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# **VITAMIN D AND MULTIPLE SCLEROSIS: EPIDEMIOLOGICAL STUDIES ON ENVIRONMENTAL AND GENETIC RISK FACTORS**

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# VITAMIN D AND MULTIPLE SCLEROSIS: EPIDEMIOLOGICAL STUDIES ON ENVIRONMENTAL AND GENETIC RISK FACTORS

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

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## To my parents

*“A cause of a disease occurrence is an event, condition, or characteristic that preceded the disease onset and that, had the event, condition, or characteristic been different in a specified way, the disease either would not have occurred at all or would not have occurred until some later time... By sufficient cause we mean a complete causal mechanism, a minimal set of conditions and events that are sufficient for the outcome to occur.”* Rothman, Modern epidemiology, 3<sup>rd</sup> edition, p. 6.

*“In the world of sense we find there is an order of efficient causes. There is no case known (neither is it, indeed, possible) in which a thing is found to be the efficient cause of itself; for so it would be prior to itself, which is impossible. Now in efficient causes it is not possible to go on to infinity, because in all efficient causes following in order, the first is the cause of the intermediate cause, and the intermediate is the cause of the ultimate cause, whether the intermediate cause be several, or only one. Now to take away the cause is to take away the effect. Therefore, if there be no first cause among efficient causes, there will be no ultimate, nor any intermediate cause. But if in efficient causes it is possible to go on to infinity, there will be no first efficient cause, neither will there be an ultimate effect, nor any intermediate efficient causes; all of which is plainly false. Therefore it is necessary to admit a first efficient cause, to which everyone gives the name of God.”* Saint Thomas Aquinas, Summa Theologica, Part 1, Question 2, Art. 3.



# ABSTRACT

**Background:** Multiple sclerosis (MS) is an autoimmune inflammatory neurological disease with complex aetiology where the causes are not completely known. The main aim of this thesis was to investigate the influence of vitamin D on the risk of developing MS.

**Methods:** The papers in this thesis are based on data from a nationwide population-based case–control study, the Epidemiological Investigation of Multiple Sclerosis (EIMS) study. The source population for the EIMS study is the Swedish population, aged 16–70 years, in defined areas of Sweden. The cases are diagnosed at neurological centres according to the McDonalds criteria, and included in the study within 2 years after diagnosis, and the controls are selected randomly from the population register and matched according to sex and age and residential area at the time of diagnosis of the case. All study participants are invited to respond to an extensive questionnaire regarding environmental and lifestyle factors and to give blood samples. The response proportion has been 91% for the cases and 70% for the controls for the questionnaire and 94% and 57% for the blood samples, respectively. The fourth paper in this thesis is based on data from the EIMS study as well as data from another Swedish case–control study, the Genes and Environment in Multiple Sclerosis (GEMS) study, and the American Kaiser Permanente Medical Plan Northern California (KPNC) study. In these studies, prevalent MS cases aged 18 years and above (and white non-hispanic individuals for the KPNC study), with a verified diagnosis according to McDonalds criteria or International Classification of Diseases (ninth revision), were invited to participate and exposure information was collected through questionnaires and blood sampling.

**Results:** Low sunlight exposure was associated with increased MS risk, where self-reported no voluntary sun exposure was associated with a 60% increased risk of developing MS compared to daily sun exposure. Low vitamin D levels were also associated with increased MS risk (odds ratio (OR) 1.4, 95% confidence interval (CI) 1.2–1.7), with no interaction with HLA-DRB1\*15. High fatty fish intake, i.e. at least once a week, which is a source of vitamin D, was significantly associated with decreased MS risk (OR 0.82, 95% CI 0.68–0.98). To investigate the timing of the exposure of vitamin D we evaluated the association between vitamin D levels in blood samples taken at birth and later risk of developing MS and did not find any sign of an association. Finally, we investigated whether or not the association seen in our studies between vitamin D deficiency and MS risk was a causal association. We calculated a genetic risk score for vitamin D levels based on three genetic polymorphisms, where a higher score corresponded to higher vitamin D levels. We found that a higher score was associated with decreased MS risk (OR 0.85, 95% CI 0.76–0.94).

**Conclusion:** Vitamin D deficiency seems to be a causal risk factor for MS, but the susceptibility period does not appear to be during the neonatal stage. Oral vitamin D intake may be protective and sunlight exposure may impact MS risk with no influence from HLA-DRB1\*15 status.

# LIST OF SCIENTIFIC PAPERS

This thesis is based on the following articles, which will be referred to in the text as papers I–IV.

- I. **Maria Bäärnhielm\***, Anna Karin Hedström\*, Ingrid Kockum, Emilie Sundqvist, Sven A. Gustafsson, Jan Hillert, Tomas Olsson\*, Lars Alfredsson\*. *Sunlight is associated with decreased multiple sclerosis risk: no interaction with human leukocyte antigen-DRB1\*15*. European Journal of Neurology 2012 Jul;19(7):955-962.
- II. **Maria Bäärnhielm**, Tomas Olsson, Lars Alfredsson. *Fatty fish is associated with decreased occurrence of multiple sclerosis*. Multiple Sclerosis Journal, 2014 May; 20(6):726-732.
- III. Peter Ueda, Farshid Rafatnia, **Maria Bäärnhielm**, Robin Fröbom, Greg Korzunowicz, Ragnar Lönnerbro, Anna Karin Hedström, Darryl Eyles, Tomas Olsson, Lars Alfredsson. *Neonatal vitamin D status and risk of multiple sclerosis*. Annals of Neurology 2014 Sep;76(3):338-346.
- IV. Brooke Rhead\*, **Maria Bäärnhielm\***, Milena Gianfrancesco, Amanda Mok, Xiaorong Shao, Hong Quach, Ling Shen, Catherine Schaefer, Jenny Link, Alexandra Gyllenberg, Anna Karin Hedström, Tomas Olsson, Jan Hillert, Ingrid Kockum, Maria Glymour, Lars Alfredsson\*, Lisa F. Barcellos\*. *Mendelian randomization provides evidence for a causal effect of low vitamin D on multiple sclerosis risk*. Manuscript.

\*These authors contributed equally.

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## LIST OF ABBREVIATIONS

25 (OH) D	25-hydroxyvitamin D
AP	Attributable proportion due to interaction
APC	Antigen-presenting cell
BMI	Body mass index
CI	Confidence interval
CIS	Clinically isolated syndrome
CNS	Central nervous system
CYP	Cytochrome P450
DBP	Vitamin D-binding protein
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein–Barr virus
EDSS	Kurtzke Expanded Disability Status Scale
EIMS	Epidemiological Investigation of Multiple Sclerosis
GC	Group-specific component
GEMS	Genes and Environment in Multiple Sclerosis
GERA	Genetic Epidemiology Research on Adult Health and Aging
GRS	Genetic risk score
GWAS	Genome-wide association study
HLA	Human leukocyte antigen
ICD	International Classification of Diseases
IFN	Interferon
IL	Interleukin
IM	Infectious mononucleosis
IMSGC	International Multiple Sclerosis Genetics Consortium
IU	International units
KPNC	Kaiser Permanente Medical Plan Northern California
LD	Linkage disequilibrium
MDS	Multidimensional scaling
MHC	Major histocompatibility complex
MS	Multiple sclerosis

NHS	Nurses' Health Study
OR	Odds ratio
PAMP	Pathogen-associated molecular pattern
PCA	Principal component analysis
PPMS	Primary progressive multiple sclerosis
RA	Rheumatoid arthritis
RERI	Relative excess risk due to interaction
RPGEH	Kaiser Permanente Research Program on Genes, Environment, and Health
RR	Relative risk
RRMS	Relapsing–remitting multiple sclerosis
SD	Standard deviation
SNP	Single-nucleotide polymorphism
SPMS	Secondary progressive multiple sclerosis
TLR	Toll-like receptors
UVR	Ultraviolet radiation
VDR	Vitamin D receptor
VDRE	Vitamin D response element

# 1 INTRODUCTION

I met a patient with multiple sclerosis (MS) for the first time during the neurology course as part of my medical studies. Soon I became interested in the disease due to, among other things, its varied symptomatology and variable disease course, as well as the expanding panorama of treatment options. Moreover, the impact of MS on the quality of life for the individuals, and on society in terms of sick leave and costs (1), further increased my interest and wish to understand this disease better.

Epidemiology is the study of the occurrence of illness and the causes of disease. Epidemiological methods are tools for performing good-quality scientific studies, and understanding of epidemiological concepts such as confounding and selection bias is necessary to be able to evaluate published scientific studies, for example interventional studies of treatments for neurological diseases. Immersing myself in the field of epidemiology and applying these epidemiological tools to MS during these years of my Ph.D. studies has been an intellectual challenge – and still is in many ways. Epidemiological concepts may seem easy at first, but seldom are when such concepts and their applications are further explored. Kenneth Rothman stated: “a commonsense approach to a simple problem can be overtly wrong, until we educate our common sense to appreciate better the nature of the problem. Any sensible person can understand epidemiology, but without considering the [epidemiological] principles..., even a sensible person using what appears to be common sense is apt to go astray” (2). The study and application of epidemiological principles during this work has presented a new world of intellectual challenge and thought-provoking ideas and has given me a better understanding (although far from exhaustive) of the complex field of disease causation with regard to MS and in many ways has “educated my common sense”.

It has been a true joy studying epidemiology and I finish this thesis knowing that I still have much more to learn and accomplish. This is the end of the beginning.

## 2 BACKGROUND

### 2.1 MULTIPLE SCLEROSIS

#### 2.1.1 Clinical features

MS is a neurological disease and one of the first clinical descriptions was written by a patient himself, the British Sir Augustus d'Este, in his diary in the early 1800s (3). In 1868, Jean-Martin Charcot provided the first histological description of the typical lesions in the nervous system, relating the histology to the clinical picture, and also suggested the first diagnostic criteria for the disease (4).

Clinically, MS presents in a variety of ways; some of the most common initial symptoms are optic neuritis, sensory disturbances and diplopia. During the course of the disease, fatigue, cognitive dysfunction, bladder disturbances, ataxia and weakness and a wide variety of other neurological dysfunctions commonly occur. The usual clinical definition of the disease is “dissemination in time and space” of lesions of inflammatory demyelinating origin, i.e. recurrence of symptoms from different locations of the nervous system (and where other causes have been ruled out), and this definition is the basis for the diagnostic criteria, the McDonalds criteria, that have been used clinically and in research for the last 15 years (5-7).

MS typically begins at 20–40 years of age, although disease onset has been reported occasionally at 60–70 years (8), with symptoms slowly increasing over a couple of days to weeks and then improving and often disappearing (9). In the majority of patients, the disease course follows a “relapsing–remitting” presentation (RRMS) where the symptoms (relapses) come and go, and usually after some years evolve into a phase with slowly increasing disability (secondary progressive MS (SPMS)). A minority presents from onset with progressive neurological dysfunction without relapses, so-called primary progressive MS (PPMS) (10). The natural history of MS has been studied for many years and this field was recently reviewed by Tremlett et al (11). This overview of natural history studies from the last 2 decades showed that the time from disease onset to reaching the SPMS phase was about 20 years and the time to reach a significant level of walking impairment (corresponding to level 6 on the Kurtzke Expanded Disability Status Scale (EDSS)) was about 25 years. Earlier studies had reported that the time to EDSS 6 was 5–20 years, indicating that disease progression might have changed, although the authors stated that this finding might have been influenced by differences among study cohorts and in diagnostic procedures over the years. Positive prognostic factors for disease progression were younger age at onset, female sex, optic neuritis as the first symptom, a relapsing–remitting disease course, full recovery from the first relapse and a lower relapse rate in the first years of the disease.

#### 2.1.2 Epidemiology

Systematic studies of the occurrence of MS in different places and ethnic groups began in the early 1900s with the work of Sydney Allison and of Charles Davenport and later with the seminal research by Geoffrey Dean in South Africa (12). These and similar descriptive

studies over several decades have given rise to the general knowledge that the prevalence of MS differs significantly worldwide, with especially high numbers in northern Europe (Scandinavia and Scotland) and lower prevalence in southern Europe, Africa and Asia. This has generally been explained by genetic differences, although the impact of environmental factors has also been recognized (13). In Sweden, the prevalence was long considered to be around 100/100 000 individuals (14, 15). However, recently the prevalence has been re-estimated and found to be 189/100 000, which is among the highest figures worldwide (16). In addition, the incidence of MS in Sweden has been re-evaluated and found to be around 10/100 000 person-years (17).

The worldwide differences in geographical distribution have for many decades been described as having a north–south gradient, with increasing MS prevalence with increasing latitude (13). Incidence rates have also shown a latitudinal variation, although less obvious (18). This gradient has been especially evident in the USA where it has mainly been investigated by Kurtzke (19, 20) but also by Hernán et al (21). Within-country differences in prevalence and incidence have been found, with higher figures at higher latitudes for example in France (22) and Australia (23), although without a clear latitudinal gradient. In a recent thorough meta-analysis (24), a latitudinal gradient was manifest in regions with populations of European descent. However the increasing prevalence with increasing latitude was only present up to 60° north, above that the association was reversed.

In studies in Scandinavia and Finland, Kurtzke (25, 26) did not find a latitudinal gradient but rather an uneven distribution with mainly higher prevalence in southern Norway and southern Sweden, and lower figures in the coastal regions of Norway. In 1995, Koch-Henriksen (27) reached the same conclusion regarding lack of latitudinal gradient, however temporal changes in MS incidence was indicative of the importance of environmental factors for MS development. Nevertheless, in the most recent prevalence study in Sweden (16), based on data from population registers and the nationwide MS register, a small but significant increase in prevalence with 1% per degree of north latitude was observed.

The geographical differences in MS distribution have long fuelled discussion about the cause of this latitudinal variation and what is most important in MS aetiology: “race or place” (28) or “nature or nurture” (29), i.e. is the development of MS determined by genetics or environment? Today, the debate is largely settled with mutual agreement that MS is due to both environmental and genetic causes, and most probably also gene–environment interactions (30, 31). Thus MS is truly a complex disease.

In addition to the latitudinal gradient in MS prevalence, the possible influence of environmental factors in MS aetiology has been corroborated by migration studies, temporal changes in MS incidence and the so-called “month of birth” effect.

Gale and Martyn (32) reviewed a large number of migration studies and found that people who migrated from countries with a high prevalence of MS to countries with a low prevalence tended to decrease their risk, and the effect was especially prominent if migration

occurred during the first two decades of life, but no substantial change in MS risk was found among those who migrated from areas of low prevalence to areas of high prevalence. However, the results of migration studies should be considered with caution because the numbers of studies and included cases and controls were sometimes small, the methodology differed between the studies and the population structure in the countries of destination was not always taken into account. Finally, there may be an inherent bias in migration studies, due to migrants being different compared to the population in the countries of origin and of destination, which may hamper the ability to draw conclusions and make generalizations.

Some authors have presented results indicating that the risk of developing MS is increasing. In Canada, Orton et al (33) found an increasing female-to-male ratio over several decades, which they interpreted as an increasing risk of MS for females and the methodology precluded sex-specific differences in time to diagnosis as an explanation of the findings. Similar results were presented from a systematic review from the USA (34), with a mean female-to-male ratio of 1.4 in 1955 and 2.3 in 2000; the authors also found a decreasing latitudinal gradient, due to increasing MS incidence at lower latitudes. In Sweden an increasing sex ratio has been reported by Westerlind et al (35) (1.7 in the 1930s and 2.7 in the 1980s) but not by Ahlgren et al (17), probably due to failure to identify all cases. The findings have been interpreted as due to changes in environmental risk factors.

The month of birth effect in MS was described in 1992 by Templer et al (36) who, using data from Denmark, found an increased risk of developing MS for people born in spring (March–June). Several authors have found the same association with increased MS risk with birth in spring or summer (May–June) in the northern hemisphere (37–39), and an inverse association in the southern hemisphere with increased MS risk in November–December (i.e. also in spring or summer, since the seasons are reversed) (40), although others have not (41, 42). In Sweden, Wiberg et al (43) and Salzer et al (44) found that more MS cases than expected were born in spring. In the latter study, based on the nationwide MS register, the results showed an increased risk of 11% for MS cases born in June, and a 5% increased risk for those born between February and July. These findings have been interpreted as indicating a seasonally variable environmental factor, such as viral infections and sunlight exposure. Maternal lack of sunlight would cause vitamin D deficiency during fetal development which has been suggested as the underlying cause (45). However, the results have been questioned with regard to the statistical methods (46) and have also been suggested to be due to confounding (47, 48).

### **2.1.3 Environmental factors**

The search for environmental factors that may contribute to the development of MS, and that may explain the peculiar characteristics of MS distribution, is as old as the field of MS research, and still on-going. Today, there is sufficient reliable evidence to conclude that smoking, Epstein–Barr virus (EBV) infection, childhood obesity and vitamin D are important for the aetiology of MS (49). However, many questions remain to be answered. Here what is



known about smoking, EBV infection and obesity in MS pathogenesis will be briefly covered. The topic of vitamin D, central to this thesis, will be discussed separately.

#### 2.1.3.1 *Smoking*

The effect of smoking on the development of MS has been the topic of several studies, in which being a smoker (defined as ever-smoker, or current/past smoker) was associated with an increased MS risk (50-52). The risk of disease progression, measured as risk of entering the SPMS phase (53) or as an increase in disability measures (increased EDSS) (54), has also been linked to smoking. Although some negative results have been reported regarding the association between smoking and MS development and progression (55, 56), smoking is now considered an established risk factor for MS, based on the large amount of results showing positive associations (57), with an increased MS risk of about 50% for ever-smokers compared to never-smokers, as well as evidence of a dose-response relationship (58). The findings are further strengthened by the fact that the risk of developing rheumatoid arthritis (RA), another organ-specific autoimmune inflammatory disease, is also associated with smoking (59). The mechanisms of action of smoking in the development of MS are not fully elucidated. Because use of moist snuff has not been associated with increased MS risk (52, 60), it seems that nicotine is not the causal factor but rather other substances in tobacco. Smoking has recently been associated with a decreased number of T regulatory cells and an increase in proinflammatory cytokines (61). It has been suggested that inflammatory reactions in the lung and oxidative stress may be involved (58).

#### 2.1.3.2 *EBV*

EBV is a herpesvirus that is very common in the general population with a seroprevalence of about 95%. It causes an asymptomatic infection in early childhood, and in some instances a fulminant clinical manifestation, infectious mononucleosis (IM), if infection occurs later in life (62). EBV and IM were first suggested to be involved in MS pathogenesis by Warner and Carp in 1981 (63) due to similarities in prevalence. Since then, numerous studies have evaluated this association, and a meta-analysis of studies of IM and MS (64) demonstrated an increased risk of MS (relative risk (RR) of 2.17, 95% confidence interval (CI) 1.97–2.39) for individuals who reported previous IM. Also, regardless of whether or not they have had IM, MS cases have been found to have higher titres of EBV antibody EBNA-1 compared to controls (65, 66). However, association is not equivalent to causation, and Levin et al (67) shed further light on the issue of causality. They found, using prospectively collected serial samples from a large US cohort, that among investigated MS cases seronegative for EBV at the start of sampling (10 out of 305 cases), all converted to EBV seropositivity before disease onset, with a mean duration between the positive EBV sample and MS onset of around 4 years. Of the EBV-negative matched healthy controls at study onset, only about 35% (10 out of 28) seroconverted. This study shows a temporal relationship between EBV infection and MS, and the fact that none of the seronegative individuals developed MS increases the probability that EBV is a causal factor for the disease. The mechanisms of action of EBV in MS pathogenesis are unclear. The finding of Serafini et al (68) of EBV in the B cell follicles

in the central nervous system (CNS) of MS patients suggested that a persistent EBV infection may contribute to a continuous inflammatory process. However, this finding has not been replicated (69) and has been much debated. Molecular mimicry has been suggested as a potential mechanism, where there is a cross-reaction between EBV antigens and some unknown antigen in the CNS, which initiates an autoimmune reaction. Another possibility is the “mistaken self” hypothesis, in which an infection such as EBV leads to upregulation of  $\alpha\beta$ -crystallin proteins, which in turn causes CD4+ T cells to attack oligodendrocytes and leads to inflammatory demyelination (62). During the last two decades of MS research, EBV has emerged as most important among all the viruses investigated, but the role of EBV – whether or not it is necessary for MS development – is far from clear, as evidenced by the recent debate in *Multiple Sclerosis Journal* (70-72).

#### 2.1.3.3 Obesity

Obesity was first reported to be associated with MS in 2009 (73); results from the Nurses’ Health Study (NHS) showed a two-fold increased risk of MS for individuals with a reported body mass index (BMI) of more than 30 kg/m<sup>2</sup> at 18 years of age whereas obesity in childhood was not associated with MS. Similar findings have been presented by other research groups (74, 75). By contrast, Gianfrancesco et al (76) also found an increased risk of MS associated with childhood obesity (at 10 years of age) independently of BMI in later life. In most studies, the association has been strongest for females. The mechanisms underlying this association are unknown, although vitamin D deficiency, known to be more prevalent in obese people (77), and obesity-related chronic inflammation (76) may be influential.

#### 2.1.4 The immune system dysfunction in MS

MS is considered an inflammatory demyelinating disease of autoimmune origin. The importance of the immune system in the disease pathogenesis is evidenced by, among other findings, the association between MS and genes involved in immune regulation (78), the presence of oligoclonal bands in the cerebrospinal fluid in MS patients (representing intrathecal immunoglobulin synthesis) (79) and the fact that therapies with immunomodulating or immunosuppressive functions (80) have been successful, at least to some extent, in treating the disease. In addition, an animal model of experimental autoimmune encephalomyelitis (EAE), which is an example of autoimmune CNS inflammation, has been used for more than 40 years to understand the pathogenesis of MS (81). Here a short overview, of the immunological dysfunction that may be important for the development of MS will be given, without any attempt to cover the entire field. Because the immunological processes may be different at onset and in later stages of the disease (82), I have focused on the initiation and early stages of MS.

The CNS inflammation that is a hallmark of MS is considered to be autoimmune, i.e. directed against the organism itself. However, despite many attempts to identify and characterize the autoantigen, it remains unknown. Initiation of the inflammation is suggested to either begin in the periphery or in the CNS (83). According to the former theory, T cells are activated against

a CNS autoantigen and transported to the CNS, where they pass across the blood–brain barrier which has become permeable, and then initiate an inflammatory process by recognizing an antigen presented by antigen-presenting cells (APCs), such as dendritic cells. The latter cell type also has the capacity to secrete cytokines and may thus modulate immune system responses. On the other hand, if the inflammatory process is initiated in the CNS (by presentation of a CNS-specific antigen), this initial reaction would induce an amplification of the autoimmune reaction, and the antigens would travel to the periphery (lymph nodes) where the adaptive immune system would be stimulated. Antigen presentation is performed by APCs with major histocompatibility complex (MHC) molecules that interact with T cell receptors on T cells, and co-stimulatory molecules, and leads to T cell activation (84). The MHC is encoded by the human leukocyte antigen (HLA) genes. CD4<sup>+</sup> T cells recognize peptides (antigens) presented by MHC class II molecules, and CD8<sup>+</sup> T cells recognize peptides presented by MHC class I molecules. The T cells are generally classified according to their repertoire of cytokine production (the major cell subsets are Th1, Th2 and Th17, which produce interferon (IFN)- $\gamma$ , interleukin (IL)-4 and IL-17, respectively (83)). MS has long been considered a Th1-mediated disease, where a shift towards a Th2-dominated cell population has been considered a major mechanism of action of some MS therapies (85). However, Th17 cells may also have an important role in the inflammatory process (83). Dysfunction of another T cell subset, T regulatory cells, which normally contribute to peripheral immune tolerance have also been found to have reduced suppressive capacity in MS (86).

Recently, the role of the B cell, the other cell type of the adaptive immune system, in MS has attracted attention. The functions of B cells range from antibody production to antigen presentation and immune system regulation through cytokine production. The intrathecal immunoglobulin production mentioned above is due to inappropriate B cell activation and its presence is associated with increased risk of conversion from clinically isolated syndrome (CIS) to MS (87). Markers of B cell activation have been associated with disease course (88) and therapies directed against B cells have shown promising results in reducing relapse rates among MS patients (89). The B cells are now considered to have a fundamental role in the immunological dysregulation in MS, although the exact mechanisms involved have not been clarified.

The above mechanisms are part of the adaptive immune system, the protective function of which normally acts by specific recognition of antigens presented by the APCs. The innate immune system functions through pattern recognition, where stereotypical molecular patterns (pathogen-associated molecular patterns (PAMPs)) in foreign organisms (bacteria and viruses) are recognized by the cells through binding to surface molecules (so-called pattern recognition receptors), among which Toll-like receptors (TLRs) are some of the most important (84). MS is usually considered to be caused by dysregulation of the adaptive immune system, but the innate immune system certainly also influences the inflammatory process. For example, the TLRs have been found, upon recognition of an antigen, to produce proinflammatory cytokines and activate APCs, and thus link the actions of the innate and

adaptive immune systems (90). The innate immune system may also have a role in the initiation and maintenance of the inflammatory process in MS, by activation of monocytes and microglia (the latter residing in the CNS) which may contribute to the inflammation and myelin degradation and have been found in MS lesions (83).

## **2.2 SUNLIGHT AND ULTRAVIOLET RADIATION**

Sunlight consists of different types of electromagnetic radiation, of which ultraviolet radiation (UVR) is especially interesting due to its effects on health. UVR comprises UV-A, UV-B and also UV-C, however the latter does not reach the Earth and will not be further considered. UV-A and UV-B have wavelengths of approximately 320–400 and 290–320 nm, respectively. The amount of UVR that reaches the surface of the Earth depends on many factors such as the ozone layer, clouds, altitude, surface reflection and solar angle. The impact of the solar angle depends on season and latitude; at higher latitudes the UV rays have to travel longer distances and on the way become attenuated. The amount of UVR that reaches the human skin is further influenced by the amount of melanin (skin pigmentation), and by clothing, sun protection and outdoor behaviour (91). Sunlight, and more specifically UV-B radiation, is necessary for vitamin D production in the body. This was discovered in the first half of the 19<sup>th</sup> century when it was demonstrated that rickets could be prevented by sunlight exposure (92). From the start of the industrial revolution rickets had become a problem for society, but due to observational and experimental studies the link between vitamin D and bone health was finally resolved and in the 1930s prevention of rickets was implemented by recommending sensible sun exposure for children (93).

However, today it is still debated how much UV-B radiation is necessary to produce sufficient amounts of vitamin D to maintain good health. Sun exposure corresponding to one minimum erythral dose (i.e. enough sun to produce slightly pink skin) has been reported to be equivalent to ingestion of oral vitamin D of 10 000–25 000 IU (250–625 µg) (91). However this measure should be interpreted with caution, as the capacity of the skin for vitamin D production depends on several factors, including age and the initial vitamin D levels (where low levels seem to enhance vitamin D production) (91).

Furthermore, UVR (both UV-A and UV-B) also has negative health effects, mainly skin cancer development, as it causes DNA breaks, produces oxidative species and influences gene expression that may enhance tumour development (94). Skin cancer accounts for 17% of all cancers reported in Sweden and is the cancer type with the highest increase in incidence (95). Because the difference between a healthy and a harmful amount of UVR may be small (91), the task of providing general sun exposure recommendations is difficult.

### **2.2.1 The immunomodulatory effects of UVR**

Since the landmark study of Fisher and Kripke (96), showing that UV-irradiated mice were immunosuppressed and therefore not capable of rejecting transplanted tumours, it has been known that UVR has immunological effects.

When UVR reaches the skin, it is absorbed by molecules, so-called chromophores (such as DNA, trans-urocanic acid, phospholipids, 7-dehydrocholesterol and tryptophan), with UVR-absorptive capacity which leads to a variety of reactions; for example, trans-urocanic acid is transformed to cis-urocanic acid which has immunosuppressive effects by causing defective antigen presentation (97). Soluble mediators such as platelet-activating factor, prostaglandins and different interleukins (IL-4, IL-13 and IL-10) are also released, which in turn influence the immune cells by downregulating Th1 and Th17 cells (98). UVR also has been found to induce T regulatory cells (99) which contribute to maintaining tolerance, as well as IL-10-producing B cells which in animal models have been found to have immunosuppressive activity (98). Another effect of UVR is DNA damage; as a consequence of this effect, dendritic cells (specifically Langerhans cells) travel to the lymph nodes where they continue to function as APCs but with reduced effectiveness, which leads to tolerance (97).

The general effect on the adaptive immune system as described here is considered to be immunosuppressive, whereas the effect on the innate immune system is to upregulate antimicrobial peptides and thus enhance protection against infections (100).

The immunosuppressive effect of UVR is dose dependent and is generally attributed to UV-B radiation (97), although UV-A may also have some influence (101). Suberythral doses (i.e. that do not cause skin redness) have mostly been used in studies and found to be efficient, but it has been suggested that higher doses may have more systemic effects. Most studies of the immunological functions of UVR have been performed in patients with skin diseases, such as contact hypersensitivity or psoriasis for which UVR is currently used as treatment (97), and much less is known regarding the systemic effect of UVR, which may be dependent to some extent on other mechanisms.

Using the animal model of MS, EAE, Becklund et al (102) showed that disease activity was suppressed with UVR although neither vitamin D nor calcium levels were increased, and similar findings have since been reported using narrow-band UVR (300–315 nm) (103). The authors concluded that UVR-mediated immunosuppression was the cause of the suppression of disease activity although this conclusion has been questioned (104, 105).

In MS patients the specific effect of UVR-mediated suppression is poorly described, to a large extent probably due to the practical difficulties of performing relevant studies. Correale et al (106) found that MS patients had lower levels of cis-urocanic acid, which would lead to less UVR-induced immunosuppression; however, the findings could be due to reverse causation (avoidance of sun due to disease) as the authors noted. At present, a randomized controlled trial is ongoing in Australia, evaluating the use of narrow-band UVR as therapy for patients who have had their first demyelinating event, with the aim of investigating whether this therapy decreases the risk of developing MS (107).

### **2.2.2 Sunlight and autoimmune diseases**

If UVR has immunosuppressive capacities as described above, its effect should be evident on several autoimmune diseases. The present state of knowledge regarding MS is discussed in

detail below. The association between UVR and other autoimmune diseases has also been studied to some extent.

The incidence of autoimmune diabetes type 1 has been reported to be increased at higher latitudes in both hemispheres in an ecological study based on data from 51 countries (108). In a study from Australia, the association between diabetes incidence and ambient UVR was mainly seen in rural but not in urban areas, and there was an association between season and date of diagnosis (the diagnosis of diabetes was more common in winter) but not season of birth. These results lend some support to an association between UVR exposure and diabetes onset.

Another autoimmune disease, RA, has been inconsistently associated with latitude, as there was no evidence of a latitudinal gradient in RA prevalence in Australia (109) but in the USA an increased risk of RA was found in northern compared to southern states although without an obvious north–south geographical gradient (110). In a study using data from the NHS (111), the risk of developing RA associated with residential UVR exposure was evaluated in both the NHS and the NHS II cohorts, comprising 1314 RA cases. In the NHS cohort a decreased RA risk was found with increasing cumulative dose of ambient UVR, but no similar association was found in NHS II. Individual information was available regarding the outcome and a number of potential covariates but not individual UVR exposure, and only general levels of UVR for each reported residential area during the study period was used for analysis.

In summary, there is some evidence for the influence of UVR on autoimmune diseases other than MS, but it is inconsistent and further study is needed.

### **2.2.3 Sunlight and MS**

It became clear during the first half of the 20<sup>th</sup> century that there are geographical differences in MS prevalence. In 1960, Acheson suggested that these latitude-related differences might be due to an association between MS and sunshine exposure. The same hypothesis was again proposed by McMichael and Hall (112) in 1997, expanding upon research conducted in the last two decades showing the immunosuppressive properties of UVR. The authors predicted, among other things, that if the association was true, occupations associated with high sun exposure would be negatively associated with MS, relapse rates would vary according to season and, with increasing sun exposure in the population, a decrease in MS incidence would follow. The following years saw an increasing number of studies investigating sunlight exposure and MS.

Occupational sun exposure was evaluated in the USA, based on death certificates, where increasing sun exposure was negatively associated with death due to MS (113), and in Sweden, using register data, where a decreased risk of MS with increasing occupational sun exposure was also found (114).

Several ecological studies, using nationwide meteorological data and prevalence or incidence figures, have been performed. In Australia, van der Mei et al (115) found an inverse association between MS prevalence in different regions and the respective ambient UVR levels. In Canada, using meteorological data on daily erythemal UVR exposure (calculated based on a Caucasian population), a significant association, although small, between UVR and MS prevalence was found (116), and similar findings have also been reported from the USA (117).

Case-control studies have the advantage in relation to ecological studies of including data on individual-level exposures and possible confounders. Several case-control studies investigating sun exposure and MS have been performed, sometimes combining data on sun exposure and vitamin D levels. A few of these are worth mentioning here. In a study performed in Tasmania (latitude 41–43° south), van der Mei et al (118) used self-reported lifetime sun exposure data from 136 MS patients (prevalent cases with mean disease duration of 12 years, and mean time since diagnosis of 9 years) and 272 control subjects. High sun exposure (at least 2–3 hours/day) before 15 years of age was associated with a 60% decreased risk of MS (age range 6–15 years; unadjusted odds ratio (OR) 0.40, 95% CI 0.20–0.80). There was no significant association between self-reported sun exposure during the years before disease onset and MS. The authors also investigated actinic skin damage, which is caused by sunlight exposure, and found an inverse association between degree of actinic damage and MS risk, corroborating the self-reported data (the latter may be subject to bias that may not affect objectively measured skin damage). In northern Norway, above the Arctic circle (latitude 66–71° north), a similar study was performed by Kampman et al (119), in which 152 prevalent MS cases (mean disease duration 14 years) and 402 matched controls were questioned about outdoor activities in childhood and adolescence. More frequent participation in outdoor activities, particularly during the summer, was associated with decreased MS risk. The effect was most apparent for the age range 16–20 years, where the pursuit of frequent outdoor activities was associated with a 45% risk reduction (OR 0.55, 95% CI 0.39–0.78).

In a North-American study (120), based on data from MS-discordant monozygotic twin pairs (prevalent cases, disease duration approximately 10 years) recruited through newspaper advertisements, information on childhood sun exposure was collected in a questionnaire. In total 79 twin pairs were disease and exposure discordant and contributed information to the analyses. The investigators evaluated nine sun exposure-related questions and constructed an index representing global sun exposure with higher values if the unaffected twin reported more sun exposure than the affected twin. The risk of MS was decreased with increasing sun exposure for several of the sun exposure measurements (e.g. sun tanning: OR 0.40, 95% CI 0.19–0.80), as well as for the global sun index where a 1-unit increase corresponded to 25% reduction in MS risk. The study design precluded confounding by genetic differences but some selection bias may have occurred.

In an international case-control study (121), EnvIMS, questionnaire data on frequency of outdoor activities (as a measure of sun exposure) were collected from prevalent MS cases (recruited from MS registries) and matched control subjects in Norway and southern Italy. The mean disease duration was 7.2 and 5.6 years, respectively. The risk of MS was inversely associated with frequency of summer outdoor activities in Norway in adolescence, and with both summer and winter outdoor activities in early childhood in Italy. When combining data from the two countries, a significant risk of MS with infrequent outdoor activity was noted for all age groups (for early childhood: OR 1.35, 95% CI 1.09–1.68; for late adolescence: OR 1.51, 95% CI 1.20–1.90) except the age range 25–30 years.

Regarding the predictions of McMichael and Hall mentioned above, the issue of seasonal variation in relapse rate was investigated by Spelman et al (122) using an MS register with patient information, including relapse rate, from 55 clinical centres in 30 countries (a total of 9811 patients with 32 762 confirmed relapses and a mean follow-up time of 8 years). There was a significant seasonal variation in relapse rate with increased rates in spring and decreased rates in autumn, in both hemispheres. The authors also evaluated ambient UVR for the location of each centre and found a lag between UVR trough and increased relapse risk, a finding they interpreted as due to the effects on the immune system causing increased inflammatory activity by either lack of UVR or vitamin D deficiency.

So far, the predictions of McMichael and Hall concerning UVR, its immunosuppressive function and its effects on MS seem to be fulfilled, pointing to a lack of UVR/sunlight exposure as a determinant of MS development. The final prediction regards changes in MS incidence with variations in sun exposure habits, and is difficult to assess. As outlined in section 2.1.2, there have been reports indicating an increase in MS incidence, especially among females. At the same time, the skin cancer incidence is rising in Sweden with an increase in age-standardized incidence among women in the last 10 years of 6.2% for all skin cancers and 5.3% for malignant melanoma (95). Sun exposure is well known to be an important cause of skin cancer, and according to reports from 2007 and 2013 (123, 124) it does not appear that sun exposure frequency habits are decreasing, or that sun protection habits (sun avoidance or use of sun screen) are increasing. MS incidence would decrease if lack of sun exposure was a major cause, and thus these findings do not suggest that sun exposure is a major determinant of MS pathogenesis.

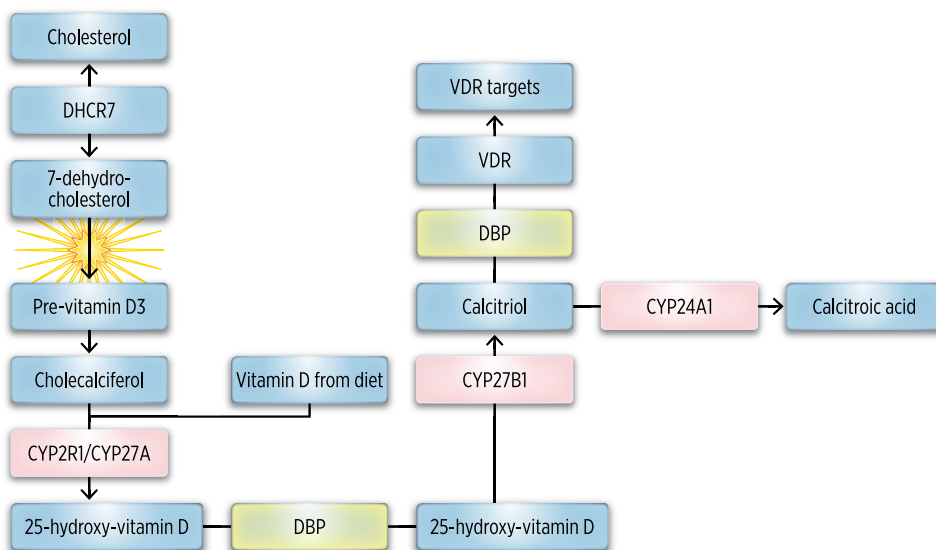
Results in this field indicate that sunlight is an environmental factor involved in the aetiology of MS. The susceptibility period (when this exposure influences MS risk) remains unclear, as occupational studies suggest an influence in adulthood, and other observational studies imply that exposure in childhood and adolescence may be relevant. Furthermore, because MS is a complex disease in which genetic markers are highly involved in disease development, possible interactions between sunlight and genetics need to be determined.



## 2.3 VITAMIN D

### 2.3.1 Production and function

The compound colloquially known as “vitamin D” exists in the body in several forms. Nowadays it is considered to be a prohormone due to its synthesis and many divergent functions in the body (125). In humans, the synthesis of vitamin D (Figure 1) begins in the skin when UV-B rays (in sunlight) cause the conversion of 7-dehydrocholesterol (a steroid compound) to previtamin D<sub>3</sub> and then heat-induced isomerization converts previtamin D<sub>3</sub> to vitamin D<sub>3</sub> (cholecalciferol). Most vitamin D in the body is produced in this way, but some is obtained through food (mainly fish) and/or supplements. The dietary vitamin D compound is vitamin D<sub>2</sub> (produced by UV-B radiation of ergosterol from yeast). In Sweden, most supplements contain vitamin D<sub>3</sub>. Through oral intake, serum vitamin D levels increase with approximately 1 nmol/L per 40 IU vitamin D (126), although this is dependent on the baseline serum level (127). Both vitamin D<sub>3</sub> and vitamin D<sub>2</sub> are metabolized in the liver, by vitamin D 25-hydroxylase (encoded by CYP2R1/CYP27A1, which belong to the cytochrome P450 family of genes/proteins) to 25-hydroxyvitamin D<sub>3</sub> and 25-hydroxyvitamin D<sub>2</sub> respectively, the functions of which do not differ significantly (128) (herein simplified to 25-hydroxyvitamin D or vitamin D). In the next metabolic step this compound is catalysed to the active substance 1, 25-dihydroxyvitamin D (calcitriol) in the kidney (by 1- $\alpha$ -hydroxylase encoded by CYP27B1). It is 25-hydroxyvitamin D that is measured in plasma, and this compound (as well as all other soluble metabolites) is transported in the circulation bound to vitamin D-binding protein (DBP) (129). The corresponding gene, group-specific component (GC), exists in several isoforms that are associated with different 25-hydroxyvitamin D concentrations (130).



**Figure 1.** Vitamin D synthesis and metabolism.

Calcitriol is the main player in calcium regulation in the body, and leads to increased plasma calcium levels through increased absorption of calcium in the intestines and kidney and through bone resorption. Calcitriol is regulated by parathyroid hormone (positive feedback leading to increased calcitriol levels) and fibroblast growth factor 23. The latter induces expression of 24-hydroxylase (encoded by CYP24A1) which causes hydroxylation of 1, 25-dihydroxyvitamin D to calcitroic acid, an inactive substance secreted in the bile (125, 129). However, 1,25-dihydroxyvitamin D also has effects on the immune system (131), on which I will concentrate in this thesis.

Calcitriol acts in the body by binding to the vitamin D receptor (VDR)–retinoid X receptor complex that in turn binds to DNA at specific sites, so-called vitamin D response elements (VDREs). These VDREs are located close to the target gene and influence gene expression (suppressing or enhancing expression) (132).

### **2.3.2 The immunomodulatory effects of vitamin D**

Vitamin D, in its active form 1, 25-dihydroxyvitamin D, has effects on both innate and adaptive immunity. Important evidence of this is that the immune cells express VDR and 1- $\alpha$ -hydroxylase (either permanently or upon induction) and hence are able to produce and be affected by calcitriol (133). When the innate immune system is activated by recognition of PAMPs by the TLRs, the latter induce expression of VDR and 1- $\alpha$ -hydroxylase, and calcitriol is synthesized. Calcitriol in turn upregulates cathelicidin, an antimicrobial peptide that is an important part of the body's defence against bacterial infections, which leads to efficient elimination of bacteria (134). Calcitriol may act on the innate immune system in several other ways (135), with the general result of enhancing innate immunity. This has led to the idea of using vitamin D as a prophylaxis against clinical infections, and a meta-analysis of 11 randomized controlled trials of vitamin D for prevention of respiratory tract infections showed a positive effect of the intervention (136). However, the studies were heterogeneous and there were signs of publication bias so the authors suggested caution in interpretation of the findings.

The effects of vitamin D on the adaptive immune system are numerous. Calcitriol inhibits T cell proliferation (137), especially Th1 cells, and the production of the cytokines IL-2 and interferon- $\gamma$  (133). It also stimulates the development of the Th2 cell subset (138) and induces T regulatory cells, as well as inhibiting Th17 cells (133).

In B cells, calcitriol has been found in vitro to decrease cell proliferation, antibody production and plasma cell proliferation (139). However, it is still not clear whether this also occurs in vivo (140), as the role of vitamin D in B cells has been less extensively investigated than the effect of vitamin D on T cells.

Calcitriol has also been found to affect dendritic cells, arresting their maturation and maintaining them in a more tolerogenic state; it also influences their cytokine production, inhibiting secretion of proinflammatory cytokines (such as IL-12, TNF- $\alpha$  and interferon- $\gamma$ ) (141).

Although there are still many questions to be answered, such as whether calcitriol affects the adaptive immune cells directly and/or indirectly via cytokine production, vitamin D can in general be considered to enhance the innate immune system and suppress adaptive immune mechanisms (142).

### **2.3.3 Vitamin D and autoimmune diseases**

By analogy with the argument for an immunosuppressive effect of UVR on autoimmune diseases, vitamin D, given its immunomodulating effects, should also have an impact on the development of autoimmune diseases.

In an animal model of autoimmune diabetes (diabetes type 1), administration of high calcitriol doses protected against disease onset (143), and CYP27B1 polymorphisms have been associated with type 1 diabetes (144), further strengthening a possible aetiological link. A meta-analysis of eight observational studies (145) investigating self-reported vitamin D supplementation in childhood and later onset of diabetes type 1 showed an association, with lower risk of disease if supplementation was reported. There were differences between the studies, with the case-control studies showing an inverse association (OR 0.68, 95% CI 0.49–0.94), compared to no significant associations in the included cohort studies. Furthermore, intervention studies conducted so far have not shown any protective effect of vitamin D supplementation (143).

In a meta-analysis (146) of three cohort studies of vitamin D intake and risk of developing RA, a significant inverse association was found (RR 0.76, 95% CI 0.58–0.94). Hiraki et al (147) used data from the NHS study in which prospectively collected blood samples were available (single measurement for each individual) and evaluated the association between plasma 25-hydroxyvitamin D level and risk of RA and found no signs of RA being influenced by vitamin D, except for a small subset of individuals where samples were drawn recently before disease onset (<4 years), where an inverse association was found.

In summary, there is some evidence for a protective effect of vitamin D against autoimmune diseases, although not conclusive.

### **2.3.4 Vitamin D and MS**

In 1974, Goldberg (148) proposed for the first time that vitamin D might be important in the aetiology of MS, basing his hypothesis on the geographical distribution of MS and its relation to sunshine exposure. In 1986, the same investigator performed a small clinical trial (149) supplementing MS patients with calcium, magnesium and vitamin D (in the form of cod liver oil) and found that the relapse rate decreased as a result of the treatment. However the trial results were far from convincing, due to the small sample size, study design and high dropout rate.

During the following decades, the topic of vitamin D and MS was extensively studied, supported by growing evidence of the immunomodulatory effects of vitamin D, including the

findings of Cantorna et al (150) in 1996 that calcitriol reversibly impeded disease progression in EAE. In the following section, only the most important studies that have been performed in this area will be discussed.

In 2004, Munger et al (151) reported a decreased MS risk with increasing vitamin D supplementation (RR 0.59, 95% CI 0.38–0.91) for intake above 400 IU/day, however vitamin D intake from food alone was not protective. Two years later, a landmark study (152) was published, in which prospectively collected serial blood samples were used to evaluate the impact of 25-hydroxyvitamin D levels on the risk of developing MS. On average, the samples were taken 5 years before disease onset, and the results showed that the risk of MS decreased by 41% for every 50 nmol/L increase in 25-hydroxyvitamin D (OR 0.59, 95% CI 0.36–0.97). The results were only significant for white people and there was no difference between the sexes. The authors also found that vitamin D levels decreased significantly after disease onset. Similarly, van der Mei et al (153) found lower vitamin D levels among MS patients than among healthy control subjects, but levels were only significantly reduced among patients with a higher degree of disability (EDSS >3) and not among those with EDSS ≤3.

These studies and others have reinforced the idea of vitamin D deficiency as a risk factor for developing MS. Because migration studies indicated that the important period for environmental exposures might be early life (childhood and adolescence), the timing of vitamin D deficiency has been investigated by several groups. In their study on outdoor exposure in childhood and adolescence above the Arctic circle (see section 2.2.3), Kampman et al (119) also evaluated intake of cod liver oil and fish consumption (sources of vitamin D) and found a protective effect of cod liver oil intake, but only among individuals who reported a low level of outdoor activities (and thus possibly low sun exposure). Frequent fish consumption (three or more times/week) was associated with reduced MS risk (OR 0.55, 95% CI 0.33–0.93). Another study investigating vitamin D intake during adolescence (154) was published in 2011. The authors used NHS data, in which MS cases and controls had reported retrospectively (at 35–65 years of age) food frequency and supplement intake during adolescence. The total vitamin D intake from food (e.g. dairy products and fish) and supplements was calculated. There was no significant association between MS and vitamin D intake from food, although a suggestive association was observed between supplement intake of vitamin D >400 IU/day and decreased MS risk (RR 0.73, 95% CI 0.50–1.07).

Due to the observed association between month of birth and MS risk, gestational period has been suggested as the window of exposure and decisive time for MS susceptibility. Vitamin D intake during pregnancy and later risk of MS in children was evaluated by Mirzaei et al (155). In a study (Nurses' Mothers' study) based on the NHS, the mothers of the participants in NHS and NHS II were asked to complete questionnaires regarding food intake during pregnancy (between 1945 and 1965). Intake of milk and dietary vitamin D and predicted 25-hydroxyvitamin D levels were then analysed with regard to risk of MS in the offspring (in total 199 MS cases among the children of the mothers included in the study). There was a significant inverse association between reported frequent milk intake during pregnancy, as well as between vitamin D intake from food, and later MS risk in the offspring but no

association with vitamin supplements. Gestational vitamin D levels in the mothers were predicted using several vitamin D-determinant covariates, and the predicted values were inversely associated with MS risk in the nurses. However, although meticulously analysed, because of the long latency between exposure and collection of exposure information and the mothers' knowledge of their daughters' disease, recall bias may have considerably influenced the results in this study.

In northern Sweden, a biobank with prospectively collected blood samples from pregnant mothers was used to identify samples from women whose children later developed MS. In total 37 MS cases were identified and matched to 185 controls (all had mothers who had donated blood to the biobank). The blood samples were collected during the first trimester. There was no association between 25-hydroxyvitamin D in these samples and subsequent risk of MS in the offspring. In the same study, the blood samples were also evaluated with regard to risk of development of MS in the donors themselves and a significant association was found between 25-hydroxyvitamin D levels above 75 nmol/L and decreased risk of MS (OR 0.39, 95% CI 0.16–0.98) but no trend was observed across quintiles. The latter result corroborates the findings of the above-mentioned study by Munger et al (152) on pre-disease vitamin D levels.

Although this thesis concerns vitamin D in the aetiology of MS, several studies investigating vitamin D and MS disease activity will also be mentioned, as it is possible that potential aetiological factors might also influence disease progression.

Vitamin D levels and risk of relapse have been evaluated in several studies. However, it has not always been possible to determine whether vitamin D levels were causally associated with relapse risk or just an epiphenomenon. In particular because it is known that MS patients have lower vitamin D levels compared to healthy people, it might be presumed that vitamin D is low due to the inflammatory activity and not the other way around (inflammation due to low vitamin D). Simpson et al (156) investigated this issue prospectively in a cohort of 145 RRMS patients, measuring 25-hydroxyvitamin D twice a year for approximately 2 years. The seasonal variation in vitamin D levels was predicted statistically and the hazard ratio of relapse was analysed in relation to 25-hydroxyvitamin D levels. The hazard of relapse was significantly reduced with increasing vitamin D levels, with a 10 nmol/L increase leading to a 9% lower risk of relapse (95% CI 3–15%), and the association was independent of immunomodulatory therapy, degree of disability and a number of other covariates. The central question today, for everyone treating MS patients, is: do vitamin D supplements reduce relapse risk or disease progression? This question was evaluated in a recent meta-analysis (157) and no effect on MS was found with intervention with vitamin D; nevertheless, the included studies had methodological limitations, differed in type of intervention and dosage and were probably underpowered, so the question remains open. At present, four ongoing clinical trials to investigate vitamin D as a therapeutic intervention in MS are registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov).

In summary, the available evidence points to vitamin D deficiency as a risk factor for the development of MS, although it cannot be completely ruled out that the observed associations may be due to disease-related decreased vitamin D levels, especially as the disease process most probably starts long before the individual notices the first symptoms. Furthermore, the

timing of the exposure (in utero, adolescence and adulthood), the possible influence on MS risk of different genetic variants in the vitamin D-related genes and, finally, the influence of gene–environment interactions between vitamin D and genetic risk factors remain to be determined.

## **2.4 GENETICS OF MULTIPLE SCLEROSIS**

The genetic contribution to MS susceptibility was identified early in the MS research history and a major breakthrough in understanding of this disease came in 1972 with the identification of HLA and its association with MS (158).

### **2.4.1 HLA associations and MS**

Since 1972, HLA-DRB1\*15 (MHC class II) has become established as an MS risk gene associated with an increased MS risk of about 3 (78); in addition, the HLA genotype HLA-A\*02 (MHC class I) (159) has been confirmed as protective with an associated OR of about 0.7. Furthermore, in recent genome-wide association studies (GWASs) it has been confirmed that other genotypes confer risk increases (although less than HLA-DRB1\*15): HLA-DRB1\*13\*03 is associated with an OR of 2.4 and HLA-DRB1\*03:01 with an OR 1.26 (78). Among the highly associated single-nucleotide polymorphisms (SNPs) identified in the latest MS GWASs, a significant proportion is located in gene transcription regions and about a third is known to be associated with immune system functions and/or other autoimmune diseases (78), lending strong support to the view of MS as an immune-mediated disorder.

### **2.4.2 Non-HLA associations and MS**

No strong evidence emerged for risk-associated genotypes other than the HLA complex (except for IL7R, the interleukin 7-receptor gene (160)) until the era of the GWAS. This type of study, with a large number of included cases and controls and the possibility of genotyping a large number of SNPs, has now identified 110 genetic loci, outside the HLA complex, that are associated with MS (78, 161). These SNPs include rs2248359 (corresponding to CYP24A1) and rs201202118 (in high linkage disequilibrium (LD) with CYP27B1). These findings enhance the probability that vitamin D has a role in the pathogenesis of MS.

## **3 AIMS**

### **3.1 OVERALL AIM**

The overall aim of this thesis was to investigate the influence of vitamin D on the development of MS.

### **3.2 SPECIFIC AIMS**

- To investigate the associations between exposure to sunlight, vitamin D levels and risk of MS, and the interaction between these exposures and HLA-DRB1\*15 (paper I).
- To investigate the association between intake of fatty fish, as a measure of oral vitamin D intake, and the risk of MS as well as the interaction between fatty fish intake and sun exposure habits with regard to MS risk (paper II).
- To examine the association between vitamin D levels at birth and the risk of later development of MS (paper III).
- To investigate the association between genotypes associated with circulating vitamin D levels, evaluated using a genetic risk score (GRS), and the risk of developing MS (paper IV).

## 4 MATERIALS AND METHODS

### 4.1 THE EIMS STUDY

The Epidemiological Investigation of Multiple Sclerosis (EIMS) study is a nationwide, population-based case–control study that began in 2005. Around 40 neurological centres from all over Sweden are recruiting MS patients to the EIMS study, in which the overall aim is to study the influence of environmental factors and gene–environment interactions in the development of MS. Data collection is on-going. Data from the EIMS study have been used for all the studies presented in this thesis.

#### 4.1.1 Case ascertainment and control selection

Patients were included in the EIMS study at their respective neurological clinic after receiving a diagnosis of MS, according to the McDonald criteria (5-7), or of CIS. At inclusion, the diagnosis should have been received a maximum of 2 years earlier and other inclusion criteria were 16–70 years of age, ability to understand Swedish and voluntary agreement to participate. Only confirmed MS cases (i.e. no CIS cases) were included in the present studies.

When a patient was selected for study inclusion, two controls were chosen from the national population register, matched for age (predefined 5-year age strata), sex and residential area (county) at the time of diagnosis of the case. Each control was contacted by post and asked to participate in the study; in case of refusal or no response a new individual was chosen in the same manner from the register until two controls had accepted. If a case did not want to participate or was excluded for some reason, the corresponding control was still retained in the study to increase power. Exclusion criteria for the controls were a diagnosis of MS and the inability to understand Swedish. The overall response proportion in EIMS has been around 91% for cases and 70% for controls with regard to the questionnaire, and 94% and 57% (of those who have answered the questionnaire) with regard to providing blood samples for cases and controls, respectively.

#### 4.1.2 Exposure assessment

The exposure information was gathered by means of an extensive questionnaire (Figure 2) that both cases and controls filled in at home. The questions regarded socioeconomic factors (education, family situation) and environmental factors such as smoking, dietary habits, sun exposure habits, alcohol intake, occupational exposures, diseases, vaccinations, physical activity and other health-related factors. In case of incompletely filled in questionnaires, trained staff at the EIMS project secretariat contacted the cases and controls by phone or post to acquire complete exposure information.

The cases gave a blood sample at their clinic and the controls at any healthcare centre of their choice after having received sample tubes by post. The blood samples are stored at the KI Biobank at between -60° C -85°C. If a control objected to blood donation, a saliva sample



was requested. A saliva sample kit was posted to the subject and returned directly to the KI Biobank for storage. If subjects did not send in their questionnaires and/or samples, they were reminded by phone or post up to four times for the questionnaires and twice for blood/saliva samples.

#### **4.1.3 Genetic and serological analyses**

The blood/saliva samples were used for genetic and serological analyses of known and possible genetic risk factors for MS.

For paper I, HLA-DRB1 genotyping was performed using sequence-specific primers (162) and OLERUP SSP™ HLA kits (Qiagen, Hilden, Germany). We also analysed 25-hydroxyvitamin D levels in blood (in papers I and II) for a subset of the EIMS study population of 2577 individuals (1173 cases and 1404 controls) included between April 2005 and December 2009. The analyses were performed using a chemiluminescence immunoassay and a LIAISON® instrument (both from Diasorin AB, Sundbyberg, Sweden) with equimolar measurement of 25-hydroxyvitamin D<sub>2</sub> and D<sub>3</sub>. Each sample was analysed for 25 (OH) D (25-hydroxyvitamin D) in duplicate. Results were arithmetic means of the duplicate measurements. The coefficient of variation of duplicates was 7%.

For paper III, 25 (OH) D<sub>3</sub> and 25 (OH) D<sub>2</sub> in dried blood spots, collected from infants in Sweden since 1975 and stored in a biobank (further details in section 4.6) were measured with highly sensitive liquid chromatography tandem mass spectroscopy. The blood spots corresponded to a blood volume of around 3.28 µL. Minimal sample cleanup and multiple reactant monitoring were used to reduce sample loss and increase specificity (163).

In paper IV the genotyping of SNPs was performed on an Illumina custom array in collaboration with the International Multiple Genetics Consortium (IMSGC) (164). Quality control measurements were performed for each marker and for all individuals, where markers with a minor allele frequency <2%, a genotyping success rate <98% or that were not in Hardy-Weinberg equilibrium ( $p < 0.0001$  among controls) were removed and individuals with >2% missing genotypes, increased heterozygosity rate, or for whom given sex did not agree with the sex chromosomes were excluded. Population outliers were identified with Eigenstrat (165) and removed, and principal component analysis (PCA) was performed to correct for population stratification.

HLA-DRB1 information was imputed using a modified version of HLA\*IMP02 (166) at four-digit resolution.



**Figure 2.** Questionnaires for the EIMS and the GEMS studies.

## 4.2 THE GEMS STUDY

The Genes and Environment in Multiple Sclerosis (GEMS) study is a case–control study of prevalent MS cases that was initiated in 2009 and concluded in 2011. The aim was to collect data on environmental and genetic risk factors, by means of questionnaires and blood sampling, to investigate their influence on the development and progress of MS.

### 4.2.1 Case ascertainment and control selection

The MS register is a national healthcare quality register for MS patients with the aim of facilitating good quality of and equality in healthcare and to supervise treatment effects as well as to enable clinical research. When GEMS was initiated in 2009, around 12 000 patients were included in the register. The inclusion process took place in May 2009 and November 2010 and in total 7706 patients were identified. Patients were contacted by post and asked to participate in the study. The inclusion criteria were a definite MS diagnosis (i.e. CIS patients were excluded), consent to research participation and age 18 years and above. For each case, one control was identified from the national population register and matched for sex, age and residential area (at the time of disease onset). Both cases and controls received an information letter about the study and a week later were invited by post to participate in the study.

In total 7706 MS cases and 8570 healthy controls received the questionnaire. The response proportions for the questionnaire were 82% for the cases and 66% for the controls, and the respective response proportions for blood sampling were 65% and 49%.

### 4.2.2 Exposure assessment

Cases and controls received by post a questionnaire (Figure 2) regarding environmental factors and tubes for blood sampling and returned both by post (blood samples were collected at the local healthcare centre). If subjects did not respond they received reminders by post

(three times for the questionnaire and twice for the blood samples). The filled in questionnaires were machine-scanned to retrieve the answers and incomplete answers were left missing.

The blood samples were stored at the KI Biobank, under identical conditions as described for the EIMS study.

### **4.2.3 Genetic analyses**

In paper IV, the blood samples were genotyped and HLA data were imputed as described for the EIMS study (see section 4.1.3 above).

## **4.3 THE KPNC STUDY**

Kaiser Permanente Medical Plan, Northern California (KPNC) is a healthcare system with a membership that comprises 25–30% of the population in the area, corresponding to around 3.2 million people, and KPNC members are largely representative of the general population (167, 168). In 2007, a research programme, the Kaiser Permanente Research Program on Genes, Environment, and Health (RPGEH), was initiated to investigate environmental and genetic disease mechanisms (169). As part of the RPGEH, a 2007 survey on demographic and health data was conducted, including 400 000 individuals. The survey participants were subsequently asked for informed consent for biobanking of saliva samples for genetic research, and 147 000 individuals consented and contributed samples. In 2009, participants in the RPGEH were invited to participate in another project, the Genetic Epidemiology Research on Adult Health and Aging (GERA) study, the object of which was genome-wide genotyping in around 100 000 individuals (170). The selected participants were asked for informed consent and 77% gave their consent. After removal for genotyping errors, 78 479 individuals were finally included for further research. The GERA cohort consists of people of different ethnicities (81% white non-Hispanic), with a generally high level of education and their average KPNC membership duration is 23.5 years.

### **4.3.1 Case ascertainment and control selection**

For paper IV, the KPNC electronic records were searched for individuals fulfilling an MS diagnosis and possible controls.

The inclusion criteria for cases were: having an MS diagnosis identified by a neurologist, according to the ninth revision of the International Classification of Diseases (ICD-9), age 18–69 years and white non-Hispanic ethnicity.

In total 3293 potential MS cases were reviewed by KPNC neurologists, who approved contact with 2823 (86%), and 1849 cases were finally considered eligible.

The controls were selected from two different sources:

- a) The KPNC: members without an MS diagnosis (or related condition such as optic neuritis or myelitis), age 18–69 years and white non-Hispanic ethnicity.

- b) The GERA/RPGEH cohort: a subset of 12 605 white non-Hispanic individuals with survey and genotyping data, without MS, aged 18–69 years.

The potential KPNC cases and controls were contacted by post with an explanation of the study procedures and a consent form (to be signed and returned by post). The response proportion regarding the questionnaire was 80% for the cases and 66% for the controls. Genetic data were available for around 80% of the study participants. The controls were matched to the cases 10:1 according to sex and age ( $\pm 2$  years). Detailed information regarding included individuals is given below in section 4.7.

#### **4.3.2 Exposure assessment**

The KPNC exposure assessment was performed through computer-assisted interviews, with questions regarding sociodemographic factors, comorbidities, smoking, IM and other environmental factors. The KPNC participants received blood sample tubes by post and donated blood at KPNC laboratories. The saliva sample tubes were received and returned by post (using Oragene kits). Blood was processed and DNA extracted according to the Gentra Puregene protocol (171). For the GERA controls, biological specimens were already collected and genotyped as part of the GERA project (170).

#### **4.3.3 Genetic analyses**

HLA-DRB1 four-digit resolution genotyping was performed as previously described (172) and SNP genotyping was performed (78) using the Illumina Infinium660K and Human Omni Express BeadChip arrays (Illumina, Santa Clara, CA, USA) for the KPNC participants and Axiom custom chips (173) (Affymetrix, Santa Clara, CA, USA) for the GERA participants.

### **4.4 PAPER I: UVR EXPOSURE AND VITAMIN D LEVELS**

*Study participants:* The study period was April 2005 to March 2010 and the cases were included from 35 neurological clinics participating in the EIMS study. There were 1326 eligible cases and 3678 controls, and response proportions were 93% and 73% respectively, resulting in 1233 cases and 2685 controls. To avoid confounding due to differences in skin colour between different ethnic groups, and to enable proper adjustment for 25 (OH) D levels, the study population was restricted to Scandinavian subjects (170 cases and 459 controls excluded) for whom plasma 25 (OH) D levels were available (50 cases and 1032 controls with no samples). Thus the study included 1013 cases and 1194 controls.

*Main exposure:* UVR exposure was measured through three questions concerning voluntary sun exposure in sunny weather, visits to sunny countries and use of sunbeds. All questions related to exposure in the last 5 years. The answer alternatives ranged from “never” to “daily/more than once a year” (see Appendix for details). Although UV-B radiation is responsible for the production of previtamin D in the skin (91), and UV-A is mainly emitted from sunbeds (174), we considered the three UVR-related exposures to reflect a general “sun-

related behaviour” and they were therefore analysed both separately and as a combined UVR exposure index. For construction of the UVR index, every answer alternative was assigned a number (1 for the lowest exposure up to 4 for the highest), and the corresponding numbers were added to obtain a score of 3–12. This index was then categorized into five groups (3; 4 and 5; 6; 7 and 8; 9–12). The other main exposure, 25-hydroxyvitamin D, was analysed in quintiles (based on the levels among the controls) as well as dichotomized with both 50 nmol/L and 75 nmol/L as cutoff values (high-level categories were used as the reference).

*Covariates:* Other covariates were vitamin D supplement intake (self-reported intake during the last 5 years, yes/no); smoking, categorized as never-/ever-smoker (the year of disease onset of the case was used as the index year, and the same year was applied to the matched controls, and an individual was considered a smoker only if smoking was reported before the index year, according to the method of Hedström et al (52)); current BMI (exposed category defined as  $\text{BMI} \geq 25 \text{ kg/m}^2$ ); fatty fish intake (exposed category defined as intake of fatty fish more than once/week); socioeconomic status (five categories: unskilled/skilled manual workers and assistant/intermediate/higher non-manual employees). HLA-DRB1\*15 heterozygosity (1 or 2 alleles versus 0) was used in the interaction analyses.

## 4.5 PAPER II: FATTY FISH CONSUMPTION

*Study participants:* In this study 40 neurological clinics recruited individuals for the study during the period April 2005 to May 2012. The response proportions were 93% and 69% for cases and controls, respectively, corresponding to 1879 cases and 4135 controls. Plasma 25-hydroxyvitamin D levels were available for a subset of 1178 cases and 1404 controls.

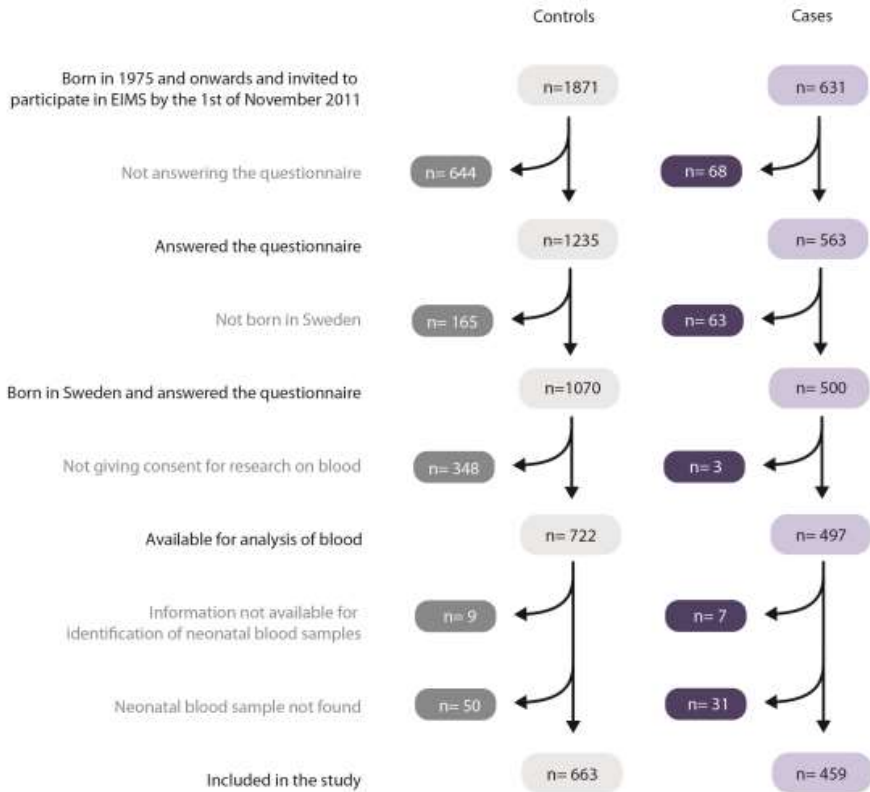
*Main exposure:* Fatty fish intake, considered as a source of vitamin D, was the main exposure but intake of lean fish was also examined. Fatty fish was defined as fish with a fat content of more than 3% (175). Exposure information was self-reported (see Appendix for details). The overall vitamin D content in fatty fish and lean fish species was calculated using information from the National Food Agency database (176), and the vitamin D content in fatty fish was approximately twice as high as that in lean fish (440 versus 220 IU/100 g). The information from the questionnaire was categorized into three groups where reported “daily” intake was collapsed with the category of “weekly” intake due to small numbers in the former category (for both fatty fish and lean fish intake). Dichotomization of fish intake (weekly intake versus monthly/seldom/never) was also used.

*Covariates:* As in paper I, the covariates used were smoking (ever/never) and UVR exposure (the same index divided into five categories and dichotomized in the same way as in paper I). We also evaluated the following covariates: current BMI and BMI at 20 years of age (in the categories  $<18.5$ ,  $18.5$  to  $<23$ ,  $23$  to  $<25$  and  $\geq 25 \text{ kg/m}^2$ , according to a modified version of the WHO (World Health Organization) classification (177)), ancestry (Scandinavian, defined as a person born in a Scandinavian country or having at least one parent who had immigrated from a Scandinavian country, yes/no), educational level (no university education/university education without qualification/university education with

qualification) and dairy vitamin D intake. Intake of vitamin D from dairy products was calculated using questionnaire information regarding daily or weekly intake of cheese, milk, yoghurt and sour milk (Swedish “filmjöl”k”) at study inclusion. The mean vitamin D content for each product was estimated (using the nutrient database of the National Food Agency (176)) and multiplied by the number of weekly servings. This variable was used both as a continuous and categorized variable (in quintiles according to levels among healthy control subjects). Levels of 25-hydroxyvitamin D were also analysed (continuous variable).

4.6 PAPER III: NEONATAL VITAMIN D LEVELS

*Study participants:* Subjects born in 1975 and onwards in Sweden and included in the EIMS study until 1 November 2011 were eligible for study inclusion, and the response proportions for the questionnaire were 89% for cases and 66% for controls, resulting in 500 cases and 1070 controls. After exclusion of individuals for several reasons (see Figure 3), 459 cases and 663 controls were included in the present study.



**Figure 3:** Flow chart for study participants in paper III. Reproduced with permission from the publishers.

Exposure information was acquired from the PKU Biobank in which dried blood samples collected from infants (during the first days of life) are stored after screening for hereditary metabolic diseases (178). The samples have been collected since 1975 and were initially stored at room temperature and after August 1981 in cold storage (4° C). The blood samples are kept without personal information about the donor, but can be identified through a unique code (the key is kept separately). The samples are stored according to the mother's personal data and the child's hospital of birth (information saved separately from the samples), consequently this information is needed for sample retrieval. We acquired information on the personal number of the mother and the hospital of birth of the child from the Swedish Medical Birth register for the eligible individuals and manually identified each person's blood sample.

*Main exposure:* 25-hydroxyvitamin D levels (nmol/L) in neonates (measured both as 25 (OH) D<sub>2</sub> and 25 (OH) D<sub>3</sub>).

*Covariates:* The same covariates as in papers I and II were considered: ancestry (having a Scandinavian parent or not), BMI at 20 years of age (continuous variable), dairy vitamin D intake (continuous variable), fatty fish consumption (dichotomized as more or less than once a week), UVR exposure (using the index created for paper I, in five categories), smoking (ever/never), education (three categories as in paper II) and plasma 25-hydroxyvitamin D (continuous). Further we also evaluated as covariates latitude of residential area of birth (54–58°, 59° and 60–69° north), postnatal age of participant at blood sampling, maternal age at birth, breastfeeding (self-reported, yes/no), socioeconomic status (manual worker versus non-manual employee) and MS heredity (self-reported, yes/no).

#### **4.7 PAPER IV: GRS FOR VITAMIN D LEVELS**

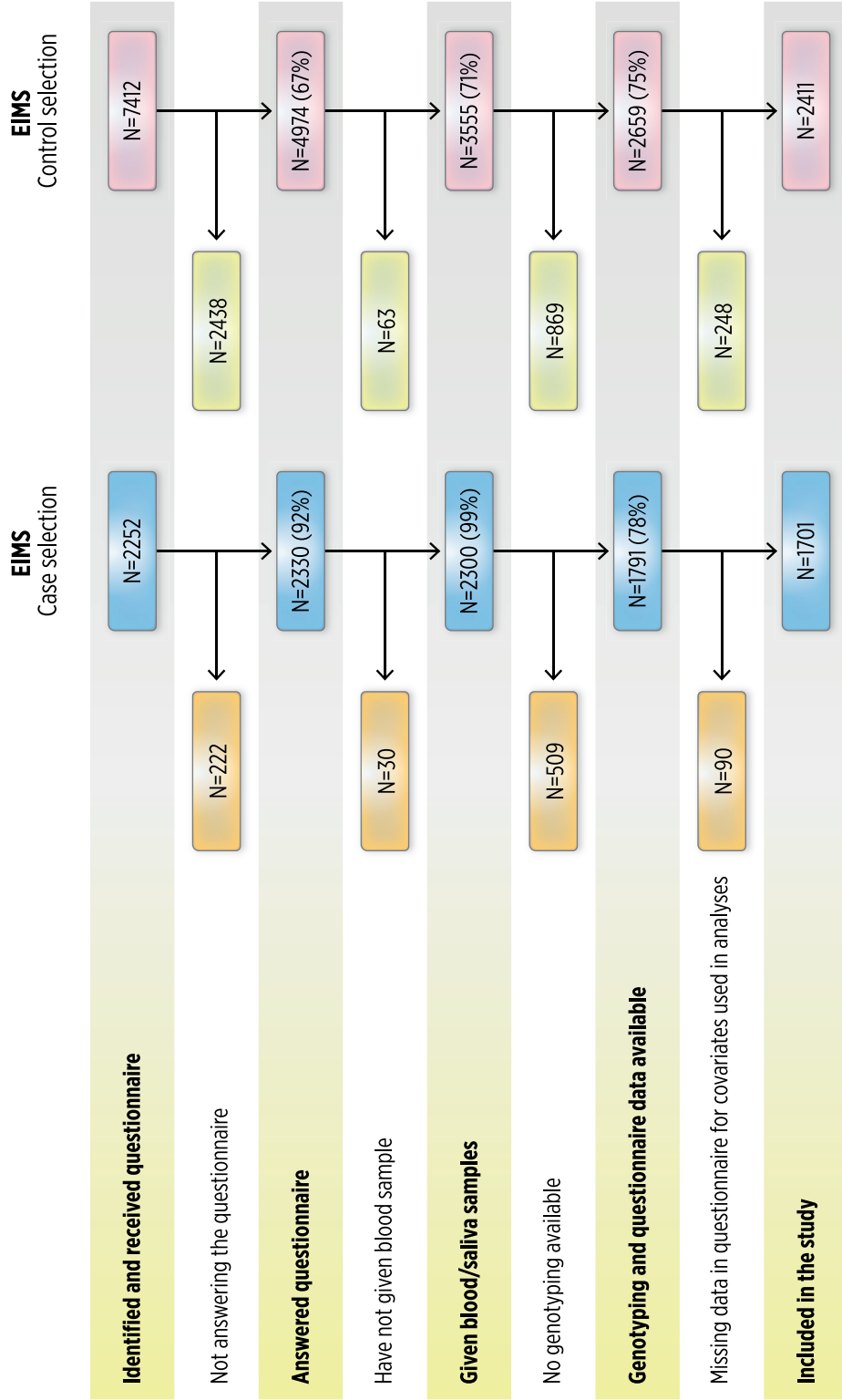
*Study participants:* The study period for the EIMS study was from April 2005 until December 2013 and for the GEMS study it was between 2009 and 2011. The final Swedish population consisted of 6335 cases and 5762 controls. For the American participants the study period was from 2007 until 2014 and the final population consisted of 1056 cases and 9015 controls. Figures 4–7 show flow charts with detailed information about selected cases and controls.

*Main exposure:* The main exposure was a GRS calculated to reflect increasing 25-hydroxyvitamin D levels. The risk score was computed from three SNPs: rs2228679-A, rs10741657-A (or in the American population rs2060793-A, in perfect LD with  $r^2=1$  in the 1000 Genomes Phase 1 European population) and rs3829251-G; all were reported to be associated with serum 25 (OH) D levels (179). All SNPs are located in close proximity to genes involved in vitamin D metabolism: rs2228679 in an intron of GC (gene ID 2638); rs10741657 upstream of CYP2R1 (gene ID 120227) and rs3829251 located in NADSYN-1 (gene ID 55191) and upstream of DHCR7 (gene ID 1717). The risk score was computed according to the method of De Jager et al (180) by multiplying the number of alleles by the weight for that allele (using weights from the GWAS by Ahn et al (179) where the weight

for each allele is the beta coefficient from the regression analysis in the GWAS) and adding the score for all three alleles.

*Covariates:* Other covariates were smoking (ever/never), HLA-DRB1\*15 heterozygosity (1 or 2 alleles versus 0), university education (yes/no) and BMI in early adulthood (measured at 18 years in KPNC and at 20 years in the Swedish population, in kg/m<sup>2</sup> as a continuous variable). Further, we also used a GRS for the 110 reported non-HLA MS risk genes (161), computed according to the method of De Jager et al (180) (information on one SNP (rs201202118) was missing for KPNC and on two SNPs (rs2028597 and rs6874308) for the Swedish population), a variable indicating study participation (EIMS versus GEMS) and a covariate for genetic ancestry. As the ancestry covariate we used PCA in the Swedish population and multidimensional scaling (MDS) in the American population (MDS is calculated differently from PCA but is used with the same function).





**Figure 4.** EIMS participants in Paper IV.

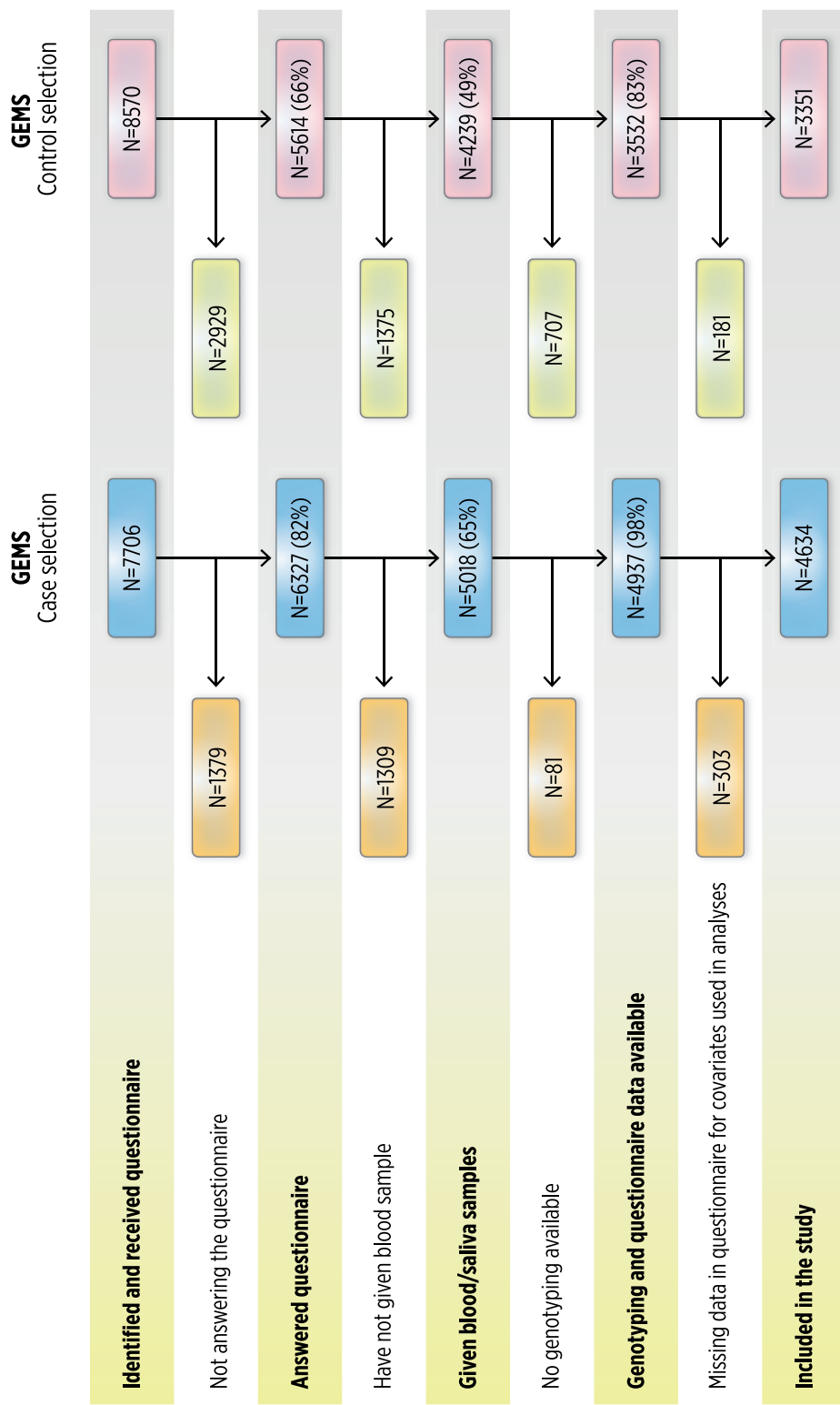
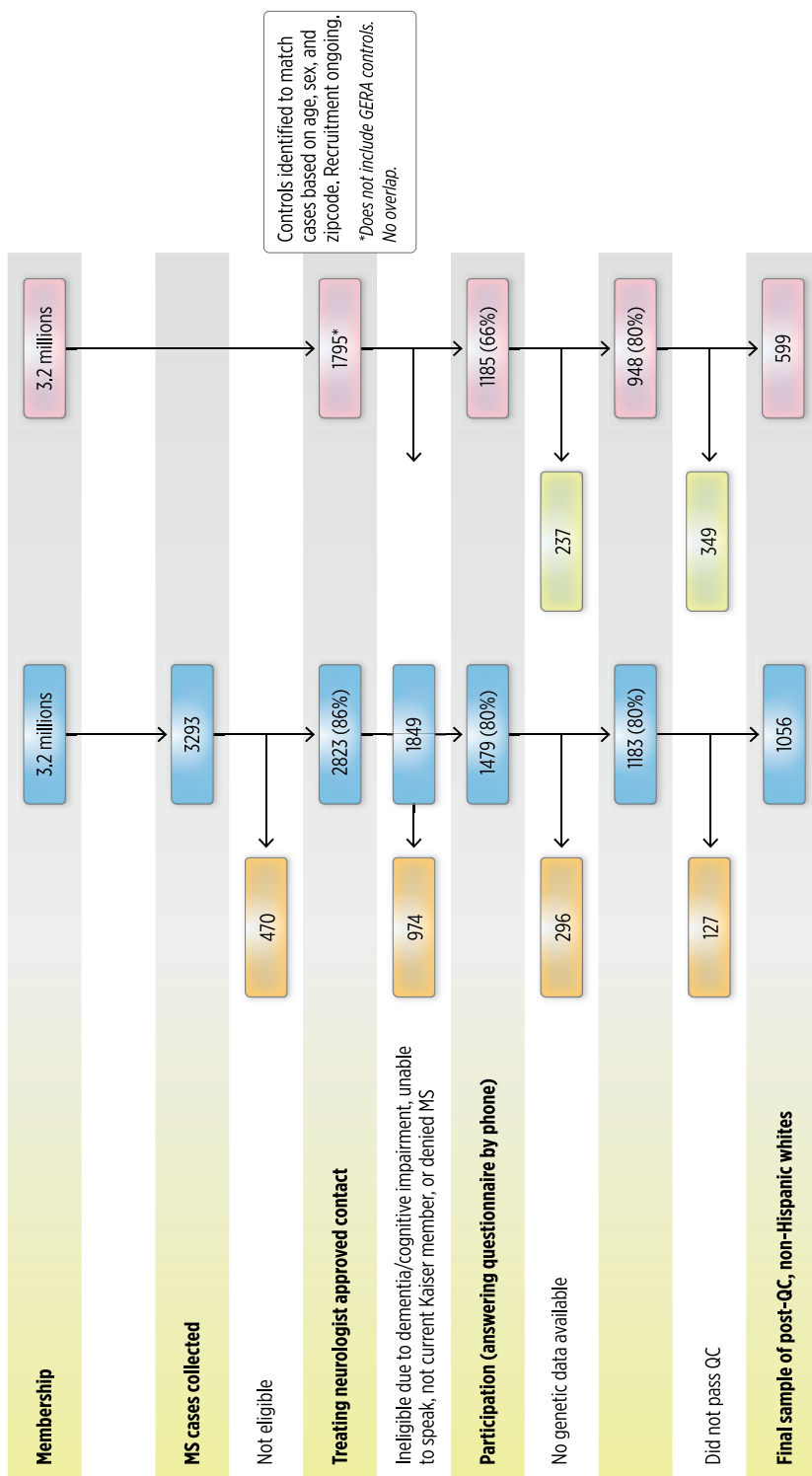


Figure 5. GEMS participants in Paper IV.



**Figure 6.** KPNC participants in Paper IV.

# RPGEH/GERA control selection

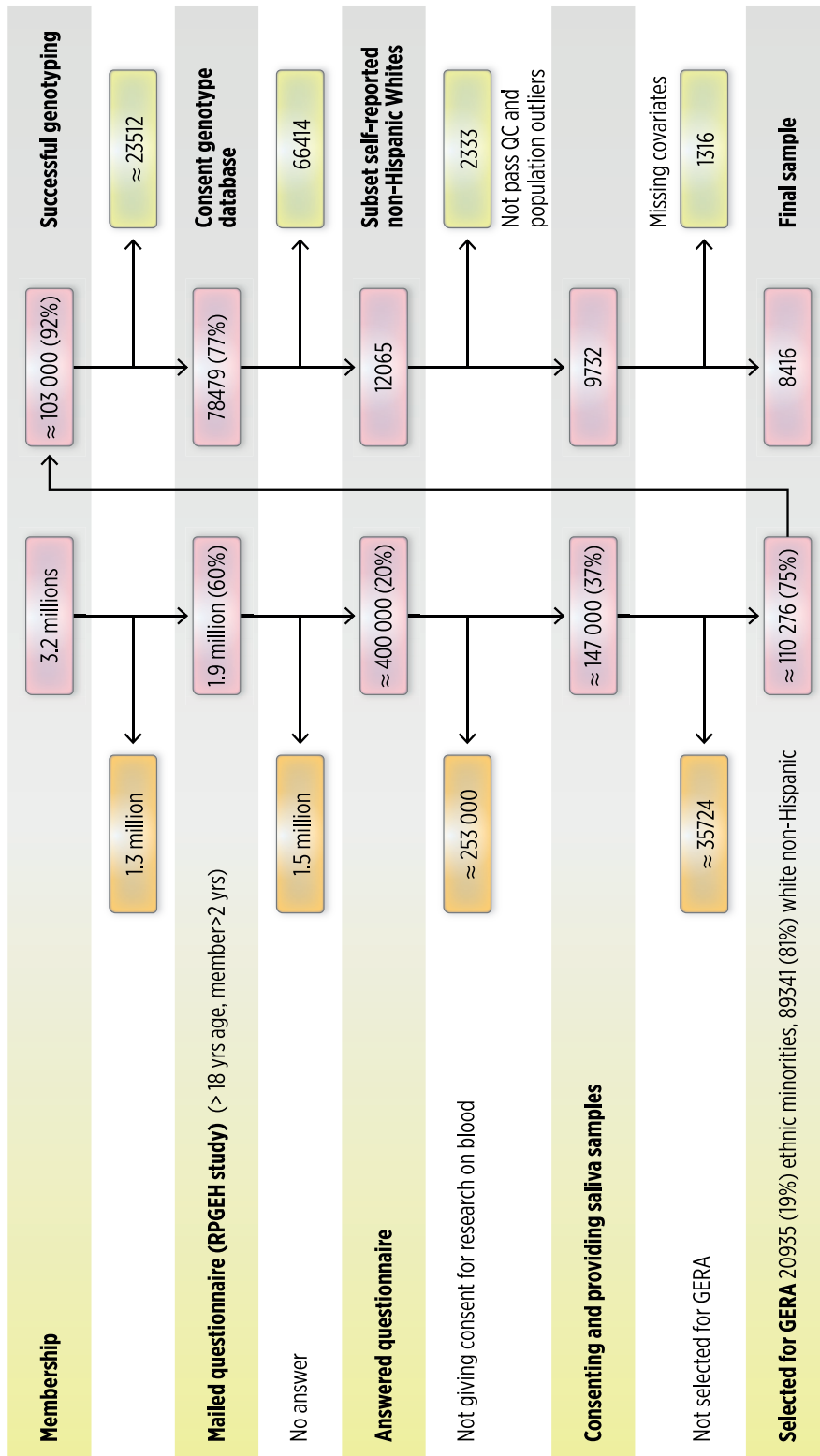


Figure 7. Additional controls from RPGEH/GERA cohort, in Paper IV.

## 4.8 STATISTICAL ANALYSES

The studies in this thesis are based on data from frequency-matched case-control studies. For some variables, such as smoking, the date of first symptom is used to classify smoking according to disease onset and then the study design can be regarded as individual-matched. The proper statistical method for an individual-matched study design is conditional logistic regression, whereas unconditional logistic regression is suitable for frequency-matched studies. In both types of multivariate logistic regression, an OR for developing MS, with 95% CI, is calculated.

In conditional logistic regression the association between exposure and outcome is analysed in the matched strata (i.e. every case is compared with the respective matched controls and only case-control pairs/triplets that are discordant in exposure contribute information regarding a possible association between exposure and disease). However, if there are missing data for a case or control, these strata cannot be used in the analysis which leads to decreased precision (wider CI values). In papers I and II we performed both conditional and unconditional logistic regression (adjusting for the matching variables sex, age and residential area). The estimated ORs were very similar but the results from the unconditional analyses had higher precision and we have chosen to present only the latter results. In papers III and IV only unconditional logistic regression was performed adjusting for the matching variables.

The matching variables were categorized according to study design: sex, age (5-year strata: <20, 20 to <25, 25 to <30, 30 to <35, 35 to <40, 40 to <45 and >45, with 25 to <30 as the reference category), and residential area (divided into counties or municipalities depending on how the controls were chosen in that area). For some analyses residential area was categorized in three geographical regions according to latitude (54–58°, 59° and 60–69° north).

Confounding is a type of bias that occurs when the true association (or lack thereof) between an exposure and an outcome is distorted by another factor that is associated with both exposure and outcome (disease) and is not a mediator between them. In the studies presented here, the covariates used in each study were evaluated as potential confounders. First we considered the possible underlying biological mechanisms that might cause confounding and, second, we introduced each covariate into the model one by one and observed the influence on the estimate, where a 10% change in the estimate was considered significant. Finally, both subject matter knowledge about MS and about the exposure in question (if it was a known risk factor for MS etc.) and the effect of the confounder on the estimate were taken into consideration when choosing the covariates to be used in the final model.

In all tests,  $p < 0.05$  was considered statistically significant. The statistical analyses were performed using SAS version 9.2–9.4 (SAS Institute, Cary, NC, USA) in papers I, II and IV, and Stata 11.0 in paper III (StataCorp, College Station, TX, USA). In paper IV PLINK (181) and R (182) were also used.

#### **4.8.1 Paper I**

For comparing cases and controls, the chi-squared test was used for categorical variables and an unpaired t-test for continuous variables.

In the final logistic regression model with UVR as exposure, we included as potential confounders the matching variables BMI, smoking, vitamin D level and blood sampling month; when vitamin D level was the exposure of interest, the model included the same variables as well as UVR exposure.

The interaction analyses were performed according to the theory of departure from additivity of effects as a sign of interaction and attributable proportion due to interaction (AP) (with 95% CI) and other measures of interaction included synergy index and relative excess risk due to interaction (RERI). However, we have only presented AP as all measures of interaction showed similar results. The AP shows the proportion of cases among those with the two factors considered (i.e. among double exposed) that are caused by the interaction per se, and a value above 0 indicates the presence of interaction.

#### **4.8.2 Paper II**

Group comparisons were performed using chi-squared test or unpaired t-test. The association between fatty fish intake and risk of MS was assessed using both conditional and unconditional logistic regression and the covariates included in the final model (as possible confounders) were BMI at 20 years of age, university education, UVR exposure, smoking and intake of lean fish.

#### **4.8.3 Paper III**

We performed linear regression of neonatal 25-hydroxyvitamin D on birth year and found that the 25-hydroxyvitamin D level increased with birth year which indicated a possible degradation in time. All analyses were therefore executed with the exposure 25-hydroxyvitamin D both as a continuous variable and categorized into year-specific quintiles. The effect of 25 (OH) D on the risk of developing MS was assessed using unconditional logistic regression, adjusted for (in the final model) the matching variables and month of birth, latitude of birth, having a Scandinavian parent, breastfeeding, sun exposure, vitamin D intake from dairy products, fatty fish consumption, smoking and BMI at 20 years of age.

#### **4.8.4 Paper IV**

We used independent samples t-test or chi-squared test, as appropriate, for group comparisons. Linear regression analyses were performed to evaluate possible associations between the GRS and possible confounding factors, and unconditional logistic regression for assessing the association between the GRS and MS. The regression model was adjusted for the matching variables, smoking, education, DRB1\*15 carriage, GRS of non-HLA MS risk genes and variables related to genetic ancestry and study type. A meta-analysis according to a random effects model was carried out in STATA.

## 4.9 ETHICAL CONSIDERATIONS

Research on humans is regulated by several laws, regulations and guidelines. Of these, the Declaration of Helsinki regarding medical research on human subjects, authored by the World Medical Association (originally in 1964 and updated in 2013), is considered among the most important. This declaration states the important principles of performing a risk–benefit assessment of a suggested research project and evaluating the risks for the individuals versus the benefit for the individuals and/or society, as well as highlighting the importance of the right to privacy of the individual and of safeguarding the confidentiality of personal information, and the necessity of obtaining informed consent.

Other laws and regulations in Sweden regarding research on humans and handling of research data are the Ethical Review of Research Involving Humans (Etikprövningslagen) (183), the Personal Data Act (regarding handling of personal information) (184) and the Biobanks in Medical Care Act (185, 186).

A risk–benefit assessment was performed before study initiation for the research projects (the EIMS, GEMS and KPNC studies) that have given rise to the data used in this thesis. The risks and burden for the individual consisted of the small disadvantages of giving blood samples, dedicating time to answer questionnaires and disclosing personal information; because the benefit of increasing knowledge about the causes of MS is enormous, the benefit far outweighed the risks. To safeguard the confidentiality of the individuals, all data have been analysed in de-identified form and initial data handling (where handling of personal, and sometimes sensitive, information is necessary) as well as later data analyses and data storage have been performed using encrypted files, virtual environments with no internet connection and other security measures for data protection.

In all studies informed consent has been obtained from all participants, through means of posted letters and fact sheets (or oral explanation in the clinic at inclusion for the MS cases involved in the EIMS study).

Paper III merits special ethical consideration. To obtain the (already collected) blood samples to analyse vitamin D levels at birth, it was necessary to retrieve information about each individual's hospital of birth as well as the mother's personal number, because the blood samples were stored according to this information. All individuals included in the EIMS study had already provided informed consent for research based on blood samples (donated at study inclusion), but not explicitly for research on blood samples donated at birth. This posed an ethical dilemma: would it be necessary to obtain informed consent specifically for this study, and what was the risk–benefit of the study? We concluded that it could be justified to presume that the individuals would agree to research on blood samples obtained at birth, as they had consented to research on blood samples obtained in adulthood, and vitamin D levels at birth might not be considered sensitive information. Also contacting the individuals again (maybe several years after inclusion in the EIMS study) to obtain new informed consent was deemed likely to elicit worries, regarding their vitamin D levels at birth and possible health

consequences, at a time when no such information could be given. Hence, our risk-benefit assessment concluded in that the risks and burden of contacting the individuals again were greater than the benefits, and obtaining new informed consent was judged unnecessary.

All Swedish studies included in this thesis have been approved by the Regional Ethical Review Board in Stockholm (diary numbers 04-252/1-4, 2008/1617-31/2, 2012/100-32 and 2012/359-32), and the data collection performed in the USA (paper IV) is part of a research project that has been approved by the corresponding ethical review board in that country.



## 5 RESULTS

An overview of the most important findings of the studies included in this thesis is presented here. For further details, the reader is referred to the publications at the end of this book.

### 5.1 PAPER I

The study population consisted of 1013 cases (72% women) and 1194 controls (75% women), and the mean age at disease onset among cases was 34.5 years.

#### 5.1.1 UVR exposure

UVR exposure, measured both separately according to the questions regarding behaviour in the sun and using the composite UVR index, showed an increasing risk for developing MS with decreasing UVR. For example, individuals who reported no voluntary sun exposure had a 60% increased risk of developing MS compared to those who reported daily sun exposure (OR 1.6, 95% CI 1.1–2.3).

In the UVR index analyses, in which the reference was the highest UVR index (corresponding to very frequent sun exposure), women and men were analysed separately and the results were similar (women: OR 2.0, 95% CI 1.2–3.3; men: OR 2.5, 95% CI 1.1–5.5).

Vitamin D is generally considered to be the causal exposure when evaluating associations between UVR and MS (where UVR level is a proxy measure for vitamin D level) but it could also be considered a mediator. To try to determine whether UVR had an independent effect on MS risk, regardless of vitamin D level, we performed analyses both adjusted for vitamin D (as a mediator) and unadjusted, and the adjustment only marginally changed the estimates (for the highest UVR index: OR 2.2, 95% CI 1.5–3.3; OR 2.0, 95% CI 1.3–3.1 when adjusted for vitamin D).

#### 5.1.2 Vitamin D

Low 25-hydroxyvitamin D levels were associated with increased MS risk, both with cutoff values of 50 nmol/L (OR 1.4, 95% CI 1.2–1.7) and of 75 nmol/L (OR 1.3, 95% CI 1.1–1.6) and using quintiles (OR 1.6, 95% CI 1.2–2.1 for MS in lowest quintile, *p* for trend 0.0002). When adjusting for UVR index, the OR decreased (OR 1.3, 95% CI 1.0–1.8 for MS in the lowest quintile, *p* for trend 0.03).

#### 5.1.3 Interaction analyses

There were no interactions between the genetic risk factor HLA-DRB1\*15 and either UVR exposure or vitamin D levels.

### 5.2 PAPER II

The study population consisted of 1879 cases and 4135 controls. The mean age at disease onset among cases was 34.4 years and the mean duration from diagnosis to study inclusion was 1.0 years.

5.2.1 Fatty fish consumption

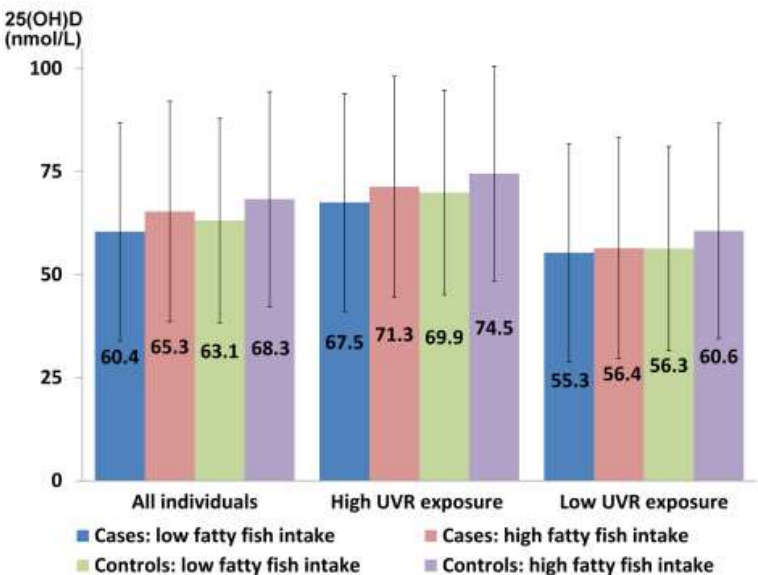
Fatty fish consumption at least once a week was significantly associated with decreased MS risk (OR 0.82, 95% CI 0.68–0.98) whereas lean fish consumption (adjusted for fatty fish consumption) had no significant effect on MS risk (OR 0.93, 95% CI 0.81–1.06).

5.2.2 Fatty fish and UVR exposure

After having concluded that lean fish intake did not influence MS risk, we further evaluated the possible effect modification of UVR exposure on the association between fatty fish and MS, and found that the risk of developing MS was decreased for fatty fish consumers who had been exposed to low UVR levels (OR 0.75, 95% CI 0.58–0.97 for the highest level of fatty fish intake) whereas there was no significant association between fatty fish and MS among those exposed to high UVR levels. We also performed interaction analyses between fatty fish consumption and UVR exposure and found that the AP was 0.19 (95% CI -0.02 to 0.40).

5.2.3 Fatty fish and vitamin D levels

Vitamin D levels were significantly lower among individuals with low fatty fish intake as compared to those with high fatty fish intake (Figure 8).



**Figure 8.** Vitamin D levels in individuals with different fatty fish intake and UVR exposure levels. Mean values (nM/L) with standard deviations. Reproduced with permission from the publishers.

### 5.3 PAPER III

The study population comprised 459 cases (76% women) and 663 controls (75% women). The mean age at disease onset among cases was 25.1 years. The mean 25-hydroxyvitamin D levels at birth were 29.4 and 29.9 nmol/L among cases and controls, respectively.

#### 5.3.1 Neonatal vitamin D levels

The level of 25-hydroxyvitamin D at birth was not associated with the risk of developing MS, when analysed as a continuous variable (OR 1.0, 95% CI 0.90–1.06 for a 10 nmol/L increase) or in year-specific quintiles. We performed analyses to determine whether a possible degradation with time of the 25-hydroxyvitamin D levels had been present, and we found that the vitamin D levels increased with birth year, and there was a significant interaction term (birth year \* month of birth). These results were interpreted as an indication of degradation with time, with a greater effect on samples with higher vitamin D levels (i.e. samples with higher levels may have degraded more rapidly). Further, we performed analyses with 25-hydroxyvitamin D (continuous and in quintiles) stratified into 5-year intervals of birth year to determine whether a possible association might have been obscured by the degradation with time. However, no significant association was found (Table 1).

**Table 1.** Risk of MS according to neonatal 25 (OH) D level, as birth year-specific quintile and per 10 nmol/L increase (OR with 95% CI).

Neonatal 25 (OH) D quintile	Total number of cases/controls	1975–1979, N=508, OR <sup>a</sup> (CI)	1980–1984, N=353, OR <sup>a</sup> (CI)	1985–1989, N=214, OR <sup>a</sup> (CI)	1990–1994, N=46, OR <sup>a</sup> (CI)
1, lowest	95/135	1.0	1.0	1.0	1.0
2	93/134	0.9 (0.51–1.55)	0.9 (0.45–1.68)	1.4 (0.57–3.32)	1.4 (0.20–9.84)
3	90/133	0.8 (0.47–1.47)	0.7 (0.35–1.36)	1.7 (0.70–4.01)	1.7 (0.19–15.73)
4	92/134	1.1 (0.62–1.89)	0.7 (0.34–1.30)	1.4 (0.59–3.43)	0.5 (0.03–9.66)
5	89/126	1.2 (0.70–2.15)	0.6 (0.31–1.22)	1.1 (0.47–2.80)	1.4 (0.16–12.90)
Per 10 nmol/L increase		1.1 (0.95–1.20)	0.9 (0.78–1.01)	1.0 (0.86–1.13)	1.0 (0.67–1.48)

<sup>a</sup> Adjusted for sex, residential area and age group.

## 5.4 PAPER IV

The study population consisted of 7391 cases and 14 777 controls, with 6335 cases (86%) and 5762 controls (39%) from the Swedish population. The population characteristics are shown in Table 2.

**Table 2.** Characteristics of cases and controls in the KPNC and EIMS/GEMS studies. Values are mean  $\pm$  standard deviation (SD) or N (%).

Characteristic	KPNC MS Cases (N=1056)	KPNC Controls (N=9015)	p-value	EIMS/GEMS MS Cases (N=6335)	EIMS/GEMS Controls (N=5762)	p-value
Females : Males	841 : 215	7328 : 1687	0.196	4162 : 1723	4378 : 1384	<0.001
Year of birth	1958 $\pm$ 8.8	1958 $\pm$ 8.9	0.246	1960 $\pm$ 13.1	1961 $\pm$ 13.3	0.021
Body mass index <sup>a</sup>	23.0 $\pm$ 4.4	21.5 $\pm$ 3.3	<0.001	22.0 $\pm$ 3.5	21.7 $\pm$ 3.2	<0.001
College graduate			<0.001			0.002
Yes	468 (44%)	3141 (35%)		1814 (29%)	1799 (31%)	
No	588 (56%)	5874 (65%)		4521 (71%)	3963 (69%)	
Smoker			<0.001			<0.001
Ever	525 (50%)	2876 (32%)		3578 (56%)	2742 (48%)	
Never	531 (50%)	6139 (68%)		2757 (44%)	3020 (52%)	
HLA- DRB1*15:01			<0.001			<0.001
0 copies	498 (47%)	6,613 (73%)		2,615 (41%)	4,095 (71%)	
1–2 copies	558 (53%)	2,402 (27%)		3,720 (59%)	1,667 (29%)	

<sup>a</sup> At age 18-20 (KPNC), or at age 20 (EIMS/GEMS).

#### **5.4.1 GRS for vitamin D levels**

The GRS for 25-hydroxyvitamin D was significantly associated with MS, with an OR of 0.79 (95% CI 0.64–0.99) for MS for a 1 unit increase in the risk score in the KPNC study; the results were similar in the Swedish population (OR 0.86, 95% CI 0.76–0.98). A meta-analysis resulted in a comparable estimate (OR 0.85, 95% CI 0.76–0.94).

## 6 DISCUSSION

The overall aim of this thesis was to investigate the influence of vitamin D on the development of MS. The studies included here show that lack of sunlight, the most important source of vitamin D, is associated with an increased risk of MS and that this risk is not influenced by the presence of HLA-DRB1\*15. Further, fatty fish intake, also a source of vitamin D, was inversely associated with decreased MS risk. Regarding the timing of the supposed risk factor, vitamin D deficiency, paper III showed that vitamin D deficiency at birth was not associated with later MS risk. Finally, in paper IV, we showed that a GRS, corresponding to increasing vitamin D levels, was positively associated with decreasing MS risk, making reverse causation a less likely explanation of the overall association between vitamin D deficiency and MS.

### 6.1 MAIN FINDINGS AND RELATION TO PREVIOUS RESEARCH

#### 6.1.1 Paper I

The main result in paper I, the inverse association between sun exposure and MS risk, has been corroborated in many other studies (see section 2.2.3), although it is difficult to draw conclusions regarding the amount of sunlight exposure that would be protective, due to methodological differences. The studies by van der Mei et al (118), Lucas et al (187) and Bjørnevik et al (121) are most similar to our study, and therefore their findings will be briefly compared with ours. Overall, the results of these studies all indicate that low exposure to sunlight/UVR increases the risk of MS, where the magnitude of the risk estimate is approximately 50%. However, there are questions, addressed by our study, worth considering:

- a) What is the time window of exposure to sunlight/UVR, i.e. when does the UVR act on the human body to influence the pathological process leading to CNS demyelination?
- b) Is the effect of sunlight/UVR due only to vitamin D production or does it have an independent effect on MS risk?
- c) Is there an interaction between vitamin D/UVR and the MS risk gene HLA-DRB1\*15?

To address question a) we measured the exposure of interest (sunlight exposure habits) using questions regarding exposure in the last 5 years, which partly corresponded to time with disease. The questions were framed in this manner to facilitate response, as questions regarding earlier life might be difficult to answer (difficult to remember). However, the problem of reverse causation inevitably may occur. In favour of our way of posing the questions, it could be claimed that it is improbable that sun exposure habits would have changed early in the disease course and the risk of reverse causation being the only explanation of the findings would be low. The measurement would then reflect earlier exposure, provided sun exposure behaviour is stable during life, and/or exposure during adulthood. The studies mentioned above all examined sun exposure habits during childhood

and adolescence, because it is generally believed that the susceptibility period for developing MS is before the age of 20, but they also examined exposure during adulthood. The results, however, differ significantly. van der Mei et al (118) and Bjørnevik et al (121) did not find any significant association between sunlight exposure in adulthood (measured 3–10 years before study inclusion or for the age range 25–30 years), but only in childhood and adolescence. However, the results of the latter study might have been influenced by selection bias as the response proportion for the controls was only 20.8% and 36.3% for participants from Italy and Norway, respectively. Lucas et al (187) investigated sun exposure and the risk of a first demyelinating event and found, surprisingly, no association at all with self-reported sun exposure in childhood/adolescence. By contrast, recent high sun exposure (during the last 3 years) was associated with decreased risk of being a case (adjusted OR 0.85–0.70 for MS with high sunlight/UVR exposure), which is in line with our findings. In conclusion, the findings are conflicting with regard to which period is important for sun exposure in influencing MS risk: childhood, adolescence or adulthood. These divergent findings are certainly to some extent due to differences in methodology (prevalent or incident cases, age ranges examined or how the questions aimed to capture sunlight exposure are formulated). However it is also possible that all findings are correct and that environmental factors may influence MS risk differently in different stages including in adulthood. A hypothesis to explain the possible causal pathways influenced by causative agents at several time points in life, including shortly before symptom onset, has been proposed by Goodin (188), and other authors have also suggested that MS development may be influenced by the environment in adulthood (189).

Question b) has only been addressed by our study and by the study of Lucas et al (187). In both studies UVR exposure and vitamin D levels were associated with MS risk (when adjusting one exposure for the other), indicating independent effects on the development of MS, as is also suggested by other experimental findings (outlined in section 2.2.1). In our study we used single measurements of vitamin D, which do not necessarily reflect the aetiologically relevant period. Lucas et al also used single measurements but examined individuals at an early moment in the disease course (CIS cases) which gives further strength to their findings. Nonetheless, neither study provides firm evidence for the conclusion of independent effects, which mainly rely on knowledge regarding the immunosuppressive functions of UVR as well as of vitamin D. Further clinical trials investigating UVR and MS, such as the one being performed in Australia (107), will be important for elucidating this relation.

Finally, question c) was initially addressed by Ramagopalan et al (190). The authors found a VDRE in the promoter region of HLA-DRB1\*15 in lymphoblastoid cells collected from Canadian patients (mainly of Caucasian/Northern European descent). This region was highly conserved (having been preserved during evolution, indicating its functional importance for the organism) and the expression of HLA-DRB1\*15 increased when adding 1, 25-dihydroxyvitamin D<sub>3</sub>, showing a functional link between this important genetic risk factor and the environmental factor vitamin D. This suggests that vitamin D deficiency would lead

to lower HLA-DRB1 expression in the thymus and subsequently lack of deletion of autoreactive T cells which would increase the risk of autoimmune disease. This study was replicated by Cocco et al (191) in Sardinia, where the MS prevalence is high but with a different genetic makeup compared to populations in other high-risk areas. In Sardinia HLA-DRB1\*15 is rare and other HLA haplotypes are more common and associated with MS (e.g. DRB1\*13:03 and DRB1\*04:05). The authors found VDREs in close proximity to several of the examined HLA haplotypes, but they did not find a consistent HLA expression increase when adding vitamin D and some of the VDREs were not functional. In summary, the findings of Ramagopalan et al were not unequivocally replicated, however this might be explained by ethnic differences in susceptibility to vitamin D deficiency.

To our knowledge, ours is the only study to address the question of interaction between vitamin D and HLA-DRB1\*15 in a non-experimental fashion. The term “interaction” means in this context, that two causal factors together/combined take part in a sufficient cause, i.e. they are constituents in a causal pathway that is different from the causal pathways that contain each of the risk factors alone (2). That is, the presence of interaction provides knowledge about the existence of a causal mechanism, where the presence of both risk factors is needed. Knowledge of different causal pathways is of course essential for understanding disease mechanisms and for disease prevention. The risk factors combined give an increased risk that is more than the sum of the separate effects (i.e. more than additive). This is illustrated by the formula:

$$RERI=RR_{AB}-RR_A-RR_B+1$$

where  $RR_{AB}$  is the relative risk of disease if both risk factors (A and B) are present,  $RR_A$  is the relative risk of disease if only one risk factor (A) is present and  $RR_B$  is the relative risk of disease if the other risk factor (B) is present. In our study we chose to present the interaction measure AP, which is calculated according to the formula:

$$AP=RERI/RR_{AB}$$

where an AP larger than 0 indicates the presence of interaction. We did not find any interaction between HLA-DRB1\*15 and the environmental exposures, neither with vitamin D levels in plasma nor with sun exposure habits/UVR exposure. This finding is difficult to reconcile with the experimental findings of Ramagopalan et al, and certainly merits further research in different populations.

### 6.1.2 Paper II

In this study we found a protective effect of weekly fatty fish consumption, with an adjusted OR for MS of 0.82 (95% CI 0.68–0.98). The exposure was measured during the previous 5 years, which partly corresponded to time with disease. The reason for using this exposure period was the same as outlined for paper I, regarding the sun exposure questions. The question of reverse causation was addressed by sub-analyses restricted to only those with a maximum of 2 years since disease onset, and similar results were obtained. Furthermore,



there was no significant difference in the number of cases versus controls reporting recent diet changes. Finally, individuals who reported high fatty fish intake had significantly higher levels of serum vitamin D. These findings support our conclusion that the observed association is not due to reverse causation or recall bias but can be attributed to the effect of vitamin D from fatty fish consumption.

Several other studies have investigated the impact of fish intake on MS risk. Zhang et al (192) used data from the large NHS cohort, and examined the association between different self-reported nutrient intakes, with a special focus on dietary fat. Cohort studies are by design less subject to biases such as recall bias and reverse causation (which may have affected our study, although we have tried to address these issues) and their results are therefore generally considered to give stronger evidence than case-control studies. Zhang et al found no association between fish intake and MS incidence, in contrast to our findings. However, the results cannot be easily compared, because the authors analysed intake of fish together with other seafood (shrimp, crayfish and lobster) with a very low vitamin D content (193), and did not separate fatty fish from lean fish. A possible association between fatty fish intake and MS may thus have been obscured.

Cortese et al (194) used the same data as Bjørnevik et al (121), but included only the Norwegian participants, and the primary exposure in their study was cod liver oil (rich in vitamin D) and its association with MS. They also analysed fatty fish consumption (although mainly as a covariate) and exposure measurements were fairly similar to ours (195), but only the age range 13-19 years was considered. They found a significant MS risk reduction (adjusted OR 0.92, 95% CI 0.86–0.99) with a 1 unit increase in their “fish intake score”, however the association was no longer significant after adjusting for cod liver oil intake. Their results are in line with ours, further supporting the view that increasing fatty fish intake is associated with decreased MS risk, at least in Scandinavian countries.

Because autoimmune diseases to some extent have overlapping genotypes (161), findings regarding associations between a certain exposure and other autoimmune diseases may strengthen the conclusion that associations seen between the exposure in question and MS are real and causal. Löfvenborg et al (196) evaluated fatty fish consumption and the risk of latent autoimmune diabetes in adults, and found a significantly decreased risk of disease with an adjusted OR of 0.51 (95% CI 0.30–0.87) for weekly fatty fish intake. Rosell et al (197) found a 20% decreased risk (OR 0.8, 95% CI 0.6–1.0) of developing RA for people who consumed fatty fish at least once a week. Consequently, these results further strengthen our findings and, given the known immunomodulatory properties of vitamin D, it seems probable that it is vitamin D that is responsible for the effect on risk, although other substances such as omega-3 fatty acids have also been suggested by both Löfvenborg et al and Rosell et al.

### **6.1.3 Paper III**

Our study on the association between vitamin D levels at birth, evaluated as a continuous variable or in quintiles, and later risk of MS did not reveal any signs of an association, even

when a suggested degradation over time was taken into account. This lack of an association may be genuine or it may be due to factors we were not able to take into account in our study, such as unknown confounding. Also, the neonatal vitamin D levels were generally low, with a mean value for the whole population of 29.7 nmol/L (SD 17.3, median 25.6 and interquartile range 17.0–38.4 nmol/L). Such values are all equivalent to vitamin D deficiency, possibly obscuring a potential association between low vitamin D values and MS, as there were almost no individuals with normal levels for comparison. To our knowledge, our study is unique in investigating neonatal vitamin D levels and MS. However two studies have investigated vitamin D levels in pregnant women and the later risk of MS in their offspring. Salzer et al (198) were able to identify 37 MS patients whose mothers had provided blood samples during pregnancy and stored in a biobank. Of these, 78% of the samples had been collected during the first trimester. The 37 mothers were matched to 185 mothers who did not have children with MS. There was no significant association between vitamin D levels in the mothers and MS risk in their children; for vitamin D levels  $\geq 75$  nmol/L (compared to  $< 75$  nmol/L), the adjusted OR was 1.8 (95% CI 0.53–5.8). The authors cautioned against drawing firm conclusions from their study due to the small sample of only 37 cases, and the wide CI values also imply that there might be a lack of power to detect a real effect. Munger et al (199) used stored blood samples from pregnant women in Finland, and matched mothers whose children later developed MS to mothers whose children did not (193 cases and 331 controls, respectively). The blood samples were also drawn mainly during the first trimester. In this study, a significant association was found between decreasing vitamin D levels in the mother and increasing MS risk in the child. The authors analysed quintiles of vitamin D levels and found an increased risk of MS of 20–90% in the lowest quintiles (equivalent to vitamin D levels of  $< 31$  nmol/L) compared to the highest quintile, although the trend was not statistically significant. When analysing vitamin D as a continuous variable, an increase of 50 nmol/L was associated with a non-significantly reduced risk (RR 0.52, 95% CI 0.22–1.19).

The findings from these three studies may seem contradictory but are not necessarily so. The sample size in the study by Salzer et al was probably too small for firm conclusions to be drawn. The negative result in our study and the positive result in the study by Munger et al are possible to reconcile by taking into account the fact that the blood samples were drawn at different times (first trimester versus at birth) in the life of the child who would later develop MS. It is known that the immune system begins to develop during the very early weeks of gestation (200) and it might be possible that vitamin D deficiency could impact the immune system differently at various time points, where the first trimester may be the crucial time and the third trimester (reflected in our study using vitamin D levels at birth) may be less important.

#### **6.1.4 Paper IV**

In this study we used the methodology known as “Mendelian randomization” to evaluate the influence of vitamin D on the risk of developing MS. We found that a GRS, composed of three genetic variants all associated with vitamin D levels (where an increasing score was

equivalent to increasing vitamin D levels), was associated with decreasing MS risk (for a 1-unit increase in score: adjusted OR 0.85, 95% CI 0.76–0.94), strengthening the causal role of vitamin D deficiency.

To our knowledge, only one study using the same methodology and evaluating vitamin D-related genotypes and MS risk has been performed. Mokry et al (201) found that four SNPS, all located in or near genes involved in vitamin D metabolism, were associated with MS, and using a meta-analysis approach they found that the pooled estimate for the combined effects of these SNPS on MS risk suggested an association between decreasing vitamin D levels and MS. In the fixed effects model, the OR for MS, for each 1-SD decrease in natural log-transformed 25-hydroxyvitamin D levels, was 2.02 (95% CI 1.65–2.96). This result is consequently in line with our findings.

## **6.2 METHODOLOGICAL CONSIDERATIONS**

### **6.2.1 Selection bias**

Selection bias occurs if the included cases and selected controls differ in their exposure status from those not selected or from those choosing not to participate, and will give rise to a different association between exposure and disease among participants and non-participants. This may occur, for example, through improper sampling of controls (if the controls are not chosen from the same source population as the cases) or through low response proportions.

In the EIMS study, the cases were included at neurology clinics located all over Sweden. Because access to healthcare is available at low cost in Sweden, and all MS patients are diagnosed and treated by neurologists, we consider it unlikely that a substantial proportion of newly diagnosed MS patients would not be identified, especially as there is a high awareness of MS, and of available effective treatments, in the general population leading people to seek healthcare. Nevertheless, we know that some MS patients are not asked to participate in EIMS, mostly because of administrative reasons such as lack of staff time. Because such reasons are not related to exposure status, biased estimates should not arise. At the time the exposure information for papers I and II was collected, it was not commonly known in the general population that sun exposure and vitamin D were associated with MS. For this reason, we consider it implausible that the non-participation of the cases in filling out the questionnaire would be related to the exposure in papers I-II, neither in paper III (neonatal vitamin D levels) nor in paper IV (vitamin D-related genes) where the participants were likewise unaware of their exposure status.

The choice of controls is important and should represent the exposure distribution in the source population giving rise to the cases. In the EIMS study the controls were chosen from the population register and matched for residential area. Access to a certain neurology clinic is dependent on residential area, which ensures that the controls come from the same source population as the cases. The response proportion among the controls was around 70% for the questionnaire and around 50–60% for the blood samples and may therefore have given rise to a selection bias. Depending on the exposure distribution among the non-respondents, the

strength of the estimated association between exposure and disease would be over- or underestimated. Nevertheless, the proportion of never-smokers among controls in papers I and II was comparable to the figure in the general population (58%) (202), suggesting that the participating controls were representative of the general population with regard to lifestyle habits.

In paper III, in which the main result was dependent on available blood samples, an analysis of non-participation among the controls was performed and showed no significant differences regarding potential confounders except for sex and education, with non-participants more likely to be men and have a low level of education. If these factors had been associated with neonatal vitamin D deficiency, non-participation would have biased the result away from the null. Because the results showed no association between MS and neonatal vitamin D levels, this is not an issue.

In paper IV, the main exposure was genetic polymorphisms in vitamin D-related genes, and we find it improbable that reasons for non-participation would be associated with this exposure. The Swedish participants included in this project mainly came from the GEMS study which recruited cases from the MS register during the period 2009–2011, when the register covered around 60% of the MS patients in Sweden (203). Theoretically, this might have caused selection bias. On the other hand, the fundamental reason for not being in the register was that the use of the register was not developed or extended to the whole country. The low response proportion, particularly among the controls and with regard to blood donation, may nevertheless have given rise to selection bias. Furthermore, the proportion of controls with a higher level of education was greater than in the general population (204) indicating that non-responders were overrepresented in lower socioeconomic classes. As education/socioeconomic status may be associated with ethnicity, which in turn is associated with genetic polymorphisms, non-participation among people with a low educational level might give rise to a biased estimate due to differential selection of individuals from ethnic groups with a higher prevalence of a high educational level. If the polymorphisms associated with vitamin D sufficiency (our exposure) were more prevalent among people from ethnic groups with a low level of education, the estimate would then, due to non-participation among the poorly educated controls, be biased away from the null; this is an example of population stratification. However, the Swedish population is fairly homogeneous, and the American population was restricted to one ethnic group (white non-Hispanic), and measures for handling population stratification were used in the analysis stage. We consider it improbable that our results would be due only to selection bias through population stratification.

### **6.2.2 Misclassification of disease**

The diagnosis of MS is made according to the internationally accepted McDonalds criteria (6, 7). During the study periods of the papers included in this thesis, two different versions of the McDonalds criteria have been used (the revisions of 2005 and 2010). These versions do not differ significantly, other than in time to confirmed diagnosis of MS which is shorter with the

latest version from 2010 (205). Misclassification of disease in the EIMS study is considered to be low, as all patients are diagnosed by a neurologist and, as reported by Hedström et al (52), the MS diagnosis was confirmed by MRI (magnetic resonance imaging) for 98% of the patients included in the study. Furthermore, at inclusion the personnel at the neurology clinics must specify whether the patient fulfils the McDonalds criteria and the type of MS. At the start of each project in this thesis, this information was checked manually and all patients not fulfilling the McDonalds criteria were excluded.

In the GEMS study, the cases were identified through an MS-specific healthcare register and cases not fulfilling the McDonalds criteria were excluded.

In the KPNC study, the possible cases were reviewed by neurologists and validated through medical records.

Still, possible misclassification of disease may have occurred (MS cases classified as such but without MS). Nonetheless, because at the start of this research project there was a lack of knowledge about important risk factors for MS, except for carriage of HLA-DRB1\*15 (which is not easily known by the physicians diagnosing MS or by the study participants), exposure status may not have influenced the diagnostic procedure. A possible misclassification of disease may accordingly have been non-differential and biased the estimates towards the null.

### **6.2.3 Misclassification of exposure**

Misclassification of exposure to some extent always occurs in observational studies. In general, if the misclassification is non-differential, i.e. not related to disease status, it leads to the estimate being biased towards the null (for exposed versus unexposed categories, i.e. in dichotomous categorization). Differential misclassification occurs when the misclassification of exposure is related to having the disease or not and may influence the estimated associations either away from or towards the null. In the observational studies used in this thesis, the environmental exposure information has been collected through extensive questionnaires in which all questions were given equal importance. When collecting exposure information after disease onset, there is always a risk of recall bias, i.e. differential reporting between patients and controls because of patients' knowledge of their own disease. However, because during the study periods there was a lack of general knowledge in the population about environmental risk factors for MS, including about the risk factors (UVR exposure, vitamin D levels, fatty fish intake) investigated in this thesis, the possibility was low that the cases specifically reported these exposures differently from the controls, although this cannot be ruled out. In addition, when possible as in paper II, the environmental exposure information on fatty fish intake has been validated by using objective data such as vitamin D levels, showing that consumers of high fatty fish intake had higher vitamin D levels. Misclassification of the main exposures in paper III (vitamin D levels at birth) and paper IV (genetic polymorphisms) is certainly not affected by disease status as the disease status of the individuals was unknown by the persons responsible for determination of vitamin D levels

and the polymorphisms. In papers I and II there is also a possibility of reverse causation, as well as recall bias. We have taken this into account by performing restricted analyses on individuals with very short disease duration (papers I and II) and consequently less probability of changing lifestyle habits due to disease or of having changed their thinking about possible causes of their disease (which might lead to differential recall between patients and healthy individuals). In paper II we also evaluated the proportion reporting changed dietary habits and found no significant difference between cases and controls, strengthening the conclusion that the observed association is not due to lifestyle changes among the cases.

#### **6.2.4 Confounding**

Using data from large observational studies with exposure information gathered from questionnaire data we were able to evaluate a large number of possible confounders (as listed in sections 4.4–4.7). Possible confounding factors were evaluated based on our knowledge of the subject and using statistical methods (see section 4.8). At the same time, residual confounding might still affect the results, either due to unknown confounding, mismeasurements of the confounding factors and confounders we did not have information about or due to insufficient adjustment, for example by the use of broad categories for the confounders.

## 7 SUMMARY

In this thesis we have presented the following findings:

- Low sunlight exposure is associated with increased risk of developing MS and this association is not influenced by HLA-DRB1\*15 status. Low sunlight exposure seems to influence MS risk even in adulthood and not only in early life or adolescence.
- UVR/sunlight exposure may influence MS risk irrespective of the immunomodulatory effect of vitamin D.
- High fatty fish intake, as a proxy for high vitamin D oral intake, decreases the risk of MS.
- Vitamin D levels at birth do not influence the risk of developing MS in adulthood.
- A GRS composed of genotypes disposing towards higher vitamin D levels is associated with MS, where a higher score corresponds to lower risk of MS development. This finding strengthens the likelihood of a causal association between vitamin D deficiency and increased MS risk.

## 8 FUTURE PERSPECTIVES

The case-control studies that have been used in this thesis, the EIMS and GEMS studies, have generated an immense amount of data on both environmental and genetic factors that may influence the risk of MS. Recent environmental epidemiological MS research has highlighted several conclusions with regard to MS and risk factors such as sunlight, smoking, obesity and IM. Some issues related to this field have been considered in this thesis, but many questions remain to be answered. The next step would be to investigate gene-environment interactions, and environment-environment interactions, with a focus on sunlight and vitamin D. The risk factors EBV seropositivity and history of IM have not been examined in the present studies, for a number of diverse reasons. EBV and/or IM have been suggested to interact with sunlight/vitamin D levels in influencing the risk of MS (206-208). In these studies, sunlight exposure has been measured using atmospheric data or latitude, and not through individual-level sunlight exposure, which is available in the EIMS and GEMS studies and which might provide more detailed knowledge regarding this association.

Further, the field of gene-environment interactions, regarding both HLA and non-HLA genotypes (such as the genotypes involved in vitamin D metabolism) and their interaction with sunlight/vitamin D, is vast, and the possibility of including information on other potential environmental risk factors such as obesity and smoking opens the way for many future studies, with the goal of more deeply understanding the causes of MS.



## 9 SAMMANFATTNING PÅ SVENSKA

MS är en kronisk neurologisk sjukdom som vanligen diagnostiseras när man är mellan 20-40 år. I Sverige har ca 17000 personer MS och varje år insjuknar ca 500 personer. Symtomen på MS är mycket varierande: nedsatt syn, nedsatt känsel, balansrubbing, nedsatt styrka, blåspåverkan, trötthet, smärtor samt minnes- och koncentrationssvårigheter. Symtomen kommer och går vanligen i skov och med tiden kan en permanent funktionsnedsättning uppstå. MS är en autoimmun inflammatoriskt betingad sjukdom där inflammation uppstår i det centrala nervsystemet (hjärna och ryggmärg). Orsaken till att inflammationen uppstår är inte helt känd men man vet att vissa gener och vissa miljöfaktorer bidrar till att sjukdomen bryter ut. Syftet med denna avhandling var att undersöka betydelsen av vitamin D för uppkomsten av MS.

Avhandlingen är baserad på data från EIMS-studien som är en svensk befolkningsbaserad fall-kontrollstudie som startade 2005 och fortfarande pågår. I denna studie inkluderas personer som inom de senaste två åren fått diagnosen MS i enlighet McDonaldskriterierna (incidenta fall) och som är mellan 16-70 år och behärskar svenska. För varje fall identifieras två friska kontrollpersoner via befolkningsregistret och matchas till fallen avseende kön, ålder och bostadsort samt tillfrågas per brev om deltagande i studien. Deltagande är frivilligt för samtliga. Fall och kontroller ombeds fylla i en omfattande enkät avseende levnadsvanor och olika miljöfaktorer samt lämna blodprov som används för genotypning. Svarsfrekvensen har varit 91% för fallen och 70% för kontrollerna avseende enkäten och 94% respektive 57% för blodprovsinlämningen. För den sista studien i denna avhandling har även använts data från två andra studier: GEMS-studien och KPNC-studien. GEMS-studien är en svensk studie på prevalenta MS-patienter som identifierats via MS-registret. Patienterna, samt friska kontroller som identifierats via befolkningsregistret, tillfrågades per brev om studiedeltagande och fick besvara en enkät och lämna blodprov. Totalt 7706 MS-patienter och 8570 kontroller tillfrågades och svarsfrekvensen var 82% för fallen och respektive 66% för kontrollerna. I KPNC-studien har patienter identifierats via journaler samt tillfrågats per brev om att lämna enkätinformation och blodprov. Svarsfrekvensen i KPNC-studien för enkätinformationen var för fall och kontroller 80% respektive 66%.

Studieresultaten i avhandlingen visade att låg solljusexponering ökade risken för MS med ca 60% (om man jämförde de som uppgav att de aldrig solade jämfört med de som solade dagligen). Låga vitamin D-halter i blod var också associerat med ökad MS-risk. Vi fann inga tecken till interaktion mellan dessa miljöfaktorer (solljus respektive vitamin D) och den viktigaste genetiska riskfaktorn HLA-DRB1\*15. Fet fisk är, förutom solljus, den viktigaste källan till vitamin D för människor. Personer som uppgav att de åt fet fisk minst en gång per vecka hade ca 20% lägre risk att utveckla MS jämfört med de som uppgav att de aldrig åt fet fisk. Vitamin D-halter i blodprov tagna strax efter födelsen analyserades avseende risken för uppkomsten av MS senare i livet och ingen association kunde ses, vilket tolkades som att vitamin D-halterna i nyföddhetsperioden inte är av betydelse för MS-sjukdomens utveckling. För att utröna om kopplingen mellan vitamin D-brist och MS, som rapporterats i flera

vetenskapliga studier, innebär ett kausalt samband så studerades associationen mellan en genetisk riskskala, uträknad utifrån tre genotyper som predisponerar för hög vitamin D-halt, och MS. Högre värden på skalan indikerade högre vitamin D-halter under hela livet, och högre värden visade sig vara signifikant associerade med lägre MS-risk, vilket tolkades som att ett kausalt samband föreligger.

Framtida forskning behövs för att utröna om interaktion föreligger mellan vitamin D och andra miljöriskfaktorer och andra kända genetiska riskfaktorer och hur detta påverkar risken att utveckla MS.

## 10 APPENDIX

### 10.1 QUESTIONNAIRE

Questions from the EIMS questionnaire, used for measuring the main exposures of interest (papers I and II). The questions have been translated from Swedish to English.

#### *Paper I: UVR/sun exposure habits:*

##### **1. How often, during the last 5 years, have you used a sunbed?**

1. Never      2. A few times a year      3. Once a month      4. Once a week

##### **2. How often, during the last 5 years, have you visited a country that is sunnier than Sweden?**

1. Never      2. More seldom      3. Once a year      4. More than once a year

##### **3. If the weather is sunny, how often do you usually sunbathe?**

1. Never      2. A few times a month      3. A few times a week      4. Daily

#### *Paper II: Intake of fish:*

**Specify how often, on average, you have eaten these classes of fish during the last 5 years.**

##### **1. Fatty fish (i.e. herring/mackerel/tuna/salmon/trout)**

1. Never/seldom      2. 1–3 times/month      3. Every week      4. Daily

##### **2. Low-fat fish (i.e. cod/pollock/haddock/whiting/pikeperch)**

1. Never/seldom      2. 1–3 times/month      3. Every week      4. Daily

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