesota, generalizable to the white population and is now 30 years old [7]. Recent studies have attempted to assess the prevalence of RA in the US, yet their generalizability to the overall US adult population is uncertain [8–10].

In addition to being outdated, there are several methodological variations among previous RA prevalence studies. The variation in the algorithms used for patient identification is a key limitation found in RA prevalence studies that utilize administrative claims databases [11–13]. When using the rheumatologist's diagnosis as the gold standard, the overall accuracy of algorithms used to identify RA cases in administrative claim-based studies differ, causing wide variations in RA prevalence rates (0.15–0.61%) [13]. Therefore, to understand US RA prevalence, additional

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✉ Theresa M. Hunter
hunter_theresa_marie@lilly.com

[1] Global Patient Outcomes and Real World Evidence, Eli Lilly and Company, Lilly Technology Center South, 1555 South Harding Street, Indianapolis, IN 46221, USA

[2] Division of Rheumatology, University of Nebraska Medical Center, Omaha, NE, USA

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studies using validated RA case identification algorithms are needed.

The purpose of this study was to assess the current prevalence of RA among commercially insured adults in the US. To do this, data from administrative insurance claims databases over the period 2004–2014 were analyzed using a validated algorithm for the identification of RA. We sought to determine the prevalence of RA among the insured US adult population and its variations according to gender, age, and geographical regions. Our findings can be used to inform the scientific and medical community on the prevalence of RA among commercially insured adults in the US and are needed to understand the economic burden of RA on the US healthcare system.

Methods

Study design

This study was an observational, retrospective, cross-sectional study based on two US administrative insurance claims databases. First, data from Truven Health MarketScan® Research database (Truven Health, Ann Arbor, MI, USA) were analyzed to assess trends in RA prevalence focusing on the 10-year period covering January 1, 2004–December 31, 2014. Prevalence rates were analyzed overall and stratified by age and gender. For the 2014 population, demographic characteristics were assessed, and the age-adjusted prevalence rate was measured overall and by gender. Additionally, for comparative purposes, prevalence rates assessed from the IMS PharMetrics Plus database (IMS Health, Waltham, MA, USA) were also reported from January 1, 2006 to December 31, 2014.

The setting for this study was US clinical practice, as reflected by the insurance claims in the databases. Truven Health MarketScan® Commercial Claims and Encounters and Medicare Supplemental databases contain de-identified data on over 50 million covered lives and capture the continuum of care in all settings including physician office visits, hospital stays, and pharmacies. The IMS PharMetrics Plus database is the largest claims database of integrated medical claims in the US and is comprised of adjudicated claims for more than 150 million unique enrollees across the US.

Study variables were defined in the Truven Health MarketScan® Research and IMS PharMetrics Plus databases using enrollment records and International Classification of Diseases, 9th Revision, Clinical Modification (ICD-9-CM) codes. All data from the databases are Health Insurance Portability and Accountability Act (HIPAA) compliant to protect patient privacy.

Identification of RA in claims database

Several published algorithms have been utilized to define RA in claims databases. When choosing a definition for our study, we assessed the published sensitivity, specificity, and accuracy of multiple potential case definitions for RA that were validated in the US [14, 15] and Canada [13, 16–18]. The RA algorithms that were assessed differed in the number of diagnostic codes and source of diagnoses (rheumatologist versus general practice physician), and varied in regards to specificity, sensitivity, and positive and negative predictive values. The algorithm chosen for this study had a sensitivity of 92.0, a specificity of 74.3, and an accuracy of 77.8 [13], which was deemed appropriate by the research team.

For each calendar year of analysis, a base cohort was assembled that consisted of all patients ≥18 years on January 1st of the calendar year with continuous enrollment in medical benefits throughout the calendar year allowing for an enrollment gap of <30 days. From these base cohorts, utilizing ICD-9 codes, the sub-set of patients with RA were identified by the following diagnostic criteria:

- two non-rheumatology physician visits with a listed RA code (ICD-9: 714.0, 714.1, 714.2) occurring at least 2 months apart;
- or at least one RA code contributed by a rheumatologist;
- or at least one inpatient hospitalization for which RA was in the diagnostic codes.

Within this definition, patients were not counted as having RA if they had at least two visits, at least two months apart, subsequent to the second RA visit above (if a second visit occurred), with two identical diagnoses of other autoimmune and connective tissue diseases [psoriatic arthritis (ICD-9: 696.0), ankylosing spondylitis (ICD-9: 720.0), and other spondyloarthropathies (ICD-9: 720.1, 720.2, 720.8, 720.9), systemic lupus erythematosus (ICD-9: 710.0), scleroderma (ICD-9: 710.1), Sjögren's syndrome (ICD-9: 710.2), dermatomyositis (ICD-9: 710.3), polymyositis (ICD-9: 710.4), primary systemic vasculitis (ICD-9: 446.0, 446.2, 446.4, 446.5, 446.7, 447.6) and other connective diseases (ICD-9: 710.5, 710.8, 710.9)] [13].

A second RA case definition was tested for comparative purposes that did not exclude comorbidities. Utilizing ICD-9-CM codes, this sub-set of patients with RA was defined by the following diagnostic criteria: two physician visits for RA at least 2 months apart [14] or at least one hospitalization where RA was in the diagnostic codes [13, 18]. These case definitions were previously tested and validated [14, 18].

Estimation of prevalence

Prevalence is defined as the proportion of individuals who have the disease of interest in a specified time period (includes both new and existing cases). In our study, annual RA prevalence was estimated using the US adult population in the US health claims databases during the period of 2004–2014. For each calendar year, a base cohort was assembled and the case identification algorithm was applied separately in each year. The numerator in the prevalence estimation was the number of patients that met the RA definition described in the previous section. The denominator was the number of patients in the base cohort.

Statistical analyses

RA prevalence was estimated for subgroups stratified by gender and age (18–34, 35–44, 45–54, 55–64, and ≥65) for each calendar year from 2004 to 2014. To account for the distortion caused by the age distributions in the datasets, we also calculated the age-adjusted prevalence of RA from 2004 to 2014 using direct standardization. The age- and gender-specific prevalence rates in 2014 were applied to the corresponding population estimates from the US Census Bureau to project the total number of persons in the US expected to have RA in 2014 and in 2020.

Results

Rheumatoid arthritis prevalence: 2004–2014

Annual RA prevalence rates ranged from 0.41 to 0.52% from 2004 to 2014 for adult US patients in the Truven MarketScan® Research database. The prevalence varied substantially by gender and age in each year and increased gradually across the years for most subgroups. Specifically, prevalence among females was more than twice the prevalence among males (Fig. 1). In the Truven MarketScan® Research database, overall prevalence in females gradually increased from 0.56% in 2004 to 0.71% in 2014, whereas the overall prevalence among males remained relativity stable over the same period (0.23% in 2004 to 0.26% in 2014) (Fig. 1). At the same time, RA prevalence increased with age among both males and females, and for most age groups the rates rose consistently across the study period (Fig. 2a, b). We also calculated the age-adjusted prevalence rates from 2004 to 2014, which ranged from 0.37 to 0.55%.

The overall RA prevalence rate for the adult US population in the IMS PharMetrics Plus database was similar to the rate in Truven Health MarketScan® Research database and ranged from 0.47 to 0.54% from 2006 to 2014. Similar to the findings in the Truven Health MarketScan® Research database, the prevalence varied substantially by gender and age in each year and increased gradually across the years for most subgroups. Rheumatoid arthritis prevalence increased with age among both males and females, and for most age groups the rates rose consistently across study years (Supplemental Figure 1A, B).

Age-adjusted RA prevalence in 2014: Truven Health MarketScan® Research database

In 2014, out of a total of 31,316,902 adult patients with continuous enrollment in the Truven Health MarketScan® Research database, there were 157,634 (0.50%) patients with RA. Of these 157,634 patients, 119, 692 (75.93%) were female and 37,942 (24.07%) were male. Mean age for overall RA population was 57.42 years [standard deviation (SD) 13.32]. A majority of patients were

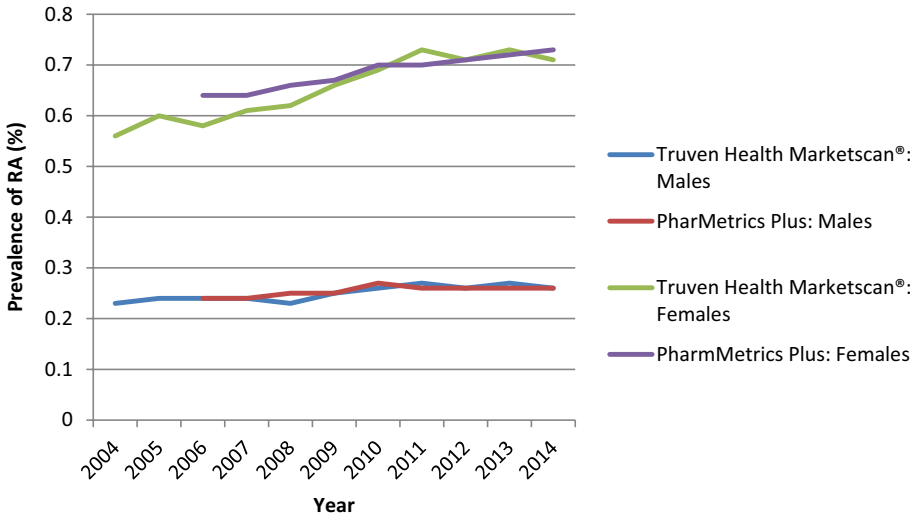

Fig. 1 Rheumatoid arthritis prevalence trends stratified by gender (2004–2014)

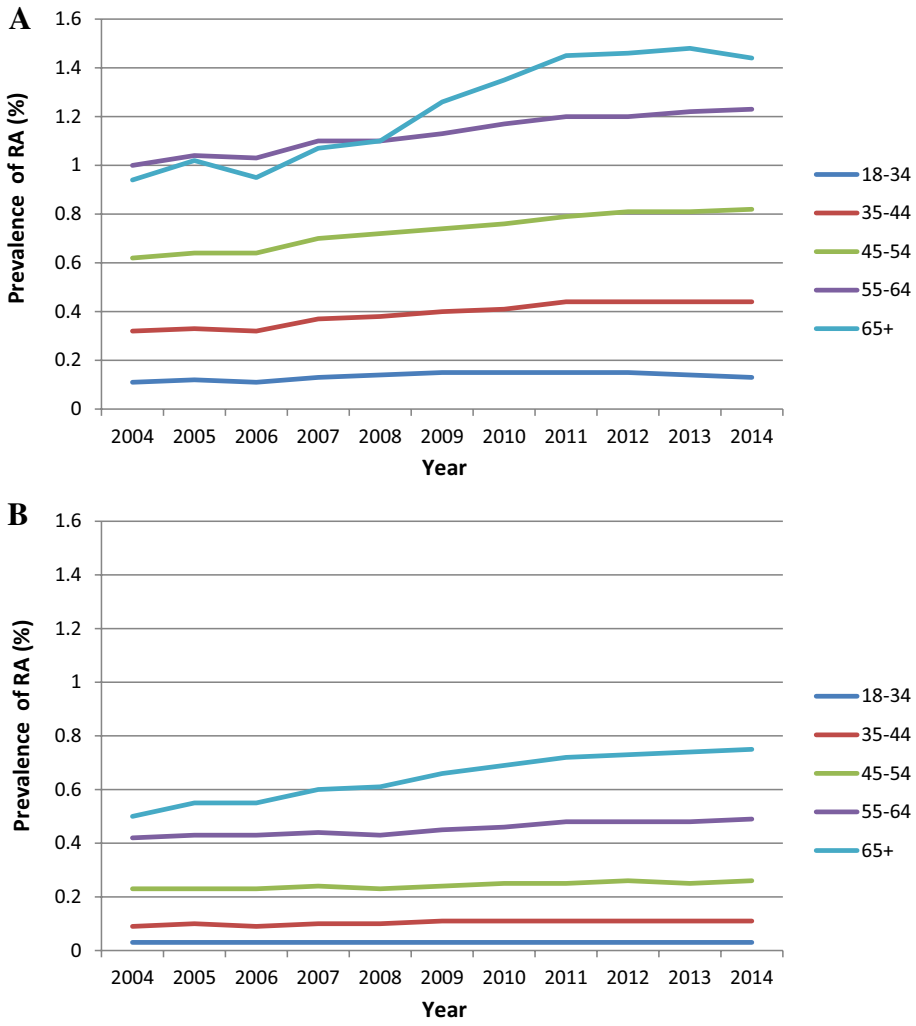

Fig. 2 **a** Rheumatoid arthritis prevalence among females stratified by age. **b** Rheumatoid arthritis prevalence among males stratified by age. Source: Truven Health MarketScan® Research Database

commercially insured and located in the Atlantic and Central US regions. The patients' demographic information is presented in Table 1.

The overall age-adjusted prevalence of RA among individuals who were aged 18 years or older on January 1, 2014 was 0.53%. Males had an age-adjusted prevalence of 0.29% and females had an age-adjusted prevalence of 0.73% in 2014.

Age-adjusted RA prevalence in 2014: IMS PharMetrics Plus database

In 2014, out of 35,083,356 adult patients in the IMS PharMetrics Plus database, there were 139,300 (0.50%) patients with RA. Of these patients, 103,442 (74.26%) were female, and 35,858 (25.74%) were male. The patients' demographic information is presented in Table 1. Mean age for the overall RA population was 56.70 (SD 12.4).

The overall age-adjusted prevalence of RA among individuals who were aged 18 years or older on January 1, 2014 was 0.55%. Males had an age-adjusted prevalence of 0.31% and females had an age-adjusted prevalence of 0.78% in 2014.

Rheumatoid arthritis prevalence—US estimates

Using population estimates from the US Census Bureau and the RA prevalence rates in 2014, it was estimated that 1.28 million (Truven Health MarketScan® Research) to 1.36 million (IMS PharMetrics Plus) US adults were affected by RA in 2014. If age- and gender-specific RA prevalence rates remain the same, it is projected that RA will affect 1.39 million US adults by 2020. Age- and gender-specific population estimates for RA are shown in Table 2.

Discussion

This study evaluated recent trends in prevalence of RA and helped to highlight the estimated burden of RA in the US.

Table 1 Baseline characteristics for patients with rheumatoid arthritis (2014)

Variables	Truven Health MarketScan® Research ($N = 157,634$)	IMS PharMetrics Plus ($N = 139,300$)
Sex		
Male	37,942 (24.07%)	35,858 (25.74%)
Female	119,692 (75.93%)	103, 442 (74.26%)
Age (years)		
18–34	7749 (4.92%)	6878 (4.94%)
35–44	16,822 (10.67%)	14,364 (10.31%)
45–54	37,332 (23.68%)	33,354 (23.94%)
55–64	54,983 (34.88%)	53,670 (38.53%)
65+	40,748 (25.85%)	31,034 (22.28%)
Insurance		
Commercial	113,215 (71.82%)	136,567 (98.03%)
Medicare	44,419 (28.18%)	1137 (0.82%)
Other	N/A	1596 (1.15%)
Geographic region divisions		
New England	6585 (4.18%)	7133 (5.12%)
Mid Atlantic	28,288 (17.94%)	19,093 (13.71%)
South Atlantic	30,484 (19.34%)	24,168 (17.35%)
East North Central	28,888 (18.33%)	27,519 (19.76%)
East South Central	10,362 (6.57%)	16,012 (11.49%)
West North Central	5952 (3.78%)	13,164 (9.45%)
West South Central	17,015 (10. 79%)	20.357 (14.61%)
Mountain	8880 (5.63%)	5728 (4.11%)
Pacific	17,633 (11.19%)	5071 (3.64%)
Unknown	3547 (2.25%)	1055 (0.72%)

Table 2 2014 US census projected RA population estimates stratified by gender and age (years)

	Truven Health MarketScan® Research	IMS PharMetrics Plus
Male		
18–34	11,429	11,231
35–44	22,175	22,242
45–54	55,705	55,357
55–64	94,677	98,811
65+	152,635	161,242
Total	336,621	365,167
Female		
18–34	47,951	51,994
35–44	89,557	92,065
45–54	180,677	180,091
55–64	255,295	261,175
65+	372,844	430,223
Total	946,324	984,084
Total	1,282,945	1,364,431

This study provided the prevalence of RA during the last decade (2004–2014) in the US commercially insured adult population using two US administrative insurance claims databases (Truven Health MarketScan® Research and IMS PharMetrics Plus). The findings from this study indicate an increase in the RA population in the US from 2004 to 2014. Based on these findings, it is estimated that approximately 1.3 million adults were affected by RA in 2014. The findings from this study can be used to inform the scientific and medical community on the prevalence of RA among commercially insured adults in the US and help providers, payers, and patients to better understand the economic burden of RA in the US.

In the US, there have been limited studies of RA prevalence. The studies that have been published differ considerably in their methods of identifying RA patients and result in a wide variation of prevalence estimates. This study utilized a validated definition [13] of RA and administrative claims data to provide consistent estimates of RA prevalence across two different databases.

The study determined that overall prevalence of RA in the US ranged from 0.41 to 0.54% and steadily increased from 2004 to 2014. When analyzing medical expenditure panel survey (MEPS) data, Simmons and colleagues found similar results: 0.40% in 2004, 0.44% in 2005, and 0.43% in 2006 [8]. These findings were lower than the rates reported by other studies. For example, based on the 2001–2005 National Ambulatory Medical Care Survey data, RA prevalence was 1.48% [10]. The widely referenced RA prevalence based on the Olmsted County cohort was reported at 0.72% in 2005 [19]. Limitations of these studies, such as lack of generalizability [19] and identification of RA patients by a single occurrence of RA diagnostic code [10], may have overestimated these rates.

The authors of the Olmsted County cohort estimated that RA affected 1.5 million US adults in 2005 [19]. Based on our estimates of national claims databases, RA prevalence in 2005 was approximately 0.44% with an estimated 0.95 million people affected. These discrepant findings may stem from differences in gender- and age-specific prevalence rates, which were higher in Olmsted County compared to the US.

Studies have consistently documented a greater prevalence of RA in women versus men; however, the relative burden differs across studies. For instance, the Olmsted County cohort found that the RA prevalence rates in women were approximately double the prevalence rates in men [19]. Our study found that RA prevalence rates among women were closer to three times higher than the rates in men, which is consistent with the results from the MEPS study [8].

It can be inferred that many of the differences in RA prevalence estimates result from the methodological variations between the previous studies. When utilizing administrative claims databases, the variations in the methods of identifying RA patients cause differentiation among estimates and results [11–13]. This study measured prevalence rates in two different large, geographically dispersed claims databases, the Truven Health MarketScan® Research and IMS PharMetrics Plus, using a robust RA case definition assessed by high sensitivity (92.0) and specificity (74.3) [13].

It is important to note that the primary purpose of insurance claims data is administrative and not research-oriented. Therefore, there are limitations to using claims data and ICD-9 codes provided for insurance claims to determine prevalence of a disease. Due to inherent limitations of claims-based data sources, there may have been a proportion of cases identified using the chosen criteria as having RA, when in fact, they might not have RA. The potential for misclassification of non-RA patients as RA patients in claims data, especially in the case of "rule-out" diagnoses (RA diagnoses coded in laboratory work-ups when RA is suspected or needs to be "ruled-out") should be taken into consideration when evaluating prevalence rates. This type of misclassification could potentially lead to overestimation of prevalence. Given that laboratory test results and medical charts review were not included in this study, confirmation of RA cases was not possible. Instead, we relied on a published RA definition with a level of sensitivity and specificity, and accuracy that we deemed acceptable [13].

Additionally, given both that the Truven Health MarketScan® Research and the IMS PharMetrics Plus populations are composed of patients with commercial insurance or Medicare supplement insurance, there are specific groups (uninsured people, military personnel, Medicaid patients, and Medicare enrollees without an employer-sponsored supplement plan) that are not represented in our analyses. Approximately, one-third of the RA patients report disease-related work disability [20, 21]. Given the limitations of the databases used in this study, unemployed RA patients would not be included in our analyses, thus resulting in a conservative estimate of RA prevalence in the US. There may also be RA patients that were not accounted for in these analyses because they did not meet the RA definition that was used in this study. This would also result in a conservative estimate of the RA prevalence in the US. Additionally, all individuals over 65 in our study are insured by an employer-sponsored Medicare supplemental plan. This population represents a very specific and relatively small proportion of the over 65 populations in the US. There is also the potential for the two data sets to overlap, but the actual overlap cannot be determined, because the databases are not linked.

Conclusion

The large sample size and dispersed geographic representation of our study enhances the validity of generalizing our prevalence estimates to the general US adult population that are commercially insured, and the consistency in rates observed in the two databases strengthens our observations. We observed that the prevalence of RA in the US appeared to increase during the period 2004–2014, affecting approximately 1.3 million adults in 2014. These results may be attributed to the increasing emphasis on early diagnosis of RA, regular monitoring of disease activity, increased life expectancy, as well as a growing elderly population.

Author contributions TMH, NNB, XZ, KS, KM, and AA all made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; participated in drafting the manuscript or revising it critically for important intellectual content; approved the final version of the submitted manuscript and agreed to

be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Compliance with ethical standards

Conflict of interest The authors declare that there are no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

Ethical approval The study has been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and later amendments.

Human or animal participants This study was a retrospective analysis of administrative claims data and does not contain any human participants or animals.

Informed consent The permission from a research ethics committee was not required and formal informed consent was not obtained.

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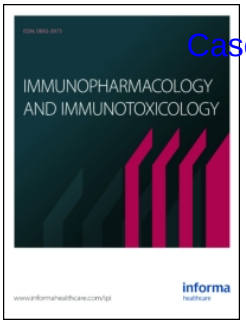
EXHIBIT 178



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REVIEW

Experimental animal models for rheumatoid arthritis

Narayan Choudhary, Lokesh K. Bhatt and Kedar S. Prabhavalkar

Department of Pharmacology, SVKM's Dr. Bhanuben Nanavati College of Pharmacy, Vile Parle (W), Mumbai, India

ABSTRACT

Rheumatoid Arthritis (RA) is an autoimmune systemic disorder of unknown etiology and is characterized by chronic inflammation and synovial infiltration of immune cells. RA is associated with decreased life expectancy and quality of life. The research on RA is greatly simplified by animal models that help us to investigate the complex system involving inflammation, immunological tolerance and autoimmunity. The animal models of RA with a proven track record of predictability for efficacy in humans include: collagen type II induced arthritis in rats as well as mice, adjuvant induced arthritis in rats and antigen induced arthritis in several species. The development of novel treatments for RA requires the interplay between clinical observations and studies in animal models. However, each model features a different mechanism driving the disease expression; the benefits of each should be evaluated carefully in making the appropriate choice for the scientific problem to be investigated. In this review article, we focus on animal models of arthritis induced in various species along with the genetic models. The review also discussed the similarity and dissimilarities with respect to human RA.

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Rheumatoid arthritis; animal models; induced models; genetic models; autoimmune systemic disorder

Introduction

Rheumatoid arthritis (RA) is a long-lasting form of autoimmune disorder, mainly affecting the joints that are identified by inflammation and swelling of the synovium of the joint[1]. The process of RA is complex and involves the synovial cell proliferation and fibrosis, pannus formation, along with cartilage and bone erosion. The inflammatory cytokines, which are involved in RA are IL-6, IL-1 and TNF-α[2]. The prevalence of RA is seen more in women than in man. There is a striking imbalance between the sexes, with females representing the majority of autoimmune disease cases. Thus, women are more affected than men; it is also true for RA, where the sex ratio is typically around 3:1. The mortality rate of RA patients is twice more than compared to that of the normal population[3]. The estimate of the worldwide prevalence of RA was published as part of the Global Burden of Disease 2010 study. This was a comprehensive effort to measure epidemiological levels and trends of 291 diseases in 187 countries[3]. RA was defined using the American College of Rheumatology (ACR) 1987 criteria for the classification of RA. The global prevalence of RA in patients, from 5 to 100 years of age, in the year 2010 was estimated to be 0.24% with 95% CI (0.23%–0.25%). The prevalence of RA was found to be approximately two times higher in females; the mean was 0.35% with 95% CI (0.34%–0.37%) than males having mean 0.13% with 95% CI (0.12%–0.13%). The value for global prevalence of RA in 2010 has not perceptibly changed from the prevalence of RA determined in 1990. In 1990, the mean was 0.25% with 95% CI (0.24%–0.26%)[3].

The genetic factors, lifestyle and sex-hormones also play a key role in disease progression. For such a disease to be understood completely, animal models are necessary, which can mimic the conditions and show resemblance to that of the human disease state. To understand the pathogenic processes of RA in humans, rodent models are helpful and essential tools. These animal models are also important for testing the novel and existing drugs for their potency, efficacy and safety. For development of novel drugs, the study of pathogenic process of RA helps us to come out with other therapeutic targets than the existing targets, which are available. The commonly used models are collagen induced arthritis, adjuvant induced arthritis. The less frequently used animal models are proteoglycan induced arthritis and streptococcal cell wall induced arthritis. There are also other models, which have been used for many years, but also differ from similarity to human RA. This review article henceforth puts forward the similarities and differences between animal models for human RA.

Animal models for RA

The models of RA are developed in a variety of animal species, but commonly rats and mice are used to study the progression and pathogenesis of RA. The rodent models are used widely because of low cost, homogeneity of the genetic background, ease of handling. Mostly, the disease in animal is developed by inducing a chemical agent with arthritogenic properties. There is no animal model, which can mimic the human condition completely so the model with least

CONTACT Lokesh K. Bhatt bhatt.lokesh@gmail.com Department of Pharmacology, SVKM's Dr. Bhanuben Nanavati College of Pharmacy, Vile Parle (W), Mumbai 400 056, India

Table 1. Animal models for rheumatoid arthritis.

Animal models for rheumatoid arthritis	
Induced models	Collagen induced arthritis
	Adjuvant induced arthritis
	Streptococcal cell wall induced arthritis
	COMP induced arthritis
	Pristane induced arthritis
	Antigen induced arthritis
	Proteoglycan induced arthritis
	G6PI induced arthritis
Genetic models	K/BxN mice
	SKG mice
	Human tumor necrosis factor gene (HTNFG) mice
	IL-1ra-/- transgenic mice

dissimilarity is preferred and research is still on way to have model with maximum similarity. Another benefit of using animal models for genetic research is the possibilities to selectively modify the genome e.g., produce congenic, knockout or transgenic strains and linkage mapping[4].

Stoerk and Pearson and Woods developed the first rat model for polyarthritis. They observed and concluded that rats when injected with complete adjuvant induced polyarthritis, possibly by a mechanism involving heat shock proteins (HSP). This model was then termed as adjuvant arthritis model and it has been used to test new drugs for inflammatory arthritis. The animal models are discussed in great detail with respect to all aspects of human rheumatoid disease. These models could be studied in a variety of animal species and can be classified into two broad categories: (A) Induced animal models for RA, (B) genetic models of RA. These animal models are listed in Table 1.

Induced rodent animal models for RA

Collagen induced arthritis (CIA)

Collagen induced arthritis is studied more extensively as animal model as it shares many similar pathological and immunological features with the human RA[5]. In CIA, an immune response is being directed against a joint antigen (collagen type II)[6]. The two important characteristics of the CIA model are breach of tolerance and generation of auto antibodies towards self and collagen. This makes CIA the gold standard *in vivo* model for RA studies[7]. This model is T helper cell mediated, in which both T helper (Th1) and (Th17) responses are induced in CIA, but Th17 cells appear to play the dominant pathological role[8].

Collagen is obtained from a variety of sources (bovine, human, porcine and chick) and responses are seen to vary with different strain and injection conditions. Not surprisingly, poor response was seen by mouse collagen. CIA is primarily an autoimmune disease of joints, which requires both T and B cell immunity to autologous type II collagen (CII) for disease manifestation[5]. The first collagen-induced arthritis (CIA) model was established by immunization of rats with type II collagen[9]. Later, the CIA model was reproduced in mouse and monkeys respectively.

Collagen induced arthritis in rats. Different types of cartilage derived proteins can be used to induce arthritis in rats: type II collagen, type XI collagen and cartilage oligomeric matrix protein. In this, we focus on arthritis induced by type II collagen as it is most widely used. An intra-dermal injection of type II collagen emulsified in incomplete Freund's adjuvant leads to the development of severe polyarthritis in DA and Lewis rats, beginning two weeks after immunization[10]. Paws of both fore and hind limbs swell, which persists for a few weeks, decreasing and then reappearing resulting in chronic arthritis with severe event such as malformations of the bone. The rat model is used for addressing the effects of compounds in the late, chronic stage of arthritis. The susceptibility to arthritis is linked to specific MHC genes. As large numbers of class II MHC cells are present in the joint, and it has been hypothesized that these antigen presenting cells (APCs) interact and activate $CD4^+$ T cells that are present in the joint, resulting in continued inflammation. TNF-α and IL-1β are key cytokines in rat CIA[10].

Collagen induced arthritis in mice. The susceptible strains DBA/1, B10.Q and B10.RIII DBA/1 mice is used widely as the CIA mice model[5]. Collagen induced arthritis is induced by immunization with heterologous type II collagen in complete Freund's adjuvant. The clinical signs of arthritis appear on the 21–25th day after the initial inoculation[7]. CIA is polyarthritis model, which can be distinguished by inflammation of the synovial fluid, cartilage destruction and bone erosion similar to human RA[10]. It has also been reported that CISA could be induced in less susceptible strains of mice as well (C57BL/6)[10].

Collagen induced arthritis in rhesus monkey. Collagen-induced arthritis (CIA) is one of the many experimental models developed for human arthritic diseases. CIA was successfully induced in Macaques, particularly belonging to old world monkey species, by immunization with heterologous type II collagen, from bovine or fowl origin[11]. This emulsion is injected into the dorsal skin, distributed over 10 spots to reduce the formation of ulcerative skin lesions[12]. The majority of CIA-affected monkeys develop a symmetrical polyarthritis, which is expressed in the metacarpal and metatarsal joints and in the inter-phalangeal joints of hands and feet. The larger synovial joints, such as knees, elbows and hips are affected at the later stage of the arthritis. Spondylitis is rarely found in CIA monkey model. Histologically, early stages of CIA are characterized by hyperplasia of the synovium, which is also penetrated by large numbers of mononuclear cells. The substantial degradation of the cartilage surface by growing pannus-like tissue is observed at that stage. The almost complete degradation of the cartilage and remodeling of bone is usually observed at the end-stage arthritis[11,13].

Adjuvant induced arthritis

According to earlier studies, it was found that in 1947, Jules Freund introduced a mixture of mineral oils, heat-killed mycobacteria and emulsifying agent, which was termed complete Freund's adjuvant (CFA). This mixture proved to be an efficient enhancer of both cell-mediated and humoral immune responses towards the antigens with which it was

emulsified. Due to repeated immunizations, mycobacteria were found to be harmful to health causing formation of persistent foci of inflammation, which was necrotic. So, sometimes, mycobacterium was omitted in the mixture, which was known as incomplete Freund's adjuvant (IFA)[14].

In another study by Lipton and Freund (1955), they demonstrated in rats that tolerance to CNS tissue could effectively be broken by immunization of the tissue together with CFA. Since then, mixtures of adjuvant and auto-antigens have been routinely used to induce a wide variety of experimental autoimmune diseases, mainly in rats and mice. At the same time, Stoerk reported in 1954 that joint lesions developed in rats after immunization with complete Freund's adjuvant and spleen tissue of rat. Stoerk suspected the spleen tissue to be arthritogenic, but Pearson demonstrated that CFA and not the spleen component was responsible for development of joint inflammation and established the adjuvant arthritis model in 1956[14].

Adjuvant induced arthritis in rats. This model is initiated in susceptible strains of rats by intradermal injection of complete Freund's adjuvant (CFA) at the base of the tail or hind paw region[15]. The genetic background of rats is important, as both major histocompatibility complex (MHC) and non-MHC genes contribute to their susceptibility to AIA; where specific trait loci are associated with the severity of the disease[16]. AIA is characterized by a rapid onset and progression to polyarticular inflammation. After 10–14 days, the symptoms of arthritis can be observed. The severity of the RA by adjuvant induction leads to permanent joint malformations, including ankylosis. In comparison to the human RA, AIA shares common symptoms like joint swelling, lymphocyte infiltration and cartilage degradation.

In rats with AIA, activated T cells can be detected in the inflamed joints. The joint is infiltrated with T cells originating from various compartments including the spleen, Peyer's patches, draining lymph nodes and the recirculating T cell pool. The immune response was seen to be induced by specific antigen heat shock protein (Hsp65), with peptide 180–186 being the responsible epitope. During the early stages of inflammation, the cytokines expressed in the joint include IL-17, IFN and TNF-α, accompanied by cytokines involved in stimulation of macrophages. As the severity of inflammation progresses in the joint, increased levels of IL-4, IL-6, monocyte chemotactic protein 1 and TGF-β can be detected. TNF-α, IL-1β, IL-21 and IL-17 are all involved in the pathology of this disease[17].

Streptococcal cell wall induced arthritis (SCWIA)

Streptococcus pyogenes produce a peptidoglycan-polysaccharide (PG-PS) polymer, which is found to possess high inflammatory activity and it also has the capacity to induce arthritis in rats. SCWIA initiates two rat models based on the fragments of PG-Ps used and the route of administration[18]. These animal models are used to study the therapeutic effects of drugs, but are used less frequently, may be due to their high costs. This model helps us to study the acute or flare reaction in arthritis, this acts as an advantage of SCW induced arthritis over the conventional model of arthritis (i.e., AIA)[10]. SCWIA could not be induced in non-human primates because they seem to be resistant to arthritis induced by bacterial antigens[19].

SCWIA in rats. *Polyarticular SCW induced arthritis.* An intraperitoneal injection of PG-PS 10 S induces polyarticular arthritis in female Lewis rats results in acute inflammation response and swelling of the joints. During the first five days, the joint inflammation progresses and it is followed by a period of respite, after which impetuous reactivation occurs, resulting in chronic arthritis. This model provides an opportunity to study the early and more chronic phases of arthritis[20]. The initial response, which was observed is not T cell dependent[10]. There is involvement of monocytes in both the acute phase and further development of arthritis[21]. During the chronic phase, the severity of arthritis can be correlated with the activation status of T cells, B cells and monocytes[21]. There is also an interrelation between the degree of inflammation and the production of TNF-α, IL-6, IL-1[22]. Some of the hallmarks of this model in similarity to human RA include synovial hyperplasia, infiltration of inflammatory cells, involvement of symmetric joints and relapsing inflammation[10,17].

Monoarticular SCW-induced arthritis. An intra-articular injection of PG-PS 100 P induces an initial surge of inflammation when it is injected to the hind ankle joint of female Lewis rats. The inflammation dies away in a few days with diffuse infiltration of the synovium containing lymphocytes and monocytes remaining in the joint. The initial intra-articular injection causes sensitization of the joint and inflammation can be reactivated by intravenous injection of PG-PS 100P[5]. This model is also known as the reactivation model of arthritis because of the challenge given to initial injection. This model is said to be mediated by Th2 cells as swelling is observed within hours[10]. The blockade of IFN and IL-10 does not affect joint swelling, whereas IL-4 was reported to be crucial in disease development[23]. The response is neutrophilic at 6 h and changes to mononuclear cell infiltrate by 48 h. In the reactivation phase of arthritis, T cells are mainly involved because depletion of T cells by monoclonal antibodies showed that rats were unresponsive to intravenously injected PG-PS. Along with T cells, IL-1, TNF-α and IL-4 are also essential in the reactivation phase of arthritis[24]. The chemokines such as CCL2/MCP-1, MIP-1 and MIP-2 are all involved in response after the reactivation[10]. This model is well suited for studying the effects of therapeutics over time, as it mimics the flares of arthritis similar to those observed in patients with RA[10]. As the disease is less severe compared to the other models of arthritis, the animals can be monitored for a longer period of time. The levels of the cytokines in the model suggest that the optimal time for evaluating the efficacy of the treatment is three days after the intravenous challenge[25].

SCWIA in mice. SCW induced arthritis mice model has been described in susceptible strains such as BALB/c, DBA/1 and C3H mice. The mice are injected once intra-articularly in one

knee joint with SCW fragments, thereby sensitizing the joint. Consecutive intravenous injection of SCW fragments in susceptible mice strains could result in a flare reaction, as seen in the rat model. Also, modified versions of this model have been described in which mice either are injected once intra-articularly or have received multiple injections, which resulted in continued inflammation[26]. The acute joint inflammation is initiated after intra-articular injection of SCW fragments, which resolves within a week and repeated injections result in the development of chronic arthritis[26]. In SCW induced arthritis in mice, macrophages and B cells play a role whereas during the reactivation phase, only T cells are involved[10]. TNF-α plays a minor role in SCW induced arthritis model and is involved in the initial joint swelling. However, IL-1β is heavily involved in inflammatory cartilage degradation and cell influx and it is important during both the acute and chronic phases of the disease[10].

Cartilage oligomeric matrix protein (COMP) induced arthritis

COMP (Cartilage Oligomeric matrix protein) is a large protein, which has a total molecular weight of 435kD. It is synthesized by chondrocytes and it is localized extracellularly. It has been detected in the nasal, tracheal and meniscal cartilage and most importantly in the articular cartilage[27] and also present in human synovial tissue[28]. For the purpose of induction of arthritis, the COMP obtained from rat origin is used and also to ensure that only the autoimmune process is involved[29]. The natured and native COMP can be used for arthritis induction.

COMP induced arthritis in rats. Severe arthritis can be induced in rat strains, mainly DA and Lewis, by immunization with native and denatured COMP in IFA (incomplete Freund's adjuvant). The solubilized COMP preparations are emulsified with an equal amount of IFA. Each rat is injected intradermally with COMP at the base of the tail[28]. In case of severe arthritis, COMP is released from the cartilage during the erosion of the tissue. This is an interesting feature of COMP and this makes it a useful marker of arthritis in RA[30]. COMP is also released in rats developing chronic arthritis after induction with pristane, a low-molecular weight adjuvant, and the levels in serum correlate strongly with the occurrence of the erosive arthritis[29].

COMP induced arthritis in mice. COMP induced arthritis mice model was performed in susceptible strains of mice[31] and it can be used as appropriate and alternate model for studying the pathogenesis of arthritis[31]. This mice model has some advantages over other various models in the mouse, such as collagen induced arthritis, glucose-6-phosphate isomerase (GPI) induced arthritis and aggrecan induced arthritis[32,33]. The arthritis onset is sudden and it is characterized by the appearance of erythema and swelling of the front and rear paws. The arthritis was observed distally in a toe joint and simultaneously flared in the ankle/wrist joints within a few days. In severe cases, it is seen to spread to the metatarsal/metacarpal region and to the knuckles and toes[31].

Pristane induced arthritis

Pristane induced arthritis (PIA) is chronic, joint-specific regulated by T cells and by MHC genes and fulfill the clinical criteria for RA. Yet, there is no evidence for autoimmune reactions[14]. This model was found to be effective and successful in mice as well as in rats. PIA model is an essential tool to figure out the mechanisms of inflammatory joint disease and efficacy of new anti-arthritic drugs can be tested in this model[17]. PIA is also used as a model for SLE due to production of autoantibodies. To understand varying manifestations of autoimmunity, studies need to be conducted for identifying the genes involved in rheumatoid arthritis and autoantibody production[14].

PIA in rats. This is a novel animal model for rheumatoid arthritis induced with the help of well-defined synthetic adjuvant oil, pristane. The rats develop severe and chronic arthritis, within two weeks, after a single intradermal injection of 150 ul of pristane. The inflammation is restricted to the joints and involved pannus formation, MHC class II expression and T lymphocyte infiltration. The disease is T cell dependent. In PIA model, the joint erosions are accompanied by elevated levels of cartilage oligomeric matrix protein. The disease is quite different from that of the mouse model induced with pristane in that no systemic abnormalities can be found and that the inflammation is restricted to the joints[34].

PIA in mice. The injection of the hydrocarbon pristane (2, 6, 10, 14-tetramethylpentodecane) i.p. into mice of susceptible strains leads to chronic arthritis, with an incidence rate of 22–100%, beginning 2–10 months after injection[35]. PIA resembles the joint inflammation and destruction seen in human RA. The similarity between PIA and human RA is synovial hyperplasia, cartilage erosions, bone abrasions, infiltration of inflammatory cells and pannus-like formation[36]. This model is easy to use and highly reproducible. The development of arthritis is highly dependent on T cell activation and is mediated through transfer of classical MHC class II restricted T cells. PIA is useful for drug validation, in particular, for T cell-related pathways leading to arthritis[37].

Antigen induced arthritis

The rodent models of AIA were first used more than 40 years ago and they are still valued[38]. Antigen induced arthritis, in simple words, is induced by immunization with a model antigen (such as bovine serum albumin or ovalbumin) intra-articularly followed by challenge with the same antigen. This generates an immune response and subsequent arthritis limited to the antigen induced joint. AIA has major advantage of applicability to multiple strains of mice, rats, guinea pigs and rabbits[39–41]. The cellular basis is similar to CIA, but with more tightly defined sensitization and it is $CD4^+$ T-cell dependent. The histopathological findings bear similarities to human RA, including synovial hyperplasia, perivascular infiltration, lymphoid follicles and pannus and cartilage erosions. ELS, similar to those seen in RA patients, are observed when repeated injections of antigen are administered. The erosiveness is related with the ability of the antigen to bind cartilage.

The antigen induced arthritis model is a mono-articular disease that affects only the injected joints. The susceptibility to AIA is not MHC class II restricted and this makes it useful for studies involving transgenic and knock-out mice[8]. The rabbit model of antigen arthritis is particularly useful when protocols require use of a larger joint[42].

AIA in mice. The susceptible strain of mice is immunized with subcutaneous or intradermal injections of antigen and then challenged with the same antigen. The antigen usually is the cationic substance such as the methylated bovine serum albumin (m-BSA), which will bind to the negatively charged cartilage and is retained in the joint. The antigen is injected into one or both joints and acute inflammation progresses rapidly leading to joint destruction. The pathogenesis of AIA involves Arthus reaction on the articular cartilage as antibodies developed in response to the positively charged antigen, which was injected and it forms complexes that activate complement locally and results in cartilage destruction[43]. Mouse models of antigen arthritis have been used extensively to study efficacy of biologics and the role of specific cytokines in the various aspects of disease pathogenesis[39].

AIA in rabbits. The guinea pigs develop arthritis when immunized twice at one week intervals with m-BSA (methylated-bovine serum albumin) in Freund's complete adjuvant and are then injected intra-articularly with 300 pg of m-BSA, three weeks after the first injection. Guinea pigs routinely develop antibodies, which will cause acute death due to anaphylaxis if the antigen is administered systemically. An injection given too deep into the joint space will sometimes connect with this vessel and the result will be instantly apparent. Agents active in human RA (cyclosporin, NSAIDs, etc) are active on the prophylactic version of the model. Guinea pigs are insensitive to the action of corticosteroids, so they cannot be used as positive control agents[42]. If the disease is allowed to progress for two weeks, histopathological evaluation reveals a highly destructive pannus, which has destroyed most of the articular cartilage[43].

Proteoglycan induced arthritis

After the development of CIA model in the late 1970s, several other proteins which are found in the joint, such as link protein, proteoglycan and gp-39 were found to induce arthritis in rodents. Proteoglycan can be used to induce arthritis in rabbits and dogs[33] as well as BALB/c mice. PG is a large molecule ($\sim 3 \times 10^6$ Da) that contains a protein core to which glycosaminoglycan side-chains including chondroitin sulfate and keratin sulfate are attached. The N- and O-linked oligosaccharides are also attached to this core region.

PGIA in rabbits. In early models of PGIA, bovine nasal septum cartilage was used for intra-articular injection. The experimental model developed spontaneous synovitis in the limb joints and rheumatoid factor-like component appeared in the sera of two rabbits from a pool of animals in the course of a long-term immunization with bovine nasal cartilage antigens. A single injection of proteoglycan antigens regularly provoked a heavy synovitis and cartilage destruction irrespective of whether the challenge injections were administered in physiological saline, or in Freund's complete adjuvant. The dose-dependent severity of arthritis demonstrated that the antibody titer against proteoglycan antigens played an important role. The four acid hydrolases (acid phosphatase, cathepsin D, hyaluronidase and beta-glucuronidase) showed an increased enzyme activity, when the synovial extract and synovial fluid of knee joints were injected with proteoglycan antigens. The degraded proteoglycans will trigger autoimmune reactions, and the process leads to chronic inflammation and joint destruction.

PGIA in mice. Proteoglycan, which is isolated from human cartilage, is used to induce arthritis in susceptible mouse strains. It can be developed in BALB/c and C3H mice strains, with vulnerability varying between mice from different suppliers[33]. This model is not MHC-specific because other mice with the same class II alleles are not susceptible to PGIA. Development of polyarthritis, immune complexes deposition and the presence of rheumatoid factor are shared features with human RA[10]. Proteoglycan, emulsified with an adjuvant, intraperitoneal (IP) injection is given on 0 and 21st day and optionally also on 42nd day. Earlier, CFA was used as the adjuvant, currently dimethyl dioctadecyl ammonium bromide (DDA) is widely used as an adjuvant; offering the advantage of early onset of arthritis and increased severity of arthritis, without tissue irritation and granuloma formation, as seen with CFA. After injection, strong B cell and T cell responses develop. Particularly, B cells have a dual role in PGIA, as they function as autoantibody-producing cells and are crucial in their role as APCs, and they also activate proteoglycan-specific T cells. Studies have demonstrated that during the effector phase of PGIA, TNF-α and IL-1β are expressed, as determined from messenger RNA (mRNA) isolated from joints. Additionally, it also showed increased levels of MIP-1α, MIP-1β, MIP-2 and MCP-1 mRNA were detected. CIA has been used more often than PGIA, due to the fact that human cartilage has to be extensively processed in order to prepare the needed proteoglycan fraction. The studies regarding proteoglycan obtained from other species has also been reported, it might facilitate the use of PGIA as a model to study new drugs for arthritis, however, the correlation efficiency may not be the same as with human proteoglycan[10,17].

G6PI induced arthritis in mice

As we all know that glucose-6-phosphate isomerase (G6PI) is a widely considered protein with multiple functions. This G6PI is an essential cytosolic enzyme in the energy cycle and has glycolytic activity, but it also expresses additional functions as an extracellular signaling molecule. G6PI is also known as autocrine motility factor and neuroleukine, which is considered to play roles in both cancer and autoimmunity[44]. Coincidentally, it was observed that G6PI plays a key role in arthritis development in mice. Several studies concluded that a bovine pancreas ribonuclease specific T-cell

receptor transgenic mouse crossed with NOD mice (the so-called K/BxN mouse) impetuously developed arthritis. Through a series of experiments, it was concluded that G6PI was recognized by transgenic T-cell receptor within the context of MHC class II molecule Ag7[45,46]. This model uses an immunization procedure similar to that used for CIA but relies mainly on a soluble auto-antigen, the ubiquinone containing glycolytic enzyme G6PI instead of collagen type II for induction of RA. As susceptibility to the disease is related with the same allelic region of the MHC II and its induction is dependent on the presence of B cells, G6PI-induced arthritis shares some common features with CIA[17].

Genetic models for rheumatoid arthritis

In addition to the induced-arthritis models, the development of arthritis occurs spontaneously in some genetically modified mice. These are mostly mice models that are either deficient in (knockout) or transgenic for a specific gene of interest. These models give information regarding the role of genes in the process of inflammation and serve as a tool to study the effect of therapeutics in mice prone to developing joint inflammation spontaneously.

K/BxN mice

One of the interesting models of arthritis to be developed in recent years is the K/BxN model of arthritis described by the group of Benoist and Mathis[47]. The KRN transgenic mouse expresses a T-cell receptor specific for an epitope of bovine pancreas ribonuclease. It was discovered serendipitously, that when KRN mice was crossed with NOD mice ($I\text{-}A^{g7}$), the resulting offspring (K/BxN) developed arthritis spontaneously at around 4–5 weeks of age. The arthritis is severe and symmetric affecting mainly the distal joints and shows resemblance to human RA in many important aspects. Further studies concluded that the arthritis development in K/BxN mice was seen to be dependent on $I\text{-}A^{g7}$ MHC class II molecules and could be blocked by administering anti-CD4 monoclonal antibody. The development of arthritis was found to require the presence of the B lymphocytes[48]. In K/BxN mice, arthritis is caused by an immune response to the enzyme glucose-6-phosphate isomerase (G6PI)[10]. The autoreactive T cells recognize a peptide derived from G6PI presented by antigen presenting cells (APCs) on I-Ag7 class II MHC molecules and activate B cells to produce G6PI-specific autoantibodies. T cells are considered important in the initiation phase of the disease, because when injection of an anti-CD4 antibody is administered within five days prior to the onset of the disease, it can block arthritis. Injection of CD4-specific antibody at a later time point does not affect the development of arthritis. IL-1 is required in this model, but TNF-α and IL-6 is not needed for development of K/BxN arthritis[10].

Human tumor necrosis factor gene (HTNFG) mice

Transgenic technology has changed the fate of cytokine biology, generating newer animal models for arthritis. For example, mice overexpressing a human TNF-α transgene, disregulated by the replacement of the 3′ AU-rich region with the 3′ untranslated region of the human β-globin gene were found by Kollias to spontaneously develop arthritis[49]. After 3–4 weeks of ages, synovial hyperplasia and inflammatory cell infiltrates can be observed and similarly mice have fully developed disease at 10 weeks of age. The features, in resemblance with human RA, observed are synovial hyperplasia, the presence of an inflammatory cell filtrate, pannus formation, cartilage destruction and bone resorption. The human TNF transgenic mice were generated by using H-2 K and H-2B, MHC haplotypes which are less favorable for arthritis development, bringing into question whether MHC has any important role in this model[10]. However, MHC can influence disease development, because backcrossing to the arthritis-vulnerable DBA/1 background resulted in earlier onset and the development of more severe disease[49]. The mice developed due to crossing human TNF-transgenic mice with RAG-1 knockout mice still develops erosive arthritis, without T cells and B cells, indicating that arthritis in this model is not dependent on T cells or B cells[50]. Further studies concluded that FLS obtained from synovium of human TNF transgenic mice can impel synovitis, cartilage damage and bone damage when transplanted into normal mice, illustrating the relative lack of involvement of immune cells in this model of arthritis[51]. This model was helpful in studying the destructive effect of excess TNF-α and the relationship between TNF and IL-1β in the pathogenesis of arthritis[52]. This model was found to be effective in assessing anti-TNF therapies but can also be used for testing other biologics and small molecules[49].

SKG mice

SKG mice were developed by the Sakaguchi Laboratory in Japan. Point mutations in the BALB/c breeding colony led to the development of BALB/c.SKG mice. This mice possessed spontaneous mutation in the SH2 domain of the ZAP-70 gene and developed spontaneous arthritis[53]. The histological observations include severe arthritis and synovitis involving the digits, ankles and frequently the base of the tail. It also reported the development of pneumonitis, dermatitis. High titers of rheumatoid factor and autoantibodies specific for type II collagen and hsp-70 were observed in the serum of infected mice, but no antibodies for DNA, as it is commonly associated with lupus-like conditions. This model could prove excellent model to study the underlying mechanisms leading to autoimmune injury of joints and other tissues. SKG mice model is best suited for understanding the mechanisms of thymic T cell selection and has yielded some insights in RA patients such as mutations in the ITAM regions of the TCR-ζ chain[53].

IL-1ra-/- transgenic mice

The most recent addition among arthritis models is the IL-1β receptor antagonist-deficient (IL-1ra-/-) mouse. It was generated on a BALB/c (but not on C57BL/6) background, this mouse spontaneously develops a polyarthropathy consisting of synovial and periarticular inflammation[54]. It also

demonstrated generation of antibodies against type II collagen, IgG and (unlike the ZAP-70-/- mouse) double-stranded DNA but not IgM rheumatoid factor[55]. The disease onset is as early as five weeks of age, with higher chances of morbidity exceeding 80% by eight weeks. This transgenic model is currently being used in mouse genome-wide microarray analysis to investigate the role of IL-1β in rheumatic disease. In addition, because IL-1β is a pleiotropic cytokine, this model also will function well in studies involving host defenses against viral and bacterial infections. Furthermore, IL-1β is known to induce IL-1α[54], IL-6 and cyclooxygenase (COX)-2, making this mouse an important tool to study COX-2 inhibitor.

Conclusion

In this review article, we have attempted to cover several important animal models of RA that are widely used or provide important observations. Still after decades of research, RA remains a disease of unknown etiology and a complex disease without a specific treatment plan. Animal models of arthritis continue to play an important role in pre-clinical research, particularly for the identification and validation of drug targets AIA and CIA models have great reproducibility and are used very frequently. Of the many available animal models, CIA, SCW and more recently, the K/BxN model have proven useful in understanding some of the mechanisms involved in RA. Although none of these models entirely rephrases clinical pathology, there is no doubt that they have provided importance to processes thought to be involved in disease development and progression. As knowledge of the etiology and pathogenesis of human RA expands, it is important to adapt and modify animal models to represent human disease. Nevertheless, despite these limitations, it is clear that animal models have provided worthwhile information relating to the pathogenesis of RA.

Disclosure statement

The authors declare that there is no conflict of interest.

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EXHIBIT 179

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Occurrence of Severe Destructive Lyme Arthritis in Hamsters Vaccinated with Outer Surface Protein A and Challenged with *Borrelia burgdorferi*

CINDY L. CROKE,[1,2] ERIK L. MUNSON,[1,2] STEVEN D. LOVRICH,[3] JOHN A. CHRISTOPHERSON,[1,2] MONICA C. REMINGTON,[1,2] DOUGLAS M. ENGLAND,[4,5] STEVEN M. CALLISTER,[3,6] AND RONALD F. SCHELL[1,2,7]*

Wisconsin State Laboratory of Hygiene[1] and Departments of Medical Microbiology and Immunology,[2] Bacteriology,[7] and Pathology and Laboratory Medicine,[4] University of Wisconsin, Madison, Wisconsin 53706; Department of Pathology, Meriter Hospital, Madison, Wisconsin 53715[5]; and Microbiology Research Laboratory[3] and Section of Infectious Diseases,[6] Gundersen Lutheran Medical Center, La Crosse, Wisconsin 54601

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Arthritis is a frequent and major complication of infection with *Borrelia burgdorferi* sensu stricto. The antigens responsible for the induction of arthritis are unknown. Here we provide direct evidence that a major surface protein, outer surface protein A (OspA), can induce arthritis. Hamsters were vaccinated with 30, 60, or 120 μg of recombinant OspA (rOspA) in aluminum hydroxide and challenged with *B. burgdorferi* sensu stricto isolate 297 or C-1-11. Swelling of the hind paws was detected in 100, 100, and 50% of hamsters vaccinated with 30, 60, or 120 μg of rOspA, respectively. In addition, arthritis developed in 57% of hamsters vaccinated with a canine rOspA vaccine after infection with *B. burgdorferi* sensu stricto. When the canine rOspA vaccine was combined with aluminum hydroxide, all vaccinated hamsters developed arthritis after challenge with *B. burgdorferi* sensu stricto. Histopathologic examination confirmed the development of severe destructive arthritis in rOspA-vaccinated hamsters challenged with *B. burgdorferi* sensu stricto. These findings suggest that rOspA vaccines should be modified to eliminate epitopes of OspA responsible for the induction of arthritis. Our results are important because an rOspA vaccine in aluminum hydroxide was approved by the Food and Drug Administration for use in humans.

Arthritis is the most frequent and the major complication of tick-borne transmission of *Borrelia burgdorferi* sensu stricto (*B. burgdorferi*) (31). Approximately 60% of individuals develop intermittent episodes of arthritis several weeks or months after infection. The brief attacks of arthritis last several days or weeks and generally occur in the larger joints (31, 32). In addition, 10% of arthritogenic patients develop antibiotic-resistant Lyme arthritis (12, 14), which can lead to permanent joint dysfunction (31). Infection with *B. burgdorferi* also causes moderate to severe arthritis in dogs (2), hamsters (13, 26), mice (4, 25), monkeys (3), and rats (5).

Recently, we showed that vaccination of hamsters with a whole-cell preparation of *B. burgdorferi* also induced arthritis, specifically severe destructive Lyme arthritis, following challenge with *B. burgdorferi* (18). Inflammation or swelling in the hind paws of vaccinated hamsters was detected 7 days after infection, peaked on day 10, and gradually decreased. A chronic synovitis characterized by hypertrophic villi, focal erosion of articular cartilage, and a subsynovial mononuclear infiltrate persisted for approximately 1 year. These findings demonstrate that *B. burgdorferi* possesses antigenic components that can induce arthritis in naturally infected humans (31, 32) and experimentally infected animals (2–5, 13, 25, 26).

Most importantly, some of these antigenic components are feasible candidates for use as a vaccine against infection with *B. burgdorferi* sensu lato (9, 11, 20, 24). The most promising candidate has been outer surface protein A (OspA) (29, 30). Recently, the Food and Drug Administration (FDA) approved the use of OspA for vaccination of humans despite indirect evidence and concerns that OspA is associated with arthritis (1, 12, 29, 30). In this study, we present direct evidence that vaccination with two preparations of recombinant OspA (rOspA) can induce severe destructive arthritis in hamsters after challenge with the Lyme borreliosis spirochete.

* Corresponding author. Mailing address: Wisconsin State Laboratory of Hygiene, University of Wisconsin, 465 Henry Mall, Madison, WI 53706. Phone: (608) 262-3634. Fax: (608) 265-3451. E-mail: rfschell@facstaff.wisc.edu.

MATERIALS AND METHODS

Hamsters. Twelve- to 16-week-old inbred LSH hamsters were obtained from our breeding colony located at the Wisconsin State Laboratory of Hygiene. Hamsters weighing 100 to 150 g were housed three or four per cage at an ambient temperature of 21°C. Food and water were provided ad libitum.

Organisms. Low-passage (<10) *B. burgdorferi* isolates 297 (from human spinal fluid), S-1-10 (from *Ixodes scapularis*), and C-1-11 (also from *I. scapularis*) were grown at 32°C in modified Barbour-Stoenner-Kelly (BSK) medium (6) until reaching a concentration of approximately 10^7 spirochetes per milliliter. Five-hundred-microliter samples were then dispensed into 1.5-ml screw-cap tubes (Sarstedt, Newton, N.C.) containing 500 μl of BSK medium supplemented with 20% glycerol (Sigma, St. Louis, Mo.), and the tubes were sealed and stored at −70°C. When needed, a frozen suspension of spirochetes was thawed and an aliquot was used to inoculate 4 ml of fresh BSK medium. Spirochetes were enumerated by dark-field microscopy, using a Petroff-Hausser counting chamber. *Escherichia coli* DH5α (Gibco BRL, Gaithersburg, Md.) was used for cloning experiments.

Amplification and cloning of the *ospA* gene. Plasmid-enriched DNA was isolated from *B. burgdorferi* isolate S-1-10 as previously described (20). The DNA was used as a template for the amplification of the *ospA* gene (GeneAmp; Perkin-Elmer Cetus, Norwalk, Conn.). The amino-terminal primer B1 (5′GCG TGGATCCATGAAAAAATATTTATTGGGAA3′) and the carboxy-terminal B2 (5′AATTCCCGGGTTATTTTAAAGCGTTTTTAA3′) were used for amplification. Primers were each used at a final concentration of 1.0 μM with an $MgCl_2$ concentration of 2.5 mM. Thermal cycling parameters were 94°C for 60 s followed by 35 cycles of (i) 94°C for 60 s, (ii) a 2-min ramp to 45°C, (iii) 45°C for 60 s, (iv) a 60-s ramp to 60°C, and (v) 60°C for 6 min. The final extension was done at 60°C for 10 min to fully extend any truncated DNA strands. Amplified DNA was purified with GeneClean (Bio 101, La Jolla, Calif.). After digestion with *Sma*I and *Bam*HI (Gibco BRL), purified DNA fragments were ligated into pGEX-2T (Pharmacia Biotech, Piscataway, N.J.). The insert and plasmid were

ligated with T4 DNA ligase (Gibco BRL), and the ligation mix was used to transform competent *E. coli* DH5α. Transformed *E. coli* cells were then plated onto 2× tryptone-yeast extract agar medium containing ampicillin (100 μg/ml; Sigma). Colonies expressing rOspA protein were identified by Western blot analysis using *B. burgdorferi* isolate B31 OspA monoclonal antibody H53332, provided by A. G. Barbour.

rOspA expression and purification. The transformed *E. coli* organisms containing the *ospA* gene were grown for 12 h at 37°C in 100 ml of 2× tryptone-yeast extract broth containing 100 μg of ampicillin per ml. Cultures were diluted 1:10 with broth medium and incubated for an additional 1 h. Isopropyl-β-D-thiogalactopyranoside (final concentration, 0.1 mM) was added, and the culture was incubated for 5 h. After incubation, the suspension of bacteria was centrifuged, resuspended in phosphate-buffered saline (PBS; pH 7.4), and lysed by three 30-s pulses with a sonicator (model W-350; Branson Sonic Power Co., Danbury, Conn.). Sonicated *E. coli* organisms were mixed with Triton X-100 (10%), diluted 10-fold with PBS, and centrifuged to remove insoluble material. The supernatant was mixed with a 50% slurry of glutathione-Sepharose beads (Pharmacia Biotech) for 5 min at room temperature and washed three times with ice-cold PBS. Fusion proteins were eluted by mixing the beads with 1 ml of 50 mM Tris-HCl (pH 8.0) containing 5 mM reduced glutathione for 2 min and collected after centrifugation for 60 s at 500 × *g*. The elution procedure was repeated four times. Fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with specific hamster serum, goat anti-glutathione *S*-transferase polyclonal antibody (Pharmacia Biotech), or monoclonal antibody H5332. Finally, fractions were concentrated by using a Centriprep-10 concentrator (Amicon, Beverly, Mass.) and dialyzed against PBS (pH 7.2) at 4°C overnight, using a dialysis cassette with a 10-kDa-molecular-mass cutoff (Pierce Chemical Co., Rockford, Ill.).

Vaccination of hamsters. Three groups of eight hamsters each were mildly anesthetized with ether contained in a nose-and-mouth cup and then vaccinated intramuscularly in each hind thigh with 0.25 ml containing 15, 30, or 60 μg of nonlipidated rOspA adsorbed to 1% aluminum hydroxide gel (Reheis Inc., Berkley Heights, N.J.) in PBS. Each hamster received a total of 30, 60, or 120 μg of rOspA. In addition, 20 hamsters were vaccinated in each hind thigh with 0.5 ml of a commercially available canine rOspA vaccine (with or without aluminum hydroxide) (Merial, Athens, Ga.). Controls consisted of nonvaccinated hamsters, hamsters inoculated with 1% aluminum hydroxide gel, hamsters vaccinated with 30, 60, or 120 μg of rOspA adsorbed to 1% aluminum hydroxide gel, and hamsters vaccinated with the canine rOspA.

Infection of hamsters. rOspA- and canine rOspA-vaccinated hamsters were mildly anesthetized with ether and challenged subcutaneously in each hind paw with 0.2 ml of BSK containing 5×10^6 viable *B. burgdorferi* isolate 297 or C-1-11 organisms. In some studies, hamsters inoculated with *B. burgdorferi* isolate 297 were reinfected with a similar inoculum 24 h after the initial infection. Nonvaccinated hamsters and those vaccinated with 1% aluminum hydroxide gel served as controls; each group was challenged with 10^7 viable spirochetes in BSK. Arthritis was not induced in hamsters vaccinated with aluminum hydroxide alone or with *E. coli* or *Staphylococcus epidermidis* in alum and then challenged with *B. burgdorferi* (8).

Assessment of arthritis. The degree of hind-paw swelling was used as an index to evaluate the inflammatory response. Prior to experimentation and cage assignment, hamsters were randomly chosen and their hind paws were measured to establish a baseline. After infection, the hind paws were measured periodically for 20 days with a dial-type Vernifer caliper (Fisher Scientific, Pittsburgh, Pa.) graduated in 0.1-cm increments. Measurements were obtained by mildly anesthetizing each hamster and carefully measuring the width and thickness of each hind paw. The daily mean group value was calculated by dividing the sum of the caliper values of each hind paw by the number of hind paws per group. This average value represented the severity of hind-paw swelling. Detection of arthritis by measurement of hind-paw swelling with a caliper is less variable when hamsters are challenged with 10^6 spirochetes or more; histopathologic examination is needed to confirm arthritis when hamsters are inoculated with 10^2 to 10^5 spirochetes.

Preparation of tissues for histologic examination. Twenty-one days after infection, hamsters were euthanized and their hind legs were amputated at midfemur, fixed in 10% neutral buffered formalin, placed in decalcifying solution (Lerner Laboratories, Pittsburgh, Pa.) for 24 h, and stored in 10% zinc formalin prior to processing. The hind legs were bisected longitudinally, placed in embedding cassettes (Fisher Scientific), embedded in paraffin, and cut into 6-mm-long sections. The sections were then placed on glass slides and stained with hematoxylin and eosin. The hind legs were randomly selected and cryptically coded for unbiased histopathologic examination by a certified pathologist.

Statistical analyses. The mean caliper values among groups were tested by analysis of variance with Minitab statistical analysis software. The alpha level was set at 0.05 before the experiments were started. The standard error of the mean for each mean caliper group value was also calculated.

RESULTS

Ability of rOspA vaccination to induce arthritis. Hamsters were vaccinated with 30, 60, or 120 μg of rOspA and challenged with *B. burgdorferi* isolate 297 at 11 and 12 days after vaccination. In addition, seven hamsters were vaccinated with a commercial canine rOspA vaccine and infected with *B. burgdorferi* isolate 297 (Table 1). Severe swelling of the hind paws was detected in 100, 100, 50, and 57% of hamsters vaccinated with 30, 60, or 120 μg of rOspA or the rOspA canine vaccine, respectively. Although slight swelling of the hind paws (mean ± standard error at baseline, 0.64 ± 0.05) occurred in nonvaccinated hamsters challenged with *B. burgdorferi* isolate 297, the degree of swelling was considerably lower than that (range, 0.91 ± 0.03 to 0.97 ± 0.04) detected in hamsters vaccinated with 30 or 60 μg of rOspA and challenged with *B. burgdorferi* isolate 297. When eight hamsters were vaccinated with 120 μg of rOspA and challenged with another *B. burgdorferi* isolate, C-1-11, that was not vaccine specific, all of the rOspA-vaccinated hamsters developed severe swelling of the hind paws. Similarly, hamsters vaccinated with 30 or 60 μg of rOspA developed severe swelling after challenge with *B. burgdorferi* isolate C-1-11. Furthermore, severe swelling of the hind paws developed in all hamsters vaccinated with the canine rOspA vaccine mixed with aluminum hydroxide and challenged with *B. burgdorferi* isolate 297. When these experiments were repeated, similar results were obtained.

TABLE 1. Development of hind-paw swelling in hamsters vaccinated with 30 μg, 60 μg, or 120 μg of rOspA or a canine rOspA vaccine and challenged with *B. burgdorferi* isolate 297[a]

Treatment group	% of animals positive for swelling[b]
OspA vaccinated	
30 μg	100
60 μg	100
120 μg	50
Canine	57
Nonvaccinated	0

[a] There were four or seven hamsters per group vaccinated with rOspA or the canine rOspA vaccine, respectively. There were five hamsters in the nonvaccinated group.

[b] Slight swelling (mean ± standard error, 0.64 ± 0.05 cm) of the hind paws occurred in nonvaccinated hamsters infected with *B. burgdorferi* isolate 297 compared to noninfected hamsters (0.42 ± 0.01 cm). However, rOspA-vaccinated hamsters developed significantly more swelling (0.87 ± 0.04 cm or more) after challenge with *B. burgdorferi* isolate 297.

Development of arthritis in rOspA-vaccinated hamsters. Two groups of three hamsters each were vaccinated with 30 μg of rOspA (Fig. 1). Eleven and 12 days after vaccination, members of one group of vaccinated hamsters were challenged subcutaneously in the hind paws with 10^7 viable *B. burgdorferi* isolate 297 organisms. Swelling of the hind paws was detected 7 days after primary challenge; increased rapidly, with peak swelling occurring on day 11; and gradually decreased. No swelling of the hind paws was detected in nonchallenged hamsters vaccinated with 30 μg of rOspA. Although swelling of the hind paws was detected in nonvaccinated hamsters challenged with *B. burgdorferi* isolate 297, the severity of swelling was considerably less than that detected in rOspA-vaccinated hamsters challenged with *B. burgdorferi* isolate 297. No swelling of the hind paws was detected in nonvaccinated, nonchallenged hamsters.

In other studies, hamsters were vaccinated with 30 μg of rOspA and challenged with *B. burgdorferi* isolate C-1-11. Swelling of the hind paws was detected on day 9, peaked on day 11, and gradually decreased. No swelling of the hind paws was detected in noninfected rOspA-vaccinated and nonvaccinated hamsters. Although nonvaccinated hamsters infected with *B.*

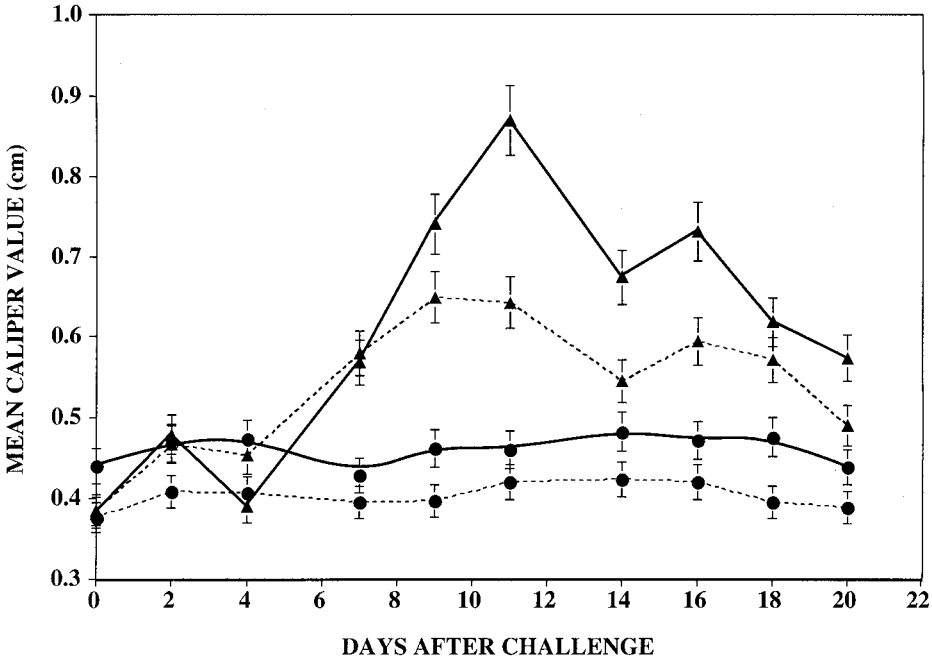

FIG. 1. Development of swelling in the hind paws of rOspA (30 μg)-vaccinated (——) and nonvaccinated (– – –) hamsters with (▲) and without (●) challenge with *B. burgdorferi* isolate 297.

burgdorferi isolate C-1-11 developed slight swelling in their hind paws, the degree of swelling was considerably lower than that detected in the rOspA-vaccinated hamsters challenged with *B. burgdorferi* isolate C-1-11.

Histopathology of hind-paw swelling. OspA-vaccinated hamsters challenged with *B. burgdorferi* isolate 297 showed a diffuse swelling of the hind paws secondary to fibroinflammatory and edematous changes of the soft tissue and joint capsule (Fig. 2A). Prominent focal tenosynovitis with subsynovial inflammation and early pannus formation (Fig. 2A) was also present. The pannus formation encroached on the periphery of the joint, causing osteoclastic reabsorption of bone as well as separation and fragmentation of the subchondrial bone of the joint. Proximal to the tibiotarsal joint, the inflammation showed further encroachment, with compression and atrophy of the bone, producing pyknosis and degeneration of osteocytic nuclei (Fig. 2B). In addition to bone erosion and distortion of the joint, a lymphoplasmacytic infiltrate admixed with a few neutrophils revealed involvement of the tendons of the hind paws. The intertarsal joints showed less inflammation. No granulomata, vasculitis, osteophytes, or loose bodies of the joints were found. By contrast, OspA-vaccinated (Fig. 2D) and nonvaccinated (Fig. 2E) hamsters not challenged with *B. burgdorferi* showed intact joints and normal capsular and pericapsular soft tissue. Nonvaccinated hamsters challenged with *B. burgdorferi* isolate 297 showed only mild soft-tissue swelling and mild tenosynovitis (Fig. 2C).

DISCUSSION

Public health concerns about the morbidity associated with Lyme borreliosis have stimulated efforts to develop an effective vaccine. Several *B. burgdorferi* sensu lato proteins, OspA (9, 20, 24), OspB (9, 24), OspC (11, 23, 24), and the 39-kDa protein (27), are capable of inducing a protective antibody response. Of these, OspA has emerged as the leading Lyme borreliosis vaccine candidate (29, 30). Two Lyme borreliosis vaccines based on rOspA have been shown to be protective in recent human clinical trials (29, 30). In addition, an rOspA vaccine has been approved by the USDA for use in dogs. Undoubtedly, these vaccines will be widely used, particularly in regions in which Lyme borreliosis is endemic, such as the upper midwestern and northeastern United States (7).

Although the Lyme borreliosis vaccines developed to date have been reported to be safe (17), there are concerns that rOspA might induce adverse effects, such as arthritis (5, 22, 29, 30). Akin et al. (1) showed that the level of anti-OspA immunoglobulin G, especially that specific to the C-terminal epitope of OspA, correlated with maximum arthritis in naturally infected patients. In addition, the cellular immune response to OspA was elevated in genetically susceptible persons, particularly those with HLA-DR4 specificity (14). These patients also had persistent arthritis despite treatment with antimicrobial agents. Furthermore, Gross et al. (12) identified an immunodominant epitope of OspA for T cells that might be responsible for the induction of treatment-resistant Lyme arthritis. Collectively, these findings suggest that OspA is involved in the induction of arthritis in patients infected with *B. burgdorferi* sensu lato.

In this study, we provided direct evidence that rOspA can induce arthritis. Hamsters vaccinated with rOspA in aluminum hydroxide (alum) developed swelling of the hind paws after infection with *B. burgdorferi* isolate 297 or C-1-11. Arthritis was detected in the hind paws of all hamsters vaccinated with 30 or 60 μg of rOspA. Histopathologic examination of the swollen hind paws confirmed the development of severe destructive arthritis. In addition, we showed that a canine rOspA vaccine primed (vaccinated) hamsters for induction of arthritis upon challenge with *B. burgdorferi* isolate 297. Fifty-seven percent of infected, canine rOspA-vaccinated hamsters developed arthritis. Furthermore, when aluminum hydroxide was incorporated into the canine rOspA vaccine, all hamsters developed arthritis after infection with *B. burgdorferi* isolate 297. These results

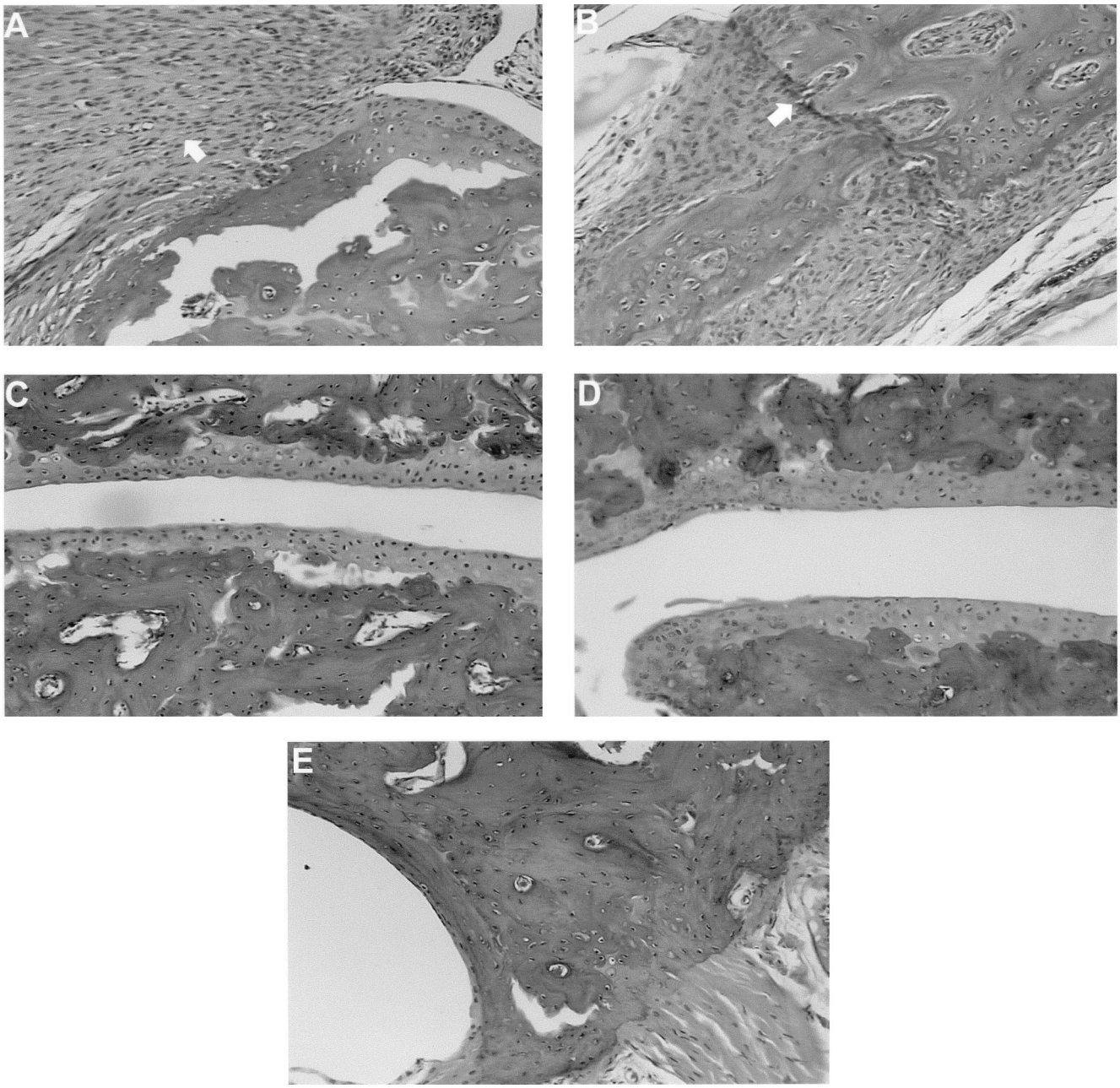

FIG. 2. Histopathology of the hind paws of rOspA-vaccinated (A and B) and nonvaccinated (C) hamsters 21 days after challenge with *B. burgdorferi* isolate 297. Controls included rOspA-vaccinated (D) and nonvaccinated (E) hamsters.

show that different preparations of rOspA can induce arthritis and that aluminum hydroxide augments the adverse response. The FDA-approved rOspA vaccine for humans contains aluminum hydroxide (30).

In other studies, 50 and 100% of hamsters vaccinated with 120 μg of rOspA developed severe destructive arthritis when challenged with the infectious vaccine-specific isolate of *B. burgdorferi* or another isolate of *B. burgdorferi* (C-1-11), respectively. Previously, we showed that humans vaccinated with 30 μg of rOspA and a booster elicited a poor anti-OspA protective borreliacidal antibody response (22) not only against the vaccine-specific agent but also against other isolates of *B. burgdorferi* sensu lato. In addition, the anti-OspA borreliacidal antibody titer waned rapidly after vaccination. Although Sigal et al. (29) and Steere et al. (30) demonstrated that rOspA was protective in human field trials, neither the level of the anti-OspA borreliacidal antibody response nor its duration of protection against *B. burgdorferi* isolates was reported. Lim et al. (18) showed that vaccinated hamsters developed severe destructive arthritis before protective borreliacidal antibodies developed and after they waned when challenged with *B. burgdorferi* or other isolates. Our results and those of Lim et al. (18) and Padilla et al. (22) suggest that rOspA primes subjects for induction of arthritis without inducing sustained high levels of anti-OspA borreliacidal antibodies. In support of this theory, several boosters of rOspA are required over a 2-year period to

obtain 68 to 78% protection against infection with *B. burgdorferi* (29, 30). Patients received a total of 90 μg of rOspA (30). Additional studies are needed in humans to determine the duration of the borreliacidal antibody response against both the vaccine-specific isolate and other isolates of *B. burgdorferi*. These studies are necessary for defining the composition of the vaccine (number of rOspA molecules) along with the number and schedule of boosters for maintaining high levels of borreliacidal antibody to prevent potential adverse effects upon challenge with homologous or other isolates of *B. burgdorferi*.

We used a challenge inoculum of approximately 10^6 viable *B. burgdorferi* organisms to elicit severe destructive arthritis in rOspA-vaccinated hamsters. The major histopathologic findings of the joint and capsule, as well as the surrounding soft tissue, resulted in swelling, pain, deformity, and selective loss of movement for the hamster. When vaccinated hamsters were challenged with fewer (10^2 to 10^4) *B. burgdorferi* cells, histopathologic responses that resulted in tenosynovitis were detected. This response in hamsters may be similar to the response that occurs in humans. Although vaccine-induced arthritis after natural infection of humans with *B. burgdorferi* has not been reported (29, 30), this does not rule out the possibility that rOspA is an arthritogenic agent. Repeated vaccinations of humans with rOspA in alum to maintain protection against infection with *B. burgdorferi* may increase the number of vaccinees reporting symptoms of arthritis. The present phase III clinical trials did not report sufficient numbers of vaccinees challenged with *B. burgdorferi* to determine whether rOspA induced arthritis. Human subjects afflicted with rOspA-related tenosynovitis before or after challenge with *B. burgdorferi* should consult a clinician. These numbers of complaints need to be determined.

The immunologic mechanism(s) by which rOspA or whole cells of *B. burgdorferi* (18) induce arthritis is incompletely understood. We showed previously that both *B. burgdorferi*-specific $CD4^+$ and $CD8^+$ T lymphocytes interacted with macrophages to induce severe destructive arthritis (8). In addition, vaccinated hamsters treated with anti-$CD4^+$ antibody failed to develop severe destructive arthritis when infected with *B. burgdorferi* (19). Other investigators (15, 16, 21) have also reported that T cells and their subsets can exert antagonistic influences on the induction of arthritis. Furthermore, rOspA may induce cross-reactive antibodies that initiate an autoimmune response. OspA has been shown to cause polyclonal activation of B cells (33). These findings indicate that components of the anti-OspA response are T-cell dependent and play a key role in the induction of arthritis. Concomitantly, T-cell-independent responses that result in the production of polyreactive antibodies which cross-react with self-components also occur (10, 28). Evidence, therefore, that several different epitopes of OspA are involved with the production of autoantibodies and protective anti-OspA borreliacidal antibodies and the induction of arthritis is accumulating. The epitopes of rOspA responsible for production of autoantibodies and arthritis must be eliminated before rOspA becomes a successful vaccine.

In conclusion, rOspA vaccination induces severe destructive Lyme arthritis. The present rOspA vaccines must be modified to eliminate potential side effects. The production of a nonarthritogenic rOspA vaccine can be readily determined by using the hamster model.

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Review

Vaccinations and risk of systemic lupus erythematosus and rheumatoid arthritis: A systematic review and meta-analysis

Bin Wang [a,b,1], Xiaoqing Shao [a,b,1], Dan Wang [c,d], Donghua Xu [e], Jin-an Zhang [a,b,*]

[a] Department of Rheumatology and Immunology, Jinshan Hospital of Fudan University, Shanghai 201508, China
[b] Department of Endocrinology, Jinshan Hospital of Fudan University, Shanghai 201508, China
[c] Department of Epidemiology and Biostatistics, School of Public Health, Hebei Medical University, Shijiazhuang 050017, China
[d] Department of Library, Hebei Medical University, Shijiazhuang 050017, China
[e] Department of Rheumatology and Immunology, The Affiliated Hospital of Weifang Medical University, Weifang 261000, China

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ABSTRACT

Background: In the past several years, more and more studies proposed some concerns on the possibly increased risk of autoimmune diseases in individuals receiving vaccinations, but published studies on the associations of vaccinations with risks of systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) reported conflicting findings. A systematic review and meta-analysis was carried out to comprehensively evaluate the relationship between vaccinations and risk of SLE and RA.

Methods: Pubmed, Web of Science and Embase were searched for observational studies assessing the associations of vaccinations with risks of RA and SLE. Two authors independently extracted data from those eligible studies. The quality of eligible studies was assessed by using the Newcastle-Ottawa Scale (NOS). The pooled relative risk (RR) with 95% confidence intervals (CIs) was used to measure the risk of RA and SLE associated with vaccinations, and was calculated through random-effect meta-analysis.

Results: Sixteen observational studies were finally considered eligible, including 12 studies on the association between vaccinations and SLE risk and 13 studies on the association between vaccinations and RA risk. The pooled findings suggested that vaccinations significantly increased risk of SLE (RR = 1.50; 95%CI 1.05–2.12, P = 0.02). In addition, there was an obvious association between vaccinations and increased risk of RA (RR = 1.32; 95%CI 1.09–1.60, P = 0.004). Meta-analysis of studies reporting outcomes of short vaccinated time also suggested that vaccinations could significantly increase risk of SLE (RR = 1.93; 95%CI 1.07–3.48, P = 0.028) and RA (RR = 1.48; 95%CI 1.08–2.03, P = 0.015). Sensitivity analyses in studies with low risk of bias also found obvious associations of vaccinations with increased risk of RA and SLE.

Conclusion: This study suggests that vaccinations are related to increased risks of SLE and RA. More and larger observational studies are needed to further verify the findings above and to assess the associations of vaccinations with other rheumatic diseases.

Contents

Abbreviations: ANA, anti-nuclear autoantibody; ASIA, autoimmune/inflammatory syndrome induced by adjuvants; CI, confidence interval; HBV, Hepatitis B virus; HPV, human papillomavirus; HR, hazard ratio; NOS, Newcastle-Ottawa Scale; OR, odds ratio; PRISMA, Preferred Reporting Items for Systematic reviews and Meta-Analyses; RA, rheumatoid arthritis; RR, relative risk; SLE, systemic lupus erythematosus.

* Corresponding author at: Department of Rheumatology and Immunology & Department of Endocrinology, Jinshan Hospital of Fudan University, No. 1508 Longhang Road, Shanghai 201508, China.
E-mail address: zhangjinan@hotmail.com (J. Zhang).

[1] B. Wang and X. Shao contributed equally to this study.

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1. Introduction

Vaccination is the most effective way of preventing infectious diseases [1]. However, recent studies suggested that it's also possibly associated with some adverse events including autoimmunity [2–6]. To increase human immune response to vaccines, adjuvants are usually added to vaccines, but they can result in the development of some autoimmune events [7,8]. Autoimmune/inflammatory syndrome induced by adjuvants (ASIA), also named as Shoenfeld's syndrome and first presented by Shoenfeld and Agmon-Levin in 2011, is a well-defined autoimmune condition caused by adjuvants [9–12]. In the past several years, more and more studies have proposed concerns about the possibly increased risk of autoimmune diseases associated with vaccinations [13–15].

Rheumatic diseases are a group of immune mediated disorders which mainly affect joints and muscles, and they have become common causes of chronic illness worldwide [16]. Rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) are two common rheumatic diseases, both of which obviously impair the life quality of affected patients and are associated with higher risk of mortality [17–19]. Though the prevalence of rheumatic diseases is relatively low, some studies suggested that the incidence rates of RA and SLE have increased in recent years [20,21]. Identifying risk factors of RA or SLE not only leads to a better understanding of their pathogenesis, but also helps us to develop some effective interventions to reduce risk of RA or SLE in those high-risk individuals.

Severe cases reports of SLE or RA following vaccinations were frequently reported in the literature, which indicated that vaccinations might be causal factors of those rheumatic diseases [22–36]. There were also a number of epidemiological studies on the associations of vaccinations with risk of RA or SLE, but they reported conflicting findings [37–49]. Some studies reported that vaccinations could increase the risk of RA or SLE [38,40,44,48,49], but other studies found that vaccinations had no obvious impact on the risk of RA or SLE [37,39,41,45,46]. Therefore, there is no definite conclusion on the relationship between vaccinations and risk of RA and SLE. To get a better understanding of the impact of vaccinations on risk of RA and SLE, a systematic review and meta-analysis was performed. The present meta-analysis was performed by the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) statement, and was registered at PROSPERO website (CRD42017059866) [50].

2. Methods

2.1. Search strategy

Pubmed, Web of Science and Embase were searched for observational studies assessing the associations of vaccinations with risk of RA and SLE. The search strategy used in Pubmed was as following: (vaccinations OR vaccination OR vaccines OR vaccine) AND (rheumatic diseases OR rheumatic disease OR rheumatoid arthritis OR lupus OR systemic lupus erythematosus OR SLE). The last time of literature search was February 15, 2017, and no language restriction was used. The bibliographies of relevant reviews and included studies were checked to find additional studies.

2.2. Selection criteria

Eligible studies must meet the following selection criteria: (1) Cohort studies or case-control studies; (2) The exposure was vaccinations, such as HBV vaccination and HPV vaccination; (3) The controls were those individuals without receiving vaccinations; (4) The outcome of interest was the risk of RA and SLE associated with vaccinations; (5) Reporting risk estimates with 95% confidence intervals (CIs) of rheumatic diseases caused by vaccinations, such as relative risk (RR), hazard ratio (HR), odds ratio (OR) and incidence rate ratios. Studies with overlapping data or without usable data were all excluded.

2.3. Data extraction

Two authors extracted data independently from those eligible studies using a predesigned form, and disagreements were resolved by discussion among all authors. Data extracted from included studies mainly contained authors' name, publication year, study design, country, characteristics of participants, selection of controls, source of information about vaccinations, length of follow-up, definitions of SLE or RA, confounding factors used for adjustment analysis, matched factors used to select controls, and risk estimates with 95%CIs of rheumatic diseases. For studies provided both unadjusted and adjusted risk estimates, only the latter one was used in the meta-analysis.

2.4. Quality assessment

We assessed the study quality according to the Newcastle Ottawa Scale (NOS). According to the NOS criteria, risk of bias of included studies was assessed on three domains: selection of participants, comparability between exposed and non-exposed participants, and the ascertainment of outcomes. Four stars, two stars and three stars were scored for those three domains respectively, and studies with total stars of <6 were deemed to have high risk of bias. Studies with 6 or more stars were deemed to have low risk of bias.

2.5. Statistical methods

The pooled RR with 95%CI was used to measure the risk of RA and SLE associated with vaccinations. To account for heterogeneity among those eligible studies, data were pooled using a random-effect meta-analysis (DerSimonian-Laird method) [51]. I^2 statistic and Cochran's Q test were utilized to test the heterogeneity [52,53]. When I^2 was >50%, meta-regression analysis was carried out to identify potential sources

of heterogeneity, and factors analyzed in the meta-regression analysis included study design (case-control or cohort), time of outcome ascertainment (<180 days or not), funding source, study quality (high risk of bias, or low risk of bias), and types of vaccines. Persson et al.'s study reported outcomes of both short vaccinated time and long vaccinated time, and we firstly used data for long vaccinated time and then performed a sensitivity analysis by using data for short vaccinated time [43]. Sensitivity analyses were also performed by excluding studies with high risk of bias, or excluding studies without outcomes for short vaccinated time (<180 days), or excluding studies funded by pharmaceutical companies. Subgroup analyses were then performed by study design (case-control or cohort), and types of vaccinations (HBV vaccine, HPV vaccine or influenza vaccine). The asymmetry of funnel plot was assessed to judge publication bias risk, and Egger's test was used [54]. Begg's rank correlation test was also utilized to assess publication bias risk [55]. STATA 12.0 (StataCorp, College Station, Texas, USA) was used in the statistical analyses, and $P < 0.05$ indicated statistical significant difference.

3. Results

3.1. Literature search and study selection

Fig. 1 showed the details of study selection in the meta-analysis (Fig. 1). Of those records identified from initial literature search, 6972 records were excluded after reading titles and abstracts for overlapping abstracts or obviously irrelevant studies. Thirty-five full-text publications were retrieved for detailed assessment [37–49,56–77], and 19 studies were further excluded [59–77]. Among those 19 excluded studies, 17 studies were excluded for irrelevant study design or lack of data on outcomes of interest [61–77], and two studies were excluded for containing overlapping data [59,60]. Therefore, 16 studies were finally considered eligible and were included into the meta-analysis [37–49, 56–58].

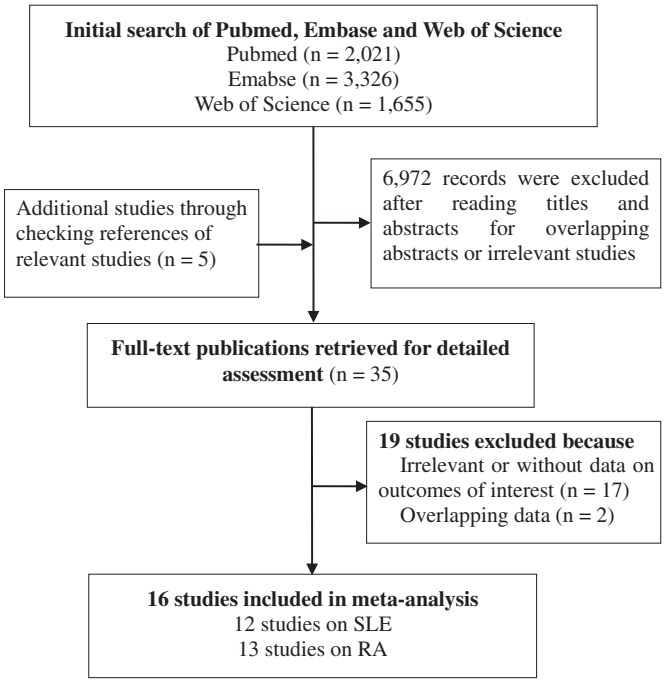

Fig. 1. Flow diagram of study selection in the meta-analysis.

3.2. Study characteristics

Table 1 showed the main characteristics of those 16 observational studies included into the meta-analysis (Table 1). Those 16 studies were published between 2002 and 2016. There were 12 studies on the association between vaccinations and SLE risk [37,38,41–49,57], and 13 studies on the association between vaccinations and RA risk [38–41,43,45–49,56–58] (Table 1). Eight studies were cohort studies [40, 41,43,46,47,56–58], and the other 8 studies were case-control studies [37–39,42,44,45,48,49]. Fourteen studies (87.5%) were performed in Europe or North America [37–43,45–49,57,58]. The vaccines used in those studies were various across different studies (Table 1). Among those 16 studies, 6 studies assessed the association of influenza vaccine with risk of SLE or RA [39,40,42,43,56,58], 5 studies assessed the association of HPV vaccine with risk of SLE or RA [41,46–48,57], 4 studies assessed the association of HBV vaccine with risk of SLE or RA [38,40,42,44]. Bardenheier et al.'s study analyzed the impact of anthrax vaccine on risk of SLE and RA [45], and Lai and Yew study assessed the impact of zoster vaccine on risk of SLE and RA [49]. Eight studies reported outcomes of short vaccinated time (no >180 days) [38,40,41,43,45,47,48, 56]. Besides, five studies received funds from pharmaceutical companies [42,46,47,57,58], but other 11 studies didn't receive funds from pharmaceutical companies [37–41,43–45,48,49,56]. There was obvious variance in the confounding factors or matched factors utilized among those 16 included studies (Table 1).

3.3. Quality assessment

Details on the risk of bias among those 16 studies were summarized in Table 2. Depending on the NOS criteria, 14 studies scored 6 or more stars and thus had low risk of bias (Table 2). Two studies had scored 5 or less stars and had high risk of bias (Table 2).

3.4. Vaccinations and SLE risk

Meta-analysis of those 12 studies on SLE [37,38,41–49,57] suggested that vaccinations significantly increased risk of SLE (RR = 1.50; 95%CI 1.05–2.12, P = 0.024; Fig. 2). When using data for short vaccinated time in Persson et al.'s study, the pooled RR of SLE related to vaccinations was 1.52 (95%CI 1.11–2.09, P = 0.01). After excluding studies with high risk of bias, there was still a significant association between vaccinations and increased risk of SLE (RR = 1.57; 95%CI 1.09–2.26, P = 0.016; Table 3). The pooled results of the meta-analysis of 8 studies without receiving funds from pharmaceutical companies also suggested an obviously increased risk of SLE related to vaccinations (RR = 1.73; 95%CI 1.06–2.82, P = 0.029). In addition, meta-analysis of five studies reporting outcomes of short vaccinated time suggested that vaccinations could significantly increase risk of SLE (RR = 1.93; 95%CI 1.07–3.48, P = 0.028; Fig. 3).

In the subgroup analyses by study design, meta-analysis of case-control studies showed a significant association between vaccinations and increased risk of SLE (RR = 1.85; 95%CI 1.03–3.32, P = 0.040), while meta-analysis of cohort studies suggested a marginally significant association between vaccinations and increased risk of SLE (RR = 1.16; 95%CI 0.99–1.37, P = 0.07). In addition, subgroup analyses by types of vaccines showed that HBV vaccine was significantly related to increased risk of SLE (RR = 2.46; 95%CI 1.09–5.52, P = 0.029) (Table 2).

Obvious heterogeneity was observed among those 12 studies on the relationship between vaccinations and SLE risk ($I^2 = 71.7\%$). Meta-regression analysis suggested that study design (P = 0.42), time of outcome ascertainment (P = 0.37), funding source (P = 0.22), study quality (P = 0.56), and types of vaccines (P = 0.31) were all not the sources of heterogeneity.

Table 1
Characteristics of 16 observational studies included into the meta-analysis.

Study [Ref.]	Study design	Country	Participants	Types of vaccines	Outcomes	Confounding factors or matched factors
Cooper GS 2002 [37]	Case-control	USA	265 SLE patients and 355 control subjects matched to patients by age, sex, and state.	Vaccinations	SLE	Age, sex, race, state, and education.
Geier DA 2005 [38]	Case-control	USA	Vaccine adverse events following HBV vaccines in comparison to controls matched by age, sex, and vaccine year.	HBV vaccine	SLE; RA	Age, sex, and vaccine year.
Verstraeten T 2008 [46]	Cohort	Multiple countries	68,512 participants involved in randomized controlled trials of AS04 adjuvanted vaccines	Vaccinations; HPV vaccine	SLE; RA	Randomized trials.
Bengtsson C 2010 [39]	Case-control	Sweden	1998 incident cases of RA and 2252 randomly selected controls matched for age, sex and residency.	Vaccinations; Influenza vaccine	RA	Age, sex and residency.
Ray P 2011 [40]	Cohort	USA	2,587,199 participants aged 15–59 years from 1997 through 1999	Vaccinations; Influenza vaccine; HBV vaccine	RA	Race, sex, and exact number of utilization visits.
Chao C 2012 [47]	Cohort	USA	189,629 women who received at least one dose of HPV vaccine between 2006 and 2008	HPV vaccine	SLE; RA	Age.
Ho TY 2012 [56]	Cohort	Taiwan	41,986 vaccinated elderly persons and 50,973 unvaccinated elderly persons	Influenza vaccine	RA	Gender, age, comorbidity, geographic region and urbanization of residence, and individual socioeconomic status.
Arnheim-Dahlstrom L 2013 [41]	Cohort	Denmark	997,585 girls and 296,826 of them received HPV vaccines	HPV vaccine	SLE; RA	Country, age, calendar year, parental educational level, parental country of birth, and paternal socioeconomic status.
Angelo MG 2014 [57]	Cohort	40 countries	31,173 adolescent girls and adult women receiving HPV vaccine and 24,241 controls involved in randomized controlled trials	HPV vaccine	SLE; RA	Randomized trials.
Zou Y 2014 [44]	Case-control	China	471 SLE cases identified from 1,253,832 individuals	HBV vaccine	SLE	Age, birth date, sweet food consumption, cooking oil consumption, fruit consumption, sunlight exposure, negative life event, and physical activities.
Persson I 2014 [43]	Cohort	Sweden	3,347,467 vaccinated with Pandemrix between 2009 and 2010 and 2,497,572 non-vaccinated individuals.	Influenza vaccine	SLE; RA	County of residence, age, gender, education, income, birth place, number of hospitalizations, and number of ambulatory care visits.
Vaughn DW 2014 [58]	Cohort	Multiple countries	22,521 participants involved in 28 randomized controlled trials	Influenza vaccine	RA	Randomized trials.
Grimaldi-Bensouda L 2014 [42]	Case-control	France/Canada	105 SLE patients and 712 controls	Vaccinations; Influenza vaccine; HBV vaccine	SLE	Age, sex, region of residence, date of recruitment, smoking, alcohol consumption, pregnancy, family history of autoimmune disorders, number of medications.
Lai YC 2015 [49]	Case-control	USA	80 SLE patients and 241 controls, and 140 RA patients and 448 controls	Zoster vaccine	SLE; RA	None.
Bardenheier BH 2016 [45]	Case-control	USA	77 RA patients and 229 controls, and 39 SLE patients and 117 controls	Anthrax vaccine	SLE; RA	Sex, age, service branch, calendar time of beginning under medical surveillance, and deployment status.
Geier D 2016 [48]	Case-control	USA	36 SLE patients and 21,998 controls, and 43 RA patients and 48,809 controls	HPV vaccine	SLE; RA	None.

(NA, Not available; HBV, Hepatitis B virus; HPV, human papillomavirus; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis.)

Table 2
Risk of bias assessment in those 16 observational studies on the associations of vaccinations with SLE and RA.

Study [Ref.]	Selection (stars awarded)	Comparability (stars awarded)	Outcome ascertainment (stars awarded)	Bias risk (total stars awarded)
Cooper GS 2002 [37]	2	2	3	Low (7)
Geier DA 2005 [38]	3	1	2	Low (6)
Verstraeten T 2008 [46]	3	1	2	Low (6)
Bengtsson C 2010 [39]	2	2	3	Low (7)
Ray P 2011 [40]	4	2	3	Low (9)
Chao C 2012 [47]	3	1	2	Low (6)
Ho TY 2012 [56]	4	2	2	Low (8)
Arnheim-Dahlstrom L 2013 [41]	4	2	2	Low (8)
Angelo MG 2014 [57]	3	1	2	Low (6)
Zou Y 2014 [44]	3	2	2	Low (7)
Persson I 2014 [43]	4	2	3	Low (9)
Vaughn DW 2014 [58]	2	1	2	High (5)
Grimaldi-Bensouda L 2014 [42]	2	2	2	Low (6)
Lai YC 2015 [49]	3	0	2	High (5)
Bardenheier BH 2016 [45]	2	2	3	Low (7)
Geier D 2016 [48]	3	0	3	Low (6)

(Acceding to the NOS, four stars, two stars and three stars were scored for those three domains respectively, and studies with total stars of <6 stars were deemed to have high risk of bias.)

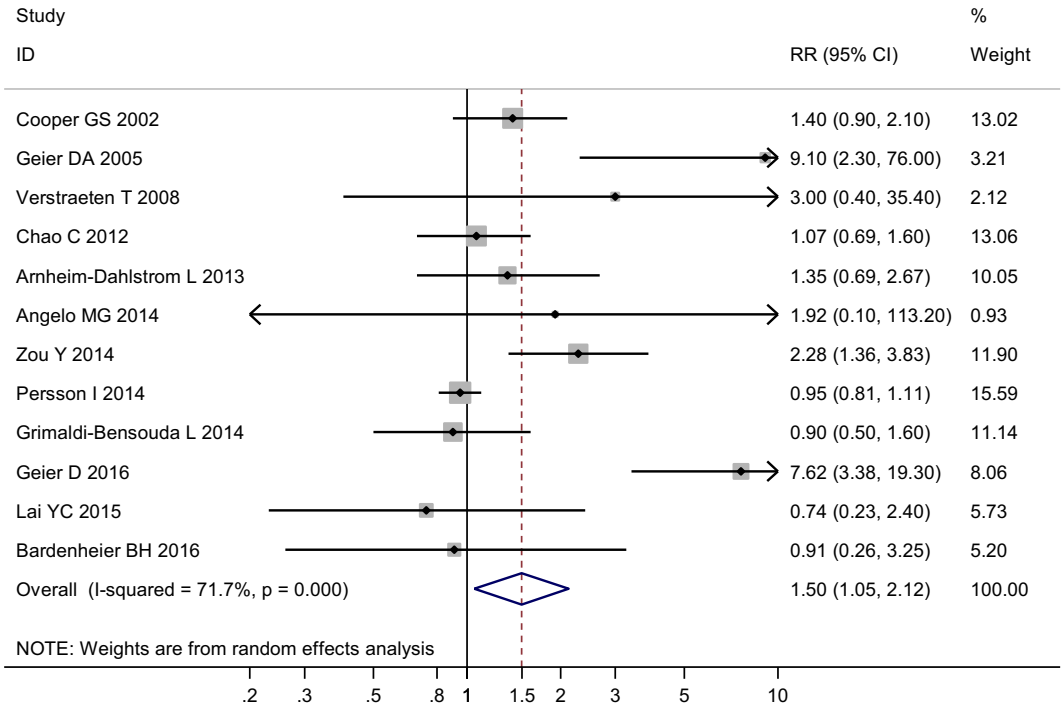

Fig. 2. Meta-analysis of 12 eligible studies showed that vaccinations significantly increased risk of SLE.

3.5. Vaccinations and RA risk

Meta-analysis of those 13 studies on RA [38–41,43,45–49,56–58] suggested that vaccinations significantly increased risk of RA (RR = 1.32; 95%CI 1.09–1.60, P = 0.004; Fig. 4). When using data for short vaccinated time in Persson et al.'s study, the pooled RR of RA associated with vaccinations was 1.36 (95%CI 1.11–1.65, P = 0.003). After excluding studies with high risk of bias, there was still a significant association between vaccinations and increased risk of RA (RR = 1.24; 95%CI 1.03–1.49, P = 0.025; Table 3). The pooled results of the meta-analysis of 9 studies without receiving funds from pharmaceutical companies also suggested an obviously increased risk of RA associated with vaccinations (RR = 1.40; 95%CI 1.14–1.72, P = 0.002). In addition, meta-analysis of 8 studies reporting outcomes of short vaccinated time suggested that vaccinations could significantly increase risk of RA (RR = 1.48; 95%CI 1.08–2.03, P = 0.015; Fig. 5).

In the subgroup analyses by study design, meta-analysis of case-control studies showed a significant association between vaccinations and increased risk of RA (RR = 2.51; 95%CI 1.13–5.57, P = 0.024), and meta-analysis of cohort studies also suggested a significant association between vaccinations and increased risk of RA (RR = 1.17; 95%CI 1.09–1.26, P < 0.001). Besides, subgroup analyses by types of vaccines showed that influenza vaccine was significantly related to increased risk of RA (RR = 1.17; 95%CI 1.09–1.25, P < 0.001) (Table 2).

Obvious heterogeneity was noted among those 13 studies on the relationship between vaccinations and RA risk (I^2 = 73.4%). Meta-regression analysis suggested that time of outcome ascertainment (P = 0.68), funding source (P = 0.35), study quality (P = 0.37), and types of vaccines (P = 0.86) were not the sources of heterogeneity, but study design (P = 0.09) was a possible source of heterogeneity.

Table 3
Summary of the main findings in the meta-analysis of the associations of vaccinations with SLE and RA.

Analyses	Number of studies	RR(95%CI)	P values	I^2 for heterogeneity
SLE				
Total studies	12	1.50(1.05–2.12)	0.024	71.7%
Studies with low bias risk	11	1.57(1.09–2.26)	0.016	74.0%
Excluding studies funded by pharmaceutical companies	8	1.73(1.06–2.82)	0.029	81.3%
Outcomes of short vaccinated time	5	1.93(1.07–3.48)	0.028	82.4%
Case-control studies	7	1.85(1.03–3.32)	0.040	75.4%
Cohort studies	5	1.16(0.99–1.37)	0.070	0%
HPV vaccine	5	2.10(0.87–5.05)	0.099	75.2%
Influenza vaccine	2	1.16(0.97–1.39)	0.105	0%
HBV vaccine	3	2.46(1.09–5.52)	0.029	38.7%
RA				
Total studies	13	1.32(1.09–1.60)	0.004	73.4%
Studies with low bias risk	11	1.24(1.03–1.49)	0.025	72.3%
Excluding studies funded by pharmaceutical companies	9	1.40(1.14–1.72)	0.002	81.5%
Outcomes of short vaccinated time	8	1.48(1.08–2.03)	0.015	75.9%
Case-control studies	5	2.51(1.13–5.57)	0.024	89.2%
Cohort studies	8	1.17(1.09–1.26)	<0.001	0%
HPV vaccine	5	1.44(0.65–3.21)	0.370	80.5%
Influenza vaccine	4	1.17(1.09–1.25)	<0.001	0%
HBV vaccine	2	3.77(0.41–34.4)	0.239	62.4%

(RR, relative risk; 95%CI, 95% confidence interval; HBV, Hepatitis B virus; HPV, human papillomavirus; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis.)

3.6. Publication bias

The funnel plots' shape did not suggest an obvious tendency of asymmetry, suggesting low risk of publication bias (Fig. 6). The P values of Egger's test and Begg's test in the meta-analysis of the association between vaccinations and SLE were 0.18 and 0.24, respectively. In addition, the P values of Egger's test and Begg's test in the meta-analysis of the association between vaccinations and RA were 0.09 and 0.58, respectively. Therefore, the findings above indicated that this meta-analysis didn't have high risk of publication bias.

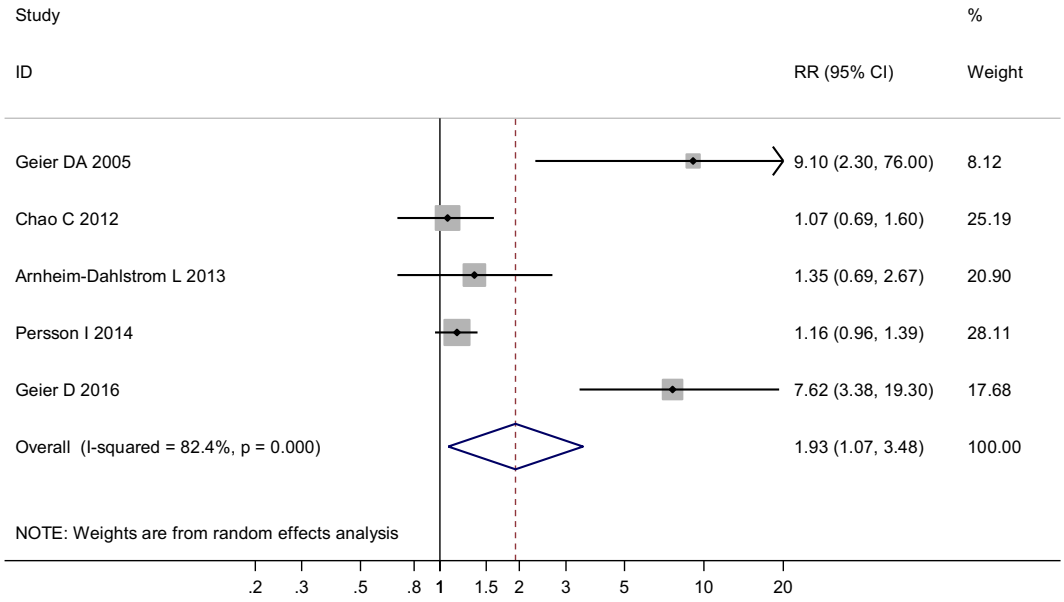

Fig. 3. Meta-analysis of studies reporting outcomes of short vaccinated time suggested a significantly increased risk of SLE associated with vaccinations.

4. Discussion

In the past two decades, the risk of autoimmune diseases following vaccinations has become a growing concern [2,4,78–80]. However, the potential associations of vaccinations with autoimmune diseases had been questioned because most published studies were case-reports or case-series [81–85]. In the past decade, a number of observational studies were conducted to assess the associations of vaccinations with SLE or RA, but those studies reported controversial findings. Currently, there is still a heated debate on whether vaccinations are risk factors of rheumatic diseases, such as SLE and RA [85,86]. Therefore, there is a clear need to perform a systematic review and meta-analysis of those published observational studies to comprehensively evaluate the impact of vaccinations on risk of SLE or RA. To the best of our knowledge, the present study is the first systematic review and meta-analysis aiming to assess the associations of vaccinations with SLE and RA, and the findings can help us get a better understanding of the impact of vaccinations on risk of SLE and RA.

This meta-analysis finally included 16 observational studies. The pooled findings suggested that vaccinations significantly increased risk of both SLE (RR = 1.50; 95%CI 1.05–2.12) and RA (RR = 1.32; 95%CI 1.09–1.60) (Figs. 2 and 4). Meta-analysis of studies providing data on outcomes of short vaccinated time also suggested that vaccinations could significantly increase risk of SLE (RR = 1.93; 95%CI 1.07–3.48)

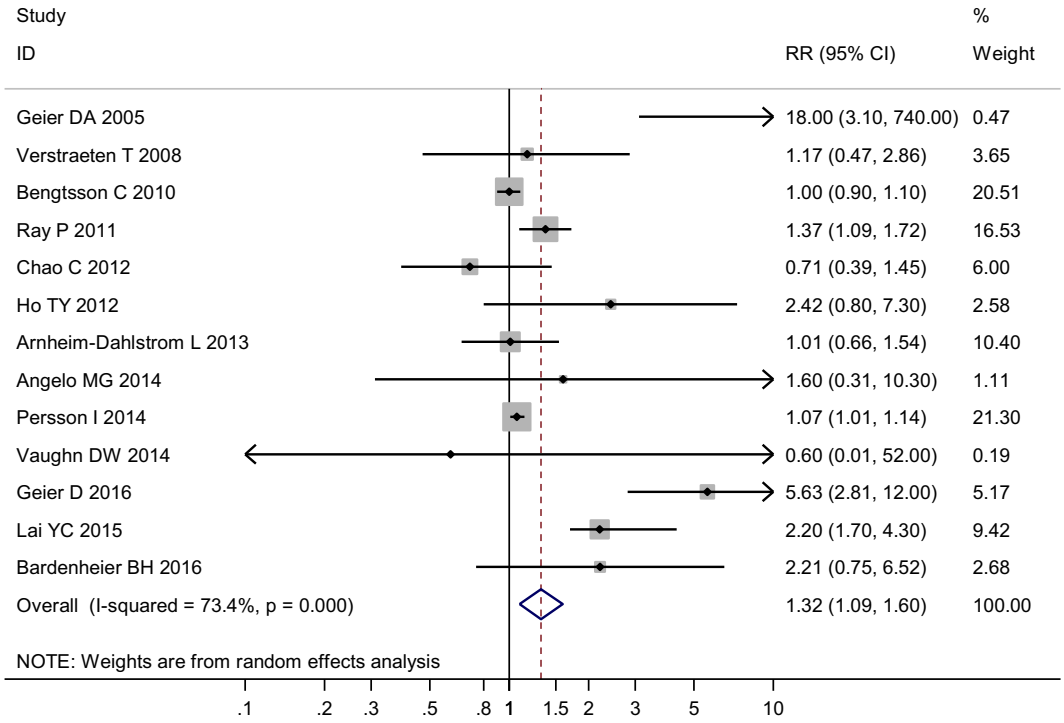

Fig. 4. Meta-analysis of 13 eligible studies showed that vaccinations significantly increased risk of RA.

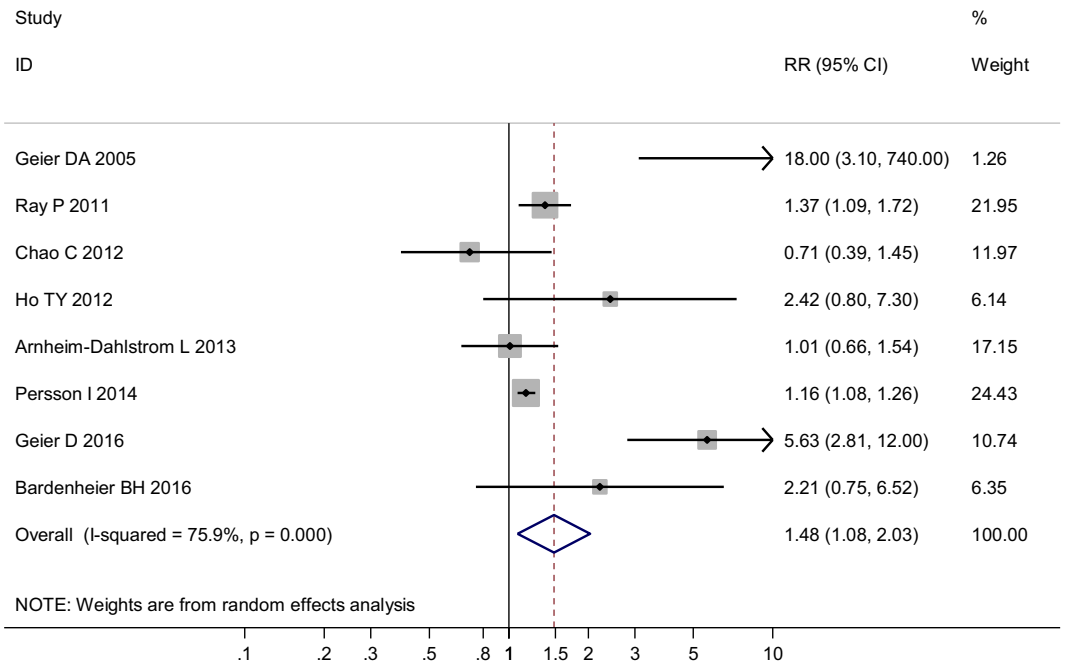

Fig. 5. Meta-analysis of studies reporting outcomes of short vaccinated time suggested a significantly increased risk of RA associated with vaccinations.

and RA (RR = 1.48; 95%CI 1.08–2.03) (Figs. 3 and 5). The obvious associations above were still significantly in other sensitivity analyses, which proved the credibility of the pooled results (Table 3). Therefore, the present meta-analysis provides epidemiological evidence for the obvious associations of vaccinations with SLE and RA, and vaccinations can increase both SLE risk and RA risk.

Our meta-analysis only assessed the relationship between vaccinations and risk of SLE and RA, but owing to the lack of data, we didn't analyze the impact of vaccinations on the risk of other rheumatic diseases, such as psoriatic arthritis and Sjögren's syndrome. A case-control study by Pattison et al. found that rubella vaccination was associated with psoriatic arthritis (OR = 12.4, 95%CI 1.2–122) [87]. Persson et al.'s study found that influenza vaccine could significantly increase the risk of Sjögren's syndrome (RR = 1.21, 95%CI 1.09–1.34), and the association was more obvious in a stratified analysis of data on outcomes of short vaccinated time (RR = 1.48, 95%CI 1.31–1.66) [43]. The findings of those studies above preliminarily suggested the possible associations of vaccinations with other rheumatic diseases. Currently, there are few published observational studies on the associations of vaccinations with other rheumatic diseases, and more and larger cohort studies are needed to explore whether vaccinations can increase risk of other rheumatic diseases.

The findings of the meta-analysis provide moderate evidence for vaccinations as risk factors of SLE and RA. The onset of SLE or RA following vaccinations from a large number of case-reports or case-series further support the causal role of vaccinations in the development of SLE and RA [22–28,30–34,36,88]. Besides, some in vivo studies also have suggested that vaccinations are causal factors of rheumatic diseases [89,90]. One study by Ravel et al. showed that HBV vaccine could increase serum IgG level and anti-nuclear autoantibody (ANA) level in female lupus-prone mice, indicating that HBV vaccine might increase risk of autoimmunity in genetically susceptible individuals [89]. Another study by Agmon-Levin et al. also found that HBV vaccine could induce SLE-like disease in a murine model, and HBV vaccine increased the level of anti-dsDNA antibodies and resulted in early onset of proteinuria [91]. Several other studies using animal models also reported that vaccinations could induce the development of rheumatic diseases [92–94].

The key strength of the present meta-analysis the inclusion of 16 eligible observational studies. The large sample size of the pooled participants was large enough to allow a reliable assessment of the association between vaccinations and risk of SLE or RA. Moreover, the consistent and obvious associations of vaccinations with SLE and RA in the sensitivity analyses further strengthened the epidemiological evidence for vaccinations as risk factors of SLE and RA. The findings of the meta-analysis are helpful to define the causal relationship between vaccinations and rheumatic diseases, and add new epidemiological evidence for vaccinations as causal factors of SLE and RA.

Several limitations of the meta-analysis should be considered. Firstly, there was obvious heterogeneity in the meta-analysis, which may come from the different study design, different characteristics of recruited participants, or different types of vaccinations. Meta-regression analysis found that study design was a possible source of heterogeneity in the meta-analysis on the relationship between vaccinations and RA risk (P = 0.09), but other possible sources of heterogeneity were not identified. The limited number of relevant studies available now may decrease the statistical power of meta-regression analysis. Therefore, more studies are needed to further evaluate the associations of vaccinations with SLE and RA. Secondly, there were only several studies exploring the impact of one particular vaccine on risk of SLE or RA (Table 1), and the findings in the subgroup analyses by types of vaccinations were not reliable enough to support a definite conclusion. To better define the impact of one particular vaccine on risk of SLE or RA, such as HPV vaccine, more and larger studies are needed in the future [95]. Thirdly, few studies have investigated the associations of vaccinations with disease activity of SLE or RA, which need to be explored in future researches. In addition, risk factors predicting the onset of SLE or RA following vaccinations are still not defined at present [96]. Identification of those risk factors is helpful to identify high-risk individuals and predict post-vaccination SLE or RA. Fourthly, most studies included into the meta-analysis were done in Europe and North America, and few studies were from Asian or African countries. Therefore, the findings from the meta-analysis cannot be generalized to Asian or African populations, and more observational studies from Asian or African countries are needed to provide epidemiological evidence for the influence of vaccinations on risk of rheumatic diseases in Asians or Africans. Finally, the molecular mechanisms underlying the associations of vaccinations with SLE and RA are still not well defined, and more researches are needed to provide adequate explanations.

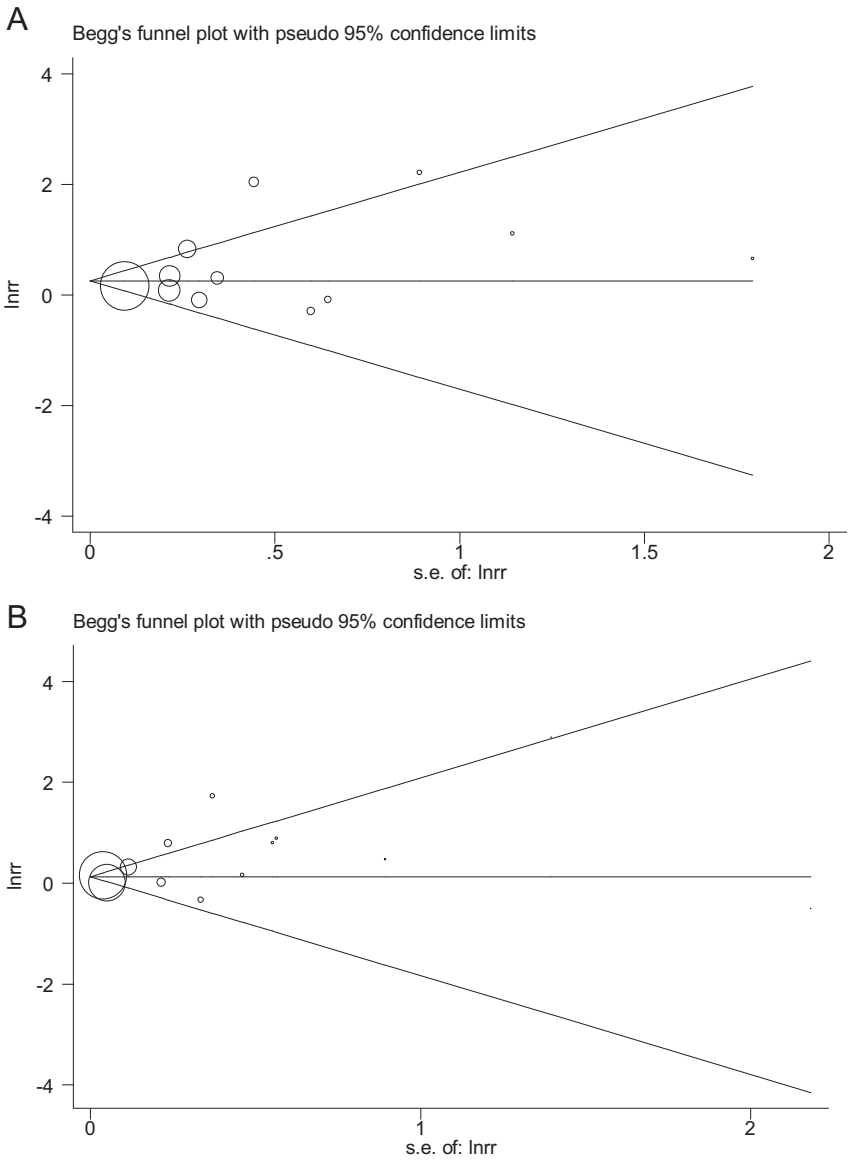

Fig. 6. Funnel plots to assess risk of publication bias in the meta-analysis. A Funnel plot in the meta-analysis of the association between vaccinations and SLE risk. B Funnel plot in the meta-analysis of the association between vaccinations and RA risk.

In conclusion, this systematic review and meta-analysis suggests that vaccinations are associated with increased risk of SLE and RA. The present study provides moderate evidence for vaccinations as risk factors of SLE and RA, but more and larger observational studies are needed to further verify the findings above. In addition, there is a clear need to perform more and larger observational studies to assess the associations of vaccinations with other rheumatic diseases.

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Conflict of interest

The authors declare that they do not have any conflict of interests.

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EXHIBIT 181

Juvenile idiopathic arthritis

Overview

Juvenile idiopathic arthritis, formerly known as juvenile rheumatoid arthritis, is the most common type of arthritis in children under the age of 16.

Juvenile idiopathic arthritis can cause persistent joint pain, swelling and stiffness. Some children may experience symptoms for only a few months, while others have symptoms for the rest of their lives.

Some types of juvenile idiopathic arthritis can cause serious complications, such as growth problems, joint damage and eye inflammation. Treatment focuses on controlling pain and inflammation, improving function, and preventing joint damage.

Symptoms

The most common signs and symptoms of juvenile idiopathic arthritis are:

- **Pain.** While your child might not complain of joint pain, you may notice that he or she limps — especially first thing in the morning or after a nap.
- **Swelling.** Joint swelling is common but is often first noticed in larger joints such as the knee.
- **Stiffness.** You might notice that your child appears clumsier than usual, particularly in the morning or after naps.
- **Fever, swollen lymph nodes and rash.** In some cases, high fever, swollen lymph nodes or a rash on the trunk may occur — which is usually worse in the evenings.

Juvenile idiopathic arthritis can affect one joint or many. There are several different subtypes of juvenile idiopathic arthritis, but the main ones are systemic, oligoarticular and polyarticular. Which type your child has depends on symptoms, the number of joints affected, and if a fever and rashes are prominent features.

Like other forms of arthritis, juvenile idiopathic arthritis is characterized by times when symptoms flare up and times when symptoms disappear.

When to see a doctor

Take your child to the doctor if he or she has joint pain, swelling or stiffness for more than a week — especially if he or she also has a fever.

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Causes

Juvenile idiopathic arthritis occurs when the body's immune system attacks its own cells and tissues. It's not known why this happens, but both heredity and environment seem to play a role. Certain gene mutations may make a person more susceptible to environmental factors — such as viruses — that may trigger the disease.

Risk factors

Some forms of juvenile idiopathic arthritis are more common in girls.

Complications

Several serious complications can result from juvenile idiopathic arthritis. But keeping a careful watch on your child's condition and seeking appropriate medical attention can greatly reduce the risk of these complications:

- **Eye problems.** Some forms can cause eye inflammation (uveitis). If this condition is left untreated, it may result in cataracts, glaucoma and even blindness.

  Eye inflammation frequently occurs without symptoms, so it's important for children with this condition to be examined regularly by an ophthalmologist.
- **Growth problems.** Juvenile idiopathic arthritis can interfere with your child's growth and bone development. Some medications used for treatment, mainly corticosteroids, also can inhibit growth.

By Mayo Clinic Staff

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Diagnosis & treatment

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EXHIBIT 182

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Genetics Home Reference | Your Guide to Understanding Genetic Conditions

Juvenile idiopathic arthritis

Description

Juvenile idiopathic arthritis refers to a group of conditions involving joint inflammation (arthritis) that first appears before the age of 16. This condition is an autoimmune disorder, which means that the immune system malfunctions and attacks the body's organs and tissues, in this case the joints.

Researchers have described seven types of juvenile idiopathic arthritis. The types are distinguished by their signs and symptoms, the number of joints affected, the results of laboratory tests, and the family history.

Systemic juvenile idiopathic arthritis causes inflammation in one or more joints. A high daily fever that lasts at least 2 weeks either precedes or accompanies the arthritis. Individuals with systemic arthritis may also have a skin rash or enlargement of the lymph nodes (lymphadenopathy), liver (hepatomegaly), or spleen (splenomegaly).

Oligoarticular juvenile idiopathic arthritis (also known as oligoarthritis) is marked by the occurrence of arthritis in four or fewer joints in the first 6 months of the disease. It is divided into two subtypes depending on the course of disease. If the arthritis is confined to four or fewer joints after 6 months, then the condition is classified as persistent oligoarthritis. If more than four joints are affected after 6 months, this condition is classified as extended oligoarthritis. Individuals with oligoarthritis are at increased risk of developing inflammation of the eye (uveitis).

Rheumatoid factor positive polyarticular juvenile idiopathic arthritis (also known as polyarthritis, rheumatoid factor positive) causes inflammation in five or more joints within the first 6 months of the disease. Individuals with this condition also have a positive blood test for proteins called rheumatoid factors. This type of arthritis closely resembles rheumatoid arthritis as seen in adults.

Rheumatoid factor negative polyarticular juvenile idiopathic arthritis (also known as polyarthritis, rheumatoid factor negative) is also characterized by arthritis in five or more joints within the first 6 months of the disease. Individuals with this type, however, test negative for rheumatoid factor in the blood.

Psoriatic juvenile idiopathic arthritis involves arthritis that usually occurs in combination with a skin disorder called psoriasis. Psoriasis is a condition characterized by patches of red, irritated skin that are often covered by flaky white scales. Some affected individuals develop psoriasis before arthritis while others first develop arthritis. Other features of psoriatic arthritis include abnormalities of the fingers and nails or eye problems.

Enthesitis-related juvenile idiopathic arthritis is characterized by tenderness where the bone meets a tendon, ligament, or other connective tissue. The most commonly affected places are the hips, knees, and feet. This tenderness, known as enthesitis, accompanies the joint inflammation of arthritis. Enthesitis-related arthritis may also involve inflammation in parts of the body other than the joints.

The last type of juvenile idiopathic arthritis is called undifferentiated arthritis. This classification is given to affected individuals who do not fit into any of the above types or who fulfill the criteria for more than one type of juvenile idiopathic arthritis.

Frequency

The incidence of juvenile idiopathic arthritis in North America and Europe is estimated to be 4 to 16 in 10,000 children. One in 1,000, or approximately 294,000, children in the United States are affected. The most common type of juvenile idiopathic arthritis in the United States is oligoarticular juvenile idiopathic arthritis, which accounts for about half of all cases. For reasons that are unclear, females seem to be affected with juvenile idiopathic arthritis somewhat more frequently than males. However, in enthesitis-related juvenile idiopathic arthritis males are affected more often than females. The incidence of juvenile idiopathic arthritis varies across different populations and ethnic groups.

Causes

Juvenile idiopathic arthritis is thought to arise from a combination of genetic and environmental factors. The term "idiopathic" indicates that the specific cause of the disorder is unknown. Its signs and symptoms result from excessive inflammation in and around the joints. Inflammation occurs when the immune system sends signaling molecules and white blood cells to a site of injury or disease to fight microbial invaders and facilitate tissue repair. Normally, the body stops the inflammatory response after healing is complete to prevent damage to its own cells and tissues. In people with juvenile idiopathic arthritis, the inflammatory response is prolonged, particularly during joint movement. The reasons for this excessive inflammatory response are unclear.

Researchers have identified changes in several genes that may influence the risk of developing juvenile idiopathic arthritis. Some of these genes belong to a family of genes that provide instructions for making a group of related proteins called the human leukocyte antigen (HLA) complex. The HLA complex helps the immune system distinguish the body's own proteins from proteins made by foreign invaders (such as viruses and bacteria). Each HLA gene has many different normal variations, allowing each person's immune system to react to a wide range of foreign proteins. Certain normal variations of several HLA genes seem to affect the risk of developing juvenile idiopathic arthritis, and the specific type of the condition that a person may have.

Normal variations in several other genes have also been associated with juvenile idiopathic arthritis. Many of these genes are thought to play roles in immune system function. Additional unknown genetic influences and environmental factors, such as infection and other issues that affect immune health, are also likely to influence a person's chances of developing this complex disorder.

Learn more about the genes associated with juvenile idiopathic arthritis

ANKRD55 CCN6 CD247 CTLA4 FAS HLA-A HLA-B HLA-DPB1
HLA-DQA1 HLA-DQB1 HLA-DRB1 HLA-G IL2RA IL2RB IL6 LACC1
MIF PTPN2 PTPN22 RUNX1 SLC11A1 STAT4 TNF TNFAIP3 TRAF1

TYK2 UBE2L3 ZFP36L1

Inheritance Pattern

Most cases of juvenile idiopathic arthritis are sporadic, which means they occur in people with no history of the disorder in their family. A small percentage of cases of juvenile idiopathic arthritis have been reported to run in families, although the inheritance pattern of the condition is unclear. A sibling of a person with juvenile idiopathic arthritis has an estimated risk of developing the condition that is about 12 times that of the general population.

Diagnosis & Management Links

Genetic Testing Information (2 links)

- What is genetic testing? (/primer/testing/genetictesting)
- Genetic Testing Registry: Rheumatoid arthritis, systemic juvenile (https://www.ncbi.nlm.nih.gov/gtr/conditions/C1858558/)

Research Studies from ClinicalTrials.gov (1 link)

- ClinicalTrials.gov (https://clinicaltrials.gov/ct2/results?cond=%22juvenile+idiopathic+arthritis%22)

Other Diagnosis and Management Resources (1 link)

- American College of Rheumatology: Juvenile Arthritis (https://www.rheumatology.org/I-Am-A/Patient-Caregiver/Diseases-Conditions/Juvenile-Arthritis)

Other Names for This Condition

- arthritis, juvenile rheumatoid
- JIA

- JRA
- juvenile chronic arthritis
- juvenile RA
- juvenile rheumatoid arthritis
- systemic juvenile rheumatoid arthritis

Additional Information & Resources

Health Information from MedlinePlus (2 links)

- Health Topic: Juvenile Arthritis (https://medlineplus.gov/juvenilearthritis.html)
- Medical Tests: Rheumatoid Factor (RF) Test (https://medlineplus.gov/lab-tests/rheumatoid-factor-rf-test/)

Genetic and Rare Diseases Information Center (1 link)

- Juvenile idiopathic arthritis (https://rarediseases.info.nih.gov/diseases/3067/juvenile-idiopathic-arthritis)

Additional NIH Resources (1 link)

- National Institute of Arthritis and Musculoskeletal and Skin Diseases: Juvenile Arthritis (https://www.niams.nih.gov/health-topics/juvenile-arthritis)

Educational Resources (7 links)

- American College of Rheumatology: Juvenile Arthritis (https://www.rheumatology.org/I-Am-A/Patient-Caregiver/Diseases-Conditions/Juvenile-Arthritis)
- Boston Children's Hospital (http://www.childrenshospital.org/conditions-and-treatments/conditions/j/juvenile-arthritis)
- JAMA Patient Page (https://jamanetwork.com/journals/jama/fullarticle/185634)

- Kids Health from Nemours (https://kidshealth.org/en/parents/jra.html)
- MalaCards: juvenile rheumatoid arthritis (https://www.malacards.org/card/juvenile_rheumatoid_arthritis)
- Merck Manual Consumer Version (https://www.merckmanuals.com/home/children-s-health-issues/juvenile-idiopathic-arthritis-jia/juvenile-idiopathic-arthritis-jia)
- Orphanet: Juvenile idiopathic arthritis (https://www.orpha.net/consor/cgi-bin/OC_Exp.php?Lng=EN&Expert=92)

Patient Support and Advocacy Resources (1 link)

- Autoinflammatory Alliance (http://autoinflammatory.org/sjia.php)

Scientific Articles on PubMed (1 link)

- PubMed (https://www.ncbi.nlm.nih.gov/pubmed?term=%28Arthritis,+Juvenile+Rheumatoid%5BMAJR%5D%29+AND+%28juvenile+idiopathic+arthritis%5BTI%5D%29+AND+review%5Bpt%5D+AND+english%5Bla%5D+AND+human%5Bmh%5D+AND+%22last+1080+days%22%5Bdp%5D)

Catalog of Genes and Diseases from OMIM (1 link)

- RHEUMATOID ARTHRITIS, SYSTEMIC JUVENILE (http://omim.org/entry/604302)

Medical Genetics Database from MedGen (7 links)

- Juvenile arthritis due to defect in LACC1 (https://www.ncbi.nlm.nih.gov/medgen/760659)
- Juvenile chronic polyarthritis (https://www.ncbi.nlm.nih.gov/medgen/140816)
- Oligoarticular juvenile idiopathic arthritis (https://www.ncbi.nlm.nih.gov/medgen/443993)
- Psoriatic Juvenile Idiopathic Arthritis (https://www.ncbi.nlm.nih.gov/medgen/855520)
- Rheumatoid arthritis, systemic juvenile (https://www.ncbi.nlm.nih.gov/medgen/346934)

- Systemic onset juvenile chronic arthritis (https://www.ncbi.nlm.nih.gov/medgen/730498)
- Undifferentiated Juvenile Idiopathic Arthritis (https://www.ncbi.nlm.nih.gov/medgen/856839)

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