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ECOLOGICAL AND EPIDEMIOLOGICAL ANALYSES OF MULTIPLE SCLEROSIS RELAPSE RATE

by

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Experience is the worst teacher -
it gives the test before presenting the lesson.

-- Vernon Law

ABSTRACT

The underlying cause of many human autoimmune diseases is unknown, but several environmental factors are implicated in triggering the self-destructive immune reactions. Multiple Sclerosis (MS) is a chronic autoimmune disease of the central nervous system, potentially leading to persistent neurological deterioration. The cause of MS is not known, and apart from immunomodulatory treatments there is no cure. In the early phase of the disease, relapsing-remitting MS (RR-MS) is characterized by unpredictable exacerbations of the neurological symptoms called relapses, which can occur at different intervals ranging from 4 weeks to several years.

Microbial infections are known to be able to trigger MS relapses, and the patients are instructed to avoid all factors that might increase the risk of infections and to properly use antibiotics as well as to take care of dental hygiene. Among those environmental factors which are known to increase susceptibility to infections, high ambient air inhalable particulate matter levels affect all people within a geographical region. During the period of interest in this thesis, the occurrence of MS relapses could be effectively reduced by injections of interferon, which has immunomodulatory and antiviral properties.

In this thesis, ecological and epidemiological analyses were used to study the possible connection between MS relapse occurrence, population level viral infections and air quality factors, as well as the effects of interferon medication. Hospital archive data were collected retrospectively from 1986-2001, a period in time ranging from when interferon medication first became available until just before other disease-modifying MS therapies arrived on the market. The grouped data were studied with logistic regression and intervention analysis, and individual patient data with survival analysis.

Interferons proved to be effective in the treatment of MS in this observational study, as the amount of MS exacerbations was lower during interferon use as compared to the time before interferon treatment. A statistically significant temporal relationship between MS relapses and inhalable particular matter (PM_{10}) concentrations was found in this study, which implies that MS patients are affected by the exposure to PM_{10}. Interferon probably protected against the effect of PM_{10}, because a significant increase in the risk of exacerbations was only observed in MS patients without interferon medication following environmental exposure to population level specific viral infections and PM_{10}. Apart from being antiviral, interferon could thus also attenuate the enhancement of immune reactions caused by ambient air PM_{10}. The retrospective approach utilizing carefully constructed hospital records proved to be an economical and reliable source of MS disease information for statistical analyses.

TIIVISTELMÄ

Monien ihmisen autoimmuunisairauksien aiheuttajia ei tunneta, mutta ympäristötekijöiden tiedetään käynnistävän omia kudoksia tuhoavia immuunireaktioita. Multippeliskleroosi (MS-tauti) on keskushermostoa rappeuttava krooninen autoimmuunisairaus. Varhaisvaiheessa MS-taudin kuvaan kuuluvat yllättävät neurologisten oireiden pahenemisvaiheet eli relapsit. Relapsien ilmaantumisen väli vaihtelee useista vuosista neljään viikkoon. Vaikka MS-taudin syy on tuntematon, tiedetään että virustulehdukset voivat käynnistää relapseja. MS-tautiin ei ole parannuskeinoa, mutta relapsien esiintymistä voidaan tehokkaasti vähentää interferonipistoshoidolla. Interferoni muokkaa immuunipuolustusta ja sillä on antiviraalisia ominaisuuksia. Tässä väitöskirjatutkimuksessa tutkin ympäristötekijöiden, väestötason virusinfektioiden ja ilmanlaadun, yhteyttä relapsien esiintymiseen sekä interferonin mahdollisia tulehduksille altistavilta tekijöiltä suojaavia ominaisuuksia takautuvasti arkistoaineistoista.

Interferonit osoittautuivat tehokkaiksi MS-taudin hoidossa, sillä relapsitiheys oli merkittävästi alempi hoitokuukausien aikana verrattuna aikaan ennen hoidon aloittamista. Tutkimuksessani havaitsin tilastollisesti merkitsevän ajallisen yhteyden MS-taudin relapsien esiintymisen ja ilman hengitettävien pienhiukkasten (PM_{10}) pitoisuuden välillä, mikä merkinnee sitä että pienhiukkaspitoisuus vaikuttaa MS-tautia sairastaviin. Altistuminen väestössä todetuille virustautitapauksille ja PM_{10}:lle kohotti pahenemisvaiheiden riskiä merkitsevästi vain niillä potilailla, jotka eivät käyttäneet interferonilääkitystä, mikä viittaa interferonin antiviraalisten ominaisuuksiensa ohella myös vaimentavan ulkoilman korkean PM_{10}-pitoisuuden aiheuttamaa immuunireaktioiden kiihtymistä. Huolellisesti kootut sairaala-arkistot osoittautuivat huokeaksi ja luotettavaksi tietolähteeksi jota voi hyödyntää MS-taudin tilastollisessa tutkimuksessa.

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ABBREVIATIONS

ACF	Autocorrelation Function
ALAT	Alanine Aminotransferase (hepatic enzyme)
APC	antigen-presenting cell
ARIMA	Autoregression Integrated Moving Average (time-series analysis method), also known as the Box-Jenkins model
BMDP	(statistical analysis software)
B cells	antibody-producing lymphocytes
CF	cerebrospinal fluid
CI	Confidence Interval
CNS	central nervous system
EAE	Experimental Autoimmune Encephalitis
EBV	Epstein-Barr Virus
EDSS	Extended Disability Status Scale
HLA	human leukocyte antigen
IFN	interferon
MA	Moving Average
MHC	Major Histocompatibility Complex
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
OR	Odds Ratio (result of logistic regression, a statistical method)
PACF	Partial Autocorrelation Function
PM_{10}	airborne particulate matter with an aerodynamic diameter of less than 10 µm
PRISMS	(an international interferon trial consortium)
RSV	Respiratory Syncytial Virus
SAS	(statistical analysis software)
SPSS	Statistical Package for Social Sciences (statistical analysis software)
SIIF	the Social Insurance Institution of Finland
T cells	cytokine-producing lymphocytes
Th1 cells	type 1 T helper cells: lymphocytes which activate and heighten antibody production by producing cytokines such as interleukin-2, interferon-gamma and tumor necrosis factor-beta
Th2 cells	type 2 T helper cells: lymphocytes which activate and heighten antibody production by producing cytokines such as interleukins 4, 5, 9, 10, and 13
UV	ultraviolet light
VZV	Varicella Zoster Virus

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals.

I Oikonen M, Laaksonen M, Laippala P, Oksaranta O, Lilius EM, Lindgren S, Rantio-Lehtimäki A, Anttinen A, Koski K, Erälinna JP: Ambient Air Quality and Occurrence of Multiple Sclerosis Relapse. *Neuroepidemiology* 2003;22:95-99

II Oikonen MK, Laaksonen M, Ilonen J, Salonen R, Erälinna J-P, Panelius M, Salmi AA: Temporal relationship between environmental Influenza A and Epstein-Barr viral infections and high Multiple Sclerosis relapse rate. *(revised version submitted)*

III Oikonen MK, Erälinna J-P: Beta-interferon protects Multiple Sclerosis patients against the enhanced susceptibility to infections caused by poor air quality. *Neuroepidemiology* 2008;30:13-19

IV Oikonen M, Erälinna J-P: Survival analysis of the relapse-free time in Multiple Sclerosis without and during interferon beta 1-a and 1-b treatment assessed retrospectively from hospital records. *(manuscript)*

V Oikonen M, Erälinna J-P: Intervention analysis of Multiple Sclerosis relapse rate without and during medication with beta-interferon. *(manuscript)*

INTRODUCTION

Activation of autoimmune reactions

Circulating self-reactive clones of T and B leukocytes, which are present in all healthy individuals, may become activated and begin to destroy healthy cells and tissues, a phenomenon which is called an autoimmune reaction (Rose et al 1998). The autoreactive T and B cells can become functionally activated by a multitude of exogenous factors, and then move from circulation to tissues by migrating rapidly through endothelia and across the blood-brain barrier (O'Connor et al 2005).

Antibodies expressed by B cells selectively target their specific self antigen (Kuby 1997). Autoimmune diseases are very variable because the antigen may be a tissue such as exocrine glands in Sjögren's Syndrome, a cell type such as splenic insulin-producing beta cells in insulin-dependent diabetes, even DNA as in Lupus Erythematosus, or a gene product such as the myelin protein layer that covers neuronal axons as is the case in Multiple Sclerosis (MS) (Rose et al 1998).

Innate immunity mechanisms are involved in the inflammatory response, which is usually triggered by tissue damage or invasion by pathogenic microorganisms (Kuby 1997). In genetically predisposed individuals, inflammation may provoke an immune reaction against self (Sadovnik 2004, Harrison et al 1998, Lafferty et al 1998). Among the events occurring during an inflammatory response is an influx of phagocytes into the tissues facilitated by an increase in capillary permeability, and the release of lytic enzymes which can damage nearby healthy cells. Thus the two immunological pathways: the B-cell-mediated humoral and the innate inflammatory response can both precipitate the production of innate proinflammatory Th1-type cytokines, which are a prerequisite for the functional activation of more T and B cells.

In many human autoimmune diseases, the underlying cause which triggers the attack against self remains unknown, but genetic factors are often implicated. Entire populations may share genes which make individuals more susceptible to self-destruction (Sotgiu et al 2008, Raivich et al 2004). The incidence of many autoimmune diseases differs between sexes, which may arise from the actions of sex hormones or genetic differences in the immune system between males and females especially in the cytokine- and MHC-encoding regions (Svejgaard 2008, Zandman-Goddard et al 2007). Autoimmune diseases share many characteristics with allergy, demonstrated by the general autoimmune disease-susceptibility locus which encodes the histamine receptor H1 (Ma et al 2002).

Exogenous modifiers of autoimmunity

Autoimmune diseases can be modified by environmental or exogenous factors which have an effect on the immune system, including contractable infections, seasonally changing climatic conditions and ambient air quality, lifestyle, diet, living conditions and family size (Franklin et al 2003). Contact with siblings in early life has been shown to contribute to the overall function of the adult human immune system.

In general, infections protect against autoimmunity, as is demonstrated by the inverse association of a general decline in the incidence of transmissible infections and an increase in the prevalence of autoimmune diseases in developed countries during the last decades (Kamradt et al 2005, Bach 2002). Protective infections can induce antigenic competition, where an immune reaction against an antigen is suppressed by the immune reaction against another, unrelated antigen; another protective mechanism of infections can be an activation of the regulatory T cells. However, infections also naturally provoke immune reactions, since the defence against microbial infections is the primary role of an efficient immune system (Harrison et al 1998).

An infection can lead to a general activation of the immune system and an inflammatory reaction. Microbial infections are also known to induce the expression of immunogenic stress proteins such as alpha B-crystallin (van Noort et al 2000). An important pathway of autoimmune activation during attacks by microbes and parasites is molecular mimicry, meaning that the infectious agents carry antigenic epitopes that resemble self-antigens, causing a cross-reaction in the immune cells (Münz et al 2009). A prolonged physical tissue damage provides the T and B cells constant stimulation with self-antigens, and the activation is sustained by the chronic inflammation. Many human lifestyle factors such as stress, diet, smoking, vaccinations or physical trauma as well as exposure to organic solvents, mercury amalgam or pets can be linked to the susceptibility to infections.

Exposure to ambient air inhalable particular matter (PM) is known to affect patients with respiratory and cardiovascular disease (Brunekreef et al 2005). The possible adverse health effects of increased inhalation of inhalable ambient air particulate matter, referred to as PM_{10} (airborne PM with a 50% cut-off aerodynamic diameter of less than 10 µm), include the lung's increased susceptibility to infectious pathogens, alveolar macrophage dysfunction after long-term exposure to particulates, a local and a systemic enhancement of the immunological functions and the possibility of a reactivation of endogenous, intracellular pathogens (Hadnagy et al 1994, Becker et al 1998 and 1999). PM_{10} and especially ultrafine particles of less than 2.5 µm are known to increase oxidative stress and directly cause lung injury (Li et al 2003 and 1997, Monn et al 1999).

PM also modifies human immunological resistance to common contractable infections. Long-term exposure to inhalable particulate matter has been observed to impair the phagocytic capacity of alveolar macrophages, which increases the risk for pulmonary infections (Hadnagy et al 1994, Lundborg et al 1999). Inhalable particulate matter has been shown *in vitro* to induce a shift in alveolar macrophage phenotypes to a more immune stimulatory state, which is a potential mechanism behind asthma exacerbations and other immune responses in the lung (Holian et al 1998). Inhaled particulate matter is phagocytosed by alveolar macrophages (Terashima et al 1997), which stimulates the recruitment of circulating leukocytes, which in turn leads to enhanced inflammation. Environmental exposure to the acidic gases sulphur dioxide (SO_2), nitric dioxide (NO_2), and nitric monoxide (NO) can impair the immunological resistance capacity of the lung and enhance susceptibility to infectious as well as endogenous pathogens.

In seasonal climates, the resistance to pathogens may fluctuate seasonally following the changes in ambient temperature and light period (Nelson et al 1995). Latitudinal gradients in solar UV and other irradiation affect immune function, because close to the equator the

intensive solar UV irradiation is immunosuppressive and away from the equator, the diminishing exposure to sunlight limits vitamin D bioavailability. The annually repeating allergenic pollen seasons could influence the occurrence of Th1-mediated autoimmune reactions via a general enhancement of immune system. Furthermore, air pollution has been shown to increase the allergenicity of airborne pollen proteins (Behrendt et al 1997, Knox et al 1997).

Multiple Sclerosis

Multiple Sclerosis (MS) is an autoimmune disease of the human central nervous system (CNS). The autoimmune reaction in MS is targeted against myelin, the lipid layer insulation along axons enabling rapid nerve impulse transfer (Arnason et al 1998). Diffuse changes in white matter, cortical lesions, and myelin and axonal loss in inflammatory lesions cause the clinical symptoms of MS, which include ataxia and sensory disturbances, impaired walking and bladder control as well as cognitive dysfunction. During the period of interest of this thesis, an MS diagnosis could have been set if a person had experienced at least two certifiable episodes of neurological activity in two anatomically distinct areas of the CNS (Poser et al 1983), yet presently the developments in imaging techniques have lead to thorough revisions of the diagnostic criteria (McDonald et al 2001, Polman et al 2005).

Early historical records of MS-like episodes include a symptom diary kept by Sir Augustus D'Este in 1822. Jean-Martin Charcot set the early criteria for MS-like disease in 1868, and Pierre Marie first suggested the role of microbes in MS (Pearce 2005, Butler et al 2003). Ever since the mid-1800s the course and cure of MS have been extensively researched. A variety of viral antibodies have been found from MS patients' peripheral blood or CSF, suggesting that several different factors and infections contracted earlier in life may be implicated in the pathogenesis (Ebers 2008, Marrie 2004, Franklin et al 2003, Marrie et al 2000, Sibley et al 1985, Salmi et al 1983).

No causative agent or pathogen has as yet been identified as a single culprit behind the disease, but infectious mononucleosis caused by the Epstein-Barr virus (EBV) is one strong candidate as the causative agent in MS: EBV has been claimed to infect all MS patients, and the timing and intensity of the EBV infection are probably connected to the risk of developing MS (Zaadstra et al 2008, Thacker et al 2006).

However, the cause of MS remains as yet unknown. The study of MS is complex due to both the long development and heterogenous symptoms of the disease, as well as the diverse environmental factors which can modify susceptibility to MS (Keegan et al 2002). A notable gender difference (female:male ratio 3:1) in the prevalence of MS highlights the complex genetic background of the disease mechanisms (Sadovnick 2009). Recently in Sardinia, Italy, a hypothesis was tested that an uncontrolled innate immunity reaction, developed during generations of evolution and directed toward pathogenic parasites could be an underlying factor in the susceptibility to and development of the autoimmune reaction in MS (Sotgiu et al 2008). The relationships between some modifiers of autoimmune reactions in MS are depicted in Figure 1.

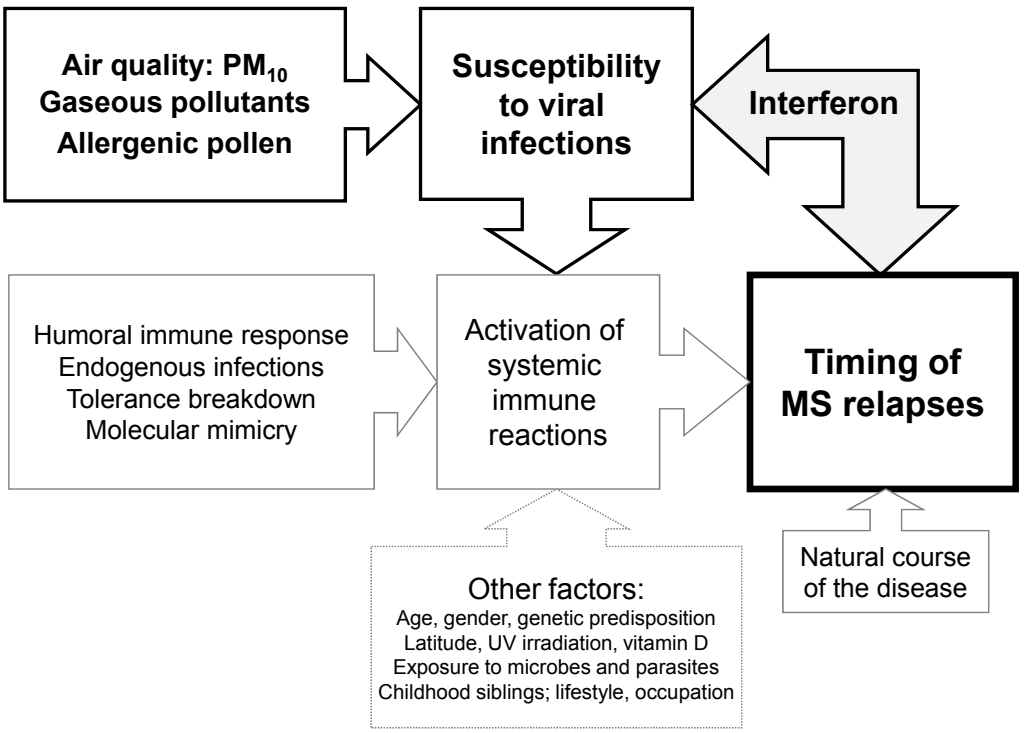

Figure 1. Schematic presentation of the relationships between some of the exogenous factors that can modify the autoimmune reactions in MS (factors studied in this thesis are written in bold letters). (Modified from O'Connor et al 2005).

An anatomic–physiologic complex at the capillary endothelium called the blood-brain barrier prevents the entry of many substances into the brain from the blood stream (Filley 2003). Although the barrier blocks the intrusion of blood-borne pathogens, T cells are able to cross the blood-brain barrier and the CNS is kept under constant immune surveillance.

The autoimmune reaction in MS is mediated by a type IV delayed immunopathological reaction, where the inflammation is dependent on Th1 lymphocytes and cells of the monocytic-macrophage system (Bartunkova et al 2009). Activated myelin-reactive T cells migrate rapidly across the blood-brain barrier into the CNS and effectively destruct the body's own myelin (Bradl et al 2003, Silverman et al 2000, Conlon 1999, Hickey 1999, Waksman 1999). Demyelination of the CNS followed by chronic antiself inflammation causes progressive brain and spinal cord scarring and atrophy.

MS is the major cause of disability in the young adult population (Noseworthy et al 2000). MS affects over 350 000 Europeans, and the prevalence in Finland is high, 98 / 100 000 people. Both the incidence and prevalence are remarkably different between geographical areas. MS incidence in Finland is among the highest in the world, up to 11.6 / 100 000 people in Southern Ostrobothnia (Sumelahti et al 2000). MS frequently starts in the age of 30-40 years and leads to persistent neurodegeneration. In 2008 there were in total 6500 patients with MS in Finland. Some of the symptoms of MS are listed in Figure 2.

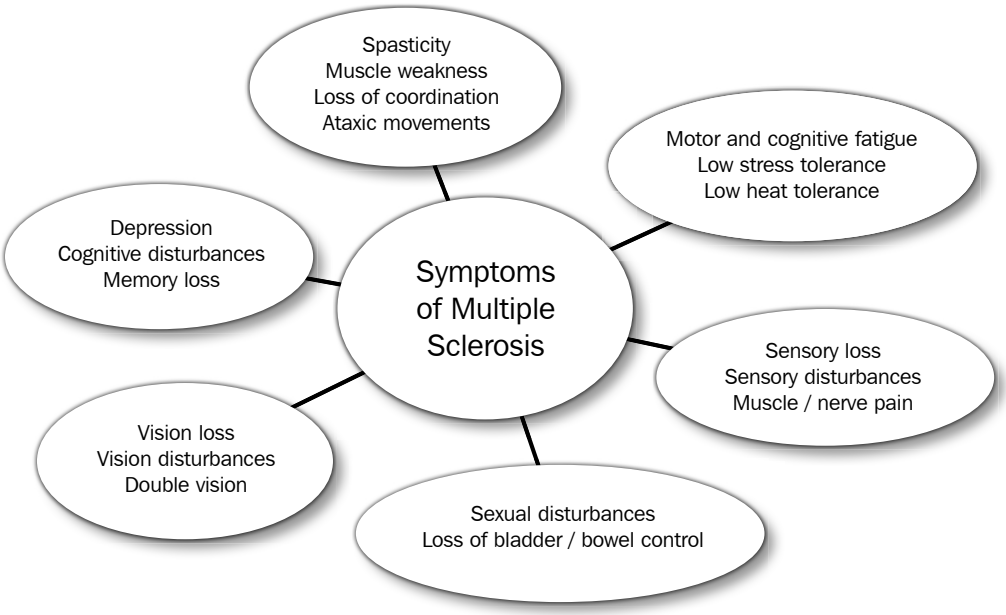

Figure 2. Some of the common symptoms in Multiple Sclerosis.

Seasonality can be seen in both the incidence of MS and the occurrence of relapses: according to a meta-analysis of MS incidence studies in the northern hemisphere, the highest frequencies of MS onset are generally detected in the spring, and lowest in the winter (Jin et al 2000). A seasonal climate causes variation in the human immune functions through affecting melatonin production of the pineal gland, which may even have implications for the incidence of MS relapses (Nelson et al 1995). Seasonal variation in the immune reaction-enhancing interferon-gamma production has been measured in patients with MS (Balashov et al 1998).

There is no conclusive evidence about the role of lifestyle factors such as vaccinations or diet in MS (Schwarz et al 2005, Franklin et al 2003). Cigarette smoking has been connected both to a higher risk of developing MS and to a faster progression of the disease, but a causality remains to be proved (Healy et al 2009, Riise et al 2003).

Environmental factors have long been suspected to play a role in the development of MS, one example being the low concordance rate of 25% among genetically identical twins (Conlon et al 1999). Also the connection between geographical area and risk of MS reflects genetic differences within a population (Marrie 2004, Tienari et al 2004). A genetical predisposition to MS has been connected to HLA alleles in the MHC II region (Hafler et al 2007). Recently, a month-of birth effect in MS patients was found to be associated with the principal MS susceptibility gene HLA-DRB1, and an as yet unknown seasonal environmental effect was suggested to take place during gestation or early childhood (Ramagopalan et al 2009).

The reasons for the observed geographical variation in the worldwide prevalence and incidence of MS are not fully understood (Noseworthy et al 2000). Hypothetically, the diminishing UV irradiation in the northern and southern latitudes is connected to an

increased risk of developing MS via the lowered amount of available vitamin D. This hypothesis is supported by two recent observations: MS patients have lower serum vitamin D levels during relapses, and supplementary intake of vitamin D protects from developing MS (Soilu-Hänninen et al 2005, Schwarz et al 2005, Munger et al 2004).

An additional protective effect of the proximity to the equator can be T-lymphocyte-mediated immunosuppression induced by the intense UV irradiation (van der Mei et al 2001). However, the hypothesis of a general north-south gradient in disease frequency has since been challenged by the observed existence of highly localized clusters in Southern Europe where MS rate is high. Migration from high-risk areas has been noticed to lower the risk of developing MS, suggesting that the physical environment is implicated in MS initiation (Kurtzke et al 2003, Franklin et al 2003, Kurtzke 2000). Also the clustered incidence of MS within Finland might indicate the influence of some locally acting environmental factors (Sumelahti et al 2003).

MS relapses

The early phase of MS is characterized by a fluctuating course, where the exacerbation of clinical symptoms occur in episodes of unpredictably recurring relapses and remissions (Confavreux et al 2000). Different MS types have been described according to the natural course of the disease: benign, relapsing-remitting (RR-MS), secondary-progressive and primary-progressive. In benign MS the patient's neurological status returns to normal between relapses, and permanent disability either never develops or is delayed. The most common MS type (comprising 80-85% of all cases) is relapsing-remitting MS (RR-MS), with no new disability between attacks. Several patients initially experience a relapsing-remitting course of the disease, but in time some 60% of the RR-MS patients develop a non-relapsing secondary-progressive form with a gradual increase in disability, where clear relapses can no longer be distinguished. Primary-progressive MS is a less common type (10%), in which the neurological symptoms constantly progress without relapses or remission from the attacks.

RR-MS relapses may cause progression of the disease towards the secondary-progressive type and eventually irreversible disability (D'Sousa et al 2008, Young et al 2006, Confavreaux et al 2000). A higher annual MS relapse frequency in the early stages of MS has been connected to a faster development of the progressive phase (Vukusic et al 2003). Furthermore, residuals of a relapse occur in approximately one third of all MS relapses, meaning an incomplete recovery which potentially can lead to cumulative disability (Lublin et al 2003). Relapses constitute a major part of the disease burden and both the advice given to patients and the treatment of RR-MS focus on the avoidance of infections to minimize risk of exacerbations. For instance, the patients are advised to use prescribed antibiotics properly and to take good care of dental hygiene. The symptoms in acute RR-MS exacerbations can be treated with high doses of intravenous glucocorticosteroids, which reduces both T cell infiltration into the CNS and the production of pro-inflammatory cytokines (Sloka et al 2005).

The ultimate reasons for the periodic worsening of symptoms are not known. Many different microbial infections can be connected to the induction of an RR-MS relapse, during an at-risk period of a few weeks from a clinical infection (Buljevac et al 2002, Edwards et al 1998, Sibley et al 1985). Microbial infections are known to activate immune

pathways that can result in MS exacerbations (Tremlett 2008, Buljevac 2002, Friedman 1999, Metz 1998, Sibley et al 1985). One possible relapse-inducing pathway could be the infection-triggered release of an immunogenic stress protein called alpha B-crystallin in the CNS (van Noort et al 2000).

The observed seasonality of RR-MS relapses might be partly caused by the seasonally fluctuating emergence of infection epidemics in the general population, which could in turn be due to the significant seasonal variation in human immune functions that is observed in cold temperate climates (Nelson et al 1995). Possible factors modulating the susceptibility to infections could be the seasonally changing UV irradiation and serum vitamin D levels, possibly acting through a similar pathway as has already been shown in RR-MS incidence studies (Soilu-Hänninen et al 2005, Schwarz et al 2005, Munger et al 2004). Recently, seasonally changing ambient sunlight was observed to be connected to a decrease in MS relapses, and upper respiratory tract infections to an increase in monthly MS relapse rate in a Tasmanian patient cohort (Tremlett et al 2008). Seasonally emerging airborne allergenic pollen could be expected to have little effect to RR-MS relapse rate, since the prevalence of Th2-mediated allergic disease has been observed to be lower among MS patients than in the general population (Oro et al 1996).

The intracellular bacterial pathogen *Chlamydophila pneumoniae* Everett (Everett et al 1999) persisting in the CNS tissue of MS patients is suggested to sustain a peripheral infection and trigger relapse onset when activated (Sriram et al 2005, 1999). Dental caries (McGrother et al 1999, Craelius 1978) and infections of the upper respiratory tract (Edwards et al 1998) and the urinary tract (Metz et al 1998) have all been considered as possible MS modulating infections. Although many resting intracellular pathogens may be reactivated by an inflammatory reaction, investigations with the endogenous viruses, human herpesvirus-6 (Rotola et al 1999) and human parvovirus B19 (Nakashima et al 1999) have demonstrated that these viruses prevail in tissues but remain in a latent stage even during an active MS exacerbation. In a small patient group, picornaviruses have been associated with MS exacerbations (Kriesel et al 2004).

The relative importance of different infectious viruses has been studied prospectively with individual patients as well as in a murine model of MS called EAE, but no concensus has been achieved as to the relative importance of the various viruses (Gilden 2005). Despite vigorous research, no pathogen species has been pointed out as the primary modulator of RR-MS disease activity (Fernandez 2004, Keegan et al 2002, Cermelli et al 2000, Noseworthy et al 2000, ter Meulen et al 1997). Consequently, any transmissible viral or bacterial infections, as well as escalations of existing inflammation can be considered as exogenous disease modifiers in RR-MS.

Disease-modifying drugs for MS

Although remyelination techniques have been studied, no cure for MS exists (Compston 2004). The self-destructive immunological reactions can, however, be partly controlled with disease-modifying medication. The purpose of systemic immunomodulatory treatment in RR-MS is to minimize inflammation that causes demyelination, axonal loss and brain atrophy. During the period of interest of this thesis, glatiramer acetate, mitoxanthrone or

natalizumab were not available, and the disease-modifying drugs regularly used in Finland were beta-interferons.

Interferons are naturally occurring cytokine proteins, produced and secreted by many cells of the immune system in response to antigenic stimulation or viral infection (Javed et al 2006, McCormack et al 2004). The pharmacological mechanism of interferons is not entirely known, but they bind to species-specific cell surface receptors and induce a cascade of signaling pathways leading to the secretion of specific gene products, which have immunomodulatory, antiviral, and antiproliferative actions (Dhib-Jalbut 2002, Garcia-Montojo et al 2007).

The anti-inflammatory effects of beta-interferons include inhibition of T-cell activation, regulation of the production of both proinflammatory and anti-inflammatory cytokines and reducing T-cell migration (Zhang et al 2002). Beta-interferons can suppress a reactivation of a latent infection in the CNS, but this mode of action has also been questioned because no evidence exists of a viral aetiology in MS (Fernandez 2004, Salmi et al 1983).

Injections of type 1-a and 1-b beta-interferons are administered either subcutaneously or intramuscularly 1-3 times per week and they effectively reduce the occurrence of MS exacerbations, which are believed to lead to disease progression (Onesti et al 2003). 2-47% of MS patients are estimated to eventually develop neutralizing antibodies to beta-interferons, which may account for an observed decrease in treatment efficacy (Bertolotto 2009, Sørensen et al 2005). In international beta-interferon drug trials, MS relapse rate has been approximately 30% lower during treatment than in patients receiving placebo.

During the period of interest of this thesis, the long-term effects of immunomodulatory medication in MS were not determined as most clinical trials had been small and brief (Noseworthy 2000). There are few reports of extended observation periods of large patient groups.

The beta-interferons beta 1-a and 1-b with the trade names Avonex, Betaferon and Rebif were regularly used in Finland since 1995. Because of the high costs of the treatment, the guidelines for beta-interferon treatment were strictly controlled. The Social Insurance Institution of Finland (SIIF) covered the costs of treatment with beta-interferon, provided that the benefit of the treatment for individual patients had been annually assessed in a neurological examination. The criteria for receiving the drug indemnity for beta-interferon products had been set by the Finnish Neurological Society: the treatment could be begun if the patient had a definite MS diagnosis, there had been at least two confirmed exacerbations during two years before the treatment is begun, and the patient was able to walk unassisted at least 100 metres. Beta-interferon treatment could be begun if there had been at least two MS relapses during the previous two years, indicating an active disease.

The drug indemnity was applied for by the patient, the application was to be repeated annually, and accompanied by a renewed medical certificate issued by a neurologist at a unit of specialized medical care. The neurological status of the patient was controlled annually to ensure that the treatment was effective, and the treatment was to be discontinued if the disease had become progressive or if the side effects were intolerable. For example, in the year 2000 there were approximately 1000 MS patients receiving treatment with beta-interferons in Finland, and the SIIF drug indemnity costs exceeded 11 million euros.

AIMS OF THE STUDY

Several environmental factors can predispose to microbial infections, among them ambient air quality and infection epidemics. Because poor air quality both activates the immune system and is expected to increase the susceptibility to airways infections, which in turn are known to be able to trigger MS relapses, it was logical to hypothesize that there could exist connections between air quality, viral infection epidemics and MS relapse occurrence. The antiviral and anti-inflammatory actions of beta-interferon could putatively attenuate these effects.

To study the effects of environmental factors and beta-interferon medication on MS relapse rate, monthly MS relapse rates were acquired retrospectively through examination of hospital archives and were studied using epidemiological methods and ecological analyses of group data.

The aims of the current study were:

- to explore the possibility of an association between air quality factors and MS relapse rate (study I),
- compare MS exacerbation rate to environmental exposure to epidemics of different viral infections in the general population (study II),
- study the effect of the proinflammatory environmental factors without and during interferon medication (study III), and
- to study the relapse rates of patients without and during interferon medication with epidemiological and ecological time-series modelling (studies IV and V).

MATERIAL AND METHODS

Patient data

In this thesis, part of the entire disease course was observed for each individual patient: relapses occurring in benign and relapsing-remitting MS, as well as from the relapsing phase of secondary-progressive MS before the disease eventually becomes progressive. The differences in the timing of relapses between the types of MS as well as the included relapsing phases in the patient data of this thesis are summarized in Figure 3.

The patient data were collected from individual patient records in hospital archives. All patients had been reliably identified before recording their information into the hospital database by a personal identity code, which has been issued since the 1960s for all Finnish citizens and foreign citizens whose residence in Finland is permanent or exceeds one year (Population Register Centre 2010). Relapses were accepted as described in the patient files. Relapse and medication data were recorded at one-month intervals.

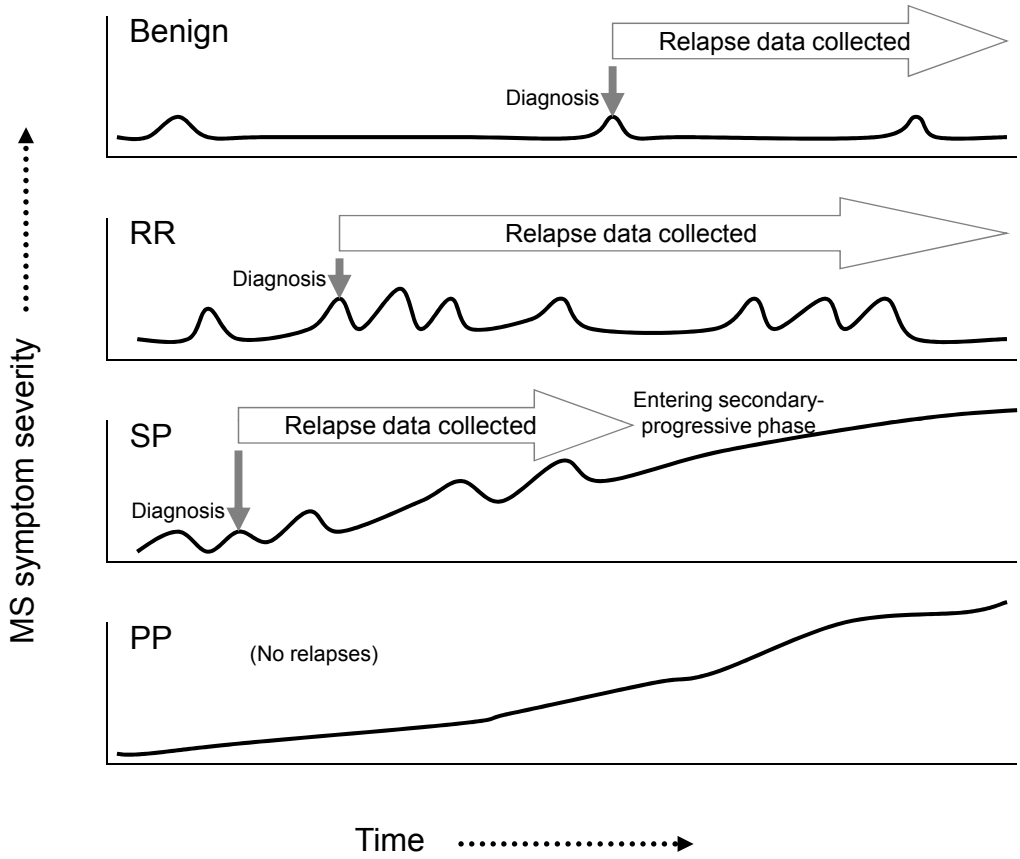

Figure 3. Schematic description of the relapse occurrence patterns of the different Multiple Sclerosis (MS) types (black curves) and the relapsing time periods included into the patient data used in this study (white arrows). Disease types: Benign = benign MS, RR = relapsing-remitting, SP = secondary-progressive, PP = primary progressive (not included). Gray arrows denote the points in time when a MS diagnosis could have been set following the second relapse. Possible beta-interferon using periods are not depicted in the graph, since they were determined individually for each patient.

The patient population in study II had been collected earlier from Turku University Hospital archives from 407 MS patients (268 with records of relapses) and covered all definite cases of MS patients treated during the years 1986-1995.

For the studies I, III, IV and V, data on MS relapses occurring monthly from January 1995 to March 2001 were collected from the Turku University Hospital, local district hospitals and the outpatient clinic of the local MS patient association patient files of those MS patients who had at least one recorded visit at the Department of Neurology of the Turku University Hospital between January 1995 and December 1999. The patient group was identified from the hospital database using the International Classification of Diseases diagnosis codes 3400A or G35 as search terms. The search result was 437 individual patient records (142 men). Two patients had refused to participate in scientific research. All of the resulting 435 patient files were read and notes were taken on the exact dates of birth and MS diagnosis, as well as the year and month of MS relapses and intravenous corticosteroid treatments. Part of the patients had no entry of a definite MS diagnosis in the papers and they were excluded. Records of a total of 359 MS patients were analysed further.

From patients who had used beta-interferon, notes on the date of the beginning beta-interferon treatment, the brand name of beta-interferon and the possible treatment discontinuation date were taken. 143 patients (97 women, 46 men) had used beta-interferon; 216 had no record of beta-interferon treatment. From January 1995 to March 2001, beta-interferon treatment was begun at the Turku University Hospital for 91 patients, 24 patients had started beta-interferon at the Turku University Hospital before 1995, and 28 started at other clinics. In the beta-interferon treated patients, on average 3,7 years had elapsed between the initial confirmation of diagnosis and the beginning of beta-interferon treatment. The mean duration of beta-interferon use was 2,56 years (sd 2,29 years). The patients were divided into beta-interferon users and non-users, and subsequently to subgroups according to the type of beta-interferon used.

Air quality data

The possible direct influence of ambient air quality on Multiple Sclerosis relapses was examined in study I. The monitored air quality parameters were the biological particles (pollen and fungal spores), abiotic particles (inhalable particulate matter) and the gaseous air pollutants sulphur dioxide (SO_2), nitric dioxide (NO_2), nitric monoxide (NO) and carbon monoxide (CO). A monthly total sum of each measured air quality parameter was used in the analyses.

Viral infection epidemics in the general population

Transmissible viral infection epidemics occurring in the general population were considered an environmental factor which can potentially modify the relapse rate in MS. The occurrence of infections in the general public and the factors known to predispose to infections were compared to the exacerbations of MS both without and during interferon medication. Monthly data on the viral infections in the general population was provided by the Department of Virology at the University of Turku. The database included the monthly

numbers of virologically confirmed diagnoses of different contractable viral infections. The Department of Virology receives patient samples from the whole area of Southwestern Finland. The viral pathogens considered in this thesis are Rhinovirus, Adenovirus, Influenza A and B, Parainfluenza 1, 2 and 3, Epstein-Barr Virus and Varicella Zoster Virus.

STATISTICAL METHODS

In this thesis, data on Multiple Sclerosis relapse occurrence was collected from hospital records to explore the effects of air quality, infection epidemics and beta-interferon medication on the occurrence of Multiple Sclerosis relapses by statistical methods. Compared to the more conventional prospective methods used in epidemiological studies, retrospective data is easier and more economical to collect over extensive time periods. A prerequisite is the reliability of the archive data: the patients must be individually recognizable, and the registry entries are consistent. All residents of Finland who were eligible for treatment at the Turku University Hospital have an individual social security number for identification. The personnel keeping the registry at the hospital archives were methodically following guidelines.

The analysis was based on ecological analysis, which compares group characteristics rather than individual-level data (Steel et al 2006, Morgenstern 1995). MS relapses are relatively rare events. According to the definition of an MS relapse, the theoretical maximum occurrence can be once per month or 12 times per year, but on the other hand, relapses can occur as sparsely as 20 years apart. Furthermore, the occurrence of relapses is non-random, yet unpredictable by disease duration or season alone. The difficulties with rare nonrandom events could be avoided by using grouped data.

The monthly numbers of MS relapses and the specifically diagnosed viral infections monitored within the same geographical area in Southwestern Finland were observed retrospectively from hospital archives. The study of the monthly occurrence of MS relapses required specific time-series analysis methods. Two distinct approaches were used: calendar-time and patient-time. In calendar-time studies, the focus was on the monthly occurrence of relapses on a conventional time scale (studies I, II and III). This allowed studying the possible effects on relapse occurrence of the concurrently occurring infection epidemics and air quality variables.

Relapse occurrence data is binary, as the event either occurs or not. Binary data are analysed with logistic regression, and the dependent factors can be binary or continuous. Multivariate logistic regression was used to study the effects of ambient air quality (study I) and epidemic episodes of specifically diagnosed viral infections in the general population (study II). The temporal association between population level viral infection epidemics and MS relapse rate was studied with logistic regression in study III. The effects of transmissible relapse-provoking infections and the potentially proinflammatory environmental factors were compared in patients under beta-interferon medication and non-users, because beta-interferons have both antiviral and anti-inflammatory properties.

The time window of a higher MS relapse risk could be several weeks (Andersen 1993, Sibley et al 1985). To include an unknown time lag caused by the fact that the infections were only diagnosed on a population level, a three-month Moving Average was chosen in study II to include a 0-3 month time lag.

The patient-time approach meant assessing the timing of the relapses on a personal lifetime scale, during the course of illness independently for each patient, starting from a specified date and ending at the end of the period of interest. The starting date in study IV

was the day of diagnosis or the day of starting beta-interferon medication, and in study V the 24-month period before starting beta-interferon medication.

The treatment groups were compared with survival analysis (study IV) and intervention analysis (study V). Survival analysis calculates risk of an event at a point in time, and the result is a hazard function. In this thesis, an event was specified as the MS relapse. If the event did not occur before the end of the period of interest, an observation was called right-censored. There are several survival analysis methods, which may compare events occurring in fixed time intervals, which could be studied using life tables or Cox regression techniques. In this thesis, the time to event was a continuous variable, in which case the only option was to use Kaplan-Meier survival analysis which is independent of time intervals (Kaplan et al 1958). The resulting survival functions could be analysed with log-rank test, where the data are divided into a rank order, and the differences between ranks is tested with Qhi-square statistic. Should the studied groups be different in their respective amounts of censoring, specific modifications of the log-rank test are used.

The relapse-free time calculated in days from the beginning of interferon treatment of the diagnosis date was studied in study IV with Kaplan-Meier time-to-event analysis, where the comparisons were based on proportions of relapse-free patients. The relapse-free time was calculated from the patient data in non-users from the diagnosis date, and in interferon users from the beginning of interferon treatment, in both cases until the first subsequent relapse or the end of the period of interest.

In study V, the effect of the beginning of beta-interferon treatment to MS relapse occurrence was studied using ARIMA intervention analysis (Box et al 1976). In time-series analysis ARIMA is preferable to regression, because time-series are not independent observations. Often time-series are autocorrelated, which would lead to biased results in linear regression. Generally at least 50 observations are required to perform an ARIMA analysis. Intervention analysis compares the level of a variable both before and after the level of a modifying factor has been changed, so that a step function was created to represent the change in interferon use. The monthly MS relapse rate was studied during the first months of interferon use by creating 24 dummy month variables. The properties of the relapse time-series were observed from a sequence chart as well as the ACF (autocorrelation function) and PACF (partial autocorrelation function) plots (Figure 4). An ARIMA model has 3 components: an autoregressive component (revealed by ACF and PACF and marked by p), an interactive component (the number of differencing steps, marked by q), and a moving average component (q) and the final model is expressed as ARIMA (p, d, q). The results are expressed as the percentage change in monthly MS relapse rate during beta-interferon medication.

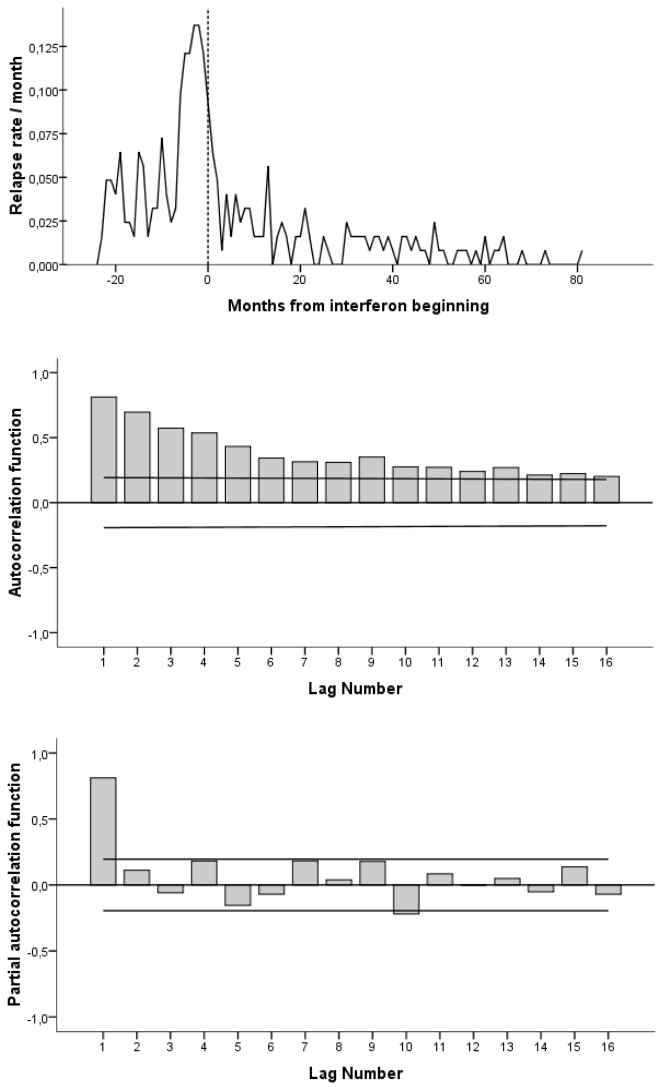

Figure 4. Sequence chart (top), Autocorrelation Function ACF (left) and Partial Autocorrelation Function PACF (right) plots of the time-series used for model identification in study V. Horizontal lines depict the confidence intervals. The ACF declined slowly and remained significant over more than half a dozen lags, indicating that the series needed differencing to reach stationarity.

Another important factor in ARIMA analysis is stationarity. If a time-series is not stationary, its overall level does not remain constant, but it is changing over time. Upon examining the slowly declining ACF plot of the series, it was evident that the time-series was not stationary and a transformation by first-order differencing was required to stabilize the series. After the transformation, a final ARIMA (0,1,1) model was selected. The data had a weak but continuous decreasing trend, so that a constant term was included in the model.

Statistical software from SAS, BMDP, Systat, Statistica and SPSS were used for the analyses.

RESULTS AND DISCUSSION

In this thesis, monthly MS relapse rates were compared to occurrence of infections in the general public and air quality factors which are known to increase the occurrence of contractable infections. Survival analysis was performed to compare the relapse-free times in different patient subgroups, with or without beta-interferon medication. Relapse rates were also compared before and during immunomodulatory treatment with beta-interferon with intervention analysis. The results and the statistical methods used in this thesis are summarized in Table 1.

Table 1. Summaries of the patient datasets, statistical methods and main results in the studies I-V.

Study	Patient dataset	Inclusion criterion	Statistical methods	Main results
I	1995-2001 N=406	All definite MS cases	Logistic regression	High PM_{10} is connected to an increase in MS relapses
II	1986-1995 N=407	All definite MS relapses	Logistic regression	Population level Influenza A and EBV infections were connected to an increase in MS relapses
III	1995-2001 N=314	Complete follow-up	Pearson correlation and logistic regression	Air quality was connected to high MS relapse rate in patients without beta-interferons
IV	1995-2001 N=309	Actively treated patients with recorded visits at the clinic between 1995-1999	Kaplan-Meier survival analysis	Beta-interferons increased the relapse-free time, and all beta-interferons were equally effective
V	1995-2001 N=135	Actively treated patients with recorded visits at the clinic between 1995-2001, and continuous observations from 24 months before to 3 months after starting interferon use	ARIMA Intervention analysis	MS relapse rate decreased by 11.5% during the first three months of beta-interferon use

Air quality, particle concentration and gaseous air pollutants

Ambient air inhalable particulate matter and pollutants are known to modify human immune reactions in general. In this thesis, the possible effects of inhalable airborne particulate matter and gaseous air pollutants to MS relapse rate were studied with multivariate logistic regression (study I). The monthly MS relapse rate was significantly different under predisposing environmental conditions. High inhalable airborne particulate matter concentrations were connected to an increase in MS relapse occurrence, as the odds ratio of the risk of a relapse onset was over four-fold (OR 4.143, $p<0.001$) when the concentration of inhalable particulate matter (PM_{10}) was at the highest quartile. The acidic gases proved to be strong confounding factors. In study III, relapses were more frequent following episodes of poor air quality in those patients who did not use interferons.

Poor air quality may enhance the seasonal changes in Multiple Sclerosis relapse occurrence through increased susceptibility to transmissible infections, since the periods of exceptionally

heavy inhalable particular matter loads in urban air are known to increase susceptibility to infections in immunologically challenged individuals. Impaired alveolar macrophage function by inhalable particulate matter might lead to activation of endogenous intracellular pathogens and subsequent inflammation, which can trigger a Multiple Sclerosis relapse without necessarily involving any exogenous infectious agents. On the other hand, the general activation of the immune system responses through mechanical irritation of the airways could lead to the onset of a relapse, or enhance the effect of a peripheral infection. A combination of air pollution and cold air, a situation not uncommon in the Finnish climatic conditions, may especially affect individuals with immunological disturbances.

A correlation between airborne allergenic pollen particles and relapse counts was detected in study I. In Southwestern Finland, the spring pollen season starts concurrently with the springtime peak in inhalable particulate matter levels; furthermore, pollen allergenicity can increase substantially through the attachment of allergen to coexisting inorganic air pollution particles (Behrendt et al. 1997). Furthermore, the allergenic particles originating from pollen and fungal spores are of respirable dimensions and they may irritate the airways mechanically. The connection between relapses and biological particles were, however, no longer detectable in the logistic regression modeling and should therefore be interpreted with caution. The prevalence of allergic disease has been considered to be lower in Multiple Sclerosis patients as compared to the general population (Oro et al 1996). However, both Th1- type MS and Th2-type atopic diseases have been noted to be significantly associated with MS, highlighting that a complex and heterogenous disease like MS cannot be explained by the Th1-Th2 balance alone (Edwards et al 2004).

Exposure to inhalable particulate matter is known to increase susceptibility to contractable microbial infections. Individuals with an immune system dysfunction are generally more susceptible to particulate matter and air pollution, and a repeated exposure to the combination of cold air and acidic gases can even have a cumulative increase in the adverse health effects on susceptible individuals (Randell 1997). However, pulmonary morbidity can be induced by various components of inhalable particulate matter such as acidity, wood smoke, latex allergens, pollen fragments and ultrafine diesel exhaust particles, which were not analysed in this study. The major contributor of airborne inhalable particles in Finland is paved road dust, a mixture of particles of abiotic origin such as fragments of tires, brakes and paving materials, detached from the road by studded tires, as well as sand, which is used to enhance traffic safety during the winter months and early spring (November-April). Thus, the size and consistence of inhalable particulate matter may be different in Finland as compared to countries where road sanding is not performed.

Carbon monoxide levels did not correlate with relapse counts in study I. The central nervous system is generally vulnerable to hypoxia caused by acute exposure to carbon monoxide (CO), but the required concentrations are rarely encountered outdoors. Furthermore, the human body is adapted to tolerate low concentrations of carbon monoxide, since trace amounts are continuously produced, for instance in the splenic cells in the degradation of heme to bilirubin.

Epidemics of contractable viral infections

Viral infections have for a long time been recognized as potent triggers of MS exacerbations. Because also MS patients are exposed to contractable viral infections

occurring in the general population, in this thesis it was hypothesized that a connection between MS relapses and viral infections could be directly studied in an ecological analysis setting, without studying the individual patients.

Stepwise logistic regression modeling was used to compare time-series data on relapse onsets for MS patients in the Turku University hospital to epidemic episodes of specifically diagnosed viral infections in the general population of Southwestern Finland from 1986-1995 (study II). The relapses were more frequent following an increase in Influenza A diagnoses: between moderate and high relapse counts the OR was 4.9 (95% CI 1.3 to 18.0) and between low and high 3.4 (1.0 to11.0). Epstein-Barr virus (EBV) infections were followed with an increase in MS relapse counts, while a clear negative correlation was found between adenovirus infections and MS relapses.

A possible pathway for the observed connection between EBV and MS relapses could be an activation of a latent EBV infection through other respiratory infections, which may initiate exacerbation in MS through cross-recognition of myelin antigens, molecular mimicry or superantigen induction (Salvetti et al 2009, Lünemann et al 2008, Haahr et al 2006). Acute EBV infection could reactivate many cross-reactive memory T cell populations specific to other previously encountered pathogens, such as Influenza A (Clute et al 2005). In contrast, vaccination does not increase MS relapses, and vaccinations against several viral infections, including Influenza A have been found to be unrelated to the onset of MS (Schattner et al 2005, DeStefano et al 2003).

In study III, logistic regression was used to assess the effects of the different viral infection epidemics and high levels of PM_{10} to the relapse rates in beta-interferon user and non-user groups. The effect of PM_{10} differed between beta-interferon users and non-users: poor air quality seemed to increase relapses in the non-user group but had no significant effect in the patients using beta-interferons. In the non-user group, PM_{10} was correlated with relapses (Pearson correlation coefficient 0.437, $p<0.002$). A marginally significant effect was detected in the time-series analysis (logistic regression odds ratio OR=1.196, $p>0.028$). Treatment with beta-interferon thus seemed to reduce the effect of ambient air inhalable particles: in the beta-interferon user group, air quality and virus infections had no effect.

In the non-user group, MS relapse rate was over twice as high following epidemics of adenovirus infections in the general population as compared to low or intermediate numbers of adenovirus diagnoses (OR=2.234, $p>0.046$). This result conflicted with the results in the study II, where a clear negative correlation between adenovirus and MS relapses was found. This might be explained by the differences in the effect of viral infections between beta-interferon users and non-users found in study III. Serologically confirmed adenovirus infections have previously been found to be positively correlated to MS relapses (Andersen et al 1993). Prospective monitoring of MS patients and serologically confirmed viral infection diagnoses might help to clarify the mechanism how beta-interferon can protect MS patients against an increased susceptibility to infections caused by inhalable particles.

A number of studies have linked the onset of MS with an initial bacterial or viral infection, but there is no conclusive evidence that any of the studied pathogen species would be the single causative agent in MS. The association between infections and MS relapses is

controversial (Confavreaux 2002). In this thesis, no virus could be pointed out as the single most important for MS relapse occurrence, but Influenza A and Epstein-Barr Virus infection epidemics in the general population were correlated with an increased occurrence of exacerbations in MS patients.

EBV is currently a strong candidate for becoming a therapeutic target in MS, as it has been shown to infect all MS patients, and patients whose first infection with EBV has occurred in adulthood have been shown to have a higher risk of developing MS (Thacker et al 2006). Possible pathways for the observed connection between EBV and MS activity could be a reactivation of a latent EBV infection through respiratory infections, which may initiate exacerbation in MS through cross-recognition of myelin antigens, molecular mimicry or superantigen induction (Salvetti et al 2009, Lünemann et al 2008, Haahr et al 2006).

In the group treated with beta-interferons, the non-significant effect of several viral pathogens can be interpreted as an indication of the antiviral action of beta-interferons. However, since many previously recognized important viruses remained insignificant also in the non-user group, individual patient testing for specific viruses probably is a more reliable way to study the effects of different viruses.

Immunomodulatory medication with beta-interferons

Treatment with beta-interferon has previously been shown to significantly decrease MS relapse rate, and beta-interferons have both anti-inflammatory and antiviral properties.

The observations in this study exceeded five years in the beta-interferon user groups. During the period of interest of this thesis, there were practically no private clinics or hospitals operating in the area, and therefore it was assumed that almost all local MS cases were diagnosed and followed in the same clinic, and that there were no socioeconomic differences between the patient groups. The occurrence of MS exacerbations during treatment with beta-interferons, compared to the two years before the beginning of the beta-interferon treatment, was reduced by more than 30% which could be expected from international clinical trials. The mean exacerbation rate was 1.10 per patient-year during the two years preceding the beginning of treatment. During beta-interferon treatment there were on average 0.39 exacerbations per patient-year, and the decrease in relapse rate was consistent during treatment (Figure 5).

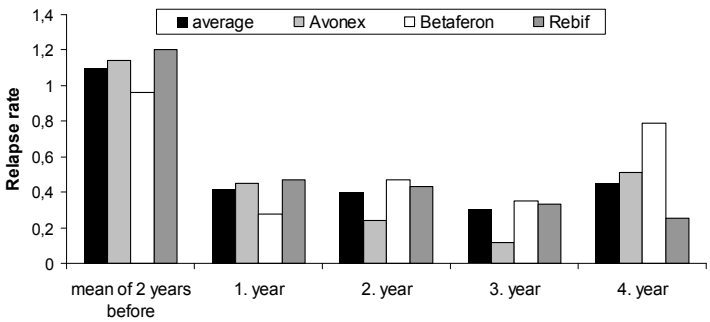

Figure 5. Relapse rate observed in the beta-interferon user groups during the two years before beta-interferon beginning and yearly during beta-interferon treatment.

A comparison between the annual relapse rates observed in this dataset and a number of international clinical trials revealed that in proportion to the decline in exacerbations, also fewer corticosteroid pulse treatments were recorded per patient during beta-interferon treatment. The severity of relapse symptoms probably remained unchanged, indicated by the reduction of corticosteroid pulse treatments in an equal proportion with the relapses, from 0.61 per patient per year to 0.31 during beta-interferon treament. However, it is not conclusive whether the frequency of corticosteroid pulse treatments can be thought of as a measure of disease activity, since there is controversial evidence of their clinical impact in MS, and the mechanism of action is unclear.

Time-series analyses of MS relapse occurrence

The first approach in the analysis of relapse occurrence was to analyse relapses in calendar-time (studies I, II and III). A time-series of MS relapse rates was constructed as relapses per patient per month from January 1995 to March 2001. The time-series plot revealed irregular monthly changes (Figure 6). Due to the chronic nature of MS, the number of patients in follow-up increased steadily toward the end of the period of interest (Figure 7).

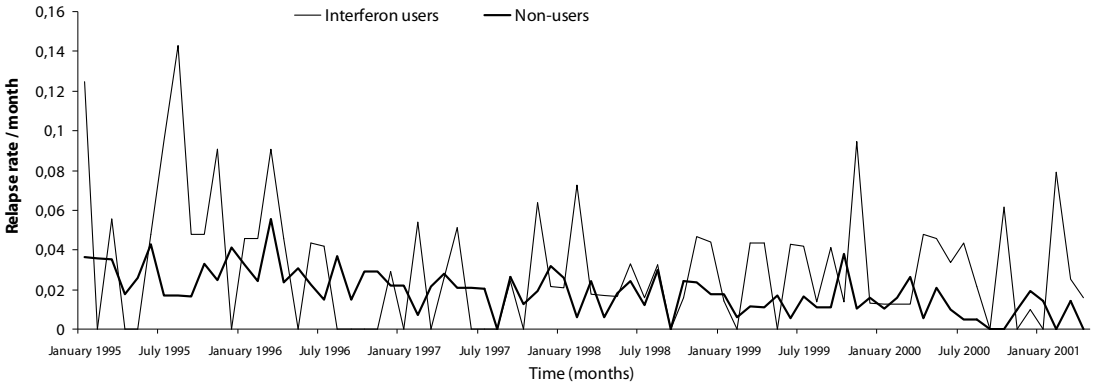

Figure 6. Time-series of monthly MS relapse rates during the period of interest from January 1995 to March 2001.

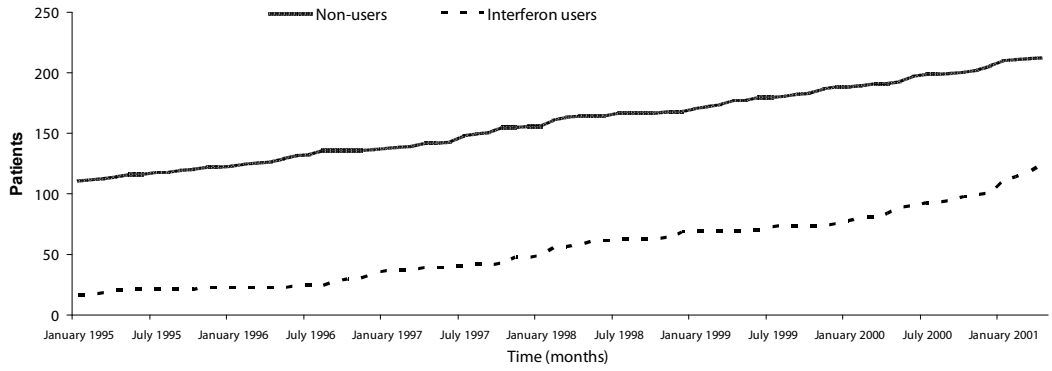

Figure 7. Numbers of patients followed during the period of interest from January 1995 to March 2001.

The second approach was to use patient-time, which allowed studying the timing of the relapses for each patient along the disease course. The event of a relapse was recorded in days from a reference date (in beta-interferon users the day of beginning of beta-interferon treatment, in non-users the diagnosis date). In study IV, the relapse-free time was studied in different patient subgroups calculated from the beginning of beta-interferon treatment of the diagnosis date and in study V the monthly relapse rate was compared before and during beta-interferon medication. Survival analysis and nonlinear mixed-effects models are the recommended analysis methods for EAE, an animal model of MS (Fleming et al 2005).

In study IV, the result of the survival analysis can be interpreted as the probability of experiencing a relapse at any time point. The differences in the survival functions for the three beta-interferon groups and those who did not use beta-interferon are depicted in Figure 8.

For comparisons between the different beta-interferon treatment groups it would be necessary to have equal numbers of censored observations in each group to avoid bias. In study IV, censoring was not equal in the different interferon groups (ranging from 13.6% to 27.4%). Although the modified Mantel-Cox log-rank test which is more tolerant of unequal censoring was used for the group comparisons, the results should be interpreted with caution. However, no significant differences were detected in the relapse-free times between beta-interferon groups and this observation fits well with earlier prospective results.

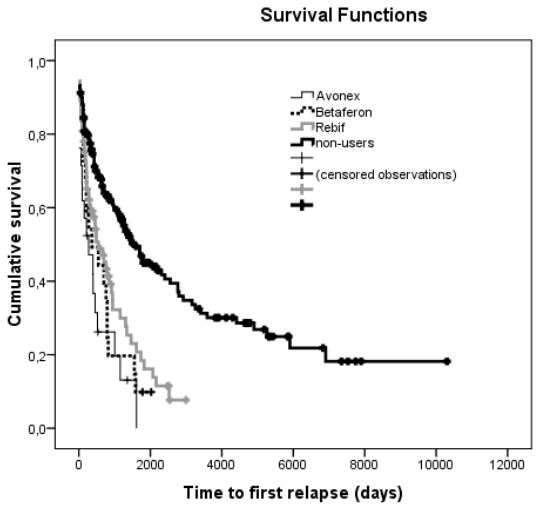

Figure 8. Schematic presentation of the differences in survival functions of the relapse-free time in beta-interferon user groups and the patients without beta-interferon (study IV).

Another time-series analysis method, the intervention analysis, was explored in study V. Relapse data were retrieved from 105 fully documented MS patients who all used beta-interferon. Monthly relapse occurrence was noted from 24 months prior to the beginning of beta-interferon treatment until the end of treatment for each patient. The mean duration of

beta-interferon use was 2.56 years (sd 2.29 years, range 100 days - 80 months). Based on the beta-interferon prescription guidelines the beta-interferon users had an annual relapse rate of >1 before medication, leading to a sharp increase in the time-series of relapse occurrence starting less than 6 months before the beginning of treatment. The effect of the treatment could be seen as a rapid decrease in the relapse occurrence curve. Intervention analysis revealed that from the beginning of beta-interferon treatment, MS relapse rate decreased by a total of 11.5% during the first three months as compared to the 24 months prior to treatment, and the relapse rate remained at the new lower level (Figure 9). This observation is lower than expected from previous clinical studies, where relapse rates have been compared to the mean relapse rate two years before beta-interferon start. One explanation may be that the monthly pre-medication relapse rate used in this study can become inflated if medication is started very soon after the required relapse rate is reached. This results could be verified on a larger patient sample and using a longer follow-up time.

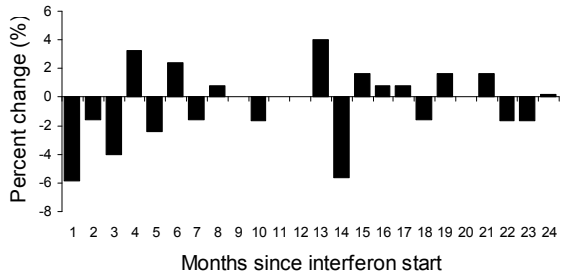

Figure 9. Histogram depicting the result of the intervention analysis with ARIMA (study V). The percentage change in relapse rate during the three first months of beta-interferon use was in total -11%.

Other observations

Detailed hospital records were available from MS patients who had passed a strict inclusion examination before receiving beta-interferon treatment. The annual examinations provided a reliable source of information on relapse rate for this long-term study on beta-interferon treatment efficacy and compliance. However, 23% of the initial patient files were excluded because of missing data, and there were not enough data on EDSS scores to enable assessment of disease progression.

Switches occurred equally between the different preparations, but the groups were too small to estimate the effect of discontinuation, switching or the patient's socioeconomic status to the outcome of treatment. The majority of switchings between beta-interferon preparations were decided by the patient, mainly due to injection site erythema and itching as well as flu-like symptoms, and some by the neurologist due to the observed ineffectiveness of treatment, leukopenia or an increase in hepatic transaminase. Other reported reasons for the switching of beta-interferon were financial difficulties, fatigue, persistent fever reaction, paraesthesis, dizziness, nausea, chest pain, anxiety and general worsening of the quality of life; injection problems: injection pain, fear of injection, lack of motivation to continue injections and discomfort connected to injections; and exacerbation of symptoms connected to the disease.

Discontinued treatment was observed for 26.4 % of the patients, which exceeds the reported dropout rate in the original beta-interferon trials and other postmarketing studies (Figure 10). Reported reasons for discontinuation of treatment were financial difficulties, ineffective treatment, injection pain, pregnancy, side effects: depression and sleeplessness, thrombocytopenia, acute hepatitis or excessive hepatic activity detected in blood samples (ALAT), fascitis, and leukopenia; difficulty in scheduling the injections into daily life or lack of motivation.

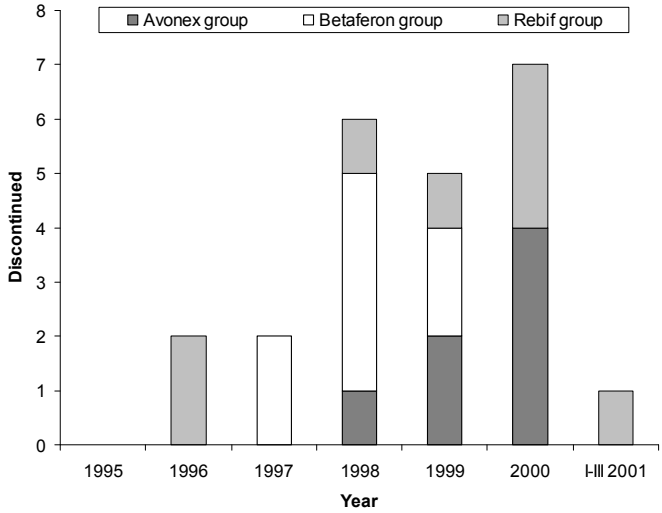

Figure 10. Yearly discontinuations of beta-interferon treatment observed in the studied MS patients.

An amount of seasonal variation was expected in this thesis in both the occurrence of MS relapses, the viral infection epidemics and in the environmental data on airborne abiotic and biological particles. The seasonal clustering of Multiple Sclerosis relapses observed in studies I and II supports the theory of some seasonally fluctuating environmental factors modifying the disease pathology. Annual cycles of changing ambient environmental conditions have long been known to have an effect on human health outcomes in the temperate climates, where the residents' immune functions fluctuate seasonally, being enhanced during winter. These fluctuations could be even more pronounced in individuals with an immune system dysfunction, such as in patients with MS.

Methodological limitations

An important limitation of ecological analysis is that as group characteristics are compared, the results cannot be interpreted to individual level (Steel et al 2006).

The main question in MS relapses is that they are not statistically independent. They are a marker of disease activity which can be modified by several factors individually for each patient. The length of observations in this thesis exceeded 5 years, and a long follow-up

can lead into another problem caused by the natural course of MS: a gradual progression of the disease eventually causes a decline in the relapse rate. The intervals between MS relapses may be several months or even years, which poses challenges in collecting enough data for prospective studies. The retrospective approach used in this thesis could be an alternative way of acquiring long-term relapse data. MS relapses often occur repeatedly, but in the Kaplan-Meier survival analysis only the first relapse and not the subsequent relapses were included. There are few analysis methods available for nonrandom repeated events.

The non-users were not a good control group for the beta-interferon user groups in study IV, because the beta-interferon groups had been *a priori* selected using strict inclusion criteria, and also because the natural history of the disease is not similar in the different groups. Choosing a suitable control group was difficult, as in this retrospective setting a placebo group or spouse controls which are commonly used in clinical trials were not applicable. Comparison to a baseline characteristic was not chosen, because the subsequent relapses are not statistically independent events, and also because the disease progression eventually causes changes in relapse rate.

It was assumed, that both beta-interferon users and non-users were equally exposed to the studied exogenous environmental factors, air quality and virus infections. Since these environmental factors were correlated with a higher frequency of exacerbations only in those MS patients who did not use beta-interferon in study III, it was interpreted as an indication of beta-interferon probably protecting against environmental factors in addition to its known antiviral properties.

Socioeconomic status has been connected to a number of autoimmune diseases (reviewed in Bach 2002). Although financial difficulties were reported as the reason for switching preparations, in this thesis it was not possible to assess the effect of the patient's socioeconomic status. Because all MS diagnoses are set at Turku University Hospital, we assumed that most MS patients in the area were also treated at the same clinic, and did not investigate private practitioners or other hospitals outside the region.

Although the studied environmental factors affect similarly all people within a geographical region, the exposure level strongly depends on lifestyle. We had no information about the patient's individual exposure to the environmental variables, including the infectious pathogens. Part of the patients probably resided in rural areas, whereas air quality parameters were only available from the urban environment of Turku. There were interruptions in the environmental measurements, but the logistic regression computations were performed only when data was simultaneously available on all sets of variables, which thus limited the number of records included in the model. The variation in the relapse counts was studied at a quite robust monthly level, although the concentrations of gases and particulates can vary significantly on a daily basis.

Two major causes of airway infections in adults, most importantly rhinoviruses were excluded from the study due to lack of reliable diagnostic methods during the first part of the study period. A role for rhinoviruses has been suggested in initiating the first episode of MS (Kriesel et al 2004, Kriesel and Sibley 2005), and their possible effect to relapse occurrence ought to be studied.

SUMMARY AND CONCLUSIONS

This was the first time a temporal connection between ambient air inhalable particulate matter and monthly MS relapse occurrence has been reported. There are several possible mechanisms how inhalable particulate matter concentrations could affect Multiple Sclerosis exacerbations. One possible pathogenetic pathway could be the lung's increased susceptibility to infectious pathogens; other MS relapse-triggering phenomena include alveolar macrophage dysfunction after long-term exposure to particulates, and the possibility of a reactivation of endogenous, intracellular pathogens. In addition, both a local and a systemic enhancement of the immunological functions should be considered.

An association between air pollutants, infection epidemics and MS relapses was only detected in the patients who did not use beta-interferons, highlighting the antiviral in addition to the anti-inflammatory properties of beta-interferons. Seasonally changing concentrations of ambient air pollutants have long been known to increase susceptibility to transmissible infections, to induce systemic immune responses, and to enhance existing peripheral inflammation. Individual exposure measurements could probably be used to assess the risk of a relapse in MS patients during episodes of high concentrations of inhalable particles.

Only a subset of the studied transmissible viral infections was temporally connected to MS relapse rate in this thesis. However, the effect of viral infections was different in patients using beta-interferons and non-users. This was the first observation of the possible protective effect of beta-interferons against air pollutants, and it deserves to be studied further. The population level viral infections did affect MS relapse rate, suggesting that MS patients should take precautions during infectious epidemic outbreaks. Seasonally fluctuating viral infections can be expected to affect the overall annual patterns in relapse occurrence, and specifically diagnosed environmental infections could be included in epidemiological models of MS relapse rate.

The statistical analyses in this thesis were based on epidemiological and ecological analysis of retrospective data. The findings were consistent with earlier prospective findings, indicating that detailed hospital records from MS patients, who have passed a strict inclusion examination before receiving beta-interferon treatment can be a reliable, readily available, economical and ethically sound source of information for long-term epidemiological studies.

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