The interplay between environmental risk factors for multiple sclerosis

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Kjetil Lauvland Bjørnevik Bergen, January 2017

Abbriviations

BMI	Body mass index
CI	Confidence interval
CIS	Clinically isolated syndrome
CNS	Central nervous system
DAG	Direct acyclic graph
DSS	Disability Status Scale
DMT	Disease modifying drugs
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EBNA-1	Epstein-Barr virus nuclear antigen 1
EDSS	Expanded Disability Status Scale
EnvIMS	Environmental Risk Factors in Multiple Sclerosis
EnvIMS-Q	Questionnaire for Environmental Risk Factors in Multiple Sclerosis
IL	Interleukin
IM	Infectious mononucleosis
IU	International units
MR	Mendelian randomization
MS	Multiple sclerosis
MRI	Magnetic resonance imaging
NEDA	No evidence of disease activity
OR	Odds ratio
PPMS	Primary progressive multiple sclerosis
PTH	Parathyroid hormone
RERI	Relative excess due to interaction
RR	Rate ratio
RRMS	Relapsing-remitting multiple sclerosis
SES	Socioeconomic status
SNP	Single-nucleotide polymorphism
SPMS	Secondary progressive multiple sclerosis

T_{reg}	Regulatory T-cells
UV	Ultraviolet
UVB	Ultraviolet B
VDR	Vitamin D receptor

Abstract

Background: Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system whose etiology is unknown. While several genetic factors and environmental exposures, including low vitamin D, smoking, infectious mononucleosis (IM) and obesity, have been consistently associated with increased MS risk, they are unlikely to fully explain the individual disease risk. Further, little is known about the underlying mechanisms by which they may affect disease risk.

Objective: The main objectives of this study were to examine how exposure to selected environmental factors in specific age periods was associated with MS risk and to disclose whether the associations varied between different populations using the same methodology. In detail, we sought 1) to examine how frequency of outdoor activity, as a proxy for sun exposure and vitamin D levels, in specific age periods from birth to disease onset was associated with MS risk, 2) to examine to which degree prior exposure to known environmental risk factors could explain the association between level of education and MS risk and 3) to examine how the interplay between smoking and IM affected MS risk in our study populations.

Methods: We used data from the large multi-national population-based case-control study Environmental Risk Factors in MS (EnvIMS), which included participants from Norway, Italy, Serbia, Sweden and Canada. For the two first articles, data from Norway and Italy was available, while for the third article data from Sweden was also available. In total, this included 1904 patients and 3694 controls. In the countries included in our analyses, patients were recruited from regional or national MS registries, while four times as many age and sex frequency-matched controls were randomly selected from population registries. All patients had been diagnosed according to the McDonald or the Poser criteria, and had clinical onset within 10 years prior to data collection. All participants were older than 18 years at time of selection. Cases and controls in each country reported on prior exposure to selected environmental factors in specific age periods of life using an identical self-administered questionnaire (EnvIMS-Q), which had been developed specifically for

our study. For the current analyses, information on outdoor activity, sunscreen use, hair color, smoking, IM, body size, cod liver oil supplementation, fatty fish intake and level of education was used. The controls were randomly assigned an index age based on the distribution of age of onset among the cases and exposure after disease onset or index age was not considered exposure. The association between disease and exposure was estimated as odds ratios (OR) with 95% confidence intervals (95% CI) using logistic regression. All analyses were adjusted for age and sex.

Results: In the first article, we found a significant inverse association between frequency of outdoor activity and MS risk in Norway and Italy. The magnitude of the association was strongest between age 16 and 18 in Norway (OR 1.83, 95% CI: 1.30-2.59), and between birth and age 5 years in Italy (OR 1.56, 95% CI: 1.16-2.10). We observed seasonal differences in the association in Norway, whereas we observed a significant association for outdoor activity during summer, but not in the winter. For Italy, the association was similar for summer and winter. In addition, we found a significant association between sunscreen use and MS risk during childhood in Norway after accounting for outdoor activity (OR 1.67, 95% CI: 1.06-2.63).

In the second article, we found an inverse association between level of education and MS risk in Norway (OR highest vs lowest level: 0.53, 95% CI: 0.41-0.68). The association remained significant after adjusting for smoking, IM, outdoor activity, cod liver oil, fatty fish consumption and body size. Further, the association remained similar after we excluded patients with early onset of disease, defined as onset before age 28.

In the third article, we found a statistical significant negative multiplicative interaction between smoking and IM in the risk of MS. Among those who reported IM, we observed no increased disease risk associated with smoking. Similarly, the effect estimates for the association between IM and MS risk were considerably lower among ever-smokers compared to never smokers. The interaction was similar in Norway, Italy, and Sweden. Lastly, we observed similar results on when estimating

the interaction on the additive scale, although they did not reach statistical significance.

Conclusion: The findings of this study add to the evidence that vitamin D has a protective effect on MS risk, and indicate that adolescence is a sensitive period for exposure. Still, exposure earlier in life might also be of importance. Further, established risk factors cannot fully explain the association between level of education and MS risk in Norway, suggesting that currently unknown environmental exposures associated with lower level of education may be important for disease risk. Lastly, our findings indicate a competing antagonism between smoking and IM in the risk of MS, which suggests that the two risk factors operate on shared biological pathways.

List of publications

I. Bjørnevik K, Riise T, Casetta I, Drulovic J, Granieri E, Holmøy T, Kampman MT, Landtblom AM, Lauer K, Lossius A, Magalhaes S, Myhr KM, Pekmezovic T, Wesnes K, Wolfson C, Pugliatti M. Sun exposure and multiple sclerosis risk in Norway and Italy: The EnvIMS study. *Multiple Sclerosis Journal* 2014; 20: 1042–1049.

II. Bjørnevik K, Riise T, Cortese M, Holmøy T, Kampman MT, Magalhaes S, Myhr KM, Wolfson C, Pugliatti M. Level of education and multiple sclerosis risk after adjustment for known risk factors: The EnvIMS study. *Multiple Sclerosis Journal* 2016; 22: 104–111.

III. Bjørnevik K, Riise T, Bostrom I, Casetta I, Cortese M, Granieri E, Holmøy T, Kampman MT, Landtblom AM, Magalhaes S, Pugliatti M,, Wolfson C, Myhr KM. Negative interaction between smoking and EBV in the risk of multiple sclerosis: The EnvIMS study. *Multiple Sclerosis Journal 2016; Sep 23 (Epub ahead of print).*

Contents

1. Introduction

1.1 Historical perspectives

In 1822, Augustus d'Este (1794-1848), the grandson of King George III of England, started a detailed diary to describe various symptoms he had been experiencing.[1] At age 28, he experienced blurred and gradually decreased vision to the extent that he could no longer read, which he thought was due to holding back his tears in a funeral. While his vision gradually improved, he experienced repeated episodes with vision loss followed by recovery over the next years. In 1827, he complained that heat was now intolerable to him, and in the following years the disease progressed and he developed numbness, increasing weakness in his legs and difficulty walking. His story is a characteristic picture of multiple sclerosis (MS), and is one of the first case reports of a disease that was not defined until 20 years after d'Este's death.

Jean-Martin Charcot (1825-1893), a French neurologist, gave a series of three lectures in 1868 where he defined a disease he referred to as "sclérose en plaque disséminées" or disseminated sclerosis,[2] which later became known as MS. Based on clinical and pathological observations, he described a disease that closely resembles how we see the disease today.[3] In the years that followed, more cases were described in the medical literature,[4] and eventually efforts to find the cause of the disease began.

1.2 Pathogenesis

During the 19th century, Charcot and others noted that the disease that later become known as MS was characterized by a selective loss and disruption of the myelin sheet,[3] a membrane structure surrounding the neuronal axon that is important both for signal transduction and protection of neurons. Areas of demyelination constitute focal lesions, and in these lesions axonal damage and degeneration may occur.[5, 6] This can lead to disruption of neuronal signaling, which is manifested in the symptoms the patient is experiencing. Charcot and others noted early that axonal pathology

correlated with disability development,[7] and it has later been widely agreed that axonal damage likely is a determinant of disease progression in MS.[5]

Immune cells specifically targeting self-tissue are likely to initiate the disease processes in MS. During the differentiation of thymocytes in the thymus, most cells targeting self-tissue are eliminated, but some mature self-reactive T-cells may still be released into the peripheral circulation.[8] These are normally kept in check by regulatory parts of the immune system, such as regulatory T (T_{reg}) cells, and remains inactivated.[8] In MS, it has been proposed that a complex interaction between genetic predisposition and environmental triggers leads to an activation of peripheral self-reactive T- and B-cells, which then may infiltrate the central nervous system (CNS) and lead to inflammation and eventually tissue damage.[9] Triggers may induce activation of these cells directly by molecular mimicry, a cross-activation of self-reactive immune cells due to structural resemblance between a foreign antigen and a self-antigen,[10] or indirectly by inducing a pro-inflammatory environment that can lower the activation threshold of self-reactive cells.[9] The precise mechanisms by which environmental exposures and genetic factor interact to eventually cause MS are still not known.

1.3 Descriptive epidemiology

There is a considerable regional variation in the distribution of MS across the world (Figure 1). In the 1950s, Kurland et al. used mortality statistics to estimate the prevalence of MS in selected regions in the US and Canada, and found a considerable higher prevalence in the north compared to the south,[11] which was consistent with observations made by Davenport already in 1922.[12] On the basis of these findings and similar findings made by others, it was suggested that an environmental exposure varying with latitude could be important for disease risk.[13] In 1964, Kurtzke published one of the first reviews of prevalence studies, and divided the world into three frequency zones; a high prevalence zone consisting of northwest Europe, southern Canada and northern USA, a medium prevalence zone consisting of southern Europe, southern USA and Australia and a low prevalence zone including

the rest of the world.[14] He noted that the prevalence seemed to be correlated with latitude, whereas the prevalence was very low in tropical areas close to the equator and increased with latitude with the highest prevalence in the most northern countries.[15] A similar pattern is described in newer studies,[16] although there are some exceptions to the suggested north-south gradient in prevalence.[17] Furthermore, it has recently been reported that the gradient in the northern hemisphere appears to be attenuated.[18]

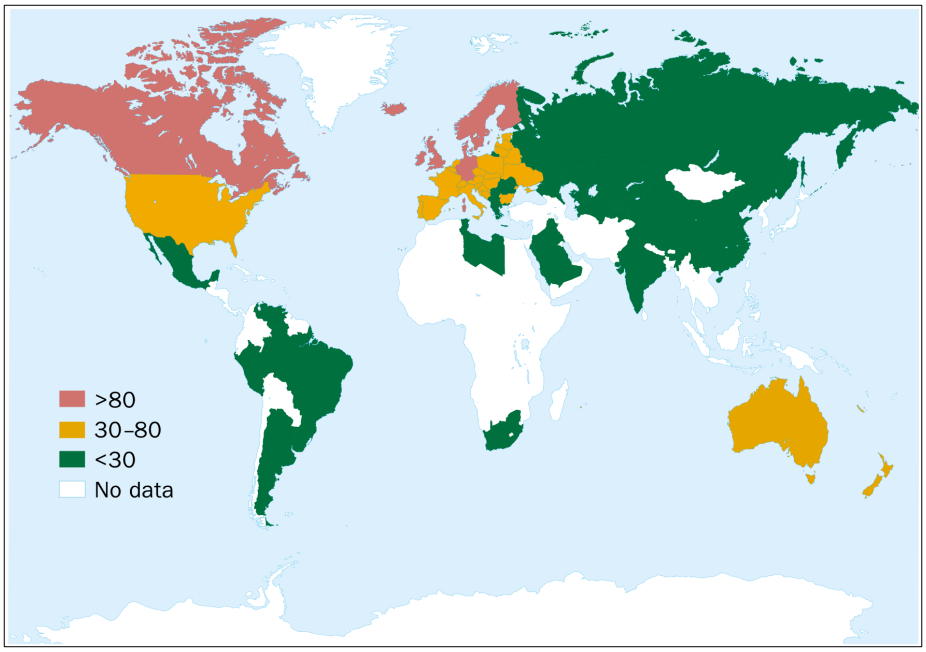

Figure 1: Worldwide prevalence of multiple sclerosis

The figure illustrates the distribution of MS across the world with prevalence reported per 100 000 population. Reprinted by permission from Elsevier: The Lancet Neurology 3: 710,[19] © Copyright 2004

MS was initially thought to be equally prevalent in men and women, or even dominated by men.[20] Early observations in the end of the 19th and beginning of the 20th century were likely affected by ascertainment bias, as some women with MS were misdiagnosed with hysteria, while men with MS tended to be more accurately

diagnosed as they dominated the work force.[21] More recent studies consistently report a higher prevalence in women than in men, which may be due to better ascertainment, but also likely to reflect a real increasing incidence in women compared to men over time.[22] The incidence of the disease is highest in young adults, and age of onset peaks between 25 and 35 years.[23]

1.4 Disease characteristics

There are two main disease phenotypes in MS, relapsing and progressive disease.[24] Relapsing disease is characterized by relapses followed by partial or complete recovery, and is referred to as relapsing-remitting MS (RRMS).[24] A relapse is defined as symptoms or signs typical of an acute inflammatory demyelinating event in the CNS with duration of at least 24 hours in the absence of fever or infection.[25] RRMS is the most common course at disease onset, and accounts for approximately 85% of the patients.[26] The first relapse is referred to as clinically isolated syndrome (CIS), which do not fulfill the criteria of MS diagnosis, but is sometimes included in the spectrum of MS phenotypes.[24] Progressive disease is characterized by a gradual accumulation of disability over time without clear periods of recovery, and is referred to as primary progressive MS (PPMS) when it is present from disease onset and secondary progressive MS (SPMS) if it occurs after an initial course of RRMS.[24]

Common initial symptoms are sensory, motor or visual disturbances consistent with demyelinating lesions in the brain, spinal cord, brainstem or optic nerve.[26] It was early suggested that a definitive diagnoses of MS should be based on dissemination of lesions in time and space,[27] which is still the case today. According to the newest diagnostic criteria, the 2010 revision of the McDonald criteria, patients with evidence of dissemination of time and space on a single magnetic resonance imaging (MRI) scan can be diagnosed with definitive MS.[25] In the study that this thesis is based on, cases with a definite or probable diagnosis according to McDonald (2005 revision)[28] or the Poser[29] criteria were included.

Further progression of the disease appears to be fairly homogenous on a population level, although there is considerable variation between affected individuals.[30] Most patients with untreated RRMS will eventually develop SPMS,[31, 32] which similarly to PPMS is associated with development of irreversible disability,[33] as illustrated in Figure 2. In prospective cohort studies of untreated patients, the median time to a score of 6 on the Disability Status Scale (DSS), corresponding to inability to walk unsupported,[34] has been reported to range from 14 to 20 years after disease onset,[26, 35, 36] although longer time intervals has been reported.[37] In patients on disease modifying treatment (DMT), the risk of reaching 6 on the Expanded Disability Status Scale (EDSS) appears to be lower compared to untreated patients,[38] although no effect of treatment on progression has also been reported.[39, 40] The overall survival among patients appears to have improved over time, but the life expectancy is still considerable lower than age-matched populations without MS.[41, 42]

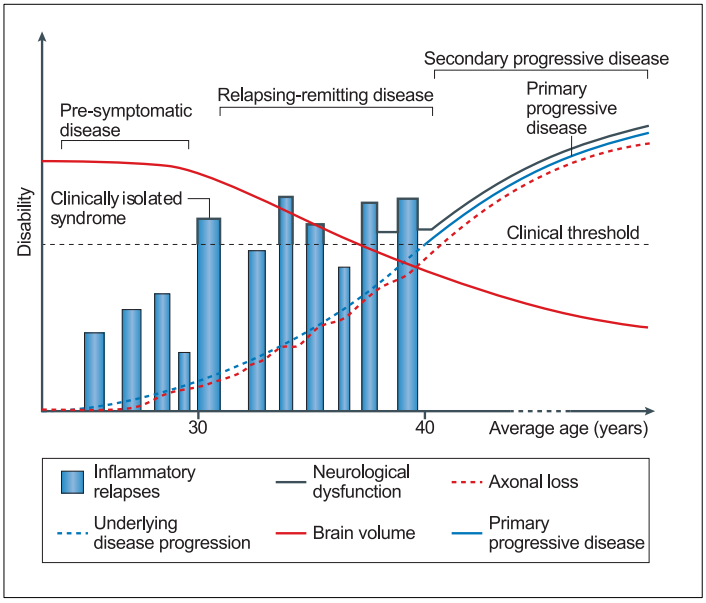

Figure 2: The heterogeneity of multiple sclerosis.

The figure illustrates the course of disease in patients with RRMS, SPMS and PPMS according to average age and disability accumulation. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology 15: 546,[9] *© Copyright 2015*

1.5 Environmental risk factors

1.5.1 Evidence for environmental risk factors

While there is strong evidence that genetic predisposition contributes to the risk of MS,[43, 44] it cannot fully explain individual disease risk. The concordance rate in monozygotic twins has been reported to be approximately 25%, with a sharp decrease in risk with increased genetic distance to the individual affected by the disease (Figure 3),[45, 46] illustrating the importance of genetic susceptibility, but also suggesting that environmental risk factors play an important role in the etiology of MS. However, as illustrated by Figure 3, the concordance rate for MS in monozygotic twins is lower than what has been reported for rickets and polio, which are both caused by environmental factors.[47] The change in risk among individuals who migrate to areas with different MS prevalence, the change in incidence and sex ratio over time and the consistent associations between specific environmental risk factors and disease risk further add to the evidence that environmental exposures contributes to individual disease risk.[48]

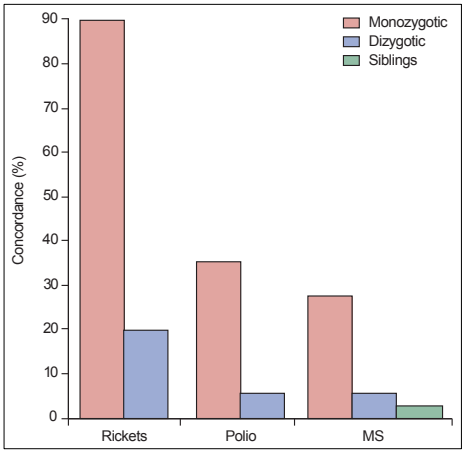

Figure 3: Twin and sibling concordance in rickets, polio and multiple sclerosis. *The figure illustrates how disease risk decreases with increasing genetic distance from the affected individual. Reprinted by permission from Elsevier: The Lancet Neurology 7(3): 271,*[47] *© Copyright 2004*

Migration studies represent a natural experiment where the population is changing their environment over a short period of time, and has been useful to understand aspects of MS.[49] In 1970, Kurtzke et al. noted that the prevalence of MS among migrants that earlier had moved from North Europe to South Africa varied by age at migration.[50] Among those who moved while they were under age 15, prevalence was about the same as that of the white native-born population, while prevalence among those aged more than 15 at time of migration was similar to that in their homeland. This suggests that environmental exposures varying in the two regions are important for disease risk, and that the age period before age 15 likely is a sensitive period. Similar findings have been documented for migrants moving from low- to high-risk areas[51, 52] and for within-country migration.[53]

Several studies have reported an increased incidence of MS over time, and the increase appears to be more pronounced in women compared to men.[22] In a systematic review of prevalence and incidence studies from 1950 to 2010, Koch-Henriksen et al. found a significant increase in incidence with time (Figure 4),[22] suggesting that the findings are not due to improved survival of MS-patients. Similarly, most studies with repeated surveys over time in the same population report increased incidence and prevalence.[22] Orton et al. calculated sex ratio in MS-patients by year in a population-based cohort over 50 years, and observed that the ratio has increased from 1.9:1 in 1931-1935 to 3.2:1 in 1976-1980,[54] which is consistent with studies in Denmark and Norway with similar follow-up time.[22, 55] While better ascertainment and more sensitive diagnostic criteria may have contributed to a higher number patients being accurately diagnosed with MS, and thus higher reported incidence rates of the disease in newer studies, it is unlikely that this can fully explain the observed changes.[22] A clear increase in incidence and a change in sex ratio over time cannot be explained by genetic predisposition alone, as the time period is too short for major changes in the genetic material to occur.

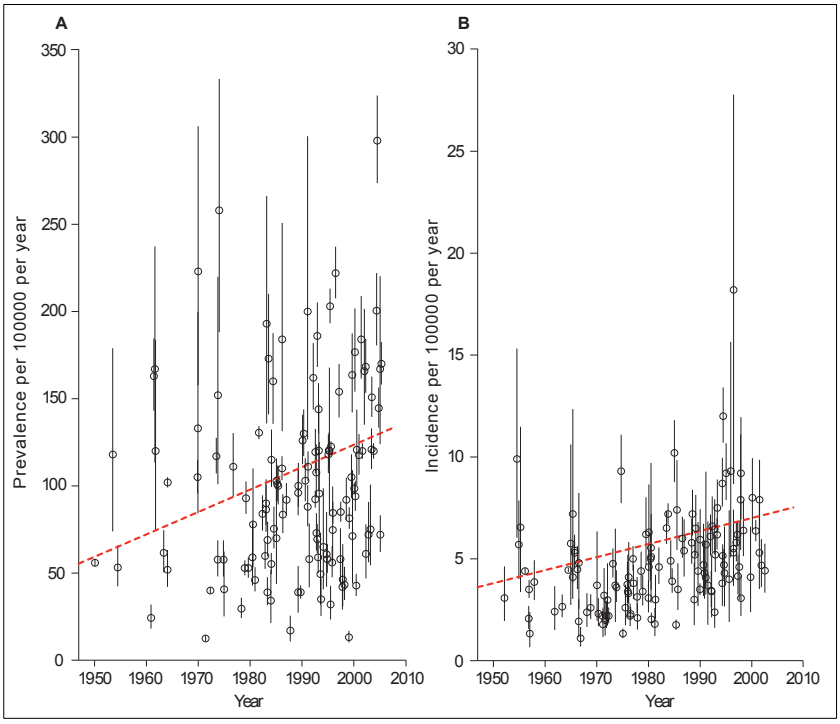

Figure 4: Prevalence and incidence rates from 1950 – 2010.

The figure illustrates the increase in prevalence (A) and incidence rates (B) in Western Europe and North America during the period 1950 – 2010. Reprinted by permission from Elsevier: The Lancet Neurology 9(5): 525,[22] *© Copyright 2010*

Lastly, several environmental exposures have consistently been associated an altered MS risk, including Epstein-Barr virus infection, vitamin D, smoking and obesity,[48] arguing for a role of these exposures in the etiology of the disease.

1.5.2 Epstein-Barr virus and infectious mononucleosis

Already in the 19th century, it was proposed by Pierre Marie, a student of Charcot, that an infection could be the cause of MS.[3] In 1975, Kurtzke et al. described MS as an epidemic disease in the Faroe Island, as the authors could not document a single patient with onset prior to 1943 among individuals who had not spent longer periods of time abroad, but observed a sharp increase in incidence after this year.[56] The

authors argued that the most likely explanation for these observations was a persistent infection introduced by British troops that occupied the island from 1940-1945, and referred to it as primary multiple sclerosis affection. Although an agent for such an infection has not been identified, and the interpretation of the findings have been questioned by other investigators,[57] the observations could argue for a role of an infectious disease in the etiology of MS.

In 1963, Poskanzer et al. noted that there were similarities between MS and poliomyelitis, as they had a similar distribution with latitude.[58] It had previously been suggested that the distribution of poliomyelitis was due to differences in sanitation, whereas poor sanitation in early life was associated with higher rate of infection with the virus, but lower rates of paralytic disease. On the other hand, better sanitation in childhood was associated with infection later in life and increased risk of paralytic disease. As sanitation varied with latitude, this could explain the geographical distribution of the disease. Similarly, if MS was due to an infection and the risk varies with age at infection, the distribution of the disease could be explained by sanitary conditions. A range of different bacterial and viral agents have been proposed to affect MS risk, but EBV is the only agent that consistently has been associated with in increased MS risk.[59] EBV has similarities with the infectious agent proposed by Poskanzer et al., as the age at primary infection varies between developing and developed countries,[60] and thus likely sanitary conditions, and an infection later in life is associated with a higher risk of symptomatic disease,[60] referred to as infectious mononucleosis (IM), which consistently has been associated with an increased MS risk.[59] Furthermore, MS risk appears to be very low among EBV negative individuals,[61] suggesting that higher sanitary condition is not a common cause for both IM and MS, but that EBV actually contributes to MS risk.

The strongest evidence for EBV in MS risk comes from prospective studies with serum samples prior to MS onset. In a nested case-control study among military personnel in the USA, Munger et al. found that serum titers prior to MS onset of a specific antibody, EBV nuclear antigen (EBNA-1), was significantly associated with subsequent MS risk, with the highest levels being associated with a 36-fold increased

risk.[62] An independent Swedish study also found associations between EBNA-1 and MS risk,[63] and together they could indicate that an altered immune response to EBV affects MS risk. Further, Levin et al. identified EBV negative individuals who later developed MS in the cohort of military personnel, and found that all of them contracted EBV before developing the MS (Figure 5), compared to 36% among those who did not develop MS.[64] This could suggest that the findings are not due to reverse causality, and that EBV may be necessary, but not sufficient for MS to develop. Still, the number of individuals that initially were EBV negative and later developed MS was low.

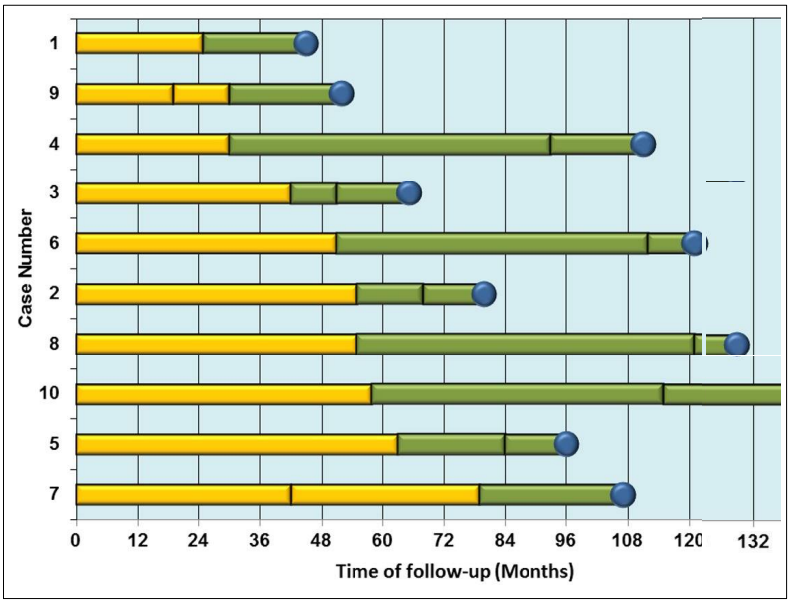

Figure 5: Time of EBV seroconversion and MS onset.

The figure illustrates the follow-up of 10 EBV negative individuals that later developed multiple sclerosis. The horizontal lines represent serum samples in individuals; Yellow bar is months as EBV negative, the green bar is months as EBV positive and the blue circle is MS onset. Reprinted by permission from Nature Publishing Group: Nature Reviews Neurology 8(12): 602,[65] © Copyright 2012

It is still unclear by which mechanisms EBV affects MS risk. It could involve cross-reactivity (i.e. molecular mimicry) between EBV-specific antigens and myelin-specific epitope, as MS patients appears to have increased levels of EBNA-1 specific CD4+ T-cells that also has a broader specificity for epitope recognition compared to EBV positive controls,[66] and these T-cells recognize myelin antigens more often than other antigens.[67]

1.5.3 Vitamin D and sun exposure

In 1960, Acheson et al. were the first to suggest that sun exposure could be protective for MS, after finding a significant correlation between hours of solar radiation and the geographical distribution of the disease.[68] Higher levels of sun exposure or more frequent outdoor activity have later consistently been associated with a lower MS risk in several case-control studies from different parts of the world, including Australia,[69] Norway[70] and Sweden.[71] It has been proposed that the association could be due to immunosuppressive effects due to suppression of melatonin[72] or through induction of T_{reg} cells and regulation of the cytokines interleukin (IL) 10 and IL-17.[73] The association is now mainly interpreted to be mediated by vitamin D, as sun exposure is an important source of the vitamin,[74] but an association between actinic damage, a marker of cumulative sun exposure, and lower MS risk independent of vitamin D levels has also been reported.[73]

Vitamin D is a fat-soluble vitamin that humans mainly get from exposure to sun, but also in smaller amounts through diet and dietary supplements,[74] as illustrated by Figure 6. The vitamin D synthesis by sun exposure is initiated when 7-dehydrocholesterol is converted to previtamin D_3 by ultraviolet B (UVB) rays penetrating the skin.[74] The amount of UVB radiation reaching the earth's surface varies markedly with season and latitude, as photons at low solar zenith angle have to travel for longer distances through the ozone level, which increases the absorption.[75] Therefore, there are marked seasonal and latitudinal variations in vitamin D synthesis by sun exposure. Previtamin D_3 is converted to Vitamin D_3 and then to 25-hydroxyvitamin D,[74] which is often is used as a marker vitamin D status due to its

long half-life and because the conversion of vitamin D_3 to 25-hydroxyvitamin D in the liver is not tightly regulated but depends more on the concentration of vitamin D_3.[76] Eventually, 25-hydroxyvitamin D can be converted to 1,25-dihydroxyvitamin D, which is the active form of vitamin D, and this conversion is tightly regulated by serum parathyoroid hormone (PTH), calcium and phosphorus levels.[74] For this reason and because of its short half-life, 1,25-hydroxyvitamin D is not a useful marker of vitamin D status.

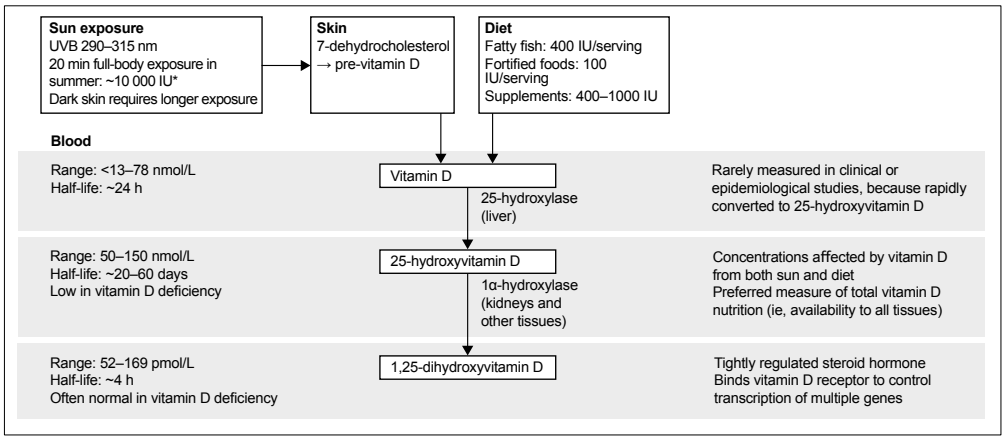

Figure 6: Metabolism of vitamin D.

The figure illustrates the metabolism of vitamin D from sun exposure and diet. Vitamin D is obtained either directly through diet, or after a conversion of 7-dehydrocholesterol to pre-vitamin D by sun exposure in the skin and a further conversion to vitamin D in the body. Vitamin D is converted to 25-hydroxyvitamin D in the liver and further to its active form 1,25-dihydroxyvitamin D in the kidneys and other organs. Reprinted by permission from Elsevier: The Lancet Neurology 9(6): 600,[77] © Copyright 2010.

Goldberg first proposed the vitamin D hypothesis for MS in 1974.[78] However, most of the evidence for a role of the vitamin in MS etiology comes from studies conducted the last 15 years. Two nested case-control studies assessed the association between preclinical serum-levels of 25-hydroxyvitamin D and MS risk, and found an inverse association suggesting a protective role of the vitamin.[79, 80] However, a

challenge with these findings is to separate the effect of vitamin D and sun exposure, as the two are highly correlated.[73] In a large prospective study of female U.S. nurses, Munger et al. found a significant inverse association between supplemental vitamin D intake and MS risk, whereas a daily intake of more than 400IU/D was associated with a 40% lower risk,[81] suggesting that vitamin D itself is protective for MS. This is consistent with some,[70, 82, 83] but not all,[69] case-control studies, where intake of supplements or food rich in vitamin D have been associated with a lower MS risk. Lastly, two studies using Mendelian randomization (MR) found an inverse association between single-nucleotide polymorphisms (SNPs) predicting vitamin D levels and MS risk.[84, 85] MR studies use genetic information as an instrumental variable, and can therefore avoid some of the bias associated with observational studies,[86] as illustrated in Figure 7. While there are methodological challenges with MR studies,[87] the results from these studies argue for a causal role of vitamin D in MS etiology and against that the association between vitamin D and MS risk is due to confounding by sun exposure.

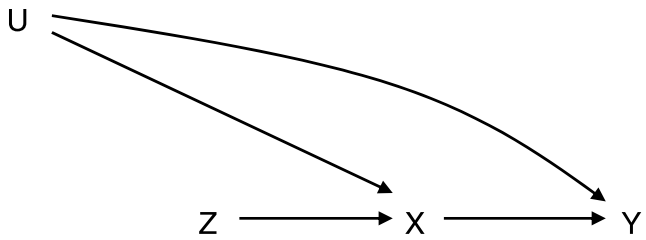

Figure 7: Instrumental variable and Mendelian randomization.

The figure is a directed acyclic graph (DAG) of an instrumental variable Z, the exposure X, the outcome Y and measured or unmeasured common causes (i.e. confounders) U. Z is an instrumental variable as it has a causal effect on X, only affects Y through X and does not share a common cause with Y.[88] In MR studies, genetic information may be interpreted as an instrumental variable, and they are thus not depending on measuring all confounders to estimate the effect of the exposure on the outcome, given that the genetic information meets all the assumptions of an instrumental variable. Reprinted by permission from Wolters Kluwer Health, Inc.: Epidemiology 17(4): 361,[88] © Copyright 2006.

Vitamin D has immunomodulatory properties that may be relevant for MS. 1,25-dihydroxyvitamin D appears to be effective in preventing and attenuating the disease progression in experimental autoimmune encephalomyelitis (EAE),[89] a commonly used animal model of MS, likely through pathways involving IL-10, T_{reg} and vitamin D receptors (VDR) in T cells.[90-92] The role of 25-hydroxyvitamin D is less clear, as it only appears to be effective in female mice.[93] Serum level of 25-hydroxyvitamin D has been correlated with frequency and function of T_{reg} cells in humans.[94, 95] This suggests that vitamin D can modulate T_{reg} activity, which have been reported to be impaired in MS.[96] Furthermore, both 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D have been associated with a reduction in the proportion of conventional Th17-cells,[97, 98] which appears to be increased in MS patients and are likely to be relevant for MS pathogenesis.[99] It has also been hypothesized that 25-hydroxyvitamin D could be directly involved in thymic negative selection, as the number of signal joint TCR excision circles, a marker of thymic output, has been reported to be inversely correlated with 25-hydroxyvitamin D levels.[100] In prospective studies of MS patients, higher levels of 25-hydroxyvitamin D have been associated with reduced disease activity and slower progression.[101, 102] The results from intervention studies have been less clear, which may be due to relatively small sample size and short follow-up, but some studies have reported significantly lower number of new lesions in the vitamin D group compared to placebo.[103, 104]

1.5.4 Tobacco smoking

The first studies on smoking and MS risk were conducted in the 1960s, after it had been reported that smoking could aggravate symptoms in MS patients.[105, 106] Antonovsky et al. first reported an association between smoking and MS risk in 1965, after finding a significant higher proportion of smokers before age of onset among MS patients compared to controls in an Israeli population-based case-control study.[13] The findings were inconsistent with a study published the year after, where no association between current smoking and MS risk was found when using control data from a national survey of smoking in Great Britain.[107] Still, as the MS patients in this study may have changed smoking habits after disease onset, the use of current

smoking as exposure makes the results from this study are less informative.[108] In the 1990s, two prospective studies on oral contraceptives and MS risk, reported findings indicating a possible association between smoking and MS risk, although the associations were not statistical significant.[109, 110]

In the years that followed, there was an increased interest in the association and several new studies were conducted. Ghadirian et al. found a significant higher risk among ever-smokers compared to never-smokers in a Canadian case-control study, and reported a clear dose-response relationship between the number of cigarettes smoked per day and MS risk.[111] However, as information on prior smoking habits was collected after disease onset, the findings could have been prone to differential misclassification by disease status, or recall bias. Hernan et al. prospectively examined the association in two cohort studies of female nurses in the US in 2001, and found a significant higher risk in current smokers compared to never-smokers and a significant dose-response relationship between pack-years of smoking and MS risk.[108] Riise et al. reported similar findings in 2003 in a study that included a large general population in Norway.[112] Consistent findings have later been reported from several different countries, including England,[113] Serbia,[114] Sweden,[115] Iran,[116] and Australia.[117] Further, elevated levels of cotinine, a marker of current smoking, measured before MS onset has also been associated with a higher MS risk.[118] Smoking is now considered to be one of the risk factors most consistently associated with the development of MS in the general population.[48, 59]

The susceptibility for MS associated with smoking seems to vary with smoking habits. In a recent meta-analysis, there was a significant difference between current smoking and past smoking, whereas current smoking was associated with an 83% increased risk and past smoking was associated with a 58% increased risk, compared to non-smokers.[119] Only a few studies have examined the association between passive smoking and MS risk,[120-122] and in the recent meta-analysis it was significantly associated with a 24% increased risk compared to individuals not exposed to smoking.[119] Further, Hedström et al. reported no significant association between smoking and MS risk 10 years after smoking cessation, regardless of the cumulative

dose of prior smoking.[120] Lastly, smoking may have different effects in men and women, as there was a significant effect modification by sex in the pooled analysis in the meta-analysis, where smoking in men was associated with a higher risk compared to in women.[119]

It is unknown by which mechanisms smoking affect MS risk, but several pathways have been suggested. In a Swedish study, tobacco smoking was associated with an increased risk, while use of the oral tobacco snuff was associated with a lower risk,[115] suggesting that mode of delivery is of importance and that the effect is not due to systemic effects of nicotine. This is consistent with an EAE study, where nicotine ameliorated symptoms, while non-nicotine components induced demyelination and microglial activation, leading to a worsening of the symptoms.[123] Interestingly, bronchus-associated lymphoid tissue in the lungs appears to be central in the activation of self-reactive T-cells,[124] which is also consistent with findings from the Swedish study. Lastly, smoking may reduce indoleamine 2,3-dioxygenase activity and increase the activity of the renin–angiotensin system, which both were associated with reduced numbers of T_{reg} in a study on MS patients.[125]

1.5.5 Obesity

Ghadirian et al. first evaluated the association between weight and MS risk in 1998, and observed that higher BMI was associated with a significant lower risk.[126] However, as the participants were asked to report weight at time of diagnosis, the findings might have been prone to reverse causation, as MS patients tend to weight less than controls after onset.[127, 128] Munger et al. prospectively examined the association in two cohort studies of female nurses, and observed a two-fold increased risk among participants reporting to be obese (body mass index [BMI] equal or above 30)[129] compared to those reporting BMI between 18.5 and 21 at age 18.[128] Similar findings were observed in a large Danish registry-based study that included children born between 1930 and 1983, where higher BMI at age 7 to 13 was significantly associated with increased MS risk.[130] The association was only significant in girls, which the authors argued to some extent could be due to power issues rather than

indicating an effect modification by sex. Three case-control studies with participants from Norway, Sweden, Italy and the US have reported findings consistent with the prospective studies.[131-133] Further, a recent MR study found a significant association between SNPs predicting BMI and MS risk,[134] which may indicate a more causal role of BMI on disease risk.

Several mechanisms have been proposed to explain the association between being overweight and MS risk. It could to some extent be mediated by vitamin D, as serum 25-hydroxyvitamin D was inversely associated with BMI in a systematic review of observational studies,[135] and SNPs predicting BMI were significantly associated with lower 25-hydroxivitamin D levels in a MR study.[136] Obesity is associated increased uptake and storage of 25-hydroxyvitamin D by adipose tissue,[137] leading to lower bioavailability of the vitamin. However, the association could also be affected by increased catabolism of 25-hydroxyviatmin D due to the presence of 25-hydroxyvitamin D-1α-hydroxylase in adipose tissue and by behavioral differences, which may lead to less sun exposure.[138, 139] Obesity is also associated with chronic inflammation that can be relevant for MS susceptibility,[140] and adipose tissue can modulate immune responses by secreting adipokines, which have been associated with lower number of T_{reg}.[141]

1.6 Timing of exposure to risk factors for MS

Converging evidence from studies on established risk factors suggest that adolescence and young adulthood are sensitive exposure periods for later MS risk.[79, 118, 128, 142] However, most studies rely on exposure assessment at one or few time points, and thus only have exposure information in limited age periods for each participant. As those reporting exposure in one age period (e.g. tobacco smoking initiated as a teenager) may be different than those reporting exposure in a different age period (e.g. tobacco smoking initiated as adult), it can be difficult to make inferences on sensitive periods from these studies. While some studies have assessed exposure in several age periods,[69, 70] studies assessing exposure from birth to disease onset using the same methodology are needed.

1.7 Socioeconomic status and MS

MS is one of few diseases where high socioeconomic status (SES) has been associated with increased disease risk. Miller et al. published one of the first studies on SES and MS risk in 1960, after observing that the disease seemed to be more common in higher-income groups.[143] The authors compared the occupation in 271 male and 388 female MS patients with the general male population in Northumberland and Durham in England, and found that a higher proportion of MS patients were in the Registrar General social class I, corresponding to professional occupations, compared to the general population, while a lower proportion of the patients were in the social class IV, corresponding to unskilled occupations, compared to the general population. It was noted that the findings could give clues on the etiology of MS if they were replicated in other studies, as findings on SES and the risk of poliomyelitis and coronary thrombosis had given clues about risk factors. Russel published similar findings in 1971, where he observed a higher proportion of MS patients in the highest social classes, compared to the general population.[144] Furthermore, in a large nested case-control study of US army veterans, Kurtzke and Page found a higher MS risk among those with more than 9 years of education.[145]

Several more recent studies do not show the same association between SES and MS risk, and some have even reported an inverse association. Ghadarian et al. found a significant lower risk among participants with more than 18 years of education in a Canadian case-control study,[111] and Riise et al. found a significant lower risk among participants with a graduate degree compared to those with only elementary school in a Norwegian registry-based study with close to 400,000 participants.[146] Briggs et al. found significant associations between several measures of low SES and higher MS risk, including education, social mobility and home ownership, which remained similar after adjusting for several risk factors for MS.[147] The conflicting results could reflect a change in the distribution of environmental exposures relevant to MS over time or geographical differences in access to education, but could also reflect methodological limitations in early studies.

SES could be a marker of exposures relevant for MS etiology. It has been proposed that the hygiene hypothesis to some extent can explain associations between high SES and higher MS risk,[148] as delayed exposure to infections early in life may affect the development of the immune system and make the individual more vulnerable altered immune response later in life. It is possible that these associations to some extent could be mediated by IM, as the risk depends on age at primary EBV infection,[60] which is likely to depend on sanitation and thus SES. On the other hand, smoking and being overweight are both associated with lower SES,[149, 150] and could mediate the association between lower SES and higher MS risk. If SES is associated with MS risk independently of known risk factors, it could give clues on currently unknown risk factors that may be important for the disease.

2. Study rationale and objective

2.1 Rationale

While several environmental exposures have consistently been associated with MS risk, the mechanisms by which they affect disease risk remain little understood. More information on these risk factors, when in life they are most important and how they interact can give clues on underlying mechanisms.

Cohort studies are usually preferred over other study designs in epidemiology, as they are less prone to bias. However, due to the age distribution and the relatively low incidence of MS, it is challenging to conduct a cohort study with sufficient power to examine relevant exposures in detail. As a case-control study observes a population more efficiently compared to a cohort study, it is useful in settings where the outcome is rare.[151] For this reason, we conducted a large multinational case-control study to examine environmental exposures suggested to be important in cohort studies in more detail.

2.2 Objectives

The objectives of this study were to:

1) Examine how frequency of outdoor activity, as a proxy for sun exposure and vitamin D levels, in specific age period from birth to disease onset was associated with MS risk.

2) Examine to which degree prior exposure to the known environmental risk factors smoking, IM, indicators of vitamin D and body size could explain the association between level of education and MS risk.

3) Examine the interaction between smoking and IM in the risk MS risk.

3. Methods

3.1 Source of data: The EnvIMS Study

3.1.1 Study design

The Environmental Risk Factors in MS Study (EnvIMS study) is a large case-control study designed to assess the association between self-reported exposure to infections (including IM), vitamin D related factors (including sun exposure, dietary intake) and lifestyle factors (including smoking and body size) and MS risk, and to disclose possible variations in risk in different populations using common methodology. Investigators from Norway, Italy, Sweden, Serbia and Canada designed and conducted the study. A detailed overview of the study has previously been published.[152]

3.1.2 Study area

The coordination and participant recruitment was conducted in each country separately. As only data from Norway, Italy and Sweden were available for this study, the description will be limited to these countries.

Norway:
Norway is a country situated between 58 and 71 degrees northern latitude. It has an estimated MS prevalence of 203 per 100,000, ranging from 142 to 275 per 100,000 in different counties.[55] Participants in the Norwegian part of EnvIMS were recruited throughout the country, and the recruitment and data collection were conducted from 2009 to 2011. Researchers from the University of Bergen coordinated the study.

Italy and Republic of San Marino:
In Italy, participants were recruited from Sardinia and Ferrara. Sardinia is an island situated between 38 and 41 degrees northern latitude and has a MS prevalence that has been reported to exceed 150 per 100,000.[153] Ferrara is a province situated in northern Italy at 44 degrees northern latitude, and has a MS prevalence that has been

reported to exceed 120 per 100,000.[154] The Republic of San Marino a small country surrounded by Italy, and is situated at 43 degrees northern latitude. It has been reported to have a MS prevalence that exceed 160 per 100,000.[155] All areas were combined in one dataset in the analysis. The recruitment and data collection were conducted from 2009 to 2010. Researchers from the University of Sassari coordinated the study.

Sweden:
Sweden is a country situated between 55 and 69 degrees northern latitude. It has been reported to have a MS prevalence of 189 per 100,000, ranging from 168 to 227 per 100,000 in different counties.[156] Participants in the Swedish part of EnvIMS were recruited from the counties of Östergötland and Värmland, and the recruitment and data collection were conducted from 2009 to 2014. Researchers from the University of Linköping coordinated the study.

3.1.3 Selection of cases

All cases included in the study were aged 18 years or older at time of selection, were diagnosed according to the McDonald[28] or the Poser[29] criteria and had clinical onset within 10 years prior to data collection. The cases were identified using population-based registries in the different countries.

Norway:
The Norwegian MS patients were recruited from the Norwegian MS registry and Biobank.[157] Among the 1368 eligible cases invited to the study, 953 (69.7%) chose to participate.

Italy:
The Italian MS patients were recruited from established regional patient registries in Sardinia, Ferrara and Republic of San Marino. Among the 1692 eligible cases invited to the study, 707 (41.8%) chose to participate.

Sweden:
The Swedish MS patients living in the counties of Östergötland and Värmland were

recruited from the Swedish MS registry.[158] Among the 381 eligible cases invited to the study, 259 (68.0%) chose to participate. 14 of these had missing on age of onset and one had disease duration of more than 10 years. The final number of cases included in the analyses were therefore 244.

3.1.4 Selection of controls

Controls were randomly selected from population-based registries in each region under study. Four controls were selected per case, and they were frequency-matched on age (within 5 years) and sex. The lists of controls were crosschecked with the registries used for selection of cases to ensure that no MS patients were selected as controls.

Norway:
Statistics Norway[159] randomly selected the Norwegian controls from the Norwegian National Registry,[160] which contains information on everyone who has or is resident in Norway. Among the 4728 individuals invited to take part in the study, 1717 (36.3%) responded and were included.

Italy:
The Italian controls were randomly selected from population-based registries in Sardinia, Ferrara and Republic of San Marino. Among the 6414 individuals invited to take part in the study, 1333 (20.8%) responded and were included.

Sweden:
Statistics Sweden[161] randomly selected the Swedish controls living in the counties of Östergötland and Värmland from the Swedish Population Register,[162] which contains information on everyone who has or is resident in Sweden. Among the 1734 individuals invited to take part in the study, 645 (37.2%) responded and were included. One of these controls missed study age. The final number of controls included in the analyses were therefore 644.

3.1.5 Assessment of exposure with EnvIMS-Q

A detailed description of the questionnaire used in EnvIMS, the EnvIMS-Q, has previously been published.[163] It was designed to be a short self-administered questionnaire that could capture detailed information on exposures likely to be relevant in MS etiology. It was first developed in English, and then translated to Italian, Norwegian, Swedish, Serbian and French, and has been tested for feasibility, acceptability and reliability in pilot studies in each of the participating countries. The questionnaire contains some core questions that are identical in each country, to allow for comparison of associations in different populations, and some country-specific questions, to allow for examination of specific hypothesis only relevant for some of the countries. The questionnaire was identical for cases and controls.

For the articles in this thesis, information on sun exposure, IM, smoking, body size, cod liver oil, fish intake and demographic variables were used. From the section on sun exposure, we included questions on frequency of outdoor activity during summer and winter (reported as 'not that often', 'reasonably often', 'quite often' and 'virtually all the time), frequency of sun cream use (Norway and Sweden; reported as 'Not so often', 'Sometimes', 'Quite often' or 'Almost always'), and frequency of sun cream use and protective clothing (Italy; reported as 'Not so often', 'Sometimes', 'Quite often' or 'Almost always'). In Norway and Sweden, the exposure was reported in age-periods adapted to the educational system (0–6, 7–12, 13–15, 16–18, 19–24, 25–30 and last 3 years), while five-year intervals were used in Italy (0-5, 6-10, 11-15, 16-20, 21-25, 26-20 and last 3 years). Further, we included information on skin colour (range from 1 to 10 with skin tones from light to dark), reaction to sun without sun cream (reported as "I always get sunburned and never tan", "I usually get sunburned and tan less than others", I sometimes get sunburned and tan like others" and "I seldom get sunburned and easily tan"), natural hair colour (reported as "dark", "dark brown", "brown", "blonde/yellow" and "red") and eye colour (reported as "black", "brown", "grey/green" and "blue"). From the section on IM, we included questions on whether the responder had experienced IM (reported as "yes", "no" and "I do not remember) and when they contracted the disease (reported using the same age periods

as for sun exposure). From the section of smoking, we used questions on whether the responder ever had been a daily smoker (reported as "yes" and "no") and age at smoking onset (the responder was asked to fill in age in years). From the section on body size, we included the question on body shape (reported using body silhouettes on Stunkard´s figure rating scale[164]) at age 5, 10, 15, 20, 25, 30 and current age. From the section on diet, we included the questions on intake of fish from the Norwegian questionnaire (included questions on intake of the specific fish species herring, mackerel, halibut, flounder, salmon and trout and was reported as "never/seldom," "1 time/ month," "2–3 times/month," "1 time/week," "2 times/ week" and "3 and more times/week" for each species) and use of cod liver oil from the Norwegian questionnaire (reported as "never/seldom," "1–3 times/month," "1 time/week," "2–3 times/week," "4–6 times/week" and "7+ times/ week" in the age period 13 to 19). From the section on demographic information, we used information on the responder's education in the Norwegian questionnaire (reported as "7 years or less" (elementary school), "8– 10 years" (middle school), "11–13 years" (high school), "14 years or more" (college/university) and "I do not know").

3.1.6 Ethical approval and patient consent

Regional ethical committees in each participating country approved the EnvIMS study. Return of the questionnaire was considered as evidence of consent.

3.2 Statistical analysis

3.2.1 Main analyses

Exposure after disease onset was not considered relevant exposure among the cases. To make sure cases and controls had similar exposure opportunities, we randomly assigned an index age to all controls based on the distribution of age at MS onset among the cases, and did not consider exposure after index age as relevant exposure.

The associations between the exposure of interest in the different articles and MS risk were estimated as odds ratios (OR) with 95% confidence intervals using logistic

regression. The exposures were included as categorical variables for the main analyses, and included as a continuous variable to test for trend. All analyses were adjusted for sex and age.

For some variables, we combined categories to ensure that there were enough participants in each category. In the second article, we combined the two lowest levels of education ("7 years or less" and "8-10 years"), and the new variable had thus three levels corresponding to compulsory education, high school and higher education (College/University). Furthermore, we generated a new variable to account for intake of any fatty fish and categorized this variable as "never", "1-2 times/month", "3-4 times/month", "5-6 times/month" and "7 or more times/month", corresponding to the categorization in a different article on fish intake and MS risk in EnvIMS.[83]

For the two first articles, the analyses were done in IBM SPSS Statistics, while in the last article STATA was used.

3.2.2 Interaction analysis

In the third article, we focused on the interaction between IM and smoking. In line with recommendations in a recent guide on interaction analyses,[165] we estimated this both on the additive scale and on the multiplicative scale. For the additive scale, we estimated the interaction as relative excess risk due to interaction (RERI), where an estimate of RERI that deviated from 0 was considered as evidence for an interaction. For the multiplicative scale, we estimated the interaction as the ratio of ORs, where a ratio that deviated from 1 was considered as evidence for an interaction.

4. Results

4.1 Article 1

In the first article, we found a significant association between infrequent of summer outdoor activity and increased MS risk in both Norway and Italy. The association was significant in all age periods in analyses combining the two countries, however the association was only significant during adolescence (13 to 18 years) in Norway and in early childhood (0 to 5 years) in Italy. Still, there were no significant differences between the two countries. In Norway, there was a significant association between frequent use of sunscreen and a higher MS risk after adjusting for frequency of outdoor activity during the same period. Lastly, we found a significant higher risk of MS among those with red and blonde hair compared to those with black hair, which remained similar after adjusting for sunscreen use and frequency of outdoor activity. The results suggest that sun exposure, and most likely vitamin D, is important for MS risk.

4.2 Article 2

In the second article, we found that smoking, IM, indicators of vitamin D (frequency of outdoor activity, cod liver and fatty fish intake) and body size could not explain the association between level of education and MS risk in Norway. Higher level of education was associated with a marked lower MS risk, and this association remained similar when cases with early onset of disease were excluded. Of all the risk factors most consistently associated with MS, only smoking confounded the association between education and MS risk. The findings suggest that currently unknown risk factors associated with level of education in Norway may be important for MS risk.

4.3 Article 3

In the third article, we found a significant negative multiplicative interaction between IM and smoking in the risk of MS. Among those who reported IM, we observed no

increased disease risk associated with smoking. Similarly, the effect estimates for the association between IM and MS risk were considerably lower among ever-smokers compared to never-smokers. The interaction was present in all three countries, although it did not reach statistical significance in Sweden. Furthermore, the direction of the estimated interaction on the additive scale was consistent with the interaction on the multiplicative scale, although the estimate did not reach statistical significance. The results indicate competing antagonism, where the two exposures compete to affect MS risk, and may thus operate on shared pathways

5. Discussion

5.1 The contribution of the findings

The findings in this thesis add to the evidence that childhood and especially adolescence are sensitive periods for exposure to environmental factors that contribute to MS risk later in life. While different studies have found associations between outdoor activity and sun exposure during prenatal period,[166] childhood[69, 167] and adolescence,[70] no prior study, to our knowledge, has examined age-periods from birth to age 30 in one single study, and thus comparing all these periods in the same population using the same methodology. We observed the most pronounced association during childhood and adolescence, which is consistent with the time period when other risk factors for MS appears to be important.[48] It is also consistent with the early migration study of Kurtzke et al. where the MS rate among individuals migration before age 15 was similar as the rate in native-born population in their new country, while the rate among those migrating after this age was similar to the rate in their home country.[50] It is also interesting to note that intake of cod liver oil, a source of vitamin D, among Norwegian participants in EnvIMS was inversely associated with MS risk only in the winter, when sun exposure is insufficient for vitamin D production, and only significant during adolescence, which is the same as observed for summer outdoor activity in Norway.[83]

In this thesis, we have also contributed with new findings that previously have not been reported. We found an association between sunscreen and MS risk in Norway, which add to the evidence linking sun exposure and vitamin D to MS risk. Sunscreen blocks vitamin D production when applied according to World Health Organization (WHO) recommendations.[168] However, it is unclear whether these findings can be translated into real-life situations, as a randomized controlled trial where participants applied the sunscreen themselves found no significant association between sunscreen use and circulating 25-hydroxyvitamin D levels.[169] Similar findings were made in a different study, and the authors speculated whether this was due to sunscreen not being applied adequately or on all sun exposure areas.[170] We only found the

association in early childhood in Norway, when sunscreen is likely to be more thoroughly applied by parents compared to later in life. It has previously been estimated that when as little as 9% of the body, corresponding to head and neck, is free of sunscreen, vitamin D production can occur.[171] This could explain why we did not find an association in Italy, as the question in the Italian EnvIMS-Q was not limited to sunscreen only, but also protective clothing.

Further, while several previous studies have examined the interaction between smoking and different measures of EBV, our study is the first to report a significant interaction. This could be due to the size of our study, as interaction analyses are particularly dependent on sample size,[172] and that we are focusing on IM and not serum antibodies against EBV, such as EBNA-1. As EBNA-1 levels do not seem to predict a positive history of IM,[173] and that the two measures of EBV infection are independently associated with MS risk,[174] they are likely to reflect different aspects of an EBV infection. Thus, studies on the interaction between EBNA-1 levels and smoking do not necessarily compare with ours. Overall, our findings suggest that IM and smoking operate on shared biological pathways in how they affect MS risk.

Lastly, while one previous study examined the association between different measures of SES and MS risk while controlling for most of the established risk factors for MS, they could not account for vitamin D,[147] which is likely to have causal role in MS etiology.[84, 85] We were able to adjust for different markers of vitamin D levels, including outdoor activity, dietary intake of vitamin D supplements and fatty fish intake, which all were associated with MS risk in EnvIMS.[83, 175] This adds to the evidence that lower SES is associated with higher MS risk independently of known risk factors, which suggests that currently unknown environmental factors associated with SES are important for disease risk.

One advantage of EnvIMS is that we are able to compare associations in different populations using the same methodology. While the interaction between smoking and IM was consistent in the three countries included in the third article, we observed some differences in the association between outdoor activity and MS risk in Norway

and Italy. This could be due to different effect of the exposures in the two countries, as smoking and IM are likely to have similar effects in Norway and Italy, while the same amount of outdoor activity in the two countries contributes to different amount of sun exposure and vitamin D production due to variation in UV radiation.[75] It is likely that sun exposure during summer contributes more to overall vitamin D levels over a year in Norway compared to Italy, as vitamin D production is possible for a longer period during the year in Italy.[75] This could explain why the association was more pronounced in Norway compared to Italy, especially during adolescence where it was not significant in Italy. Further, the differences could also be due to variation in sun seeking behavior in the two countries, as very few participants reported infrequent amount of outdoor activity in early childhood in Norway, but close to 10% reported this in Italy. Sunscreen behavior could be more important during this period in Norway, as very few spend time inside. Lastly, we observed that frequency of outdoor activity was inversely associated with MS risk during the last age period in Italy (26-30), while the association was least pronounced, and not significant, in Norway in this period. Disease processes in MS are likely to start several years before the first symptom of the disease and heat sensitivity is a common symptom among patients.[176, 177] Therefore, these findings could to some extent reflect reverse causality as this age period is close to the reported mean age of onset among the Italian participants. Heat sensitivity may also be a larger problem in Italy than in Norway due to higher overall temperatures.[177] Still, it is important to note that we did not find any significant differences in the two countries when testing for heterogeneity in the effect estimates in the two countries, and the observed differences could thus be due to random error.

5.2 Methodological considerations and limitations

5.2.1 Direct acyclic graphs (DAGs)

DAG is a specific type of graph developed by Judea Pearl, and is useful to classify assumptions and problems in study designs and models.[178] I will use DAGs to illustrate methodological considerations and limitations in the articles included in this thesis, and will therefore briefly describe some of the theory behind these here.

A detailed description of DAGs has been published by Pearl.[179] In short, a graph consists of nodes representing variables and edges denoting the relationship between the variables. A graph is a DAG when it is both directed, which means that all edges denote a direction (i.e. they are arrows), and is acyclic, which means that the graph does not include any cycles implying that a variable cannot cause itself. Several variables connected by arrows form a path, and information in a DAG can flow between these variables depending on the direction of the relationship between them. Information can flow along a path if all arrows point in the same direction or if an intermediate variable is a common cause, as long as we do not condition on any of the variables involved in these paths.

Figure 8.1

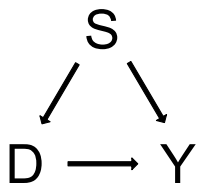

Figure 8.2

In Figure 8.1, we assume that all the effect of sun exposure (S) on MS (Y) is mediated by vitamin D (D), and thus the information flows from S to Y through D. In Figure 8.2 we assume that both sun exposure (S) and vitamin D (D) affects MS risk

(Y), and that sun exposure (S) is a common cause of both vitamin D (D) and MS (Y). Information will thus flow from D to Y and from D to Y through S. In this DAG, the association between D and Y will be a combination of the information on flowing on path D to Y and D to Y through S.

An important concept in DAGs is directional-separation (*d*-separation), which is used to determine whether two variables are independent of each other.[179] Two variables are *d*-separated, or independent, if conditioning (e.g. by stratifying or adjusting in a regression model) on a mediator or a common cause, blocks every path between the two variables.

Figure 9.1

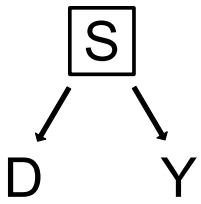

Figure 9.2

In Figure 9.1, we have conditioned on vitamin D (D), which is denoted by a square around the variable, and have therefore blocked all paths from sun exposure (S) to MS (Y). If this DAG is correct, we would not expect any association between sun exposure and MS if we condition on vitamin D, and the two variables are thus *d*-separated conditional on vitamin D. In Figure 9.2, we have conditioned on the common cause sun exposure (S). If this blocks all paths from vitamin D (D) to MS (Y), then vitamin D and MS are *d*-separated conditional on sun exposure, indicating that any initial association was due confounding by sun exposure. If there is still an association after conditioning on sun exposure, then vitamin D is associated with MS independent of sun exposure, as we have blocked the path from vitamin D to MS through sun exposure.

Lastly, two variables are *d*-separated if there is a common effect on the path between them, which is often referred to as a collider. In Figure 10.1, the path between genetic predisposition for MS (G) and vitamin D (D) is blocked by the common effect MS (Y), and we would not expect any association between them given that the DAG is correct. However, if we condition on a common effect, as illustrated in Figure 10.2, we open up the path between D and G. Thus, within MS patients there may be an association between G and D, as we have stratified (i.e. conditioned) on disease status.

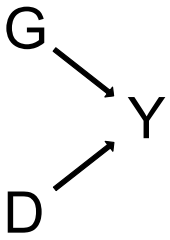

Figure 10.1

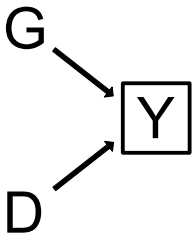

Figure 10.2

5.2.2 Selection bias

Selection bias is a bias that occurs when we condition on a common effect.[180] In a study, we condition on participation as we only observe those who chose to take part in the study (i.e. we stratify on participation). As exposure and disease can be associated with the likelihood of participating, we may induce an association between them by conditioning on the common effect and thus get biased results. This can be illustrated by the association examined in the second article in this thesis, the association between level of education and MS risk (Figure 11).

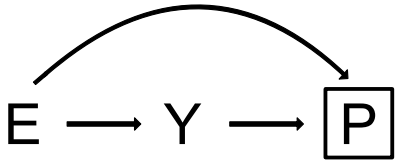

Figure 11

In this DAG, there is an arrow from MS (Y) to participation (P), as those affected by a disease often are more motivated to take part in a study,[180] which was also the case in EnvIMS. There is also an arrow from level of education (E) to participation (P), as those with a higher level of education are more likely to be motivated to take part in research compared to those with lower level of education.[181] By conditioning on the common effect participation (P), we open up the path between level of education (E) and MS (Y) through P, and can therefore induce an association between education and MS. Thus, the observed association in this article may be due to selection bias rather than representing an association that also would be present in the source population.

While we cannot exclude that the results from the articles in this thesis are due to selection bias, it is unlikely that it can fully explain them. The results from the article on education and MS risk are consistent with two prospective registry-based studies in Norway,[146, 182] which are unlikely to be affected by selection bias. This is because the participants were free of disease at study start, which makes it unlikely that there is an association between the outcome they eventually develop and the likelihood to take part in the study in the first place. The effect estimate in our study (OR 0.53, 95% CI: 0.41-0.68) was similar to the one reported in the largest study on education and MS risk in Norway by Riise et al. (rate ratio [RR] 0.48, 95% CI 0.53-0.88),[146] and our results are consistent with the findings of Bjørnevik et al. in a registry-based sibling study.[182] Further, findings of the environmental risk factors most consistently associated with MS risk have been reproduced in EnvIMS, which makes it unlikely that our findings can be fully explained by selection bias.

5.2.3 Recall bias

Information in epidemiological studies is unlikely to be measured perfectly, and there is therefore measurement error or misclassification in our variables. This can be non-differential or differential by disease status, i.e. whether the misclassification varies with disease status. Case-control studies are prone to differential misclassification by disease status, as exposure is retrospectively recalled after the development of the outcome, and patients may recall prior exposure differently than controls.[183] This is referred to as recall bias, and can be illustrated by the first article in this thesis, the association between outdoor activity and MS risk (Figure 12).

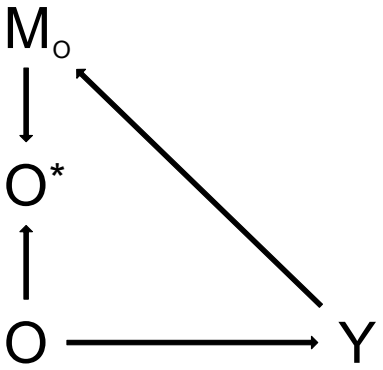

Figure 12

We were interested in the association between outdoor activity (O) and MS (Y). The frequency of outdoor activity reported in the study (O*) is affected by measurement error (M_O), which again is affected by disease status MS (Y), as patients may recall prior exposure differently than the controls. Since there is an open path from O* to M_O to Y, we are not able to isolate the association of O* through O to Y. The results from our study may thus be affected by recall bias.

We cannot exclude that the results in this thesis are affected by recall bias, but similarly to selection bias, it is unlikely that it can fully explain them. First, it is unlikely that we would observe age-specific associations, like those observed for outdoor activity, as recall bias would have to selectively affect recall of exposure in

some age-periods and not in others. Further, it is unlikely that recall bias would vary across strata of another risk factor, and thus explain the findings of an interaction in the third article in this thesis. Lastly, the associations we found between vitamin D related factors, smoking, body size and MS risk are consistent with results from prospective studies, which are not affected by recall bias as information on exposure is collected before the participants develop the outcome.

5.2.4 Confounding

Confounding is a bias that occur when an exposure and an outcome share a common cause.[180] One of the main problems with making causal interpretations using observational studies is that confounders that we do not have data on can affect the associations we observe. This means that the criteria of exchangeability, which means that the risk of the outcome in the unexposed group would have been the same as in the exposed group if we would have switched the exposure status,[180] is unrealistic in observation studies.

It is likely that our findings are affected by unmeasured confounding. Still, MR studies on vitamin D and obesity suggests a causal role of these factors in MS risk,[84, 85, 134] and these findings are consistent with the findings in EnvIMS, suggesting that unmeasured confounding cannot fully explain these specific associations in EnvIMS. Although consistent results with other observational studies do not exclude the possibility that unmeasured confounding can fully explain our findings, it makes it less likely.

5.2.5 Additive and multiplicative interaction analysis

If the effect of one exposure on an outcome depends on the presence of another exposure, then there is an interaction between the two exposures.[165] Within the counterfactual framework, it is common to limit the term interaction to potential interventions, to differentiate the term from effect modification.[180] Interactions are further complicated by the fact that they are scale-dependent, which means that the presence of an interactions depends on the scale they are analyzed on.[151] This makes

it possible to have an interaction on the additive scale, but not on the multiplicative scale, and also possible to have an interaction on the multiplicative scale, but not on the additive scale. Furthermore, when both exposures have an effect on the outcome, there will always be an interaction on at least one scale.[151] The main argument for the use of an additive scale is that it is a better scale to assess the public health importance of interventions and interactions.[151] On the other hand, it may be more natural to assess interactions on the multiplicative scale when using multiplicative statistical models, such as logistic regression, as the model is exponential and thus multiplicative. Furthermore, interactions on a multiplicative scale are often easier to estimate. As arguments can be made for both scales, it is best to report the interactions on both.[165] In the third article included in this thesis, we found an interaction both on the additive and the multiplicative scale, which adds weight to the findings.

6. Further perspectives

In this thesis, we have contributed with new knowledge on established risk factors, including how the association between outdoor activity, and thus likely vitamin D exposure, and MS risk vary with age. This adds to the evidence that childhood and especially adolescence are sensitive periods for exposure to environmental factors that contribute to MS risk later in life. This would be important to consider in a study aiming to assess the effect of primary prevention of MS through modification of known risk factors.

We have also contributed with novel findings that previously have not been reported, including the findings on sunscreen use, SES and the negative interaction between IM and smoking. Our findings suggest that currently unknown environmental factors contribute to disease risk, and that these may be associated with lower SES. This could be useful to consider in studies aiming to identify new risk factors for the disease. Further, if smoking and IM affects MS risk on shared biological pathways, as suggested by the third article, this would be important to consider in studies aiming to identify the mechanisms contributing to the pathogenesis of MS. As discussed above, it is unlikely that the new findings can be fully explained by selection and recall bias, but this cannot be excluded until the findings are replicated in prospective studies.

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