Umeå University Medical Dissertations, New Series No 1545

# Environmental risk factors for multiple sclerosis

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Umeå 2013

Responsible publisher under swedish law: the Dean of the Medical Faculty

ISBN: 978-91-7459-545-1
ISSN: 0346-6612
Cover image: The sun over the Gulf of Bothnia just south of Umeå, at noon the 25th of December 2011 (4 days after the winter solstice). Photograph by Jonatan Salzer.
Electronic version available at http://umu.diva-portal.org/
Printed by: Print & Media, Umeå University
Umeå, Sweden 2013

*"Whoever wishes to investigate medicine properly should proceed thus: in the first place to consider the seasons of the year, and what effects each of them produces. Then the winds, the hot and the cold, especially such as are common to all countries, and then such as are peculiar to each country. In the same manner, when one comes into a city in which he is a stranger, he should consider its situation, how it lies as to the winds and the rising of the sun; for its influence is not the same whether it lies to the north or the south, to the rising or the setting of the sun. One should consider most attentively the waters which the inhabitants use, whether they be marshy and soft, or hard and running from elevated rocky situations, and then if saltish and unfit for cooking, and the ground, whether it be naked and deficient in water, or wooded and well watered, and whether it lies in a hollow and confined situation, or is elevated and cold; and the mode in which the inhabitants live, and what are their pursuits, whether they are fond of drinking and eating to excess, and given to indolence, or are fond of exercise and labor."*

(Hippocrates, the father of modern medicine, approximately 400 BC; citation widely available online)

# Publications and manuscripts

I Salzer J, Svenningsson A, Sundström P. Season of birth and multiple sclerosis in Sweden. Acta Neurol Scand 2009;121:20-23. Corrected by Erratum 2010;122:70-73.

II Salzer J, Hallmans G, Nyström M, Stenlund H, Wadell G, Sundström P. Vitamin D as a protective factor in multiple sclerosis. Neurology 2012;79:2140-2145.

III Salzer J, Hallmans G, Nyström M, Stenlund H, Wadell G, Sundström P. Vitamin A and systemic inflammation as protective factors in multiple sclerosis. Mult Scler 2013 [Epub ahead of print, DOI: 10.1177/1352458512472752].

IV Salzer J, Hallmans G, Nyström M, Stenlund H, Wadell G, Sundström P. Smoking as a risk factor for multiple sclerosis. Mult Scler 2012 [Epub ahead of print, DOI: 10.1177/1352458512470862].

V Salzer J, Nyström M, Hallmans G, Stenlund H, Wadell G, Sundström P. Epstein-Barr virus antibodies and vitamin D in prospective multiple sclerosis biobank samples. Submitted manuscript.

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# Abstract

**Background**
Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system. It usually strikes during young adulthood, and 2.5 million individuals are estimated to have the disease worldwide. The causes of MS are not known, but several factors have been shown to be associated with the risk of the disease, including certain genes, vitamin D, smoking and Epstein-Barr virus infection. Little is known about how/if these factors interact.

**Methods**
Study I: The risk of MS by month of birth was investigated using MS cases from the Swedish MS registry and using general population controls. Studies II–V: We identified MS cases who had donated blood prior to disease onset, and MS cases whose mothers had donated blood during pregnancy, by cross-linking a database of MS cases, and a database of mothers of MS cases, to two local biobank cohorts. One of them consisted of blood samples collected during early pregnancy, and one with samples collected during health controls. Levels of 25(OH)D (25-hydroxyvitamin D), RBP (retinol binding protein, a surrogate marker for vitamin A), CRP (C-reactive protein), cotinine (a nicotine metabolite) and anti Epstein-Barr virus nuclear antigen-1 (EBNA-1) antibodies were measured in cases and matched controls. The risk of MS by categories of these exposures was estimated in bi- and multivariable matched logistic regression models.

**Results**
Subjects born in spring had a higher risk of MS, but no influence of early gestational levels of the measured risk factors on the risk of MS in the offspring was observed. In prospective samples from MS cases and controls, 25(OH)D levels ≥75 nmol/l, intermediate RBP levels, and elevated CRP levels in young were associated with a decreased risk of MS. Elevated cotinine levels (suggestive of smoking) and high antibody reactivity against EBNA-1 were associated with an increased risk of MS. All factors but RBP were more clearly associated with MS in young subjects.

**Conclusion**
All factors analyzed in prospectively collected samples were associated with the risk of MS, and taken together, the data indicate that the key etiopathological events that lead to MS occur before the age of 20–30. Study II provides support for trials exploring the primary preventive potential of oral vitamin D supplementation.

# Glossary

| | |
|---|---|
| 1-OHase | 25-hydroxyvitamin D-1α-hydroxylase |
| $1,25(OH)_2D$ | 1,25-dihydroxyvitamin D |
| 25(OH)D | 25-hydroxyvitamin D |
| AU | Arbitrary Units |
| BBB | Blood brain barrier |
| CNS | Central nervous system |
| CSF | Cerebrospinal fluid |
| DNA | Deoxyribonucleic acid |
| EBNA-1 | Epstein-Barr nuclear antigen-1 |
| EBV | Epstein-Barr virus |
| FDE | First Demyelinating Event |
| HLA | Human leucocyte antigen |
| hs-CRP | Highly-sensitive C-reactive protein |
| IgG | Immunoglobulin G |
| IOM | Institute of Medicine |
| IU | International Units |
| MBR | Medical Birth Registry |
| MHC | Mayor histocompatibility complex |
| MRI | Magnetic resonance imaging |
| MS | Multiple sclerosis |
| NBHW | National Board of Health and Welfare |
| NMO | Neuromyelitis optica |
| NSHDS | Northern Sweden Health and Disease Study cohort |
| NSMC | Northern Sweden Maternity Cohort |
| PML | Progressive multifocal leucoencephalopathy |
| PPMS | Primary progressive multiple sclerosis |
| PRMS | Progressive relapsing multiple sclerosis |
| RA | Retinoic acid |
| RAR | Retinoic acid receptor |
| RARE | Retinoic acid response element |
| RBP | Retinol binding protein |
| RCT | Randomized controlled trial |
| RDI | Recommended daily intake |
| RRMS | Relapsing-remitting multiple sclerosis |
| SPMS | Secondary progressive multiple sclerosis |
| UL | Tolerable upper intake level |
| VDRE | Vitamin D responsive element |
| VDR-RXR | Vitamin D receptor–retinoic acid x-receptor complex |

# Kort sammanfattning på svenska

**Miljöfaktorers betydelse för multipel skleros**

Multipel skleros (MS) är en inflammatorisk sjukdom som drabbar det centrala nervsystemet (hjärna och ryggmärg). Sjukdomen debuterar vanligen mellan 20 och 40 års ålder, och kan leda till symtom såsom förlamning, känselstörningar, synstörningar, smärtor och balansbesvär. Symtomen kommer och går i skov hos majoriteten av de drabbade.

Man vet idag inte vilka orsakerna till MS är, men de flesta är överens om att det krävs en kombination av gener och miljöfaktorer för att sjukdomen ska utvecklas.

Vi har i Studie I analyserat risken för MS baserat på födelsemånad. I Studierna II–V har vi i norra Sverige sökt efter individer med MS som donerat blod till biobanker vid Umeå universitet innan sjukdomsdebut. Vi har också sökt efter blodprover donerade under graviditet där barnet senare utvecklat MS. Hos dessa, samt hos matchade kontroller utan MS (eller där barnet ej utvecklat MS), har vi sedan analyserat nivåer av D-vitamin (som i huvudsak bildas vid solljusexponering), RBP (en markör för A-vitamin), CRP (en markör för infektion/inflammation), cotinine (en nikotinnedbrytningsprodukt) samt antikroppar mot körtelfebervirus. Vi har sedan jämfört risken att utveckla MS baserat på halter av dessa faktorer.

Resultaten från studierna visade att höga D-vitaminnivåer samt höga CRP-nivåer (hos unga) kunde kopplas till en minskad risk för MS, medan höga nivåer av cotinine och körtelfebervirusantikroppar kunde kopplas till en ökad risk för MS. Risken för MS baserad på RBP-nivåer varierade U-format vilket antyder att normala (inte för höga och inte för låga) A-vitaminnivåer kunde kopplas till en minskad risk för MS. Vidare kunde vi också konstatera att samtliga faktorer förutom RBP var starkare kopplade till MS-risk hos unga individer, vilket antyder att den immunologiska grunden för sjukdomen läggs tidigt i livet, sannolikt innan 20–30 års ålder. D-vitaminnivåerna var överlag mycket låga. Detta kan till stor del kan förklaras av den låga mängden solljus vi exponeras för i norra Sverige, vilket illustreras av omslagsbilden som är tagen mitt på dagen en vinterdag i Umeå. Att vara född på våren innebar en något förhöjd risk för MS, men i det begränsade materialet från tidig graviditet (där effekt av årstidsvariationer dock ej kunde studeras) gick det inte att finna någon orsak till detta.

Mer forskning behövs för att klarlägga om D-vitamintillskott till befolkningen kan minska risken för MS. Det behövs också ytterligare studier som belyser samvariationen mellan de olika riskfaktorerna.

# Background

### Multiple sclerosis overview

Multiple sclerosis (MS) occurs with a yearly incidence (the number of new cases in the population each year) of about 5/100,000 in the western world. The prevalence (the number of subjects with MS alive at a specific date in the population) is about 100/100,000. There are indications that both those figures are increasing with time, and both increase with increasing distance from the equator.[1] Approximately 2.5 million individuals are estimated to have the disease worldwide.[2]

Since the first written clinical description of MS around 200 years ago, much has become known regarding the epidemiology, etiology, pathogenesis and treatment of the disease. MS is 2- to 3-fold more common among women than men, and strikes in the pro-, and reproductive ages between 20 and 40 years. The disease causes much suffering and disability, and it induces a need for fundamental changes in the way of life for many.[2]

Untreated, the disease may lead to stepwise, or gradually accumulating neurological disability, which makes it impossible to walk even short distances without crutches or other similar devices within a few years to decades, depending on the subtype and the level of disease activity.[3] The impact of MS on working ability is striking; among the $n$=399 MS cases in Västerbotten county in 1997, almost half were sick listed full-time.[4]

During the last 15 years, starting with the interferons, many new drugs have been developed, and the prognosis for an individual with newly diagnosed MS today differs markedly from that of a few decades ago.[5] Parallel to the astonishing pace at which new drugs are emerging, great progress has been made in the understanding of the epidemiological hallmarks of MS. Today we believe we know a great deal about how, and in some cases why, the occurrence of the disease varies over the world and between families. Several risk factors for MS have been proposed, although many of them have been refuted some time later. However, some remain and have been repeatedly shown to influence the risk of MS. Among these are genetic variations, most notably within the HLA complex, Epstein-Barr virus (EBV) infection, smoking and vitamin D levels.[6-8]

Even though patients, doctors, pharmaceutical companies, and stockholders benefit from the recent advances in the realm of MS treatment, prevention of the disease would undoubtedly be the most effective way to reduce the impact of MS on mankind. To even begin to design primary preventive MS studies, we first need to know the causes of the disease, and – for environmental factors – at what age in life they influence the disease risk.

**Pathogenesis, clinical and para-clinical features**

The neurological deficits seen clinically as relapses (attacks) in MS are mostly the result of white matter demyelination (loss of the protective fatty sheath) and axonal damage (nerve transection) at the sites of inflammatory plaques within the central nervous system (CNS). However, later pathological studies have shown lesions also within the grey, non-myelinated matter.[9]

Active lesions are enriched with lymphocytes, mainly T-cells and microglia, and these lesions arise when immune cells leave the blood vessels, pass through the blood-brain barrier (BBB), and enter the CNS.[2] It is currently not known why the BBB is compromised in MS, or what antigen within the CNS is recognized by the immune cells, but the myelin or the myelin-producing oligodendrocytes may be the target. With time, the lesions become inactive and show astrogliosis and hypocellularity. The demyelinated nerve fibers conduct electric impulses at a reduced velocity, and this can give rise to symptoms such as impaired muscle control, impaired muscle strength or sensory deficits. Demyelinated nerve fibers can also fire spontaneously or when stretched, causing radiating pain (Lhermitte's sign), or "cross-talk" (ephaptic transmission) causing paroxysmal symptoms such as trigeminal neuralgia. Other clinical manifestations of MS include loss of visual acuity (due to optic neuritis), dysarthria, diplopia, ataxia and more.[2]

In progressive disease, the axonal damage is more pronounced, and it was earlier believed that the inflammatory component no longer played a major role in causing disability,[2] although this dogma has been questioned lately. In recent overviews, one generally sees the loss of neurological function during the progressive phase of the disease as being, at least in part, driven by inflammation behind the BBB.[10]

The anatomical sites involved in the cerebrum are typically the juxtacortical and periventricular areas. MS also causes inflammatory damage to the anterior parts of the visual pathways, and to infratentorial sites (the brainstem, the cerebellum and the spinal cord). An active MS lesion lights up on MRI upon administration of gadolinium contrast, an effect of the compromised BBB and the inflammatory milieu, and usually has ill-defined margins, probably due to peripheral edema and inflammation. The inactive MS plaque is usually quite small (around 3–10 mm across), well circumscribed, often has an ovoid shape, and is usually arranged at right angles to the corpus callosum (following the projection of myelinated nerve fibers and venules). The spinal plaques seldom transect the spinal cord, but rather engage smaller parts of it and extend vertically, in the affected tract, seldom longer than one segment.[11]

Investigation of the cerebrospinal fluid (CSF) of individuals with MS often (in approximately 95% of cases) reveals oligoclonal (two or more) banding and/or an elevated IgG index.[2,12] Whether these IgG antibodies are merely a

by-product of the disease, or are a part of the MS pathogenesis remains unclear.[2]

### *Sub-types*

During the natural course of the disease different clinical pictures appear. MS typically starts as a relapsing-remitting disease (RRMS) with attacks of neurological disability that appear in a sub-acute manner over days or weeks. The symptoms then gradually decline over weeks to months. Sometimes the symptoms resolve completely and sometimes they cause persistent disability, accumulating over time with each subsequent relapse. After a period of time, from a few years to decades, in the majority of cases, the disease converts into a progressive phase with gradually increasing disability (secondary progressive MS [SPMS]).[3] Figure 1 depicts a schematic representation of the temporal evolvement of the disease according to clinical subtype. In some individuals (around 10-20%), more often old than young, without female preponderance, the disease is progressive from clinical onset with (progressive relapsing MS [PRMS]) or without (primary progressive MS [PPMS]) superimposed relapses.[2]

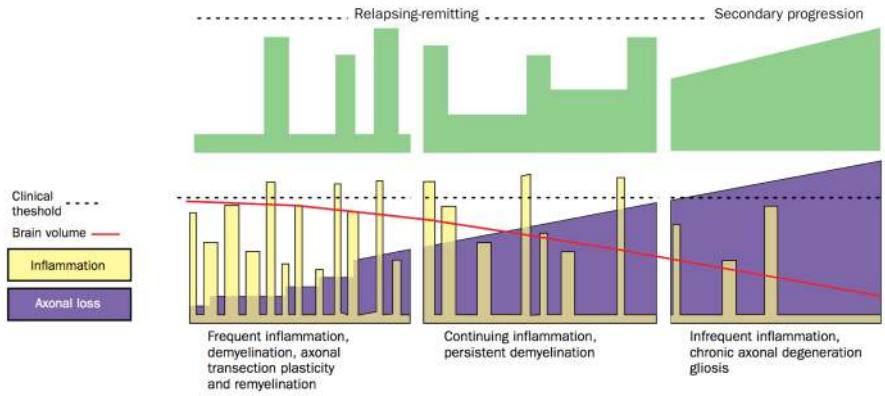


**Figure 1.**[2] Schematic representation of the temporal relationship between clinical symptoms (the top panel), inflammatory activity, brain volume changes and axonal loss (the bottom panel), according to clinical subtype in multiple sclerosis. © Elsevier. Reprinted with permission.

## Establishing a multiple sclerosis diagnosis

The diagnostic criteria for MS have varied over the years.[13-16] In general, with better imaging techniques, specific tests for differential diagnoses such as the NMO-antibodies in neuromyelitis optica, and new criteria, it has become easier to diagnose MS at an early stage. The key to diagnose MS is to demonstrate dissemination in time and space (DIT and DIS), which can be

done either by MRI, by clinical investigation, or both. The latest revisions, the 2010 revisions of the McDonald criteria, sometimes referred to as "any new, any two" have simplified the diagnostic procedure.[16] According to these criteria, after a clinical attack suggestive of MS, any new lesion appearing on a subsequent MRI, or the presence of contrast enhancing and silent lesions on the same MRI, is sufficient to establish DIT. Any two MS-like lesions, in the typical MS-locations (juxtacortical, periventricular and infratentorial), are sufficient to establish DIS.[16] Clinical evidence of DIT and DIS follows the same line of logic, where a history of two separate relapses (separate both in time and space) with corresponding clinical evidence is sufficient for the diagnosis. The use of CSF analyses varies, both nationally and internationally, and the results from such analyses are no longer included in the diagnostic criteria for MS.[16] The presence (or absence) of elevated IgG index and/or oligoclonal banding can be helpful, and CSF cell counts can argue in favor of, or against, possible differential diagnoses.

**Treatment**

Important aspects of MS care include spasticity treatment, pain alleviation, treatment of fatigue, pregnancy counseling, psychosocial care, and towards the end, nursing. Hopefully, the need for the latter will decrease considerably during the years to follow, as the new and emerging MS disease-modifying treatments will probably delay the time to accumulation of neurological deficits, as discussed in more detail below. These aspects of MS care are facilitated by so called MS teams, which are multidisciplinary teams of nurses, physiotherapists, speech therapists, counselors, dietitians and physicians, all working together to provide high quality care for MS patients. Among the symptomatic treatments that might be helpful are medicines that reduce or alleviate fatigue, urinary incontinence, pain, constipation, and spasticity, and medicines for psychiatric conditions.

The discovery of disease-modifying drugs in the 1990s has dramatically changed the landscape of MS care. First-line treatment of relapsing-remitting MS include the beta interferons and glatiramer acetate, which reduce the risk of subsequent clinical relapses by roughly 30%, reduce the number of new lesions seen on MRI, and prolong the time to sustained disability progression.[5,11] The side-effects of these drugs include injection site reactions, depression, anxiety, palpitations, dyspnea, elevated liver enzymes and leukopenia. No serious (i.e. life-threatening) long-term negative effects have been reported.

When first-line drugs fail to suppress the disease activity, or when an individual has a very active disease from the start, second-line treatments are warranted. These currently include intravenously administered monoclonal antibodies directed against lymphocyte surface molecules,[17,18] and one orally taken immunomodulatory pill preventing egress of

lymphocytes from lymph nodes.[19] All second-line regimens have a more pronounced effect on the number of clinical relapses, the MRI disease activity markers, and disease progression, compared with the first-line drugs. As expected, some grave side-effects are present. These include the potentially fatal condition progressive multifocal leukoencephalopathy (PML), autoimmune disorders, and infections. Many new drugs are being developed and will soon enter the clinical setting, presenting clinicians with a diverse flora of immunomodulatory drugs to use against the CNS inflammation in MS.[20] Efforts to find an effective treatment for progressive MS have been largely unsuccessful. The exception being subjects below 50 years of age with neuroradiological signs of active inflammation where treatment with a monoclonal B-cell depleting antibody may have a beneficial effect.[21]

## Epidemiology and risk factors

### *Background*

MS is a relatively rare disease, as mentioned above, and therefore the terms risk and odds will be used synonymously throughout this thesis. Most data regarding risk factors derive from calculations using odds and odds ratios.

The epidemiological features in MS are striking and probably hold clues to the etiopathogenesis to an even greater extent than presently understood. For example, we know that MS is more common among women than men, but we are not sure why. A deeper understanding of this might provide more clues to MS etiology. There is a latitude-dependent gradient in the occurrence of the disease, both in the northern and the southern hemispheres, with incidence and prevalence rates increasing with the distance from the equator, both between,[1,7] and within countries.[1,22] This pattern, together with a multitude of studies showing that a high degree of exposure to sunlight is associated with a lower risk of MS, points towards high vitamin D levels as a potential protective factor.[8] Vitamin D is presented in more detail below.

Migrational studies have shown that moving from a high- to a low-risk area in childhood reduces the risk of MS to a level close to that of the final residence, while moving during young adulthood only reduces the risk to an intermediate between the two areas. Migration in the opposite direction, however, does not seem to increase the risk substantially until the next generation, whose risk is close to that of their birthplace.[7,23] These data suggest an environmental factor, acting early in life, to determine MS risk. When compiling the evidence in 1995 from all the then available studies on MS risk and migration, Gale and Martyn concluded that an individual's risk of MS is largely established during the first two decades of life.[23] According to the hygiene hypothesis, having many infections early in life confers a long

lasting protection against autoimmunity, and this fits quite well with the pattern described above.[24]

***Epstein-Barr virus***

The only infectious agent that has been repeatedly shown to be associated with MS is the Epstein-Barr virus (EBV). A history of infectious mononucleosis (IM) – symptomatic EBV infection – is associated with a 2- to 3-fold increased risk of MS compared with the absence of IM. Seropositivity is associated with a 20-fold increased MS risk compared with seronegativity.[7,25] A recent review even suggested that no adult EBV seronegative MS cases exist when using proper laboratory methods for assessing EBV status.[26] A schematic illustration of the connection between MS and EBV is shown in Figure 2.

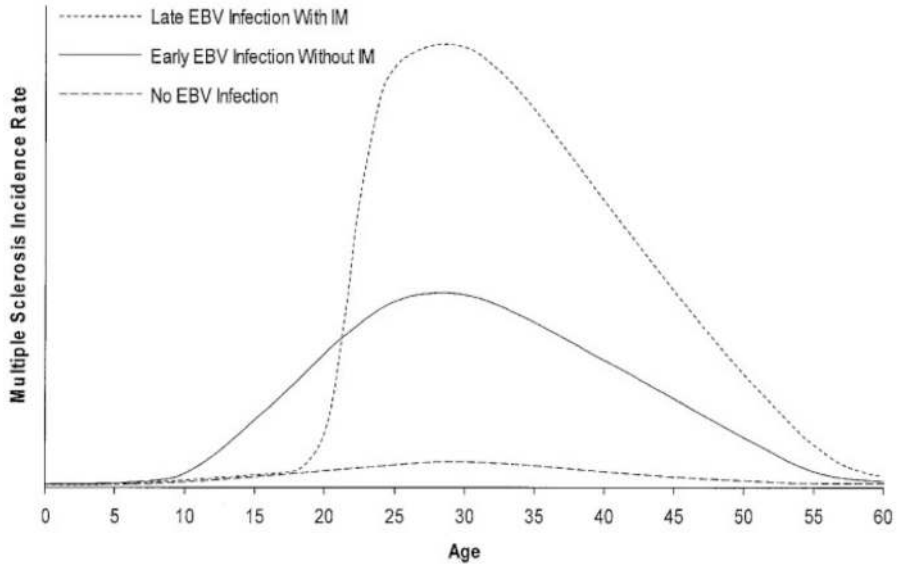


**Figure 2**.[25] Schematic representation of multiple sclerosis incidence according to Epstein-Barr virus infection status. This figure was published before reference 26, which suggests that the "No EBV Infection" group might have zero risk of developing MS. © John Wiley and Sons. Reprinted with permission.

EBV is highly prevalent in low-MS-risk areas, where most people are seropositive before two years of age, while half of the high-school students are seronegative in high-MS-risk areas.[27] As both early EBV infection, *and* seronegativity, seems to be associated with a lower MS risk, the hygiene hypothesis does not seem to apply entirely to MS etiopathogenesis in this context. The piece of this puzzle that is not explained by the hygiene

hypothesis is that the few adults not infected by EBV (about 5% of the adult population), who share the same hygienic environment as those with late infection, have virtually no risk of developing MS.[7,26] The interpretation of this may be that past EBV infection is a prerequisite to develop adult onset MS, and that the timing of the seroconversion is essential.

The role of EBV in MS pathogenesis has been reinforced by studies showing that high antibody titers against Epstein-Barr nuclear antigen-1 (EBNA-1), and particularly against one segment of the protein (amino acids 385–420), are associated with an increased risk of MS.[28-33]

In conclusion, past EBV infection seems to be necessary, but by no means sufficient, to develop adult onset MS, and more factors are obviously of interest when studying MS epidemiology.[34]

### *Smoking*

A history of cigarette smoking has in prospective questionnaire studies been shown to be associated with an increased risk of MS with risk estimates (relative risks) of 1.3–1.8.[35-38] Several retrospective questionnaire studies have also been performed,[39-46] and all but three[41,45,46] found an association between smoking and an increased risk of MS. A dose-response relation seems to be present, with a higher risk of MS with higher levels of smoke exposure.[8,44] Although two of the retrospective studies, which showed an increased risk of MS among ever smokers, investigated tobacco habits shortly after diagnosis,[43,44] thereby reducing the risk for recall bias, this potential disease-related influence on recall patterns cannot be completely excluded in the retrospective study design.[47] Furthermore, a certain measure of subjectivity is always present in questionnaire studies, and validation of subjects' answers by comparison with objective measures is usually necessary.

An objective way of measuring tobacco use is to measure levels of cotinine, a nicotine metabolite, which is a recognized marker for tobacco use with a half-life of 20 hours.[48] In a retrospective study elevated levels of cotinine have been associated with MS – even at modest levels suggestive of passive smoking.[49] A possible confounder in the context of determining the association between serum cotinine levels and MS is the use of smokeless tobacco (such as Swedish snuff), which also gives rise to increased levels of cotinine. Swedish snuff use may actually *decrease* rather than *increase* the MS risk.[44,50] Therefore, it is essential to establish whether the increased cotinine levels in this context depend on smoking or on smokeless tobacco use. Cotinine levels in prospectively collected MS samples have not been measured previously.

Regarding MS disease progression, smoking, as estimated by questionnaire data, seems to worsen MS prognosis by accelerating transition from relapsing-remitting to secondary progressive MS.[38,51]

### *Season of birth*

A season-of-birth effect has been shown in MS, with a higher risk of the disease with spring births, and a conversely lower risk with autumn births in the Northern hemisphere (Figure 3).[52-55] These findings are mirrored by a risk increase with November and December births compared with May and June births in Australia.[56] The effect is quite small, with risk effects around 10% (odds ratios around 1.1), however consistent between different studies on different populations in different settings. Seasonal fluctuations of gestational vitamin D levels have been suggested to explain the season-of-birth effect in MS.

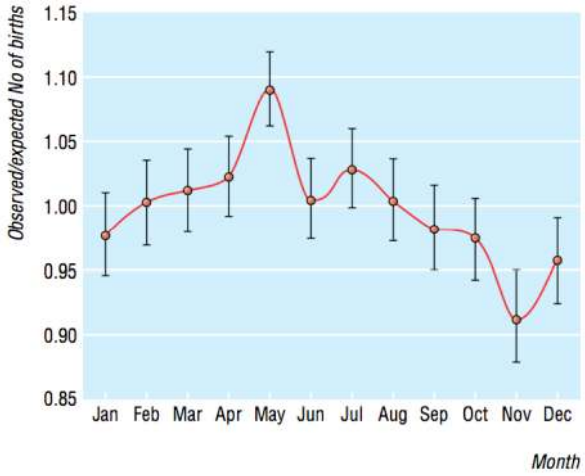


**Figure 3**. Odds ratios of multiple sclerosis by month of birth. Figure from a pooled analysis[52] on studies from Scotland, Denmark, Canada and Sweden on 42,045 cases. © BMJ Publishing Group Ltd. Reprinted with permission

### *Vitamin D*

Vitamin D is needed for maintaining serum calcium levels and bone mineral density and high levels have been associated with a reduced risk of autoimmune diseases and cancer.[57-60] Humans get vitamin D from three sources: the diet, dietary supplements, and from cutaneous 7-dehydrocholesterol which converts to previtamin $D_3$ and then vitamin $D_3$ upon UVB radiation of the skin. It is then metabolized in the liver to 25-hydroxyvitamin D (25[OH]D), the primary circulating form of vitamin D, which travels the body bound to vitamin D binding protein. When 25(OH)D reaches its target cells it gets converted to the biologically active form 1,25-dihydroxyvitamin D ($1,25[OH]_2D$) by 25-hydroxyvitamin D-1α-hydroxylase (1-OHase). This happens mainly in the kidneys, but it has lately been shown that several other tissues and cell types (macrophages, parathyroid glands, breast, colon, prostate) possess 1-OHase and can convert 25(OH)D to $1,25(OH)_2D$. This metabolite then acts on the target cells as a transcription factor together with the vitamin D receptor-retinoic acid x-receptor complex

(VDR-RXR) by binding to vitamin D-responsive elements (VDREs) on the DNA.[61] The effects of $1,25(OH)_2D$ on epithelial cells in the small intestines and osteoblasts result in a rise in serum calcium levels by increased intestinal calcium absorption and increased osteoclast activity.[57]

In 2006, Munger et al. showed that levels of 25(OH)D, in prospectively collected serum samples, were associated with MS risk. Levels >99 nmol/l (the highest quintile) were associated with a 62% reduced risk of MS compared with levels <63 nmol/l (the lowest quintile), and there was a trend over quintiles suggesting the presence of a dose-response relationship.[59] Further support for the association between vitamin D and MS came from a study that showed an increased MS risk among subjects with rare loss-of-function mutations in the CYP27B1 gene, the gene encoding the 1-OHase enzyme.[62] These mutations lead to a decreased ability to convert 25(OH)D to the biologically active form $1,25(OH)_2D$.

The possible mechanisms of action regarding the association between vitamin D and MS risk are unknown. However, vitamin D exerts non-skeletal effects, and one of these is regulation of the immune system, including shifting the T-cell repertoire from a pro-inflammatory $Th_1$-dominated state towards higher $Th_2$ and $T_{reg}$ activities.[57,63] Sufficient vitamin D levels are also needed for proper macrophage function,[57] and Ramagopalan and colleagues have demonstrated $n=2776$ different VDREs in the genome of B-cells. The genetic loci associated with MS are more enriched with VDREs than non-MS loci.[64] Also, it seems that the expression of the MS-associated HLA haplotype DRB1*15:01 may be regulated by vitamin D.[65]

### *Vitamin A*

Vitamin A (retinol) is necessary for the making of rhodopsin, the light-sensitive structure in the retina that mediates visual information to the brain. It is also needed for the structural integrity of the epithelium in the eyes, the gut, and the airways, and the vitamin plays a major role for the immune system.[66,67] Vitamin A acts as a transcription factor in the nuclei of immune cells by binding to retinoic acid response elements (RAREs) on the DNA after forming complexes with the retinoic acid receptor (RAR) and RXR, forming RAR-RXR heterodimers.[61] The sources of vitamin A are dietary, and early in life breastfeeding is essential for maintaining adequate levels. Vitamin A can also be found in liver, eggs, milk, and cheese, and ß-carotene, which converts to vitamin A after ingestion, can be found in carrots, green leaves, and mangos and other orange-yellow vegetables and fruits.[68] Vitamin A is consumed by different inflammatory pathways during infections, and vitamin A deficiency (compared with sufficiency) is associated with worse outcomes during HIV infection, measles and diarrheal diseases.[67] This is a major health concern in the developing countries.

However, in the industrialized parts of the world, severe vitamin A deficiency is uncommon.[68]

No studies have assessed whether the serum levels of vitamin A influence MS risk. In two prospective questionnaire studies within the Nurses' health study cohorts, no influence of vitamin A intake and MS risk has been found.[69,70] However, there are data indicating important functions of vitamin A regarding MS-related immunological pathways. For example, retinoic acid, a derivate of vitamin A, is important for the survival of thymus T-cells, the development of regulatory T-cells, and the inhibition of EBV DNA synthesis and viral reactivation.[71-74] A possible link between vitamin A and MS has also been suggested based on the observation of lower serum retinol levels in MS cases compared with controls.[75,76] Furthermore, vitamin A derivates have been successfully used to treat Experimental Allergic Encephalomyelitis (EAE), the animal model of MS.[77,78] WHO defines vitamin A deficiency as serum levels <0.70 μmol/l, and sufficient vitamin A levels as serum levels ≥1.05 μmol/l.[68]

***The genes***

Many studies have shown that relatives to persons with MS have a higher risk of the disease compared with subjects without MS in the family, and that environmental factors play little role for this.[2,79] Through Genome-Wide Association Studies (GWAS) and other genetic studies, many different genes have been shown to affect MS risk, most of them with immunological functions. Recently, an international collaborative study was published in Nature presenting all now known alleles affecting the risk of MS, which are more than $n$=50.[6] Two genes within the MHC complex (HLA DRB1*15:01 [OR 3.1] and HLA-A*02:01 [OR 0.73]) are among the most well studied ones and have repeatedly been shown to exert robust effects on MS risk. For both HLA DRB1*15:01 and HLA-A*02:01, statistical interaction has been shown with smoking and EBV,[80,81] although the results have been conflicting.[82]

Interaction studies take epidemiological associations to a new level, suggesting possible mechanisms of action, and are the logical extension of all risk factor studies on diseases believed to be caused by a mix of environmental risk factors and genes. Apart from gene-environment interactions, an environment-environment interaction between smoking and EBV has also been proposed,[82] although this has not been replicated.[83]

# Specific aims

**Study I.** To estimate the risk of MS by month of birth in the Swedish MS population as registered in the Swedish MS register and using general population controls.

**Study II.** To estimate the risk of MS by levels of 25(OH)D in prospectively collected blood samples from MS cases and controls, and to determine the risk of MS in the offspring by levels of 25(OH)D during pregnancy. Secondary objectives included comparing estimates of risks of MS by 25(OH)D levels during gestation and for different age strata later in life.

**Study III.** To estimate the risk of MS by levels of vitamin A (estimated by the surrogate marker RBP) in prospectively collected blood samples from MS cases and controls, and to determine the risk of MS in the offspring by levels of vitamin A during pregnancy. Secondary objectives included comparing estimates of risks of MS by vitamin A levels during gestation and for different age strata later in life.

**Study IV.** To examine the risk of MS by levels of cotinine in prospectively collected blood samples from MS cases and controls. Secondary objectives included comparison of risk estimates for different age strata, assessment of whether there was a dose-response relation present with regards to cigarette smoking and the risk of MS, and to investigate whether exclusion of snuff-users influenced the ORs based upon cotinine data.

**Study V.** To investigate the association between MS and 1) the antibody reactivity against EBNA-1 using a commercial ELISA, and 2) the antibody reactivity against our locally produced recombinant EBNA-1 fragments, and 3) to test for interaction between these antibody reactivities and 25(OH)D status regarding MS risk.

# Methods

## Season of birth and multiple sclerosis in Sweden (Study I)

### *Study population and databases*

In the first study we examined the risk of MS by month of birth using all MS cases as registered in the Swedish MS registry in the spring of 2008 ($n$=9,361).[84] The median year of birth for cases was 1957 (range 1912–1995). As controls, all births in Sweden between 1900 and 2007 ($n$=12,116,853) were used. Data on month of birth for the general population between 1900 and 1964 came from a study by Wiberg et al.,[53] and data on month of birth from 1965 and onwards were supplied by Statistics Sweden (Statistiska centralbyrån, SCB). Since the data from Wiberg et al. were not stratified by year of birth, it was not possible to exclude controls born before 1912, and consequently, to treat both ends of the spectrum in the same manner, the controls born after 1995 were also included in the publication. We also present the data with controls truncated at 1995 in the Appendix section in this thesis (Appendix, supplementary Table 3).

## Risk factors of multiple sclerosis (RoMS) and Gestational risk factors of multiple sclerosis (GRoMS) (Studies II–V)

### *Study population and sample databases*

To find the prospective cases for RoMS in the second, third, fourth and fifth studies, we performed a search for all MS cases in the four northern counties in Sweden (Norrbotten, Västerbotten, Västernorrland and Jämtland) by use of the following sources: 1) medical records from relevant departments (neurology, neurosurgery, ophthalmology, internal medicine, geriatrics, and pediatrics) at all twelve hospitals in the region, 2) medical charts from the primary health care centers in Västerbotten county, 3) the national patient registry and the causes of death registry at the national board of health and welfare (NBHW, Socialstyrelsen), and 4) the Swedish MS registry. From all sources, we asked for a search from 1968 and onwards (ICD-8, 9, and 10), however most instances delivered diagnostic codes from the ICD-10 era only, which started 1997. See Table 1 (published as supplementary Table e-1 in Study II) for details.

The database of cases obtained was then cross-linked to two local biobank cohorts: 1) the Northern Sweden Maternity Cohort (NSMC), and 2) the Northern Sweden Health and Disease Study cohort (NSHDS). The NSMC contained approximately $n$=124,000 serum samples collected between 1975 and 1999 from pregnant women, drawn at the first visit to the antenatal care clinic. The NSMC samples have been stored in -20°C freezers, and were heat-

**Table 1. Outline of the International Classification of Diseases (ICD) codes used for case identification**

| | ICD-8 | ICD-9 | ICD-10 |
|---|---|---|---|
| **Years** | 1968–1986 | 1987–1996 | 1997– |
| **Multiple sclerosis** | 340.99 | 340 | G35.9 |
| **Neuromyelitis optica** | 341 | 341A | G36.0 |
| **Hemorrhagic leukoencephalitis** | | | G36.1 |
| **Acute disseminated encephalomyelitis** | | | G36.8, G36.9 |
| **Demyelinating disease of the central nervous system** | 341.08, 341.09 | 341W, 341X | G37.8, G37.9 |
| **Optic neuritis** | 367.02 | 377D | H46.9 |
| **Optic nerve atrophy** | 367.03 | 377B | H48.0, H48.1, H48.8 |

The diagnostic codes above were included in the search for MS and adjacent diagnoses. Most instances choose not to provide data for the ICD-8 and ICD-9 time periods. ©AAN Enterprises. From Study II (supplementary data online, Table e-1). Slightly modified. Reprinted with permission.

treated during 1975–1987 to enable complement binding analyses. When these studies were performed, the NSHDS contained approximately *n*=185,000 plasma samples from three different cohorts: 1) The Västerbotten Intervention Program (VIP) cohort which started in 1985 and is a population-based health promoting project with *n*=113,000 samples from individuals aged 30, 40, 50 and 60 years in Västerbotten county, 2) The Mammography Screening Cohort which started in 1997 and contained *n*=54,500 samples from women aged 50 to 69 years in Västerbotten county, and 3) The Monitoring Trends and Determinants in Cardiovascular Disease (MONICA) project which contained *n*=17,400 random blood samples from individuals aged 25 to 64 years. The latter cohort was not included in our study since the aims of our study were not deemed to harmonize with the purpose of MONICA. The samples in the NSHDS cohort have been stored in -70°C freezers. When adding up the different cohorts included in our study, they contained a total of approximately *n*=291,500 samples from *n*=164,000 unique individuals.

After identifying the biobank samples, we went on to exclude retrospective, missing, and non-MS samples as described to the right in Figure 4 (from Study II) ending up with *n*=192 pre-symptomatic MS samples eligible for inclusion. Two controls for each case were selected, matched for biobank, sex, age at sampling, and date of sampling.

*GRoMS case ascertainment*

The gestational samples (GRoMS) in studies II and III were found by cross-linking the database of MS cases (only those born 1975 and onwards were used since the NSMC was established in that year) to the medical birth

registry (MBR, medicinska födelseregistret) at NBHW, which contains information on kinship. This created a database of mothers of MS cases. This database was then cross-linked to the NSMC, and we excluded missing and non-MS samples as shown to the left in Figure 4. We found *n*=37 samples drawn during gestation from mothers whose children had later developed MS. Five control mothers from NSMC for each case mother were selected, matched for age and date of sampling.

*Ascertainment of multiple sclerosis diagnoses*.
The diagnosis of MS was established by a medical records review focused on identifying clinical symptoms and signs suggestive of MS, and establishment of dissemination in time and space. If necessary, support from magnetic resonance imaging (MRI) findings were used, as described in the 2010 revisions of the McDonald criteria for MS.[16] The date of onset was established as the date of first symptom(s) suggestive of MS recorded in the medical records.

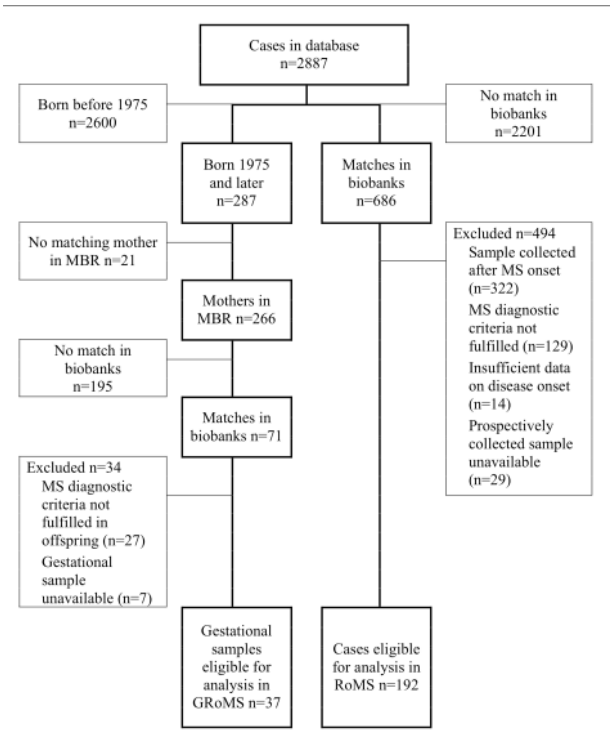


**Figure 4**. Flowchart of case ascertainment. To identify prospectively collected MS samples and samples from pregnancies where the offspring had later developed MS, a search for MS and adjacent diagnoses in northern Sweden was performed. The initial search yielded *n*=2887 cases. To the left is shown how subsequent exclusion (working order) of cases yielded *n*=37 samples drawn from women during pregnancy where the offspring had later developed MS, used in Gestational Risk factors of Multiple Sclerosis, GRoMS. To the right is shown how the *n*=2887 initial cases were reduced to *n*=192 MS cases with prospectively collected samples, used in Risk factors of Multiple Sclerosis, RoMS. MS = Multiple Sclerosis. MBR = Medical Birth Registry. © AAN Enterprises. From Study II (Figure 1). Reprinted with permission

### *Other sources of data*
Since vitamin D levels vary depending on skin color, which also connects to MS risk, and the protective effect of vitamin D on the risk of MS has been

shown in white subjects only, ethnicity needed to be included as a potential confounder in Study II.[59] There has been a low influx of migrants from outside the Nordic countries to northern Sweden (Statistics Sweden), and this is probably why no data on skin color was included in the biobank data. In an effort to adjust for possible effects from immigration, and to enable exclusion of subjects with a higher probability for dark skin color, a local database was used to obtain information on country of birth. Cases and controls were categorized into those born in any of the five Nordic countries (Sweden, Norway, Denmark, Finland and Iceland), and those born elsewhere. This allowed for a post hoc analysis on cases and controls born in one of the Nordic countries only.

To enable exclusion of subjects with high cotinine levels due to smokeless tobacco use in Study IV, data on smoking habits and smokeless tobacco consumption was collected retrospectively using a questionnaire that was mailed out to all participants. The subjects were asked eight questions regarding tobacco consumption, as detailed in Study IV. The index year was defined as the year when the case in each set first experienced neurological symptoms suggestive of MS (disease onset). By use of this questionnaire data, subjects were categorized into ever smokers, never smokers, and ever users of Swedish snuff before or during the index year. Smoke exposure before and during the index year was also quantified (number of pack-years) by use of questionnaire data. A pack-year of cigarettes was defined as the consumption of one package of $n$=20 cigarettes per day for one year. Figure 1 in Study IV describes the process of data collection through questionnaires.

### *Measurements and laboratory methods*

Vitamin A is light-sensitive and proper sampling routines include immediate protection from light.[85] Since this was not done in NSHDS and NSMC, retinol binding protein (RBP), an excellent surrogate marker for vitamin A in the absence of inflammation, was measured instead.[86] One mol RBP weighs 21,000 g, and it has an equimolar relationship to vitamin A in the blood. This allows for a simple equation to convert RBP levels to expected corresponding vitamin A levels: c(RBP) (mg/dm$^3$) = 21*c(vitamin A) (μmol/dm$^3$). Thus, dividing the measured RBP levels in mg/l by 21 yields the expected vitamin A level in μmol/l. The degree of inflammation was assessed by measuring the levels of highly-sensitive C-reactive protein (hs-CRP), and an inflammatory response was considered to be present with hs-CRP levels ≥10 mg/l.

The levels of 25(OH)D, RBP and hs-CRP were measured using commercially available ELISAs from Immundiagnostik AG, Bensheim, Germany. The EBNA-1 antibody reactivity was measured using a commercially available ELISA from Biotest, Dreieich, Germany. The anti-EBNA-1 fragment analyses were performed using in-house developed ELISAs. All ELISA analyses were performed by Maria Nyström, biomedical

analyst, who was blinded to the case or control status of the samples. The 25(OH)D ELISA analyses were performed according to the manufacturer's instructions. The commercial EBNA-1, the RBP, and the hs-CRP ELISA analyses were not run in duplicates. Apart from this, the manufacturers' instructions were followed. The 25(OH)D ELISA correlates to a high degree to established liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods with similar median, mean and dispersion values (Roth, HJ, personal communication 2011, Appendix, supplementary Table 1, supplementary Figure 1).

The levels of the nicotine metabolite cotinine were measured by Kemiskt Laboratorium, Norrlands Universitetssjukhus using the kit "Nicotine Metabolite" by Diagnostic Products Corporation (DPC), Los Angeles, California, U.S.A., analyzed on the immunoassay Immulite 2000 immunoassay system, Siemens, New York, U.S.A. The method was quantitative for levels ≥10 ng/ml, <500 ng/ml.

The recombinant EBNA-1 fragments (amino acids 1–90, 61–90, 402–502, 385–420 and 421–449) were generated and purified according to a previously described method.[29] To quantify the binding of IgG to these EBNA-1 fragments, an in-house ELISA was used as described in Study V. The antibody reactivity against the EBNA-1 domains was reported in arbitrary units (AU), as a percentage of the absorbance of the positive control.

The quality of the NSMC samples was visually examined by the laboratory analyst, who for each sample made a note describing the appearance of the sample. These were then divided into six different categories, including the "normal appearing sample" category. The mean levels of 25(OH)D, RBP, cotinine and EBNA-1 of all other categories were then compared with normal appearing samples by use of the independent samples *t*-test, and the categories with significantly higher, or lower, means were excluded from the post hoc analyses on good quality samples only. Only control samples were included in this comparison to avoid possible MS-related bias. The tables showing the mean levels and *p*-values are included in the appendix section of this thesis (Appendix, supplementary Tables 2a–d).

Analyses on trends for RBP and CRP levels over time were done on NSMC controls only, to avoid MS-related bias and problems arising from pooling cohorts with samples collected at different ages, during different time periods and stored under different conditions. For 25(OH)D time trends, only NSMC controls from any of the Nordic countries with samples of good sample quality were included, for the same reasons as above, and to avoid possible bias due to immigration and skin pigmentation.

### *Statistical methods*

For statistical analyses we used the SPSS software, versions 19 through 20. A *p*-value of <0.05 was considered significant. All statistical analyses were two-sided. The independent samples *t*-test was used to test for differences between means, and the Mann-Whitney *U* test or the Kruskal-Wallis test were used to test for differences between medians. Chi-square statistics and Fischer's exact test were used to test for differences between proportions.

In Study I, we analyzed the month-specific risk of MS compared with the other 11 months in terms of odds ratios (ORs) and 95% confidence intervals (CIs) using the 2x2 chi-squared test comparing the observed with the expected number of births for each month. Significant *p*-values for single months were corrected for the 12 comparisons using the Bonferroni correction (the *p*-values multiplied by the number of tests, i.e. 12).

In Studies II–V, we first compared means and medians of the measured risk factors among cases and controls (data not shown). After getting acquainted with the dataset, we went on to estimate the risk of MS (ORs and 95% CIs) using matched logistic regression for the following exploratory pre-defined 25(OH)D and RBP groups: Above and below median, stratified into tertiles, quartiles, and quintiles. For 25(OH)D we also stratified subjects into two groups with ≥75 vs. <75 nmol/l and ≥50 vs. <50 nmol/l, the two different cut-offs suggested to define sufficient levels.[87,88] The 25(OH)D levels in nmol/l can be transformed to ng/ml by dividing the concentrations in nmol/l with 2.5.[57] Seventy-five nmol/l thus equates 30 ng/ml, and 50 nmol/l equates 20 ng/ml. For RBP, we also stratified subjects into two groups (calculated as above - equation) with insufficient vitamin A levels (RBP <14.70 mg/l) vs. higher, and sufficient vitamin A levels (RBP ≥22.05 mg/l) vs. lower.[68]

A cotinine level of ≥10 ng/ml has been used to discriminate smokers from non-smokers and passive smokers.[89] We stratified subjects according to cotinine levels <10 vs. ≥10 ng/ml, <10 ng/ml vs. below median and above median among those with elevated levels, <10 vs. ≥10 ng/ml, excluding subjects who had used Swedish snuff before or during the index year, and <10 ng/ml vs. below median and above median among those with elevated levels, excluding subjects who had used Swedish snuff before or during the index year. We also examined the risk of MS by smoking history data obtained in the questionnaires using unmatched logistic regression adjusted for age at index year, and sex, stratifying subjects as detailed above. In this analysis, we included only subjects who had provided complete responses to the questionnaire. The data on tobacco use obtained via questionnaires were also analyzed quantitatively, by stratifying users into five groups according to the number of pack-years of smoking,[44] to assess whether or not a dose-response relation regarding the risk of MS was present.

The antibody reactivity against EBNA-1 and the EBNA-1 domains was stratified into tertiles (pre-defined).[28] In the interaction analyses, the antibody reactivity against EBNA-1 and the fragments were subdivided at median among controls with 25(OH)D levels <75 nmol/l, and ≥75 nmol/l respectively, to assess the possibility of an additive interaction. It was not possible to use tertiles of antibody reactivity because the lowest tertile among subjects with 25(OH)D levels ≥75 nmol/l contained no cases for three of the fragments, and because calculation of additive interaction measures demanded four exposure categories. The lowest antibody reactivity category among subjects with 25(OH)D levels ≥75 nmol/l was used as the reference category, as this category would yield the lowest risk of MS according to earlier publications.[29,59] Attributable proportion due to interaction (AP) and 95% CIs were calculated using multinomial regression as described by Andersson et al.[90]

For the subgroup analyses by age at blood sampling (for 25(OH)D status, cotinine levels, CRP levels and EBNA-1 antibody reactivity) we evaluated the heterogeneity of the stratum-specific OR estimates using the chi-square test of heterogeneity in the WinPepi statistical software, version 11.24. Pearson's correlation coefficient ($r$) was used to test for linear correlations between continuous variables. Summer was pragmatically defined as May–October, winter as November–April. Probability is denoted by $p$.

### *Ethical considerations*

Ethical permission for Study I was not sought, as no person-specific data was handled (only birth year and month) and no human experiments were performed. For RoMS and GRoMS (Studies II–V), all aspects were approved by the local ethics committee in Umeå (Dnr: 08-135M). As these studies involved biobank samples, in some cases donated >30 years ago (and possibly forgotten by many), special care was taken to inform all participants in a clear and concise manner about the study and its aims. All participants did receive an information letter (Appendix, supplementary Data 1) outlining the aims of the study, the measurements that were intended, and why they had been selected for participation. In this letter, information on how to opt out was also provided. Two subjects contacted us and asked for their sample to be destructed, but did not ask to be excluded from the present study, and we complied with their requests. Together with the information letter was a request to fill out a questionnaire exploring the participants' tobacco use habits for Study IV. Three-hundred and twenty-one of the participants in the prospective study arm (RoMS) were also asked if they were willing to donate a saliva sample for DNA extraction. For the remaining $n$=255 subjects, DNA was already available in the NSHDS biobank and in national MS-projects, as further elaborated under the heading "Future prospects".

Permission to use the NSMC serum samples for medical research was not explicitly sought when collecting the samples. This presented us with a dual ethical dilemma where we had to consider both the possible integrity violation by analyzing the samples for research purposes, and the possibility of loosing valuable information if the samples were not included in the project. In our approach, we presumed that the participants would give their consent, but gave clear instructions on how to opt out. This represented a way of harnessing as much as possible of the material available in the biobanks while still allowing exclusion of those who wished to be excluded.

# Results

### Season of birth and multiple sclerosis in Sweden (Study I)

The risk of MS was 11% higher for persons born in June compared with those born during the rest of the year (OR 1.11, 95% CI 1.03–1.19). This remained significant after correction for multiple comparisons with the Bonferroni method. Conversely, the risk of MS was 8% and 10% lower if born in December (OR 0.92, 95% CI 0.85–1.00) or January (OR 0.90, 95% CI 0.84–0.98) compared with the rest of the year. This was not significant after Bonferroni correction (see Table 1 in Study I). When comparing seasons, there was a 5% higher risk of MS if born in February–July (OR 1.05, 95% CI 1.01–1.09) compared with August–January. When analyzing the data with controls truncated at 1995, the ORs were identical and *p*-values slightly lower (Appendix, supplementary Table 3).

### Risk factors of multiple sclerosis (RoMS, Studies II–V)

#### *Clinical and demographic characteristics*

The cases and controls in RoMS were well matched regarding the matching variables, as shown in the table in Study II. The samples were drawn median (range) nine years (two months–32 years) prior to disease onset. Since most (86%) of the samples were found in the maternity cohort (NSMC), as much as 92% of the subjects were female. One of the more important matching variables were date of sampling, as 25(OH)D levels vary over the year due to seasonal changes in UVB exposure. The matching accuracy for this variable was high, with a median sampling day difference between cases and controls of 0 days, range 0–20 days. Ninety-six percent of the controls were sampled within two days of the case in each set. According to the latest information in the medical charts, *n*=120 (63%) of the cases had relapsing-remitting MS, *n*=45 (23%) had secondary-progressive MS, *n*=23 (12%) had primary progressive or progressive relapsing MS, and for *n*=4 (2%) data on disease subtype were missing.

#### *Vitamin D (Study II)*

The levels of 25(OH)D were overall very low; only 29.2% of cases (56/192) and controls (112/384) had 25(OH)D levels ≥50 nmol/l, 3.6% (7/192) of the cases and 7.8% (30/384) of the controls had levels ≥75 nmol/l, and 1.0% (2/192) of the cases and 1.8% (7/384) of the controls hade levels ≥100 nmol/l.

Levels of 25(OH)D ≥75 nmol/l (vs. <75 nmol/l) were associated with a decreased risk of MS (OR 0.39, 95% CI 0.16–0.98, Table 3). This was also true when only cases and controls born in any of the Nordic countries with

good quality samples (Appendix, supplementary Table 2a) were analyzed (OR 0.33, 95% CI 0.12–0.89). In young (below median age at sampling, <26.4 years) the decrease in MS risk with 25(OH)D levels ≥75 nmol/l (vs. <75 nmol/l) was even more pronounced, although not significant (OR 0.16, 95% CI 0.02–1.3, Table 4). Among older subjects (above median age at sampling ≥26.4 years) the OR for MS with 25(OH)D levels ≥75 nmol/l (vs. <75 nmol/l) was 0.58 (95% CI 0.20–1.7) (*p* for heterogeneity = 0.28) None of the other pre-defined 25(OH)D-strata yielded significant findings.

***Vitamin A (Study III)***

RBP levels ≥22.05 mg/l indicate sufficient vitamin A levels (≥1.05 μmol/l) and most cases (86.5%) and controls (90.9%) had levels above this threshold. The risk of MS varied across RBP quintiles in a U-shaped manner; having RBP levels in the second quintile compared with the first, was associated with a lower risk of MS (OR 0.45, 95% CI 0.24–0.85), and the ORs for the higher quintiles gradually increased towards 1.0 (Table 3). None of the other pre-defined RBP strata yielded significant findings. In young individuals (below median age at sampling, <26.4 years), the OR pattern for RBP quintiles was the same although the decreased risk associated with quintile two did not reach statistical significance (Table 4). Excluding samples of suboptimal quality (one case, one control, Appendix, supplementary Table 2b) did not alter the ORs or CIs (data not shown).

*C-reactive protein (Study III)*

In the subgroup of young subjects (below median age at sampling, <26.4 years), hs-CRP levels ≥10 mg/l were associated with a lower risk of MS (OR 0.39, 95% CI 0.16-0.93, Table 4). This was not the case in subjects ≥26.4 years old at sampling (OR 1.3, 95% CI 0.68–2.7) (*p* for heterogeneity = 0.035).

***Smoking (Study IV)***

Cotinine levels ≥10 ng/ml, or having smoked before or during the index year according to the questionnaire, were associated with a 50% increased risk of MS, (OR 1.5, 95% CI 1.01–2.1 for cotinine [Table 3] and OR 1.5, 95% CI 0.98–2.3 for questionnaire data). The association between cotinine categories and MS was present in young (<26.4 years old at sampling) individuals (OR 2.2, 95% CI 1.3–3.8, Table 4), but not among older subjects (OR 0.88, 95% CI 0.52–1.5) (*p* for heterogeneity = 0.02). The ORs became slightly higher, and the 95% CIs slightly narrower when excluding ever snuff-users from the analyses by cotinine categories. There were no clear dose-response relations present (Tables 1 and 2, Study IV). Excluding samples of suboptimal quality (one case, one control, Appendix, supplementary Table 2c) did not alter the ORs or CIs (data not shown).

### *Gestational samples, GRoMS (Studies II, III and cotinine data)*

The GRoMS cases and controls were well matched (Table, Study II). The majority of the case mothers' samples (78%) were drawn during the first trimester (data not available for controls). The median sampling day difference between case and control mothers was 0 days, range 0–12.

In the gestational samples, no effect on the risk of MS by any of the pre-defined 25(OH)D (Study II) or RBP (Study III) strata was found. Neither gestational CRP levels ≥10 mg/l (vs. lower) (Study III), nor gestational cotinine levels ≥10 ng/ml (vs. lower) (unpublished data) affected MS risk. This did not change when excluding samples of suboptimal quality and/or those born outside the Nordic countries.

### *EBNA-1 antibodies and their relation to 25(OH)D levels (Study V)*

There was an increasing risk of MS across tertiles of antibody reactivity against EBNA-1 (Table 3), EBNA-1$^{402–502}$ and EBNA-1$^{385–420}$ (Figure 5A and Appendix, supplementary Table 4) ($p$ trend for all three <0.001). The strongest effect on the risk of MS was seen for the EBNA-1$^{385–420}$ domain in those below median age (<26.4 years) at sampling (Figure 5B and Appendix, supplementary Table 5).

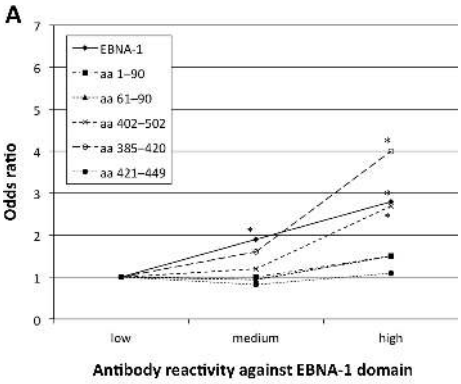


**Figure 5A**. The antibody reactivity against different EBNA-1 domains in all subjects ($n$=192 cases and $n$=384 matched controls), and the odds ratios of multiple sclerosis in prospectively collected biobank samples. Odds ratios were calculated with matched bivariate logistic regression. An asterisk (*) denotes significant associations ($p$ < 0.05). aa = amino acid. EBNA-1 = Epstein-Barr nuclear antigen-1. From Study V, Figure.

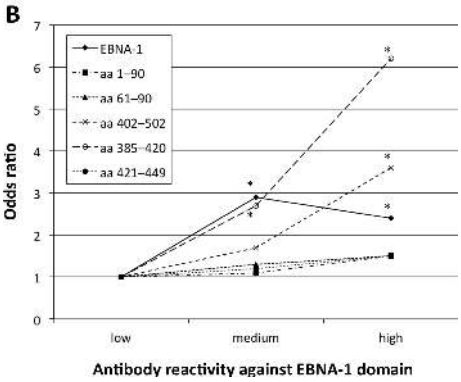


**Figure 5B.** The antibody reactivity against different EBNA-1 domains in young subjects (below median age at sampling, <26.4 years, $n$=96 cases and $n$=192 matched controls), and the odds ratios of multiple sclerosis in prospectively collected biobank samples. Odds ratios were calculated with matched bivariate logistic regression. An asterisk (*) denotes significant associations ($p$ < 0.05). aa = amino acid. EBNA-1 = Epstein-Barr nuclear antigen-1. From Study V, Figure.

Among these young subjects the OR of MS associated with antibodies against the EBNA-1$^{385–420}$ domain within the highest tertile (vs. the lowest) was 6.2 (95% CI 2.8–14), and among older subjects (≥26.4 years at sampling) the OR was 2.8 (95% CI 1.5–5.4) (*p* for heterogeneity = 0.13). No signs of interaction on the additive scale between the different antibody reactivities and 25(OH)D levels were found (Appendix, supplementary Table 6). Among subjects below median age at sampling, levels of 25(OH)D and antibody reactivity against EBNA-1, EBNA-1$^{402–502}$, and EBNA-1$^{385–420}$ correlated inversely. This negative correlation became even more pronounced in those aged <20 years at sampling (Study V, Results). Excluding samples of suboptimal quality (one case, one control, Appendix, supplementary Table 2d) did not alter the ORs or CIs (data not shown).

### *Trends over time*

In post hoc analyses on the NSMC controls born in any of the Nordic countries with no signs of suboptimal sample quality (*n*=479), a decrease in the prevalence of 25(OH)D levels ≥75 nmol/l over time was evident (Figure 2, Study II). This finding remained when including all NSMC controls (data not shown). The correlation between 25(OH)D levels and sampling year was only seen during summer, not during winter, as presented in Table 2 below, where the correlation coefficients for all other laboratory parameters are also shown, both among all NSMC controls, and among NSMC controls born in any of the Nordic countries with no signs of suboptimal sample quality. Only 25(OH)D levels and EBNA-1 antibody reactivity showed statistically significant negative correlations with sampling year. CRP levels, on the contrary, increased during the study period.

**Table 2. Pearson's correlation coefficients (*r*) and *p*-values for the correlations between sampling year and different variables for two selections of NSMC control samples during winter[a] and summer[b].**

| Variable | 515 NSMC controls[c] | | | | 479 NSMC controls[d] | | | |
|---|---|---|---|---|---|---|---|---|
| | Summer (*n*=244) | | Winter (*n*=271) | | Summer (*n*=228) | | Winter (*n*=251) | |
| | *r* | *p* | *r* | *p* | *r* | *p* | *r* | *p* |
| **25(OH)D** | **-0.24** | **<0.01** | 0.05 | 0.41 | **-0.22** | **<0.01** | 0.08 | 0.19 |
| **RBP** | -0.10 | 0.13 | 0.02 | 0.69 | -0.07 | 0.29 | 0.03 | 0.61 |
| **Cotinine** | -0.04 | 0.59 | -0.04 | 0.53 | -0.09 | 0.19 | -0.01 | 0.85 |
| **EBNA-1** | **-0.15** | **0.02** | -0.02 | 0.72 | **-0.17** | **0.01** | 0.01 | 0.94 |
| **CRP** | **0.24** | **<0.01** | **0.31** | **<0.01** | **0.24** | **<0.01** | **0.32** | **<0.01** |

Bold figures indicate significant ($p<0.05$) findings. [a]Winter was defined as November – April. [b]Summer was defined as May – October. [c]All NSMC controls. [d]NSMC controls born in any of the Nordic countries with good sample quality according to 25(OH)D levels. NSMC = Northern Sweden Maternity Cohort; 25(OH)D = 25-hydroxyvitamin D; RBP = Retinol Binding Protein; EBNA-1 = Epstein-Barr Nuclear Antigen-1; CRP = C-Reactive Protein; RoMS = Risk factors of Multiple Sclerosis; GRoMS = Gestational Risk factors of Multiple Sclerosis.

### *Adjusted OR estimations*

As the articles were published sequentially, adjustment for all possible confounders in all analyses was not deemed feasible. This would have demanded very long elaborations on the rationale behind the analyses and interpretations of the results. When all other manuscripts were prepared, we included an adjustment for the well-known risk factors (EBNA-1, smoking and 25[OH]D levels) in the manuscript on vitamin A and CRP and MS risk, as the two risk factors analyzed in that manuscript were new, and thereby possibly the subject of meticulous scrutiny by reviewers. In this thesis, as all articles have been submitted and/or published, we perform estimations of ORs and 95% CIs using matched logistic regression with adjustments made for all variables. As shown in Table 3 for all, and Table 4 for young subjects, the OR and 95% CI estimations from the original publications were essentially replicated, and no clear signs of confounding were seen, except for the following findings: in the multivariable analysis among all subjects, the protective effect associated with 25(OH)D levels, and the increased risk of MS associated with cotinine levels ≥10 ng/ml, failed to reach statistical significance (Table 3). Among young, the OR for MS with elevated cotinine levels became slightly lower in the multivariable analysis (Table 4). Regarding 25(OH)D, significance was regained in the multivariable analysis when excluding samples of suboptimal quality (Appendix, supplementary Table 2a) and samples from subjects born outside the Nordic countries (OR 0.34, 95% CI 0.12–0.98). In the case of cotinine, it was the addition of EBNA-1 tertiles to the multivariable analyses that induced the change in ORs and 95% CIs, both in all subjects (Table 3), and among young individuals (Table 4). When excluding EBNA-1 tertiles from the analysis in Table 3, the OR for MS associated with elevated cotinine levels was 1.5 (95% CI 1.002–2.1), and when including only cotinine and EBNA-1, the OR of MS with elevated cotinine levels was 1.3 (95% CI 0.91–1.9). Among young subjects the results were similar; thus adding EBNA-1 tertiles attenuated the association between cotinine and MS, and when removing EBNA-1, the OR of MS with elevated cotinine levels were unchanged compared to the bivariable analysis.

**Table 3. Odds ratios of multiple sclerosis for categories of 25(OH)D, RBP, CRP, cotinine and EBNA-1 antibody reactivity in prospectively collected samples (*n*=192 cases and *n*=384 matched controls).**

| Variables | Categories | Number of | | Bivariable analysis | | Multivariable analysis[a] | |
|---|---|---|---|---|---|---|---|
| | | Cases | Controls | OR | 95% CI | OR | 95% CI |
| **25(OH)D (nmol/l)** | <75.0 | 185 | 354 | 1.0 | (referent) | 1.0 | (referent) |
| | ≥75.0 | 7 | 30 | **0.39** | **0.16–0.98** | 0.39 | 0.15–1.01 |
| **RBP quintiles (mg/l)** | ≤25.7 | 50 | 77 | 1.0 | (referent) | 1.0 | (referent) |
| | 25.8–30.9 | 26 | 77 | **0.45** | **0.24–0.85** | **0.38** | **0.19–0.74** |
| | 31.0–38.2 | 34 | 77 | 0.65 | 0.37–1.1 | 0.60 | 0.33–1.1 |
| | 38.3–51.9 | 40 | 77 | 0.77 | 0.42–1.4 | 0.61 | 0.32–1.2 |
| | >51.9 | 42 | 76 | 0.85 | 0.46–1.6 | 0.76 | 0.39–1.5 |
| **CRP (mg/l)** | <10.0 | 168 | 325 | 1.0 | (referent) | 1.0 | (referent) |
| | ≥10.0 | 24 | 59 | 0.77 | 0.46–1.3 | 0.68 | 0.38–1.2 |
| **Cotinine (ng/ml)** | <10.0 | 109 | 251 | 1.0 | (referent) | 1.0 | (referent) |
| | ≥10.0 | 83 | 133 | **1.5** | **1.01–2.1** | 1.3 | 0.90–1.9 |
| **EBNA-1 antibody reactivity** | low | 35 | 128 | 1.0 | (referent) | 1.0 | (referent) |
| | medium | 70 | 128 | **1.9** | **1.2–3.1** | **1.9** | **1.1–3.1** |
| | high | 87 | 128 | **2.8** | **1.7–4.7** | **2.8** | **1.7–4.8** |

See Table 4 legend

**Table 4. Odds ratios of multiple sclerosis for categories of 25(OH)D, RBP, CRP, cotinine and EBNA-1 antibody reactivity in prospectively collected samples from young (below median age at sampling, <26.4 years) subjects (*n*=96 cases and *n*=192 matched controls).**

| Variables | Categories | Number of | | Univariable analysis | | Multivariable analysis[a] | |
|---|---|---|---|---|---|---|---|
| | | Cases | Controls | OR | 95% CI | OR | 95% CI |
| **25(OH)D (nmol/l)** | <75.0 | 95 | 181 | 1.0 | (referent) | 1.0 | (referent) |
| | ≥75.0 | 1 | 11 | 0.16 | 0.02–1.3 | 0.16 | 0.19–1.3 |
| **RBP quintiles (mg/l)** | ≤26.2 | 22 | 38 | 1.0 | (referent) | 1.0 | (referent) |
| | 26.3–30.7 | 14 | 39 | 0.47 | 0.18–1.2 | 0.42 | 0.16–1.1 |
| | 30.8–35.9 | 17 | 38 | 0.77 | 0.34–1.7 | 0.74 | 0.30–1.8 |
| | 36.0–47.5 | 19 | 39 | 0.84 | 0.36–1.9 | 0.63 | 0.25–1.6 |
| | >47.5 | 24 | 38 | 1.2 | 0.54–2.8 | 0.87 | 0.33–2.3 |
| **CRP (mg/l)** | <10.0 | 88 | 157 | 1.0 | (referent) | 1.0 | (referent) |
| | ≥10.0 | 8 | 35 | **0.39** | **0.16–0.93** | **0.36** | **0.14–0.95** |
| **Cotinine (ng/ml)** | <10.0 | 47 | 130 | 1.0 | (referent) | 1.0 | (referent) |
| | ≥10.0 | 49 | 62 | **2.2** | **1.3–3.8** | **2.1** | **1.2–3.7** |
| **EBNA-1 antibody reactivity** | low | 16 | 64 | 1.0 | (referent) | 1.0 | (referent) |
| | medium | 43 | 64 | **2.9** | **1.4–6.0** | **2.7** | **1.2–6.0** |
| | high | 37 | 64 | **2.4** | **1.1–5.2** | **2.4** | **1.1–5.4** |

Matched logistic regression was used to calculate ORs and 95% CIs. Bold figures indicate significant ($p<0.05$) findings. [a]Adjusted for the variables in this table. 25(OH)D = 25 hydroxyvitamin D; RBP = retinol binding protein; CRP = C-reactive protein; EBNA-1 = Epstein-Barr Nuclear Antigen-1; OR = odds ratio; CI = confidence interval.

# Discussion

**Season of birth (Study I) and gestational risk factors of multiple sclerosis (GRoMS) (Studies II, III and cotinine data)**

We have shown a slightly increased risk of MS with spring births compared with the rest of the year. When comparing the results from our study to the results from the larger study by Willer et al.,[52] there was a displacement by one month (our study showed a risk increase for June births and a risk decrease for December and January births before Bonferroni correction while they found an increased risk of MS for May births and a decreased risk of MS for December births). There was also a more pronounced irregularity between the risk estimates for different months in our study, with no apparent visual sinusoidal shape of the risk curve. We believe these differences result from chance and the fact that our study was smaller.

The kind of information derived from such research as performed in Study I can only be used to generate hypotheses, and to suggest associations that need to be further explored. It is reasonable to assume that environmental factors account for the differences observed, but establishing one single causative factor is not easily done. The timing of the environmental risk influence could range from conception, through pregnancy to peri- and postnatal. Seasonal fluctuations in vitamin D status have been proposed to account for the increased MS risk with spring births, and one diet questionnaire study within the Nurses' health cohort study supports this, finding a 43% decreased risk of MS with high maternal vitamin D intake vs. low intake (quintile five vs. one) during pregnancy.[91] As the season-of-birth (SOB) studies show an increased MS risk with spring births, the gestational vitamin D nadir in late winter probably coincides with the third trimester in these pregnancies. This suggests that if gestational vitamin D influences MS risk in the offspring, it may be active in late pregnancy. An interaction between the SOB effect and HLA-DRB1*15 has been demonstrated, with a more pronounced SOB effect in HLA-DRB1*15-positive individuals than in those without this allele.[92] If vitamin D in fact regulates the expression of this gene as suggested,[65] this finding strengthens the idea that vitamin D is responsible for the SOB effect. Also, a recently published meta-analysis indicates that the SOB effect is stronger at higher latitudes in the northern hemisphere, although study size rather than latitude may account for this finding as the larger studies with a higher power to detect differences were performed further to the north.[55] However, this latitude gradient was also present in one very recent Norwegian study, further strengthening this notion.[93] At a first glance, one might interpret this as supporting the vitamin-D hypothesis, however seasonal fluctuations of other variables possibly of interest in this sense may also be more pronounced at higher latitudes. Some

of the other possible explanations for the SOB effect include seasonal variations in infections, possibly connected to vitamin D levels[94,95] although the data are conflicting,[96] and diet.[97] For example, the dietary intake of antioxidants is at its lowest during fall, when most MS cases are conceived according to our data, possibly resulting in oxidative damage to fetal cells during early developmental stages.[98] It is also possible that those born in spring are less protected against wintertime infections during their first year of life due to cessation of breastfeeding during winter.[99] They might thus suffer from more frequent and perhaps more serious infections during their first year of life. However, this proposal is somewhat paradoxical since the hygiene hypothesis suggests that early infections protect against autoimmunity.[24]

Our data from the GRoMS study arm do not suggest an effect by early gestational levels of the measured risk factors and MS risk in the offspring. This lack of findings in the GroMS material could be due to low power, as only $n$=37 case mothers were identified. Owing to the matching for date of sampling, the differences that account for the observed SOB effect were removed, and thereby these studies only allow for analysis of differences between case and control mothers unrelated to time of year (i.e. not allowing for analysis of UVB-derived cutaneous vitamin D, or seasonally varying infections). However, the diet questionnaire study mentioned earlier, suggests that orally taken vitamin D during pregnancy protects the offspring against MS, and this finding cannot be confirmed in our dataset.[91] Finally, as our GRoMS samples were drawn in early pregnancy, we cannot exclude associations between late pregnancy levels of the measured risk factors and MS risk in the offspring.

**Vitamin D in prospectively collected samples (Study II)**

In Study II, we showed an association between vitamin D levels in samples drawn a median nine years prior to disease onset, and MS risk. The decrease in MS risk associated with 25(OH)D levels ≥75 nmol/l was 61% in all samples, and 67% if individuals from outside the five Nordic countries, and individuals with suboptimal sample quality were removed. In the multivariable analysis on the entire cohort (Table 3), the protective effect by 25(OH)D levels ≥75 nmol/l (vs. lower) did not reach statistical significance. However, when removing samples of suboptimal quality and cases and controls born outside the Nordic countries, significance was regained. The latter analysis may be considered more appropriate regarding risk estimates for MS by 25(OH)D levels, as the association between vitamin D and MS risk has been shown in white subjects only.[59] The decrease in MS risk among young individuals (below median age at sampling), with 25(OH)D levels ≥75 nmol/l was even more impressive, 84%, although not statistically significant.

These findings are quite similar to an earlier report from the U.S.A., with regards to the magnitude of the effect on MS risk. However, the cut-off level for 25(OH)D above which a statistically significant protective effect was seen in their study was approximately 100 nmol/l.[59] This cut-off could not be studied in our material due to overall low 25(OH)D levels. Interestingly, in their study the majority (68%) of cases were of male sex since the biobank used was the Department of Defense Serum Repository in which serum samples from US military personnel is stored. Our cohort contains mainly women (92%) due to the fact that the NSMC contains serum samples from pregnant women only. Therefore these two studies complement each other, and the combined evidence support a role for high vitamin D levels as a protective factor in MS in both sexes in geographically different areas, with the strongest effect in younger adults.

### *Possible mechanisms of action*

Vitamin D has been shown to be immunomodulatory, inducing a shift towards a more anti-inflammatory immune response with enhanced regulatory T-cell function and decreased Th1 cytokine production.[63] This could be of potential interest in MS as Th1 activity seems to be important for both the initial steps and the long-term evolution of MS.[100] Figure 6 depicts schematically the influence of vitamin D on the inflammatory response, as presented by Smolders et al.[63]

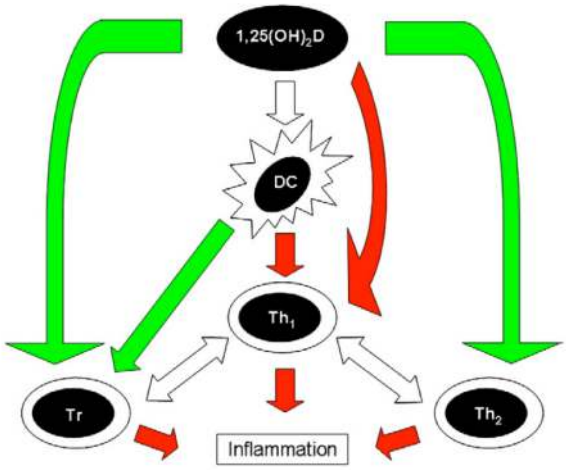


**Figure 6**.[63] Schematic representation of the effects of the active vitamin D metabolite 1,25 dihydroxyvitamin D ($1,25[OH]_2D$) on the immune system in vitro. Green arrow: positive influence. Red arrow: negative influence. White arrow: interaction. DC = Dendritic cell; Th1 = T helper type 1 lymphocyte; Th2 = T helper type 2 lymphocyte; Tr = regulatory T lymphocyte. © Elsevier. Reprinted with permission.

It is also possible that vitamin D influences MS risk independently of T-lymphocytes, as shown by Wergeland et al.[101] In that study, the authors demonstrate that high dietary doses of vitamin D can prevent cuprizone (a copper chelator) induced toxic demyelination in mice. This effect may be due to vitamin D effects on microglia/macrophages in the CNS, two cell-types

which possess both the VDR and the 1-OHase, thus being capable of converting 25(OH)D to its biologically active form.[102,103] Vitamin D may also have direct effects on neuronal and glial cells.[101] The mechanism of action by which vitamin D is believed to influence MS risk (either through lymphocytes, macrophages/microglia or neuronal or glial cells) is via gene transcription regulation. This is supported by the presence of vitamin D responsive elements (VDREs) close to genes related to MS. In a recent overview, Disanto and colleges conclude that there are at least 2776 different VDREs in the B-cell genome, and that genetic loci associated with MS are strikingly enriched for vitamin D receptor binding sites.[64] Vitamin D has also been shown to augment the expression of HLA-DRB1*15:01, the allele that increases MS risk the most of all now known genes associated with MS.[65] However, this finding may seem counterintuitive, since *increasing* the expression of a gene that *increases* the risk of MS seems like something that would rather *increase* than *decrease* the risk of MS. The authors argue that vitamin D might influence MS risk selectively in HLA-DRB1*15:01 positive individuals.[65] However, such an interaction was not seen in a study of $n$=302 children with a first demyelinating event (FDE).[104] It has not been tested whether such an interaction exists in a prospective material.

### *Possible confounders*

*Sunlight*

Circulating levels of 25(OH)D depend mainly on cutaneous UVB radiation-derived vitamin D.[57] UV radiation in itself has immunomodulatory effects, e.g. enhancing regulatory T-cell function. It is possible that the levels of 25(OH)D merely act as a surrogate marker for the amount of UV radiation each individual has experienced, and that the association between vitamin D and the risk of MS seen in our material is in fact an association between solar radiation and MS.[105,106]

One Australian study measured 25(OH)D levels at the time of an FDE, and quantified the cumulative amount of sun exposure before the FDE by use of a questionnaire, and silicon casts of the skin, measuring the degree of actinic damage, which correlates to the amount of UVA exposure.[107] They found that the protective effect from UV exposure remained after correction for 25(OH)D levels and concluded that UV exposure has a protective effect on MS risk independent of 25(OH)D. However, the UV exposure preceding the FDE might actually be a better proxy for 25(OH)D levels during this period than a single serum concentration of 25(OH)D at the time of the FDE. Therefore, it is still unclear whether the UV exposure in itself, or the higher levels of 25(OH)D that is generated from such exposure, confers the protective effect seen.[108]

In EAE, the animal model of MS, whole-body UV-light exposure has been shown to suppress disease activity, which, again, could be related to increased levels of 25(OH)D.[109] However, there are indications that orally or intraperitoneally administered $1,25(OH)_2D$ also has the same positive effects on EAE risk and severity.[110,111] One way to test whether it is the UV radiation in itself, or the increased levels of vitamin D that result from such radiation, that is responsible for the association between 25(OH)D and MS risk in humans is to perform a large scale primary preventive vitamin D supplementation study, as will be discussed below (see "Future prospects").

*Genes*

The MS-related genes represent another possible confounder regarding the findings of an association between the risk of MS and 25(OH)D levels. There are a number of genes involved in the vitamin D metabolism that may also affect MS risk, possibly through other pathways than those involving vitamin D.[112] A recent attempt to elucidate the interplay between these genetic predictors of 25(OH)D levels and MS risk was made by Simon et al.[113] They report only modest effects on MS risk by the investigated alleles, and these genetic determinants of 25(OH)D cannot, even if added together, explain the relatively large reduction in MS risk seen with 25(OH)D levels ≥75 nmol/l in Study II. Also, mutations in 25(OH)D-related genes with larger effects MS risk has been found, although these are extremely rare variants.[62] Therefore, we conclude that although genetic factors, which might differ between cases and controls in our cohort, might explain a small fraction of the reduced risk of MS associated with high 25(OH)D levels, the now known MS-related genes do not represent a major confounder. It is actually more probable that the genes related both to vitamin D metabolism and MS exert their effects on MS risk through vitamin D.[62]

### *Definition of vitamin D sufficiency*

There is debate as to what 25(OH)D levels should be considered as sufficient. Currently, the National Food Administration in Sweden (Livsmedelsverket) and the Institute of Medicine (IOM) in the U.S.A. claim that levels of over 50 nmol/l are sufficient.[88,114] However, there are indications that at least 75 nmol/l is needed to maintain calcium and parathyroid hormone homeostasis, according to the American Endocrine Society.[57,87] Even if accepting the lower cut-off, less than one third of the subjects in our study had sufficient 25(OH)D levels. It is not possible to establish the normal vitamin D requirements in humans by looking at dietary intake since our (non-enriched) diet does not contain enough vitamin D even to prevent rickets and osteomalacia.[115] Humans must therefore have been dependent on sunlight-derived cutaneous vitamin D during evolution. By moving to more temperate climates, wearing sun-screen and clothing, and spending most

time indoors behind UV-shielding windows, we have changed the conditions radically, and this in a relatively short time period compared with how long we as a species have existed. When comparing the vitamin D levels in non-human primates with those of humans with and without abundant sun exposure, Reinhold Vieth concludes that a modern human living in a temperate climate has extremely low levels (Figure 7).[115] This is also illustrated by the cover image of this thesis, showing the solar elevation at noon the 25th of December just south of Umeå at latitude 63° N. During winter in northern Sweden, the sun is only briefly seen each day, and the solar radiation travels through much atmosphere before reaching the earth due to the low elevation angle. Because of this, no vitamin D production occurs in the skin at these latitudes between October/November and March.[116]

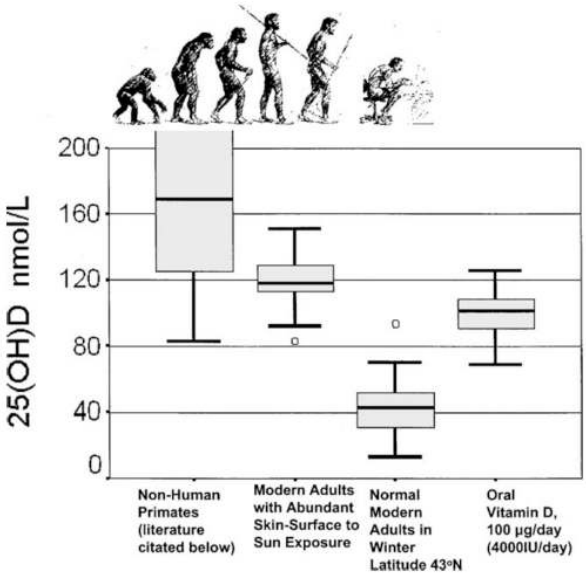


**Figure 7.**[115] The cartoon across the top of the figure represents stages of human evolution and development. The whiskers show the range, the boxes the inter-quartile range and the lines within the boxes show median values of 25(OH)D levels. ©Elsevier. Reprinted with permission.

Due to the arguments outlined above, it seems rational to supplement vitamin D in areas with little sunlight during winter, and this is done in infants up to two years of age in Sweden, who are recommended 400 IU (10 µg) cholecalciferol (vitamin $D_3$) each day. Vitamin D in high doses has had a reputation for toxicity, however this has been overcome lately.[57,115] In one small study on MS cases evaluating the safety profile of high doses of vitamin D supplementation, the active study arm ($n$=23) on a dose-escalating vitamin D regimen received on average 14,000 IU (350 µg) each day for up to 52 weeks without adverse effects or changes in serum levels of calcium, hepatic enzymes or creatinine, or urinary calcium, even though the mean levels of 25(OH)D were above 250 nmol/l, the cited toxic value, for more

than 18 weeks.[117] No clear benefits regarding the disease activity were seen in this small study, and this was expected as it was not powered to show differences in these measures. One Finnish randomized controlled trial (RCT) with *n*=62 participants, given either placebo or 20,000 (500 μg) IU vitamin D/week, has recently shown fewer new T1-enhancing lesions in the vitamin D study arm. At least seven larger RCTs are ongoing as this is written.[118,119] The IOM recently published an extensive review of the currently available data regarding vitamin D and calcium intake and associations with different health outcomes.[88] They conclude that even though observational data supporting a benefit from higher 25(OH)D levels exist, the lack of RCTs showing a clear reduction in the risk of disease and the possible presence of a reversed J-shaped relationship between 25(OH)D levels and health outcomes warrant caution when establishing a recommended daily intake (RDI). According to this report, the RDI for people aged 0–12 months is 400 IU (10 μg), for people aged 1–70 years 600 IU (15 μg) and for those above that 800 IU (20 μg). The upper tolerable level however, below which no adverse effects are likely to occur, even with life long substitution, is set with a safety margin of 20% at 2000 IU (50 μg) for all between 1–8 years of age, and 4000 IU (100 μg) for all above 8 years of age.[88]

**Vitamin A in prospectively collected samples (Study III)**

The association between RBP levels, used as a surrogate marker for vitamin A, and MS risk is an intriguing finding. In Study III, we demonstrated a U-shaped relationship between circulating levels of RBP and the risk of MS. We found a 55% decreased MS risk for those in the second RBP quintile vs. the first, and the ORs gradually increased towards 1.0 for the higher quintiles. One interpretation of this may be that adequate levels of vitamin A are needed for proper immune function, but that higher levels might increase MS risk by competitively blocking the binding of 25(OH)D to RXR, and thereby antagonizing the protective effects conferred by high 25(OH)D levels.[61,120] This hypothesis is to some extent supported by the finding of a U-shaped risk curve over vitamin A quintiles regarding the risk of hip fracture, also possibly related to 25(OH)D antagonism.[121] Interestingly, when analyzing the risk of MS by 25(OH)D strata in our material, excluding the subjects with RBP levels within the highest quintile, the OR of MS with 25(OH)D levels ≥75 nmol/l was 0.09, 95% CI 0.01–0.70 (post hoc analysis).

***Possible mechanisms of action***

As described briefly in the background, vitamin A has important functions regarding immune function. The vitamin A metabolite, retinoic acid (RA), is involved in T-cell differentiation and the immune response against EBV.[71-74,122] Recently, Correale and colleges investigated MS cases with and without

Helminth parasite infection, and found that infected cases had fewer relapses and lower MRI activity than matched, uninfected cases.[123] They interpret this as a result from the immunomodulatory effects that these parasites have, including the induction of regulatory T-, and B-cells. Interestingly, the pathways by which these immunomodulatory effects take place all include RA.[124] This suggests a connection between RA and the immune system with effects on MS disease activity, which is further supported by one study showing that vitamin A levels correlated inversely to MRI activity in $n$=88 RRMS cases.[125] Vitamin A in MS has gained interest lately, as indicated by a recent review by Torkildsen et al.[126]

**CRP levels in prospectively collected samples (Study III)**

The post hoc finding of an association between CRP levels and MS risk in young subjects, with a 61% lower risk of MS with CRP levels ≥10 mg/l, warrants further investigation. Interpreted within the frames of the hygiene hypothesis, which has been mentioned earlier, this finding might support the notion that a heavier infectious load early in life confers a long-lasting protection against autoimmunity.[24,127] This is also supported by a recent report which shows a lower risk of pediatric onset MS among those seropositive to cytomegalovirus,[128] a virus which has been linked to elevated CRP levels in some,[129] but not all studies.[130]

It is also possible that the decreased risk of MS with higher CRP levels derive from an impaired oxidative burst in phagocytic cells that has been implicated in autoimmune diseases.[131,132] A third alternative is that the lower CRP levels in cases reflect the EBV latency pattern typical for MS, with fewer EBV reactivations.[28]

***Possible confounders and limitations***

Due to a limited supply of biobank serum and plasma samples, no RBP or CRP duplicates were run. This probably reduced the precision of the estimates of the concentrations of these factors, and renders the cut-offs inexact. Therefore, the levels found to be associated with MS risk should be taken cautiously and need to be replicated. However, this does not render the main findings of the U-shaped association with MS risk across the RBP quintiles, and the lower MS risk with elevated CRP levels, less reliable. Rather, assuming that reduced precision increases the risk of false negative findings (type II errors), one can argue that the findings of these associations, despite this shortcoming, suggests that these are robust associations that would have been ever more clearly evident if duplicates had been analyzed. Apart from the matching variables and the variables used to control for possible confounding, no other epidemiological and individual data were available, and residual confounding from such factors cannot be ruled out, as discussed below (see “General advantages and limitations”).

**Smoking and MS risk (Study IV)**

We demonstrated an association between smoking (assessed both by cotinine levels in prospectively collected samples and by questionnaire data) and an increased risk of MS. The size of the effect (around 50% increased risk of MS) harmonizes with findings from earlier prospective studies.[35-38] Interestingly, the association between cotinine and MS was *only* seen in young subjects (below median age at sampling), suggesting that smoking during the first two–three decades of life is associated with an increased MS risk, but smoking later in life is not. As shown in the supplementary Table 7 in the Appendix, this finding could not easily be explained by an increased amount of smoking exposure among those below median age at sampling. This is further explored below (see "The age-related influence on MS risk"). In the multivariable analyses, the association between cotinine and MS risk was slightly attenuated after the addition of the anti-EBNA-1 IgG reactivity tertiles (Tables 3 and 4). There was a positive, albeit very weak, correlation between anti-EBNA-1 IgG reactivity and cotinine ($r$=0.08; $p$=0.044, $n$=576). These findings are to some extent consistent with the findings of a meta-analysis from 2010, which pooled the results from three studies.[82] In that report, the authors showed that, when adjusting the logistic regression model for anti-EBNA-1 IgG reactivity titers, the risk of MS was no longer influenced by smoking history. The effect of adding anti-EBNA-1 IgG reactivity in that study was more pronounced compared with the present study; their OR estimates went from 1.4 (95% CI 1.1–1.8) to 1.1 (95% CI 0.8–1.4).[82] In the same study, the authors showed that the increased risk of MS with elevated anti-EBNA-1 reactivity was stronger among smokers than among non-smokers, suggesting an interaction between those two risk factors. One failed attempt to replicate this finding was recently published,[83] and therefore this putative interaction between smoking and EBV has yet to be confirmed.

***Possible mechanisms of action***

The possible mechanism(s) by which smoking may increase MS risk have not been elucidated. As suggested by Simon et al., it is possible that EBV and smoking share a common biological pathway whereby smoking modulates either the EBV infection in itself or the host's response to the EBV infection.[82] This would possibly explain why the risk-increasing effect mediated by cotinine was attenuated when adjusted for anti-EBNA-1 IgG reactivity tertiles. Current hypotheses also include neurotoxic effects, EBV-independent immunomodulatory effects and post-translational protein modification, among others.[8,80] The fact that Swedish snuff use seems to be associated with a *decreased* risk of MS implies that it is not the serum nicotine levels, but rather some other factor associated with cigarette smoking that is responsible for the association seen.[44,50] It is possible that the

nicotine administration route plays a so far unknown role here, so that nicotine administered mainly via the airways has effects that nicotine administered mainly via oral and gut mucosa does not.

***Limitations***

No clear dose-response relations between cotinine levels and MS risk, and between questionnaire data on smoking and MS risk were seen. As for serum cotinine levels, a relative lack of correlation between these and the number of cigarettes smoked per day has been shown,[48] and this might be why we did not see any dose-response effect. That the questionnaire data also lacked this effect might be due to low power or inexact reporting of quantities, as discussed in Study IV. It would have been interesting to add genetic markers to this analysis, as an interaction between certain HLA genotypes and smoking has been shown,[80] however these analyses were not completed when these manuscripts were prepared, and the data will probably be ready for analysis during 2013 as mentioned below (see "Future prospects").

**EBNA-1 antibodies and their relation to 25(OH)D levels (Study V)**

The risk of MS increased across tertiles of IgG antibody reactivity against EBNA-1. The association was most pronounced for antibody reactivity against the EBNA-1 domains EBNA-1$^{385-420}$ and EBNA-1$^{402-502}$, as was the case in two earlier publications.[29,133] No signs of confounding by 25(OH)D status were found. We searched for signs of interaction between EBNA-1 antibody reactivities and 25(OH)D status using both an additive (Appendix, supplementary Table 6), and a multiplicative model (data not shown), as there is no consensus regarding which model is more appropriate.[82,83,134] No signs of interaction were detected. However, in young subjects, we did observe inverse correlations between: 1) 25(OH)D levels and antibody reactivity against EBNA-1; and 2) 25(OH)D levels and antibody reactivity against the two most MS-specific EBNA-1 domains.

***Interpretation***

The higher antibody reactivity against EBNA-1 and the EBNA-1 domains among MS cases compared with controls might be explained by molecular mimicry, i.e. that the antibodies directed against this/these EBV epitope(s) cross-react with a human CNS antigen and thus causes an immune attack against myelin, the oligodendrocytes or some other CNS structure.[29] However, no signs of intrathecal anti-EBNA-1 antibody production has been observed,[135,136] and the putative CNS antigen has not been identified.

***Limitations***

This study, which focuses on the possible interaction between EBV and vitamin D, suffers from two main drawbacks regarding the study population:

The first drawback is the high median age at sampling. As the correlation between EBNA-1 antibody reactivity and 25(OH)D levels was only seen in younger individuals, the potential biological interaction between the two risk factors probably occurs at a younger age, and a larger sample of young subjects would have been desirable. The other drawback is the overall low 25(OH)D levels. Only seven cases had 25(OH)D levels ≥75 nmol/l, and this hampered the interaction analyses as both cases and controls are needed in all categories to allow calculation of ORs and attributable proportion due to interaction. As there were no signs of additive interaction, a multiplicative interaction assessment using interaction terms (the cross-product of the two variables) did not add any important information and was not included in this thesis. Also, analyses of heterogeneity of effects of 25(OH)D and EBV antibody reactivity across strata of each other did not prove informative due to small exposure groups, and were not included in this thesis. In the subgroup of individuals below median age at sampling only one case with a 25(OH)D level ≥75 nmol/l was found, which prevented interaction analyses in this subgroup. As in most previous studies on EBNA-1 and MS risk, the age at EBV seroconversion, and the time-lapse between this event and sampling, were unknown. It is possible that the antibody reactivities are merely markers of age at seroconversion, which may in itself be important in determining MS risk.

**The age-related influence on multiple sclerosis risk**

In all four studies on the RoMS cohort, there were age-at-sampling specific effects of the exposure variables on the risk of MS. The associations with MS risk were more pronounced in those below median age at sampling (<26.4 years old) for 25(OH)D status, and for antibody reactivity against EBNA-1$^{385–420}$ and EBNA-1$^{402–502}$. For cotinine and CRP, the associations with MS risk were *only* seen in those below median age at sampling.

The interpretation of this may be that the environmental risk factors of MS studied in this project mainly influence the risk of MS early in life. This is supported by migration data indicating that the risk of MS is largely established during the first two decades of life,[23] and studies showing that infectious mononucleosis, which mainly strikes during adolescence in the western world, is associated with an increased risk of MS.[25]

Table 5 displays a comparison between the two groups of cases with sampling age below vs. above median. From this table, it is apparent that there are differences between the two groups, apart from age at blood sampling. To test whether the age-at-sampling specific ORs for the different exposure variables were dependent on sex, clinical disease subtype at onset and/or biobank, we homogenized the two groups by excluding all NSHDS cases and controls (this biobank contained all males), and all cases with progressive disease at onset, and their corresponding controls. This left $n$=93

**Table 5. Comparison of characteristics of cases (*n*=192) below and above median age-at-sampling (26.4 years).**

| Variable | Cases <26.4 years old | Cases ≥26.4 years old | *p* |
|---|---|---|---|
| **Female *n* (%)** | 96 (100) | 81 (85) | <0.001 |
| **Age at sampling** | 23 (16–26) | 31 (26–59) | - |
| **Sample season *n* (%)[a]** | | | |
| **Summer** | 55 (57) | 39 (41) | |
| **Winter** | 41 (43) | 57 (59) | 0.02 |
| **Age at disease onset** | 32 (19–54) | 41 (27–66) | <0.001 |
| **Year of sampling** | 1985 (1976–2005) | 1988 (1976–2005) | <0.001 |
| **Clinical onset *n* (%)** | | | |
| **RR** | 93 (97) | 72 (78) | |
| **Progressive** | 3 (3) | 20 (22) | <0.001 |
| **MSSS** | 3.0 (0.1–10) | 4.7 (0.1–10) | 0.001 |
| **Biobank *n* (%)** | | | |
| **NSMC** | 96 (100) | 69 (72) | |
| **NSHDS** | 0 (0) | 27 (28) | <0.001 |
| **Lab parameters** | | | |
| **25(OH)D (nmol/l)** | 35 (0–113) | 42 (13–122) | 0.06 |
| **RBP (mg/l)** | 35 (1–173) | 36 (7–173) | 0.97 |
| **CRP (mg/l)** | 1.3 (0–79) | 1.0 (0–79) | 0.45 |
| **Cotinine (ng/ml)** | 12 (0–>500) | 0 (0–>500) | 0.17 |
| **EBNA-1 (AU)** | 149 (0–195) | 143 (1–187) | 0.001 |

All figures are median (range) unless stated otherwise. The Mann-Whitney *U* test was used to test for differences between medians, and chi-square statistics were used to test for differences between proportions.
[a]Summer was defined as May – October, winter as November – March.
RR = Relapsing-Remitting; MSSS = Multiple Sclerosis Severity Score; NSMC = Northern Sweden Maternity Cohort; NSHDS = Northern Sweden Health and Disease Study cohort; 25(OH)D = 25-hydroxyvitamin D; RBP = Retinol Binding Protein; CRP = C-reactive Protein; EBNA-1 = Epstein-Barr Nuclear Antigen-1; AU = Arbitrary Units

cases and *n*=186 controls in the young group, and *n*=51 cases and *n*=102 controls in the old group (Table 6). The proportions "Sex", "Clinical onset", and "Biobank" were now identical between the age groups. The MSSS medians were more similar than before, but the "Sample season" proportions difference became non-significant due to lower power. As age-at-sampling was strongly correlated to age-at-disease-onset ($r$=0.68; $p$<0.001), and to year-of-sampling ($r$=0.39; $p$<0.001), these two variables remained different between the groups in Table 6. When attempting to truncate the young group in Table 6 to raise age-at-disease-onset and year-of-sampling to medians similar to the older group, it was not until only *n*=34 young cases were left that the variables harmonized. This cohort was to small to perform any meaningful analyses. Another attempt to investigate whether these variables were more important predictors of the size of the risk factor effects than age-at-sampling was made by dichotomizing the entire cohort at median year-of-sampling and at median age-at-disease-onset, to create two new stratifications. However, due to the fact that these variables were so

**Table 6. Comparison of characteristics of cases (*n*=144) below and above median age-at-sampling (26.4 years). Subjects from the NSHDS and subjects with progressive onset were excluded to harmonize the age-at-sampling groups.**

| Variable | Cases <26.4 years old | Cases ≥26.4 years old | *p* |
|---|---|---|---|
| **Female *n* (%)** | 93 (100) | 51 (100) | - |
| **Age at sampling** | 23 (16–26) | 30 (26–39) | - |
| **Sample season *n* (%)**[a] | | | |
| **Summer** | 53 (57) | 21 (41) | |
| **Winter** | 40 (43) | 30 (59) | 0.07 |
| **Age at disease onset** | 32 (19–54) | 40 (27–54) | <0.001 |
| **Year of sampling** | 1985 (1976–2005) | 1988 (1977–2005) | 0.02 |
| **Clinical onset *n* (%)** | | | |
| **RR** | 93 (100) | 51 (100) | |
| **Progressive** | 0 (0) | 0 (0) | - |
| **MSSS** | 3.0 (0.1–10) | 3.9 (0.1–10) | 0.16 |
| **Biobank *n* (%)** | | | |
| **NSMC** | 96 (100) | 51 (100) | |
| **NSHDS** | 0 (0) | 0 (0) | - |
| **Lab parameters** | | | |
| **25(OH)D (nmol/l)** | 35 (0–113) | 38 (13–122) | 0.49 |
| **RBP (mg/l)** | 34 (1–173) | 41 (12–173) | 0.16 |
| **CRP (mg/l)** | 1 (0–79) | 2 (0–79) | 0.49 |
| **Cotinine (ng/ml)** | 11 (0–>500) | 0 (0–>500) | 0.18 |
| **EBNA-1 (AU)** | 149 (0–195) | 142 (1–187) | 0.02 |

All figures are median (range) unless stated otherwise. The Mann-Whitney *U* test was used to test for differences between medians, and chi-square statistics were used to test for differences between proportions.
[a]Summer was defined as May – October, winter as November – March.
RR = Relapsing-Remitting; MSSS = Multiple Sclerosis Severity Score; NSMC = Northern Sweden Maternity Cohort; NSHDS = Northern Sweden Health and Disease Study cohort; 25(OH)D = 25-hydroxyvitamin D; RBP = Retinol Binding Protein; CRP = C-reactive Protein; EBNA-1 = Epstein-Barr Nuclear Antigen-1; AU = Arbitrary Units

highly correlated to age-at-sampling, the resulting stratifications were almost identical to the age-at-sampling stratification, and further logistic regression analyses using these stratifications did not help to differentiate between the three entangled variables. It was therefore not deemed possible to avoid differences between the age-at-sampling groups regarding these two variables.

We then went on, using the Table 6 cohort, to perform matched logistic regression analyses on the risk of MS for the different exposure categories regarding all studied risk factors and protective factors in all the subjects, in young subjects only, and in old subjects only. The ORs, CIs, and OR differences between the age groups were in these analyses (the Table 6 cohort) nearly identical to those when the entire cohort (the Table 5 cohort) was analyzed (data not shown). This analysis could therefore not support the idea that the variables controlled for here were responsible for the age-at-sampling specific differences in ORs. Also, as argued above and in Study IV,

no difference in the amount of exposure to cigarette smoke between the two age groups according to questionnaire data was found (Appendix, supplementary Table 7). Whether there are other differences between the two age groups, apart from those already known, that were not controlled for cannot be ruled out. Given that earlier publications support the hypothesis that the fundamental etiopathological event(s) that lead to MS occur at a young age,[23,25,59,137] and the lack of association between early gestational 25(OH)D, RBP, cotinine, and CRP and MS risk, our findings with a more pronounced effect on MS risk in those sampled at younger age point towards the first two–three decades of life as being crucial in this sense.

Hypothetically, in adolescents, a combination of suboptimal vitamin D levels, smoking, a relatively inexperienced (few infections) but mature (beyond childhood) immune system, and genetic predisposition act together to promote an inadequate immune response to EBV at the time of seroconversion, which subsequently could lead to MS. Perhaps suboptimal vitamin A levels during life might help promote this inadequate immune response, and the development of MS.

## General advantages and limitations

### *Reversed causation*

In risk factor epidemiology, it is essential to find ways to minimize the risk of reversed causation, where the outcome causes the exposure rather than the opposite. For example, reversed causation might cause the vitamin D levels in MS cases to become either lower (less time spent outdoors due to impaired walking ability), or higher (more common with vitamin D supplementation in MS cases),[138] depending on timing and cohort characteristics. The study design used in this project with prospectively collected samples reduces the possibility of reversed causation, even though earlier occurring neurological symptoms, not found in the medical records review undertaken for each case in RoMS, cannot be completely excluded.

### *Matching and confounding*

Due to the amount of samples available in the biobanks (approximately $n$=291,500 from $n$=164,000 individuals when the cross-linkage between the case database and the biobanks was performed), a high degree of matching accuracy was achieved (Table, Study II). We also show only small changes in estimated ORs and CIs after adjusting for possible confounders, further strengthening the results (Tables 3 and 4).

The possibility of confounding by factors not addressed in the matching strategy or the multivariable analyses cannot be excluded. One such factor might be shift work at a young age, although uncommon, which has been shown to be associated with increased MS risk in one study of two cohorts.[137]

It is possible that shift work is associated with one or more of the exposures, which may be non-causally associated with MS, and thereby shift-work might represent a confounder. However, the opposite (that shift work is in fact a proxy for low vitamin D levels or perhaps higher smoking prevalence which in turn are causally associated with MS) seems more likely.[139] Nonetheless, adjustment for multiple possible confounders, including sun exposure, vitamin D status after disease onset, and smoking, were undertaken in the study on shift work and MS, and the results seemed robust. Among the suggested mechanisms for the association seen are circadian disruption and sleep deprivation, associated with melatonin-related effects on the immune system.[137] Other factors that have been associated with MS risk are obesity during adolescence,[140,141] exposure to organic solvents,[142-146] certain occupations,[147] educational level,[148] dietary factors,[149,150] psychological stress,[151] vaccinations,[152] sex hormones,[153] and many more.[8] For some of these factors, such as vaccinations and intake of dietary fat, the results have been conflicting,[8] and some studies suffer from methodological shortcomings, such as a high probability of confounding.[149]

In summary, several possible confounders, which are not controlled for in this thesis, might exist. The role in MS etiopathogenesis for many of these remain unclear, and some might actually be proxies for other factors. E.g., obese persons have been shown to have lower 25(OH)D levels due to decreased bioavailability of vitamin D because of its deposition in body fat compartments.[154,155] Many of the possible confounders would have required extensive, preferably prospective, questionnaires to be properly measured. It was therefore not deemed feasible to include all of them in the present project.

### *Laboratory methods*

We have used ELISAs to measure the levels of 25(OH)D, RBP, hs-CRP, and antibody reactivity against EBNA-1 and against the EBNA-1 domains in this study. For the EBNA-1 domains, we see no alternative method. The absolute values of the other ELISA-measurements may not be as accurate as if more exact methods had been used. However, this was not an option, as the methods employed in such analyses generally require much larger sample volumes than the ELISAs used here. We were asked to minimize the estimated expenditure of plasma and serum and were ultimately granted only 180 μl from each stored sample. It is proper and wise from the point of view of the biobank representatives to act in this way, since minimizing sample volume usage has high priority, making it possible to perform more studies of this kind on stored samples. The drawback was reduced precision due to the fact that more inexact methods had to be used. However, this is not a major problem when it comes to comparing levels of factors in case and control samples analyzed together on the same day and plate, and using the

same methodology. As for our 25(OH)D estimates, there is a theoretical possibility that a systematic laboratory method-related bias has influenced the results and contributed to the overall very low vitamin D levels found in our study. On the other hand, one study from Finland reported median 25(OH)D levels of 30 nmol/l in *n*=106 inpatients and 41 nmol/l in *n*=99 outpatients, and one study from Canada reported mean 25(OH)D levels of 41 nmol/l in *n*=61 healthy hospital workers.[156,157] When comparing the 25(OH)D status in our study with that in those two studies, it seems plausible that the levels we report are representative of the overall levels in populations at these latitudes. The method for assessing vitamin D levels in both of the studies mentioned above was an established radioimmunoassay (RIA) from Diasorin, Stillwater, MN, USA, which is the same assay used in the prospective MS-study by Munger et al. performed at lower latitudes showing higher vitamin D levels in biobank samples.[59]

**Trends over time**

In NSMC controls, we reported a decreasing prevalence of 25(OH)D levels ≥75 nmol/l over the time period 1976–2005 by comparing ORs for having 25(OH)D levels ≥75 nmol/l for different time periods, and an inverse correlation between 25(OH)D levels and sampling year during the summer months. If we had expected to see this decrease in 25(OH)D levels over time, we would have added sodium levels ($Na^+$) to the analyses as this electrolyte is used as an indicator of sample concentration, and thereby the degree of evaporation/dilution could have been properly estimated. However, the results were interpreted after sample re-freezing, and we did not choose to re-thaw and add sodium to the analyses. As shown in Table 2, the finding of decreasing 25(OH)D levels could not easily be explained by evaporation in older samples, as such a phenomenon probably would have resulted in similar findings for the other variables as well. In that table we show that CRP levels, on the contrary, *increased* during the same time period, EBNA-1 antibody reactivity decreased, but to a lesser extent than 25(OH)D levels, and RBP and cotinine concentrations remained unchanged. Taken together, this implies that the 25(OH)D levels in pregnant women has decreased during the 30 years over which this study spans. As elaborated in Study II, this might be due to altered behavior during summer, such as increased use of sun-screen or less time spent outdoors,[87] and it is not impossible that the increasing incidence and prevalence of MS seen over the years in the industrialized parts of the world is an effect of decreasing 25(OH)D levels.[1]

# Summary

In conclusion, by analyzing serum and plasma samples collected before disease onset in cases and matched controls, these studies have shown that:

1) high vitamin D levels were associated with a decreased risk of MS,

2) the vitamin D levels in pregnant women decreased during 1976–2005,

3) intermediate vitamin A levels, compared with low levels, seemed to be associated with a decreased risk of MS,

4) elevated CRP levels in young subjects were associated with a decreased risk of MS,

5) smoking was associated with an increased risk of MS,

6) high IgG antibody reactivity against EBNA-1, and especially the domains EBNA-$1^{385-420}$, and EBNA-$1^{402-502}$, were associated with an increased risk for MS,

7) the EBNA-1 antibody reactivity and vitamin D levels correlated inversely in young subjects, suggesting that one of these might influence the levels of the other,

8) compiled evidence from all these analyses suggest that the initial etiopathological event(s) that later lead to MS may occur during the first 2–3 decades of life and,

9) these studies have also shown that although spring-births were associated with a small increase in MS risk, this could not easily be explained by early gestational vitamin D, CRP, cotinine or vitamin A levels.

Astonishingly, clues to most of what we have learned from these studies can be found in the Hippocrates quote at the beginning of this thesis: the impact of seasons (*...consider the seasons of the year...*), latitude (*...peculiar to each locality...*) and sunlight (*...and the rising of the sun...*) on 25(OH)D levels, the hygienic aspect which connects to infections (*...the waters which the inhabitants use...*), and habits, such as smoking (*...what are their pursuits...*). Who would have thought that a bloke walking the earth 400 BC could inspire research being performed in the 21st century?

# Future prospects

DNA from *n*=255 of the participants in the prospective study arm has been identified in the NSHDS biobank and in national MS projects, and a mailable saliva collection kit for DNA extraction has been sent to the remaining *n*=321, of which *n*=148 responded. DNA has been extracted from the saliva samples by a local laboratory and HLA haplotype (HLA DRB1*15:01 and HLA A*02:01) will be determined in collaboration with Prof. Tomas Olsson's group, Karolinska Institutet. These data, which are expected during 2013, will allow for interaction analyses on smoking and HLA, vitamin D and HLA, and EBV and HLA.

An extension of these studies, entitled DEMOS (vitamin D and Epstein-Barr virus – infectious MOnonucleosis – in multiple Sclerosis etiology) has been launched. This study includes more Swedish biobanks with samples collected further south (with potentially higher 25[OH]D levels) and from younger individuals. Most of the permissions have been granted and data collection has begun. We estimate that approximately 1000 prospectively collected MS samples will be found in this study, making it by far the largest MS study on prospectively collected biobank samples.

Another logical extension of our findings would be an interventional study exploring the primary preventive potential, regarding MS, of oral vitamin D supplementation. However, as the annual MS incidence in the general population is quite low, around 5/100,000 in Västerbotten county,[158] such a study targeting the general population would require a very large cohort, and a long follow-up. One way of reducing these numbers would be to enrich the study population with genetically predisposed subjects, such as first-degree relatives to MS cases,[159] however, this approach entails additional ethical and logistic difficulties.

# Acknowledgements

Anna, for your love, and for sharing (and sometimes even outmatching) my anticipation and nervousness awaiting editorial decisions. And for allowing me to be periodically lost in my universe of exposures, outcomes, and confounders.

My daughters: Isa, Stina and Kajsa, for not allowing that, but literally dragging me back into your wonderful realms of imaginative elves, trolls, sorcerers, unicorns, princesses, and cuddly cats.

Peter Sundström, my main supervisor. Thank you for your never ending patience, mirroring my eagerness, and your razor-sharp reviews of my texts. And for believing in me.

Anders Svenningsson, my co-supervisor, for being such an inspiration and for regarding me as determined and executive, rather than haphazard and hasty.

Hans Stenlund, my own SOD (statistician-on-demand). Your advice has been invaluable.

Göran Hallmans, your foresight and devotion in building and maintaining the biobanks has been absolutely crucial for this thesis.

Göran Wadell, who started collecting NSMC samples almost 40 years ago, the samples that have been invaluable for this project.

Maria Nyström, working evenings, nights and mornings in the lab. With meticulous accuracy. Great work!

Jörgen Andersson, your efforts in the freezers are much appreciated.

Viktor Grut, who did a great job with case-ascertainment.

Barbara Frankel, thank you for chopping up my sentences into smaller, more readable, bits. I know, I am a comma-junkie. I am working on it…

Jan Malm, your long experience in the research community has taught me a lot. And your critical commentaries likewise.

Gull-Britt Widefjäll, thank you for running up and down between the outpatient clinic and the medical charts storage room with old, dusty paper charts for me.

Johan Jacobsson, my clinical supervisor and personal idol.

Hans Lindsten, the boss. I have still not managed to find a piece of paper that you have hesitated to sign.

All personnel involved in creating and maintaining the biobanks used in this study. A lot of work has been put into this by you, without which these studies would not have been possible.

All you who have donated blood to the biobanks, and generously consented to contribute to these studies.

All colleagues and personnel at the Neurology department for support.

My family for love and support.

The studies in this thesis were funded by grants from The Swedish Association of Neurologically Disabled (N.H.R.); The Department of Clinical Neuroscience at Umeå University; Biogen Idec; Sanofi Aventis; Merck Serono; Bayer-Schering Pharma; and through a regional agreement between Umeå University and Västerbotten County Council on cooperation in the fields of Medicine, Odontology, and Health (A.L.F.).

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# Appendices

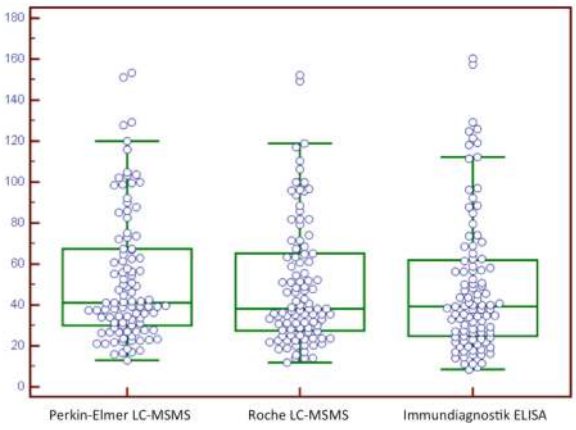
## Supplementary Table 1

**Supplementary Table 1. 25(OH)D levels in *n*=100 randomly selected serum samples (*n*=61 female, *n*=39 male) from end-stage-renal-disease patients according to three different methods. The correlation coefficients for the LC-MSMS methods compared to the ELISA are also shown.**

| Method | Median (range) 25(OH)D | Mean (SD) 25(OH)D | Correlation coefficients (*r*) |
|---|---|---|---|
| **Perkin Elmer LC-MS/MS** | 41 (13–153) | 45 (32) | $r$=0.89, $p$<0.0001 |
| **Roche LC-MS/MS** | 38 (12–152) | 42 (30) | $r$=0.89, $p$<0.0001 |
| **Immundiagnostik ELISA** | 39 (9–160) | 40 (34) | - |

Data on file, kindly provided by HJ Roth 2011. Printed with permission.
25(OH)D = 25-hydroxyvitamin D; LC-MSMS = Liquid chromatography-tandem mass spectrometry; ELISA = Enzyme linked immunosorbent assay; SD = Standard Deviation

## Supplementary Figure 1




Supplementary Figure 1. This figure shows the agreement between the 25(OH)D ELISA used in Study II, and two established LC-MSMS methods. The y axis denotes 25(OH)D levels in nmol/l. One small circle represents each sample. The boxes show the interquartile range, the lines within the boxes show the median values and the whiskers show the range excluding outliers. Data on file, kindly provided by HJ Roth 2011. Printed with permission. 25(OH)D = 25-hydroxyvitamin D; ELISA = Enzyme linked immunosorbent assay; LC-MSMS = Liquid chromatography - tandem mass spectrometry.

## Supplementary Tables 2a–d

**Supplementary Table 2a. Number of samples, mean 25(OH)D levels and *p*-values for comparison of means in each category to the mean of normal appearing samples among 515 RoMS and GRoMS controls from the NSMC**

| Categories | No. of samples | Mean (SD) 25(OH)D | *p* |
|---|---|---|---|
| **Normal appearance** | 460 | 43 (20) | (referent) |
| **Hemolysis** | 7 | 130 (139) | <0.001 |
| **Low sample volume** | 8 | 41 (17) | 0.83 |
| **Watery appearance** | 2 | 0 (0) | 0.003 |
| **Partially clotted** | 24 | 46 (20) | 0.48 |
| **Not properly sealed** | 14 | 57 (39) | 0.016 |

The independent samples *t*-test was used to test for differences between means. 25(OH)D level unit nmol/l.
25(OH)D = 25-hydroxyvitamin D; RoMS = Risk factors of Multiple Sclerosis; GRoMS = Gestational Risk factors of Multiple Sclerosis; NSMC = Northern Sweden Maternity Cohort; SD = Standard Deviation. 
From Study II (supplementary data online, Table e-2). Slightly modified. Reprinted with permission.

**Supplementary Table 2b. Number of samples, mean RBP levels and *p*-values for comparison of means in each category to the mean of normal appearing samples among 515 RoMS and GRoMS controls from the NSMC**

| Categories | No. of samples | Mean (SD) RBP | *p* |
|---|---|---|---|
| **Normal appearance** | 460 | 43 (30) | (referent) |
| **Hemolysis** | 7 | 44 (49) | 0.9 |
| **Low sample volume** | 8 | 60 (23) | 0.1 |
| **Watery appearance** | 2 | 1 (2) | 0.048 |
| **Partially clotted** | 24 | 48 (39) | 0.5 |
| **Not properly sealed** | 14 | 43 (18) | 0.9 |

The independent samples *t*-test was used to test for differences between means. RBP unit mg/l.
RBP = Retinol Binding Protein; RoMS = Risk factors of Multiple Sclerosis; GRoMS = Gestational Risk factors of Multiple Sclerosis; NSMC = Northern Sweden Maternity Cohort; SD = Standard Deviation.

**Supplementary Table 2c. Number of samples, mean cotinine levels and *p*-values for comparison of means in each category to the mean of normal appearing samples among 330 RoMS controls from the NSMC**

| Categories | No. of samples | Mean (SD) cotinine | *p* |
|---|---|---|---|
| **Normal appearance** | 297 | 77 (136) | (referent) |
| **Hemolysis** | 1 | 0 (-) | 0.6 |
| **Low sample volume** | 6 | 57 (97) | 0.7 |
| **Watery appearance** | 1 | >500 (-) | 0.002 |
| **Partially clotted** | 13 | 42 (84) | 0.4 |
| **Not properly sealed** | 12 | 78 (121) | 1.0 |

The independent samples *t*-test was used to test for differences between means. Cotinine unit ng/l.
RoMS = Risk factors of Multiple Sclerosis; NSMC = Northern Sweden Maternity Cohort; SD = Standard Deviation.

**Supplementary Table 2d. Number of samples, mean EBNA-1 antibody reactivites and *p*-values for comparison of means in each category to the mean of normal appearing samples among 330 RoMS and GRoMS controls from the NSMC**

| Categories | No. of samples | Mean (SD) EBNA-1 antibody reactivity | *p* |
|---|---|---|---|
| **Normal appearance** | 297 | 123 (48) | (referent) |
| **Hemolysis** | 1 | 119 (-) | 0.9 |
| **Low sample volume** | 6 | 146 (19) | 0.2 |
| **Watery appearance** | 1 | 2 (-) | 0.013 |
| **Partially clotted** | 13 | 118 (38) | 0.7 |
| **Not properly sealed** | 12 | 125 (56) | 0.9 |

The independent samples *t*-test was used to test for differences between means. EBNA-1 antibody reactivity unit AU. EBNA-1 = Epstein-Barr Nuclear Antigen-1; RoMS = Risk factors of Multiple Sclerosis; NSMC = Northern Sweden Maternity Cohort; SD = Standard Deviation; AU = Arbitrary Units

## Supplementary Table 3 (alternative analysis of Study I data)

**Supplementary Table 3. Observed number of cases of multiple sclerosis in Sweden compared with expected number, according to month of birth. Cases identified through the Swedish multiple sclerosis register; controls, truncated at 1995, from Statistics Sweden.**

| Month | All Births 1900–1995 *n*=10,952,005 | Observed no. of cases *n*=9,361 | Expected no. of cases | OR (95% CI) | *p* |
|---|---|---|---|---|---|
| **Jan** | 931,400 | 723 | 796 | 0.90 (0.84–0.97) | 0.007[a] |
| **Feb** | 875,313 | 791 | 748 | 1.06 (0.99–1.14) | 0.10 |
| **Mar** | 1,023,006 | 831 | 874 | 0.95 (0.88–1.02) | 0.12 |
| **Apr** | 997,609 | 867 | 853 | 1.02 (0.95–1.09) | 0.61 |
| **May** | 976,605 | 848 | 835 | 1.02 (0.95–1.09) | 0.63 |
| **Jun** | 913,057 | 860 | 781 | 1.11 (1.04–1.19) | 0.003[b] |
| **Jul** | 914,412 | 788 | 782 | 1.01 (0.94–1.09) | 0.81 |
| **Aug** | 887,909 | 763 | 759 | 1.01 (0.93–1.09) | 0.88 |
| **Sep** | 913,608 | 791 | 781 | 1.01 (0.94–1.09) | 0.71 |
| **Oct** | 896,365 | 714 | 743 | 0.96 (0.89–1.03) | 0.27 |
| **Nov** | 795,012 | 713 | 680 | 1.05 (0.98–1.14) | 0.18 |
| **Dec** | 854,709 | 672 | 731 | 0.91 (0.85–0.99) | 0.024[c] |

[a]0.084 with Bonferroni correction
[b]0.036 with Bonferroni correction
[c]0.288 with Bonferroni correction

## Supplementary Tables 4, 5 and 6 (from Study V)

**Supplementary Table 4. Odds ratios (OR) and 95% confidence intervals (CI) of developing multiple sclerosis (MS) for different antibody reactivity categories against Epstein-Barr nuclear antigen-1 (EBNA-1) and different EBNA-1 regions in prospectively collected samples from *n*=192 multiple sclerosis cases and *n*=384 matched controls.**

| EBNA-1 domain | Categories (AU) | No. Of | | Bivariate analysis | | Multivariable analysis | | Trend |
|---|---|---|---|---|---|---|---|---|
| | | Cases | Cont. | OR | 95% CI | OR | 95% CI | *p* |
| **1-90** | Low (<18) | 55 | 128 | 1.0 | (ref) | 1.0 | (ref) | |
| | Med (18–65) | 55 | 128 | 1.0 | 0.65–1.5 | 1.0 | 0.64–1.6 | |
| | High (>65) | 82 | 128 | 1.5 | 0.97–2.2 | 1.4 | 0.95–2.2 | 0.09 |
| **61-90** | Low (<13) | 56 | 128 | 1.0 | (ref) | 1.0 | (ref) | |
| | Med (13–54) | 53 | 128 | 0.94 | 0.60–1.5 | 0.93 | 0.60–1.5 | |
| | High (>54) | 83 | 128 | 1.5 | 0.97–2.2 | 1.5 | 0.96–2.2 | 0.07 |
| **402-502** | Low (<33) | 38 | 128 | 1.0 | (ref) | 1.0 | (ref) | |
| | Med (33–71) | 49 | 128 | 1.2 | 0.74–2.0 | 1.2 | 0.74–2.0 | |
| | High (>71) | 105 | 128 | 2.7 | 1.7–4.3 | 2.7 | 1.7–4.2 | <0.01 |
| **385-420** | Low (<21) | 28 | 128 | 1.0 | (ref) | 1.0 | (ref) | |
| | Med (21–60) | 47 | 128 | 1.6 | 0.95–2.7 | 1.6 | 0.93–2.7 | |
| | High (>60) | 117 | 128 | 4.0 | 2.5–6.7 | 4.0 | 2.5–6.6 | <0.01 |
| **421-449** | Low (<7) | 64 | 128 | 1.0 | (ref) | 1.0 | (ref) | |
| | Med (7–29) | 53 | 128 | 0.82 | 0.52–1.3 | 0.82 | 0.52–1.3 | |
| | High (>29) | 75 | 128 | 1.1 | 0.77–1.8 | 1.1 | 0.74–1.7 | 0.28 |
| **EBNA-1** | Low (<120) | 35 | 128 | 1.0 | (ref) | 1.0 | (ref) | |
| | Med (120–147) | 70 | 128 | 1.9 | 1.2–3.1 | 2.0 | 1.2–3.2 | |
| | High (>147) | 87 | 128 | 2.8 | 1.7–4.7 | 2.8 | 1.7–4.6 | <0.01 |

ORs and 95% CIs were estimated in matched bivariate and multivariable (adjusted for 25-hydroxyvitamin D <75 vs. ≥75 nmol/l) logistic regression analysis. From Study V, supplementary Table 1. Slightly modified.

**Supplementary Table 5. Odds ratios (OR) and 95% confidence intervals (CI) of developing multiple sclerosis (MS) for different antibody reactivity categories against Epstein-Barr nuclear antigen-1 (EBNA-1) and different EBNA-1 regions in prospectively collected samples from *n*=96 multiple sclerosis cases and *n*=192 matched controls. All subjects were below median age at sampling (<26.4 years old).**

| EBNA-1 domain | Categories (AU) | No. Of | | Bivariate analysis | | Multivariable analysis | | Trend |
|---|---|---|---|---|---|---|---|---|
| | | Cases | Cont. | OR | 95% CI | OR | 95% CI | *p* |
| **1-90** | Low (<19) | 26 | 64 | 1.0 | (ref) | 1.0 | (ref) | |
| | Med (19–70) | 29 | 64 | 1.1 | 0.59–2.1 | 1.2 | 0.63–2.2 | |
| | High (>70) | 41 | 64 | 1.5 | 0.90–3.0 | 1.6 | 0.86–2.9 | 0.28 |
| **61-90** | Low (<14) | 25 | 64 | 1.0 | (ref) | 1.0 | (ref) | |
| | Med (14–69) | 34 | 64 | 1.3 | 0.72–2.5 | 1.3 | 0.70–2.5 | |
| | High (>69) | 37 | 64 | 1.5 | 0.83–2.8 | 1.4 | 0.77–2.6 | 0.43 |
| **402-502** | Low (<33) | 16 | 64 | 1.0 | (ref) | 1.0 | (ref) | |
| | Med (33–72) | 26 | 64 | 1.7 | 0.81–3.5 | 1.6 | 0.78–3.4 | |
| | High (>72) | 54 | 64 | 3.6 | 1.8–7.2 | 3.3 | 1.6–6.7 | <0.01 |
| **385-420** | Low (<21) | 10 | 64 | 1.0 | (ref) | 1.0 | (ref) | |
| | Med (21–59) | 25 | 64 | 2.7 | 1.1–6.5 | 2.7 | 1.1–6.4 | |
| | High (>59) | 61 | 64 | 6.2 | 2.8–14 | 6.0 | 2.6–14 | <0.01 |
| **421-449** | Low (<6) | 26 | 64 | 1.0 | (ref) | 1.0 | (ref) | |
| | Med (7–31) | 32 | 64 | 1.2 | 0.65–2.3 | 1.2 | 0.65–2.4 | |
| | High (>31) | 38 | 64 | 1.6 | 0.82–2.9 | 1.5 | 0.77–2.8 | 0.47 |
| **EBNA-1** | Low (<121) | 16 | 64 | 1.0 | (ref) | 1.0 | (ref) | |
| | Med (121–154) | 43 | 64 | 2.9 | 1.4–6.0 | 2.9 | 1.4–6.1 | |
| | High (>154) | 37 | 64 | 2.3 | 1.1–5.2 | 2.3 | 1.1–5.0 | 0.01 |

ORs and 95% CIs were estimated in matched bivariate and multivariable (adjusted for 25-hydroxyvitamin D <75 vs. ≥75 nmol/l) logistic regression analysis. From Study V, supplementary Table 2. Slightly modified.

**Supplementary Table 6. Odds ratios (OR) and 95% confidence intervals (CI) of developing multiple sclerosis for different antibody reactivity categories to Epstein-Barr nuclear antigen-1 (EBNA-1) regions according to 25-hydroxyvitamin D (25[OH]D) status, and analyses of additive interaction, in prospectively collected samples from *n*=192 multiple sclerosis cases and *n*=384 matched controls.**

| 25(OH)D status | Antibody reactivity categories | Arbi-trary units | No. of | | Interaction analysis | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Cases | Controls | OR | 95% CI | AP | 95% CI |
| **Fragment EBNA-1$^{1–90}$** | | | | | | | | |
| ≥75 nmol/l | Low | <26 | 4 | 15 | 1.0 | (referent) | | |
| ≥75 nmol/l | High | ≥26 | 3 | 15 | 0.75 | 0.14–3.9 | | |
| <75 nmol/l | Low | <32 | 67 | 177 | 1.4 | 0.46–4.4 | | |
| <75 nmol/l | High | ≥32 | 118 | 177 | 2.5 | 0.81–7.7 | 0.53 | -0.08–1.1 |
| **Fragment EBNA-1$^{61–90}$** | | | | | | | | |
| ≥75 nmol/l | Low | <20 | 3 | 15 | 1.0 | (referent) | | |
| ≥75 nmol/l | High | ≥20 | 4 | 15 | 1.3 | 0.25–7.0 | | |
| <75 nmol/l | Low | <27 | 71 | 177 | 2.0 | 0.56–7.1 | | |
| <75 nmol/l | High | ≥27 | 114 | 177 | 3.2 | 0.91–11 | 0.27 | -0.37–0.92 |
| **Fragment EBNA-1$^{402–502}$** | | | | | | | | |
| ≥75 nmol/l | Low | <56 | 1 | 15 | 1.0 | (referent) | | |
| ≥75 nmol/l | High | ≥56 | 6 | 15 | 6.0 | 0.64–56 | | |
| <75 nmol/l | Low | <52 | 58 | 177 | 4.9 | 0.64–38 | | |
| <75 nmol/l | High | ≥52 | 127 | 177 | 11 | 1.4–83 | 0.08 | -0.53–0.69 |
| **Fragment EBNA-1$^{385–420}$** | | | | | | | | |
| ≥75 nmol/l | Low | <45 | 1 | 15 | 1.0 | (referent) | | |
| ≥75 nmol/l | High | ≥45 | 6 | 15 | 6.0 | 0.64–56 | | |
| <75 nmol/l | Low | <39 | 50 | 177 | 4.2 | 0.55–33 | | |
| <75 nmol/l | High | ≥39 | 135 | 177 | 11 | 1.5–87 | 0.19 | -0.38–0.76 |
| **Fragment EBNA-1$^{421–449}$** | | | | | | | | |
| ≥75 nmol/l | Low | <15 | 3 | 15 | 1 | (referent) | | |
| ≥75 nmol/l | High | ≥15 | 4 | 15 | 1.3 | 0.25–7.0 | | |
| <75 nmol/l | Low | <17 | 86 | 177 | 2.4 | 0.69–8.6 | | |
| <75 nmol/l | High | ≥17 | 99 | 177 | 2.8 | 0.79–9.9 | 0.01 | -0.75–0.78 |
| **EBNA-1** | | | | | | | | |
| ≥75 nmol/l | Low | <138 | 1 | 15 | 1.0 | (referent) | | |
| ≥75 nmol/l | High | ≥138 | 6 | 15 | 6.0 | 0.64–56 | | |
| <75 nmol/l | Low | <139 | 63 | 177 | 5.3 | 0.69–41 | | |
| <75 nmol/l | High | ≥139 | 122 | 177 | 10 | 1.3–79 | 0.00 | -0.64–0.64 |

ORs and 95% CIs were estimated in unmatched multinomial logistic regression analyses. The antibody reactivity towards the EBNA-1 fragments were dichotomized (median) among controls with 25(OH)D levels ≥75 nmol/l (*n*=30), and among controls with 25(OH)D levels <75 nmol/l (*n*=354), respectively. Attributable Proportion due to interaction (AP) was calculated as described elsewhere.[9o] From Study V, supplementary Table 3. Slightly modified.

**Supplementary Data 1 (in Swedish)**

*Information letter to all participants. Note that the point about saliva was omitted if we had already found DNA in the biobanks or in the national MS projects. Instead, we informed that HLA analyses would be performed on already biobanked material, otherwise the letters were identical.*

”Vi vill härmed informera Dig om ett pågående forskningsprojekt där vår avsikt är att använda blodprov som Du för flera år sedan donerat till den s.k. Maternitetskohorten som ingår i Medicinska biobanken, Norrlands Universitetssjukhus. Det aktuella projektet gäller olika miljöfaktorers betydelse för uppkomsten av inflammatorisk sjukdom i nervsystemet, särskilt multipel skleros.

Med detta brev vill vi informera Dig om att vi avser att använda Ditt donerade blodprov för följande analyser:
Tecken på genomgången virusinfektion, särskilt körtelfeber, tecken på tobaksbruk och halter av vitamin A och D.

Då vävnadstyp och tobaksbruk i hög grad påverkar tolkningen av dessa analyser önskar Vi också be Dig om att Du:

1. **Lämnar ett salivprov i bifogat provrör** för bestämning av vävnadstyp, s k HLA-typ. samt att Du i så fall skriver under samtyckesformuläret.
2. **Besvarar bifogad enkät kring tobaksbruk**. Posta allt i bifogat svarskuvert!

- Umeå Universitet är ansvarigt för personuppgifter, adress: Personuppgiftsombudet, Umeå Universitet, 901 87 Umeå. Org nr 202100-2874.
- Undertecknad är kontaktperson för registret.
- Du har som deltagare rätt att skriftligen ansöka om information, s k registerutdrag, och få rättelse avseende eventuellt felaktiga uppgifter.
- Du har självklart rätt att tacka nej till att dessa analyser görs på det blodprov Du donerat, och/eller tacka nej till att lämna salivprov och/eller välja att inte besvara bifogad enkät. Du behöver ej heller ange skäl till detta.
- Studien har godkänts av Regionala etikprövningsnämnden, Umeå Universitet.

Med vänlig hälsning!

Peter Sundström
Överläkare, medicine doktor
Neurologklin, NUS
901 85 Umeå
Tel 090-7851942

Jonatan Salzer
ST-läkare, doktorand
Neurologklin, NUS
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## Supplementary Table 7 (from Study IV)

**Supplementary Table 7. Comparison of smoking questionnaire data, and of effects of cotinine status on MS risk according to questionnaire response status, among cases and controls by age at sampling.**

| | <26.4 years old at sampling | | | | ≥26.4 years old at sampling | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Cases | | Controls | | Cases | | Controls | | *p* |
| | *median* | *n* | *median* | *n* | *median* | *n* | *median* | *n* | |
| **Age at debut of smoking**[a] | 16 | 45 | 16 | 55 | 17 | 39 | 17 | 70 | 0.07[c] |
| **Duration of smoking (years)**[a] | 13 | 45 | 13 | 55 | 12 | 39 | 13 | 70 | 0.76[c] |
| **No. of packyears**[b] | 5.2 | 44 | 6.3 | 51 | 6.0 | 37 | 5.6 | 68 | 0.74[c] |
| | *n cases/controls (%)* | | | | *n cases/controls (%)* | | | | |
| **Proportion smokers during sampling year according to questionnaire**[d] | 35/72 (49) | | 44/106 (42) | | 20/69 (29) | | 35/148 (24) | | <0.01[e] |
| | *OR (95% CI)* | | | | *OR (95% CI)* | | | | |
| **OR for MS for "ever smokers" before or during index year**[d] | 1.5 (0.83–2.8) | | | | 1.4 (0.81–2.6) | | | | 0.87[g] |
| **OR for MS with cotinine levels ≥10 ng/ml among questionnaire responders**[d] | 1.9 (1.02–3.5) | | | | 1.1 (0.59–2.0) | | | | 0.22[g] |
| **OR for MS with cotinine levels ≥10 ng/ml among non-responders**[f] | 2.6 (1.02–6.8) | | | | 0.55 (0.21–1.4) | | | | 0.02[g] |

Only smoking data before index were included. Logistic regression, adjusted for sex and age at index, was used to calculate ORs and 95% CIs.

[a] *n*=209 had smoked before the index year and provided data on year of smoke debut, and smoking cessation.

[b] *n*=200 had smoked before the index year and provided data on the number of packyears smoked.

[c] *p*-values calculated with the Kruskal-Wallis test.

[d] *n*=395 were questionnaire responders (had provided information on smoking before or during the index year, and had provided information on smoking status during the sampling year).

[e] *p*-value calculated with the chi-square test.

[f] *n*=181 did not respond to the questionnaires.

[g] *p* for heterogeneity of ORs across strata.

From Study IV (supplementary data online). Slightly modified. No permission was needed to reprint this table.