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TOBACCO AND MULTIPLE SCLEROSIS SUSCEPTIBILITY

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**Karolinska
Institutet**

Stockholm 2015

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Printed by E-Print AB.

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ISBN 97891-7676-148-9

ABSTRACT

Multiple sclerosis (MS) is a chronic immune-mediated disease of the central nervous system (CNS) that arises from a combination of a complex genetic predisposition and environmental factors. For northern Europeans, the lifetime risk of MS is 1:400, making it the most common non-traumatic cause of disability in young adults. The strongest genetic associations with MS are located within the human leukocyte antigen (HLA) complex and in recent years, a large number of non-HLA risk loci that influence disease susceptibility have been identified. The contribution of lifestyle and environmental factors is more difficult to study. However, it is important to identify these factors since they are potentially preventable and may also lead to hypotheses on critical pathogenic events.

This thesis focuses on the impact of tobacco on MS risk. We replicated the finding of an association between smoking and MS risk and demonstrated that the risk of developing the disease increases with cumulative dose of smoking. However, snuff use, which leads to exposure to high doses of nicotine, was associated with decreased MS risk (paper I). In paper II, we showed an inverse dose-response correlation between cumulative dose of snuff use and disease risk. Nicotine has been shown to be protective in several models of inflammatory diseases, and may thus exert anti-inflammatory and immune-modulating effects in a way that reduces the risk of developing MS.

In paper IV, we investigated the association between smoking and MS in more detail. Both duration and intensity of smoking contribute independently to the risk of developing the disease. Smoking affects MS risk regardless of age at exposure, in contrast to many other environmental risk factors which seem to act mainly during adolescence. The detrimental effect of smoking slowly abates after smoking cessation regardless of the timing of smoking and regardless of the cumulative dose of smoking.

In paper III, we demonstrated that exposure to passive smoking among never smokers is associated with increased risk of MS.

Tobacco smoke, but not tobacco consumption in the form of moist snuff, increases MS risk, suggesting that the critical effects of smoking may be caused by irritation in the lungs.

Smoking increases pro-inflammatory cell activation and induces post-translational modifications of proteins in the lungs. In paper V, an interaction was demonstrated between carriage of HLA-DRB1*15, absence of HLA A*02, and smoking in the development of MS. We hypothesize that smoke-induced lung irritation, in the context of MS risk HLA genes, may generate post-translationally modified peptides which are cross-reactive with CNS antigens, promoting a CNS-directed autoaggressive immunity that results in MS. Further studies would be valuable in order to investigate whether other forms of lung irritation contribute to the triggering of MS.

Apart from generating data of importance for preventive measures, our finding of an interaction between smoking and HLA genotype emphasizes the need to include data on environmental exposures in genetic analyses of complex diseases and vice versa.

LIST OF SCIENTIFIC PAPERS

- I. *Tobacco smoking, but not Swedish snuff use, increases the risk of multiple sclerosis.* Hedström AK, Bäärnhielm M, Olsson T*, Alfredsson L*. *Neurology* 2009;73:696-701.
- II. *Nicotine might have a protective effect in the etiology of multiple sclerosis.* Hedström AK, Hillert J, Olsson T*, Alfredsson L*. *Multiple Sclerosis* 2013;19:1009-1013.
- III. *Exposure to environmental tobacco smoke is associated with increased risk for multiple sclerosis.* Hedström AK, Bäärnhielm M, Olsson T*, Alfredsson L*. *Multiple Sclerosis* 2011;17:788-793.
- VI. *Smoking and MS susceptibility.* Hedström AK, Hillert J, Olsson T*, Alfredsson L*. *Eur J Epidemiol* 2013;28:867-874.
- V. *Smoking and two human leukocyte antigen genes interact to increase the risk for multiple sclerosis.* Hedström AK, Sundqvist E, Bäärnhielm M, Nordin N, Hillert J, Kockum I, Olsson T*, Alfredsson L*. *Brain* 2011;134:653-664.

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CONTENTS

1	Introduction	5
1.1	Multiple sclerosis	5
1.2	The etiology of MS	6
1.2.1	Genetics	6
1.2.2	Environment and lifestyle	7
1.2.2.1	Sun exposure habits and vitamin D	8
1.2.2.2	Epstein-Barr virus and infection and mononucleosis	9
1.2.2.3	Smoking habits and exposure to passive smoking	10
1.2.2.4	Other environmental factors	10
1.3	The pathophysiology of MS	11
2	Epidemiological studies	13
2.1	Cohort studies	13
2.2	Case-control studies	13
2.3	Statistical methods in epidemiological studies	14
2.3.1	Logistic regression	14
2.3.2	Measures of association	14
2.3.3	Interaction	15
3	Aims of thesis	16
4	Materials and methods	17
4.1	EIMS	17
4.2	GEMS	17
4.3	Data collection	18
4.3.1	Definition of exposures	18
4.4	Genotyping	18
4.5	Statistical analysis	19
4.6	Confounding factors	19
5	Results and interpretation	21
5.1	Paper I	21
5.2	Paper II	21
5.3	Paper III	23
5.4	Paper IV	23
5.5	Paper V	27
6	Discussion	29
7	Summary of findings	32
8	Concluding remarks	33
9	Acknowledgements	35
10	References	37

LIST OF ABBREVIATIONS

AP	Attributable proportion due to interaction
APC	Antigen-presenting cell
BMI	Body mass index
CIS	Clinically isolated syndrome
CNS	Central nervous system
CD	Cluster of differentiation
CI	Confidence interval
EAE	Experimental autoimmune encephalitis
EIMS	Epidemiological Investigation of Multiple Sclerosis
EBNA	Epstein-Barr virus-determined nuclear antigen
EBV	Epstein-Barr virus
ELISA	Enzyme-Linked ImmunoSorbent Assay
GEMS	Genes and Environment in Multiple Sclerosis
GWAS	Genome-wide association study
HLA	Human leukocyte antigen
IL	Interleukin
IM	Infectious mononucleosis
LD	Linkage disequilibrium
MHC	Major histocompatibility complex
MOB	Month of birth
MRI	Magnetic resonance imaging
PCR	Polymerase chain reaction
PPMS	Primary progressive MS
RERI	Relative excess risk due to interaction
SAS	Statistical Analysis System
SPMS	Secondary progressive MS
SI	Synergy Index
OR	Odds ratio
UVR	Ultraviolet radiation

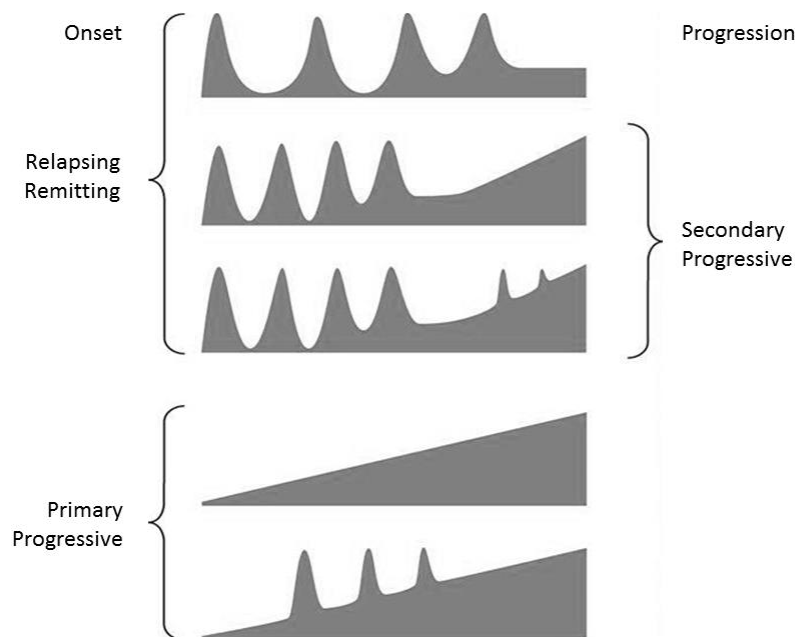
1 INTRODUCTION

1.1 MULTIPLE SCLEROSIS

MS is an inflammatory disease of the central nervous system, characterized by myelin destruction, axonal degeneration and sclerotic plaques¹. Approximately 2.5 million people are estimated to suffer from MS worldwide. In Sweden, the prevalence of MS has been estimated to be 188.9/100,000, with a female-to-male ratio of approximately 2.35:1². The disease occurs with an annual incidence of approximately 5/100,000.

MS typically starts as a relapsing remitting disease (RR MS) characterized by bouts of disease activity between periods of remission. The symptoms vary considerably depending on the location of the lesions within the CNS. During relapses, patients commonly experience symptoms from the motor, sensory, visual and autonomic systems. A relapse usually appears in a sub-acute manner over days or weeks followed by a gradual decline of the symptoms over weeks to months. Many lesions are clinically silent and magnetic resonance imaging (MRI) studies have indicated that lesions appear seven to ten times more frequently than clinical relapses. Ultimately the disease usually converts into secondary progressive MS (SPMS) with gradually increasing disability. In a low proportion of people affected by MS, the disease is progressive from onset without preceding clinically recognizable relapses (primary progressive MS; PPMS) (figure 1).

Figure 1. Common disease phenotypes of MS.



The diagnosis of RRMS is based on relapse history, findings on MRI and exclusion of differential diagnoses. The first neurological manifestation is usually referred to as a clinically isolated syndrome (CIS). For an MS diagnosis to be made according to the McDonald criteria a second event, separate in time or space, is required. A diagnosis of PPMS is based on clinical history, MRI findings, and cerebrospinal examination³.

The therapeutic options for MS have expanded in the past two decades. Several disease-modifying treatments are available today that reduce the number of clinical relapses and lesions seen by MRI, and oral compounds have recently been introduced. The first-line treatment of MS includes interferon-beta products and glatiramer acetate, which have similar long-term efficacy⁴⁻⁵. However, neutralizing antibodies to interferon β products may develop during treatment and reduce clinical efficacy⁶. When first-line drugs fail to suppress disease activity or in cases of high disease activity at onset, second-line treatments may be used. These include intravenously administered monoclonal antibodies directed against lymphocyte surface molecules⁷, and orally taken immunomodulatory drugs⁸⁻⁹. Most of the second-line drugs act broadly on the immune system with potentially serious side effects.

1.2 THE ETIOLOGY OF MS

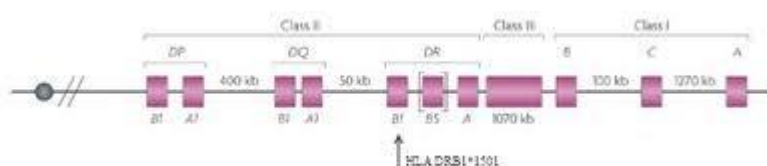
Numerous studies have shown that MS is a complex disease in which both genetic and environmental factors contribute to disease susceptibility. The concordance rate has previously been estimated to be around 30% for monozygotic twins and approximately 7% for dizygotic twins¹⁰. Recent population based studies have given more certain and lower estimates of heritability, with age adjusted concordance rates of about 17 % for monozygotic twins, as opposed to about 2 % for dizygotic twins and siblings in general¹¹. The substantially increased risk of developing MS in relatives of affected individuals gives a solid base for gene variants contributing to susceptibility, whereas the monozygotic concordance rates of about 17% is a strong argument for an important role of lifestyle and environmental factors in determining the risk of MS.

1.2.1 GENETICS

As in most organ specific inflammatory diseases of suspected auto-immune origin, the strongest genetic associations with MS are located within the human leukocyte antigen (HLA) complex (figure 2). The association between the HLA gene complex and MS was first described in 1972¹². The full sequence of the region was completed in 1999. Determining the role that specific HLA genes play in MS has been difficult since genes in this region are often passed down in tightly linked combinations, making it difficult to distinguish their individual effects. The proportion of specific HLA alleles differs between ethnic groups. The HLA complex contains around 200 genes, the vast majority coding for proteins involved in immune function. In organ specific inflammatory diseases, the HLA class I- and class II-encoded molecules are of particular importance. These are cell-surface glycoproteins that

display and present short antigenic peptide fragments to specific T-cells which can then become activated by a second stimulatory signal and initiate an immune response. Class I molecules present peptides to CD8+ T cells, whereas class II molecules present peptides to CD4+ T cells. The class II allele HLA-DRB1*15 increases the risk of developing MS in almost all populations, with an odds ratio (OR) around 3¹³. Other HLA-DRB1 alleles have recently been independently associated with MS risk¹⁴. Genes in the class I region have also been implicated in MS, such as the class I allele HLA-A*02, which has a protective effect in many populations with an OR of approximately 0.7¹⁵⁻¹⁷.

Figure 2. The HLA complex



Genome-wide association studies (GWAS) have identified a large number of gene regions outside the HLA complex that influence disease susceptibility. These studies have, at this moment, unequivocally uncovered associations with over a 100 susceptibility loci¹⁸⁻¹⁹. Each of the non-HLA locus has a smaller impact on MS risk with ORs less than 1.2. Furthermore, it is possible that a number of rare variants or susceptibility loci with effect sizes below the significance threshold in GWAS may contribute to the disease. Most of the disease-associated non-HLA genes are involved in regulating immune functions, which suggests that the critical disease mechanism involves immunological pathways. Despite the large number of gene loci associated with MS risk, they only explain a fraction of the heritability. One of several potential reasons for the missing heritability is interactions between risk genes and lifestyle/environmental factors.

1.2.2 ENVIRONMENT AND LIFESTYLE

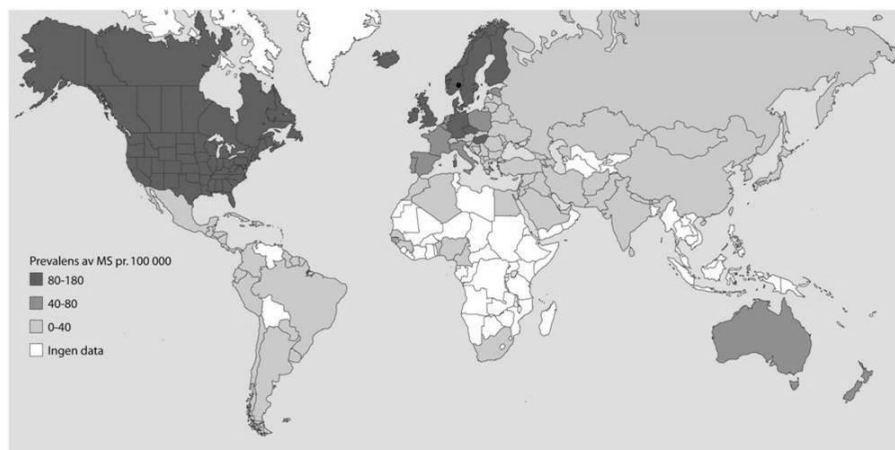
Migration studies have shown that when people move from a high- to a low-risk area in childhood this reduces the risk of MS to an intermediate level, between the level of their birth country and that of their final residence. Migration in the opposite direction does not consistently increase the risk of MS until the next generation whose risk is close to that of their birthplace²⁰⁻²¹. These data suggest that environmental exposures during childhood and adolescence are of essential importance for disease risk.

Lifestyle factors and environmental exposures are more difficult to study than genetic factors. However, it is important to identify these factors since they, as opposed to risk genes, are potential targets for prevention. Environmental factors that have been repeatedly shown to be associated with the risk of MS are sun exposure habits and vitamin D status, Epstein-Barr virus (EBV) infection and mononucleosis (IM), adolescent body-mass index (BMI), and smoking.

1.2.2.1 Sun-exposure habits and vitamin D

Both the incidence and prevalence of MS increase with the distance from the equator (figure 3). Latitudinal gradients have been identified throughout the world including Europe, North America, Australia, and New Zealand²². It has been suggested that this latitude-dependent gradient in MS occurrence is caused by less exposure to sunlight or decreased levels of vitamin D²³.

Figure 3. The prevalence of MS associated with latitude.



There is evidence suggesting that frequent exposure to ultraviolet radiation (UVR) confers a protective effect against developing MS²⁴⁻²⁶, and vitamin D has been proposed to be the major mediator of this protective effect²⁷⁻²⁸. The intensity of UVR exposure varies with latitude and season, and lower intensity of UVR in winter may be insufficient to support vitamin D synthesis in some locations²⁹. Vitamin D is involved in the regulation of the immune system by binding to vitamin D response elements in the regulatory region of immune genes³⁰⁻³¹. Furthermore, in several GWAS and candidate studies, an association has been observed between MS risk and markers in the CYP27B1 and CYP24A1 genes, the latter of which encodes an enzyme involved in vitamin D metabolism³²⁻³³.

However, it has been observed that low levels of sun exposure may have an impact on MS risk even after adjustment for vitamin D status, suggesting that the association between sun exposure and MS risk cannot be fully explained by vitamin D-mediated mechanisms³⁴.

There are a number of pathways whereby UVR may affect immune functions that are independent of vitamin D production³⁵. UVB appears to up-regulate the secretion of TNF- α , IL-10, and regulatory T-cells³⁶, and UVA radiation has a complex dose-related immunomodulating effect where the underlying mechanism is not fully investigated. In experimental autoimmune encephalomyelitis (EAE) studies, UVB exposure influenced systemic immune reactions and attenuated systemic autoimmunity via the induction of skin-derived tolerogenic dendritic cells and regulatory T cells³⁷. Vitamin D status may thus not be the only mediator of a latitude effect related to UVR exposure.

Another aspect discussed in the context of UVR and vitamin D is when insufficient exposure exerts its effect on the risk for MS, and if there is an interaction with MS risk genes. Several reports of a month of birth effect (MOB) have been published³⁸⁻³⁹. Children born in the spring in the northern hemisphere run an increased MS risk later in life. However, the authors of a recent study suggest that the claims of an association between MS and MOB may be false positive due to inadequate control for confounding factors⁴⁰. In our own studies of vitamin D status of newborn, there was no difference in vitamin D status between those who later developed MS and matched controls⁴¹.

Childhood and adolescence may be the most critical period for insufficient exposure. In an EAE study, vitamin D supplementation in the adolescent period improved the disease outcome whereas the same supplementation regimen had no influence when the administration took place during pre- and postnatal development or in adult age⁴².

An interaction with the HLA locus has been suggested⁴³. However, we found no such interaction in our case control cohort³⁴.

1.2.2.2 Epstein-Barr virus infection and mononucleosis

EBV infection is usually asymptomatic in childhood, and in countries where MS is rare, early infection with EBV is almost universal. However, in countries where primary infection is delayed beyond the early years of childhood and the infection more commonly results in IM, the prevalence of MS is high. Several studies have examined the association between IM and MS, with consistent results. People who have had IM have about a twofold increased risk of developing MS compared to those who were infected during childhood, whereas people who remain uninfected with EBV have an extremely low risk of developing the disease⁴⁴⁻⁴⁵. A meta-analysis of eight published studies found that the overall OR for MS was 13.5 (95% CI 6.3-31.4) when comparing EBV-seropositive and EBV-seronegative people⁴⁶.

By measuring anti-EBV titers before and after MS onset in 305 cases, Levin et al demonstrated that 100% of MS cases that were initially EBV seronegative had seroconverted prior to MS onset⁴⁷. Several studies have observed a significant increase in antibody titers many years prior to MS onset^{46, 48-49}. Nielsen et al found that the increased risk of MS following IM is independent of age, gender and infection severity, and may persist for decades⁵⁰. The consistent findings that EBV infection and elevation of anti-EBNA antibody titers precede MS onset suggest that EBV may be a necessary factor in MS development.

Data from several studies suggest that HLA status and either IM or high anti-EBV titers synergistically increase the risk of MS⁵¹⁻⁵². In the largest study on this topic, those who were positive for HLA-DRB1*15, negative for HLA-A*02 and with high EBNA:385-420 titers had a 16-fold higher risk of MS than those who did not carry any of these factors⁵². The observed interaction between HLA status and anti-EBV titers suggests that the mechanism through which HLA genes influence the risk of MS may involve the immune control of EBV infection. However, it cannot be completely ruled out that a dysregulated immunological response to EBV infection may be a consequence of the underlying

pathophysiology and genetic predisposition of MS.

1.2.2.3 Smoking habits and exposure to passive smoking

The first detected association between smoking and MS risk was reported in the 1960s⁵³. However, other studies found no impact of smoking on MS risk⁵⁴. In the 1990s, smoking was found to be associated with MS risk in two prospective cohort studies⁵⁵. Several studies investigating the link between smoking and MS susceptibility have been published during the last decade and almost all have detected a significant detrimental effect⁵⁶⁻⁵⁹. In a study using banked blood samples, Sundström et al found that cotinine levels, indicating recent exposure to tobacco smoke, were increased in MS cases compared with controls⁶⁰. There is also evidence of a dose-response correlation between cumulative dose of smoking and the risk of developing the disease⁵⁹.

Data has been inconsistent regarding the influence of passive smoking. A French case-control study found an association between exposure to parental smoking at home and early onset MS⁶¹. The risk increased with longer duration of exposure. However, no effect of maternal smoking during pregnancy on MS risk in offspring has been observed⁶²⁻⁶³. In the study by Montgomery et al⁶², information regarding maternal smoking during pregnancy was recorded prospectively, thus eliminating the problems associated with differential reporting bias. However, many women who smoke during pregnancy inaccurately report themselves as non-smokers⁶⁴⁻⁶⁵. Furthermore, maternal smoking during pregnancy may not be a sufficiently sensitive measure of later parental smoking at home.

The molecular pathways responsible for the association between smoking and MS are not yet known, but several plausible hypotheses regarding the mechanism have been put forward. Both humoral and cell-mediated immunity are affected by smoking⁶⁶, and smokers have increased levels of important markers of inflammation such as C-reactive protein and Interleukin (IL)-6⁶⁷. Serum concentrations of cyanide are strongly correlated with the level of tobacco consumption, and chronic cyanide intoxication may lead to widespread demyelination⁶⁸⁻⁶⁹. Some evidence points to a potential role of the free radical nitric oxide. Exposure to nitric oxide has been shown to cause axonal degeneration or block axonal conduction⁷⁰⁻⁷¹. Finally, smoking may increase the risk of MS by increasing the frequency and persistence of respiratory infections.

1.2.2.4 Other environmental factors

Other lifestyle or environmental factors that have been associated with MS are high BMI in adolescence⁷²⁻⁷³, shift work⁷⁴, and exposure to organic solvents⁷⁵, among others.

1.3 THE PATHOPHYSIOLOGY OF MS

The main function of the immune system is to protect the host from harmful pathogens, which is done by the recognition of self and response to non-self. The immune response has been divided into innate and adaptive immunity. The main components of the innate immune system, ready to fight microbes at the site of infection, are physical epithelial barriers, phagocytic leukocytes, dendritic cells, natural killer cells and circulating plasma proteins. The adaptive immune system is further divided into humoral immunity, mediated by antibodies produced by B cells, and cell-mediated immunity, mediated by T cells. CD4⁺ T cells have been regarded as helper T cells, since they are required for B-cell mediated antibody production and effective cellular responses. CD8⁺ T cells differentiate into cytotoxic cells that are crucial in the protection from virus infections and tumour cells.

T-cell progenitors migrate from the bone marrow to the thymus where they undergo differentiation. Only those that bind MHC products with low affinity survive (positive selection). In a second step, T-cells that are self-reactive are eliminated (negative selection). The selection events result in a T-cell population efficient in fighting foreign antigen and tolerant to self. However, in all individuals, potentially self-reactive T-cells slip through negative selection and enter the periphery. Peripheral tolerance is normally maintained by clonal elimination, anergy, and suppression of autoreactive cells by regulatory T cells⁷⁶.

Antigen recognition by T cells requires that the antigen has been picked up by an antigen-presenting cell (APC) and split into small peptide fragments. The peptides then form a complex with MHC class I or class II proteins, which is transported to the surface of the APC. CD4⁺ T cells recognize antigen peptides bound to MHC class II molecules, whereas CD8⁺ T cells recognize antigen peptides bound to MHC class I molecules. Antigen-specific activation of T-cells also requires co-stimulatory signals.

Following activation by APC, naive CD4⁺ T cells differentiate into different subtypes depending on the local cytokine environment. T helper 1 (Th1) T cells are responsible for cell-mediated immunity, and type T helper 2 (Th2) T cells are responsible for inducing antibody production by B cells and allergic responses. T helper 17 (Th17) T cells are produced in parallel to Th1 and also have the capacity to induce T cell mediated inflammation⁷⁷. Regulatory T cells are a subpopulation of T cells that suppress immune responses of other cells and are important in maintaining tolerance to self antigens.

It has been debated whether the primary mechanism of MS is inflammatory or neurodegenerative⁷⁸. However, autoimmunity and inflammation are both important manifestations of the disease and have a central role in both the fluctuating symptoms in RRMS and in the accumulating CNS injury. One central hypothesis of the initiation of MS is that T cells specific for myelin antigens are activated in the periphery and migrate to the CNS, yet the mechanism of peripheral activation remains largely unknown. Once activated, the T cells express adhesion molecules and can actively migrate across the blood-brain

barrier⁷⁹⁻⁸⁰. In the CNS, antigen-presenting cells reactivate the T cells, which in turn leads to targeted destruction of the myelin sheath.

The majority of infiltrating lymphocytes in the CNS are T cells and they include both CD4+ and CD8+ T cells. B cells and macrophages are also recruited and involved in the pathogenic process leading to the destruction of myelin sheaths. In addition, the blood brain barrier is compromised, which results in protein leakage into the CNS⁸¹.

2 EPIDEMIOLOGICAL STUDIES

2.1 COHORT STUDIES

Studies on how environmental and genetic factors influence MS risk are associated with several methodological and practical problems. The two major methods, cohort studies and case-control studies, have provided the majority of our current knowledge on environmental factors in MS.

In a cohort study, the included study subjects are classified with regard to exposure and then followed over a period of time with regard to occurrence of disease. Cohort studies can be classified as prospective or retrospective based on when outcomes occurred in relation to the enrolment of the cohort. An advantage of prospective cohort studies is that a chronological order of events can be defined, thereby reducing the risk of recall bias and the problem of reverse causation.

Due to the low incidence for many chronic diseases such as MS, a cohort study would need to be very large in order to generate enough cases during the course of a reasonable follow-up period. The need to obtain information on exposures from a large number of subjects often makes cohort studies very expensive to carry out.

2.2 CASE-CONTROL STUDIES

As is the case with cohort studies, case-control studies investigate the association between exposure and disease within a defined study base. Properly carried out, case-control studies provide information that mirrors what could be learned from the corresponding cohort study performed in the same study base, but more efficiently, using sampling. Cases that occur in the study population during the study period should be identified and included in the study as cases and controls should be randomly sampled from the study base to provide information on the exposure frequency in the study base. The controls may be frequency matched or individually matched on different characteristics of the cases (e.g. age and gender) in order to provide a more efficient statistical analysis. Information regarding different exposures and other characteristics among cases and controls is usually collected retrospectively. The exposure among cases is then compared to the exposure among controls in order to compute the OR as a measure of association. If the controls are drawn according to the principles of incidence density sampling, the estimated OR will be an approximation of the incidence rate ratio. The case-control design is well suited to investigate rare diseases or outcomes. In comparison with cohort studies, case-control studies are relatively inexpensive and considerably less time consuming.

Bias refers to systematic errors that result in an incorrect estimate of the association between exposure and outcome. In case-control studies, selection bias occurs when controls are not representative of the population that generated the cases with regard to the exposure of

interest. If the response rate is less than ideal, selection bias can also be introduced if the likelihood of responding is related to both the exposure and the outcome. The most significant drawback in case-control studies with retrospectively collected exposure information relates to the difficulty of obtaining reliable information on previous exposures. Recall bias occurs when the validity of the exposure information differs between cases and controls. One strategy to minimize this problem is to identify and investigate the cases as soon as possible after disease onset. Cohort studies are usually less subject to both these biases but often have a low power in uncommon diseases such as MS, particularly when there is a long follow-up period. Optimally, results from the two approaches should be combined.

2.3 STATISTICAL METHODS IN EPIDEMIOLOGICAL STUDIES

2.3.1 LOGISTIC REGRESSION

Logistic regression is a frequently applied method of analysis in medical sciences. Univariate logistic regression is used to explore associations between one outcome and one exposure whereas multivariate logistic regression succeeds in controlling confounding effects since the effect of each exposure is unconfounded by the others when put in the same model, given no misclassification of confounders. Logistic regression can thus be used to estimate the occurrence of a disease as a function of a risk factor, taking other risk factors into account. In case-control studies, matching on potential confounding factors may introduce selection bias, which must be taken into account in the analysis. When controls are frequency matched to cases, the matching variables should be included in an unconditional logistic model, whereas conditional logistic regression, in which each matched set forms a stratum, should be used when controls are individually matched to cases. However, losses may be substantial when data contain incomplete matched sets. Data from an individual matched case-control study may also be analyzed with unconditional logistic regression as long as the variables used to form the match are included in the model.

2.3.2 MEASURES OF ASSOCIATION

The incidence rate ratio is defined as the ratio of the disease incidence in the exposed group divided by the corresponding disease incidence in the unexposed group. The OR is the primary measure of association in logistic regression and represents the odds that an outcome will occur given a particular exposure, compared to the odds of the outcome occurring in the absence of that exposure. The interpretation of the OR calculated from a case-control study depends on how the controls have been sampled. If controls are sampled according to the principles of incidence density sampling, the estimated OR can be interpreted as an approximation of the incidence rate ratio.

An OR of 1 indicates that the odds of the outcome are equally likely for both groups under comparison. A 95% confidence interval is used to estimate the precision of the OR, and gives a range of values within which there is a 95% probability that the true value can be found.

2.3.3 INTERACTION

Interaction between two exposures is present when the effect of one exposure on the outcome is different across strata of the other exposure. Interaction on an additive scale means that the combined effect of two exposures differs from the sum of the individual effects of the exposures whereas interaction on a multiplicative scale means that the combined effect differs from the product of the individual effects. Biologic interaction should preferably be assessed on an additive scale⁸²⁻⁸³ and the interaction can be assessed by using different measures such as the relative excess risk due to interaction (RERI), the attributable proportion due to interaction (AP), and the synergy index (SI). In the case of preventive factors, these should be recoded into risk factors by considering the absence of the preventive factor to be the cause before calculating measures of interaction on an additive scale⁸⁴.

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

where RR_{AB} is the relative risk (RR) of disease if both factors A and B are present, RR_A is the RR of disease if factor A but not factor B is present, and RR_B is the RR of disease if only factor B is present. RERI can be interpreted as the excess risk due to interaction relative to the risk without exposure. A RERI greater than zero indicates the presence of interaction.

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

AP is the proportion of disease that is due to the interaction per se among individuals with both exposures. An AP greater than zero indicates the presence of interaction.

3 AIMS OF THESIS

The overall aim of this thesis was to investigate the impact of smoking habits, and the potential interaction between smoking and genetic risk factors with regard to MS risk. The influence from environmental tobacco smoke as well as moist snuff use was also of interest. Each project is described in more detail below.

Paper I: Previous studies with limited case numbers have provided evidence of an association between smoking and MS. We replicated the association between smoking and MS risk and investigated the effect of cumulative dose of smoking, stopping smoking and snuff use.

Paper II: Use of moist snuff is common in Sweden and leads to exposure to high doses of nicotine. We aimed to investigate whether snuff use is associated with MS risk, taking smoking habits into consideration.

Paper III: The influence of passive smoking on MS risk has been investigated in two previous studies, with inconsistent results. Our aim was to examine whether exposure to passive smoking influences MS risk among subjects who have never smoked.

Paper IV: We investigated how age at smoking debut, smoking duration and intensity, and the cumulative dose of smoking and smoking cessation influence the association between smoking and MS risk.

Paper V: The strongest genetic associations with MS are located within the HLA complex. In this paper, we focused on the potential interaction between smoking and the MS risk HLA genotypes HLA-DRB1*15 and absence of HLA-A*02 alleles, with regard to MS risk.

4 MATERIAL AND METHODS

All papers were based on data from the ongoing project Epidemiological Investigation of Multiple Sclerosis (EIMS). Papers II and IV also included subjects from the project Genes and Environment in Multiple Sclerosis (GEMS). Both EIMS and GEMS are Swedish population-based case-control studies, approved by the ethical review board of Karolinska Institutet. All participants in both studies provide written informed consent to participate.

4.1 EIMS

This project was designed as a population-based case-control study using incident cases of MS, with a study population comprising the Swedish population aged 16-70. Since 2005, cases have been recruited via hospital-based and privately run neurology units. In total, 40 study centers, including all university hospitals, report newly diagnosed cases of MS to the study. Each case was examined and diagnosed according to the McDonald criteria⁸⁵ by a neurologist located at the unit where the case was entered.

For each case, two controls were randomly selected from the national population registry, matched by age in five-year strata, gender and residential area. If information could not be obtained from the control selected, then another control was chosen using the same principles. Personal information and information on exposures, such as tobacco consumption, was collected using a standardized questionnaire given to the cases shortly after they had received their diagnosis and sent by mail to the controls. All questionnaires were answered at home. Incompletely answered questionnaires were completed by mail or by telephone. All participants who answered the questionnaire were asked to provide a blood sample.

The response rate for the questionnaire was approximately 98% among cases and 73% among controls. We received blood samples from 98% of the cases who completed the questionnaire and from 58% of the controls. All samples were stored at the Karolinska Institutet Biobank.

4.2 GEMS

In GEMS, prevalent cases, distinct from those in EIMS, were identified from the Swedish National MS-Registry. All cases fulfilled the McDonald criteria. For each case, one control was randomly selected from the national population register, matched for age, gender, and residential area at the time of disease onset. All participants filled out a questionnaire, similar but not identical to the EIMS questionnaire. The response rate was 82% for the cases and 66% for the controls.

4.3 DATA COLLECTION

Information on smoking was obtained by asking about current and previous smoking habits including duration of smoking, average number of cigarettes smoked per day, and type of cigarettes. The questions regarding snuff use were asked in a similar fashion as for smoking. Information on passive smoking was obtained by asking if the subject had been exposed daily to passive smoking at home or at work, including duration of exposure. The questions on tobacco consumption and exposure to passive smoking were identical in EIMS and GEMS.

4.3.1 DEFINITION OF EXPOSURES

For each case, the time at the initial appearance of symptoms indicative of MS was used as an estimate of the disease onset and the year in which this occurred was defined as the index year. Tobacco consumption and exposure to passive smoking were only considered prior to the index year in the cases and during the same period of time in the corresponding controls.

In all papers but paper II and V, subjects who had smoked during the index year were defined as current smokers, those who had stopped smoking prior to the index year were defined as past smokers, and subjects who had never smoked before or during the index year were defined as never smokers. In order to analyze the influence of a cumulative dose of smoking, we further categorized the smokers into groups based on the number of cigarettes smoked prior to index. One pack year is defined as smoking 20 cigarettes daily for one year.

In paper V, current smoking was defined as above. However, subjects who had stopped smoking within five years prior to index were excluded in paper V whereas subjects who had never smoked or had stopped smoking more than five years prior to index were defined as non smokers. In paper II, current smoking was defined as smoking during the index year without stopping smoking during this year. Past smoking was defined as having stopped smoking before or during the index year.

Subjects who had used snuff before or during the index year were defined as snuff users. The subjects were then categorized into groups based on the duration of snuff use (years) and the cumulative dose of snuff (packet-years) before index. One packet-year is the equivalent of consuming one packet of snuff daily for one year.

4.4 GENOTYPING

The blood samples in EIMS were genotyped with sequence-specific primers using Olerup SSP™ HLA kits⁸⁶. After polymerase chain reaction (PCR) amplification, the products were run on an agarose electrophoresis gel stained with GelRed, and visualized on a UV-table. The wells with a positive signal were interpreted according to a chart and the HLA-DRB1 and HLA-A genotypes were established at the two-digit level. Genotypes from EIMS were used in paper V.

4.5 STATISTICAL ANALYSIS

In all papers, logistic regression was performed to assess associations between exposure and MS risk, using Statistical Analysis System (SAS) version 9.2.

The lifestyle- and environmental factors investigated in this thesis were considered prior to the index year in the cases and during the same period of time in the corresponding controls. In principle, this calls for a matched analysis. We performed both matched analyses based on all available case-control pairs or triplets and unmatched analyses based on all available subjects. In all papers, the results from the unmatched analyses were in close agreement with those from the matched analyses but had a higher degree of precision. Matching led to a loss of cases and controls in the analyses which is why the results from the unmatched analyses were presented in the papers.

In paper IV, a trend test for a dose response relationship regarding cumulative dose of smoking and risk of MS was performed by using a continuous variable for cumulative dose of smoking, expressed as pack-years, in a logistic regression model. In order to determine whether intensity or duration of smoking contributes most to the risk of MS, we examined the components comprising pack-years in the same logistic regression model.

In paper V, the linkage disequilibrium (LD) between HLA-A*02 and HLA-DRB1*15 was estimated in Unphased 3.0 (Dudbridge, 2003) by considering presence and absence of HLA-A*02 and HLA-DRB1*15 alleles. We applied multivariate logistic regression in order to distinguish specific HLA genotypes contributing to MS susceptibility, using a model with all HLA-DRB1 and HLA-A alleles with frequencies above 10% among controls. Alleles with a frequency of less than 10% among controls were grouped together into DRB1X and AX, respectively. We then investigated the possible gene-gene interaction between alleles that had a significant influence on MS risk and also the possible interaction between these alleles and smoking. The interaction analyses were performed using departure from additivity of effects and were evaluated by calculating AP together with 95% confidence intervals (CI).

4.6 CONFOUNDING FACTORS

In the context of unmatched logistic regression, the analyses were adjusted for the matching variables. All analyses were adjusted for ancestry. Assessment of ancestry was based on whether the subject was born in Sweden or not, and whether either of the subject's parents had immigrated to Sweden. A subject who was born in Sweden, whose parents had not immigrated, was classified as Swedish. When EIMS and GEMS data were analyzed together, adjustment was made for study.

All analyses were further adjusted for a broad range of potential confounding variables. In paper I, adjustments were made for educational level (university degree or not), BMI at inclusion in the study (<19 or >19 kg/m²), parity (yes or no), and oral contraceptive use (ever or never). BMI was obtained by dividing self-reported weight in kilograms by self-reported height in meters squared. However, these factors had minor influence on the results and were not adjusted for in the final analyses.

In paper II, the final analyses were adjusted for educational level, and when appropriate, smoking habits (0, 1–5, 6–10, 11–15 or >15 pack years of smoking). Adjustments were also made for heredity (having a first- or second-degree relative with MS or not), passive smoking (yes or no), BMI at age 20 years (≤ 27 or > 27 kg/m²), and socioeconomic status. Detailed information was obtained regarding current and previous professions and work places. Each occupation was given an occupational class code according to a Swedish classification system of occupations adjusted to international standards⁸⁷. Based on the occupational codes, study subjects were classified according to occupational class (skilled and unskilled workers, assistant, intermediate, and higher non-manual employees). These factors had minor influence on the results and were not included in the final analyses.

In paper III, which comprised never smokers, the final analyses were adjusted for heredity, educational level, socioeconomic status, BMI at inclusion in the study, self-reported history of mononucleosis (yes or no), Epstein-Barr virus-determined nuclear antigen 1 (EBNA1) immunoglobulin G (IgG) titres, UVR exposure habits, and vitamin D status. EBNA1 IgG and vitamin D were measured in samples included between April 2005 and December 2009. EBNA1 IgG levels were measured with ELISA kits and categorized into high level (more than the median among controls), low level (less than the median of controls), or unknown level. Based on three questions regarding UVR exposure where each answer alternative was given a number ranging from 1 (the lowest exposure) to 4 (the highest exposure), we constructed an index by adding the numbers together and thus acquired an index value between 3 and 12. UVR exposure was dichotomized into high or low exposure (index values more or less than 6). Vitamin D status was measured as 25(OH)-vitamin D in plasma and categorized into sufficient levels (50 nmol/l or higher), vitamin D deficiency (less than 50 nmol/l), or unknown.

The analyses on smoking and MS risk in paper IV were adjusted for a broader range of potential confounding factors than the analyses in paper I; heredity, educational level, BMI at age 20 years, UVR exposure habits during the last 5 years, self-reported history of infectious mononucleosis, passive smoking, and snuff use (yes or no). These factors had minor influence on the results of the study. Only passive smoking was retained in the final analyses.

In paper V, further adjustment for educational level, HLA-DRB1*07, HLA-DRB1*X, and HLA-A*03 status only marginally influenced the results and these factors were not retained in the final analyses.

5 RESULTS AND INTERPRETATION

5.1 PAPER I

Our analyses of smoking and MS risk included 902 cases and 1855 controls. The study period for this report was April 2005 to October 2008. Compared with never smokers, the OR for MS among smokers was 1.5 (95% CI 1.3-1.8) which was in accordance with the result of a pooled analysis of nine previous studies on smoking and MS risk. We thus replicated the finding of an association between smoking and MS risk, and found clear evidence of a dose-response correlation between cumulative dose of smoking and the risk of developing the disease (p value for trend <0.0001). We further observed that the increased risk of MS associated with smoking abates a few years after smoking cessation (table 1).

Table 1. Adjusted OR with 95% of developing MS for different categories of smokers compared with never smokers.

Smoking	ca/co*	OR (95% CI)
Never smoker	385/967	1.0 (reference)
Ever smoker	517/888	1.5 (1.3-1.8)
Current smoker	322/530	1.6 (1.3-1.9)
Past smoker	195/358	1.4 (1.1-1.8)
5 years since smoking cessation	74/107	1.5 (1.1-2.0)
≥5 years since smoking cessation	120/251	1.0 (0.8-1.3)

* number of exposed cases and controls

adjusted for age, gender, residential area, and ancestry.

Both among ever and never smokers, snuff use was associated with decreased risk of developing MS. However, the number of exposed subjects was limited. All analyses were adjusted for age, residential area and ancestry, and when appropriate, gender.

5.2 PAPER II

Since a large proportion of the Swedish population uses moist snuff, which leads to exposure to high doses of nicotine, we set out to investigate the impact of snuff on MS risk using both EIMS and GEMS data (7883 cases and 9437 controls). Compared with subjects who had never used snuff, the OR of developing MS was 0.83 (95% CI 0.75-0.92) for snuff users, and

a significant trend showed an inverse risk of MS with higher dose of snuff use (table 2).

Table 2. OR with 95% CI of developing MS for snuff users compared with subjects who have never used moist snuff, by cumulative dose of snuff use.

Packet-years	ca/co*	OR (95% CI)#	OR (95% CI)¤	p	p value for trend
0	7095/8363	1.0 (reference)	1.0 (reference)		
<5	516/642	0.95 (0.83–1.07)	0.85 (0.75–0.97)	0.02	
5-10	181/251	0.84 (0.68–1.03)	0.77 (0.63–0.95)	0.01	
>10	91/181	0.59 (0.45–0.77)	0.57 (0.44–0.74)	<0.0001	<0.0001

* exposed number of cases and controls

adjusted for age, gender, residential area, educational level, and ancestry.

¤ adjusted for age, gender, residential area, educational level, ancestry, and smoking.

The results remained similar but had a lower degree of precision when the analysis was restricted to never smokers. There were no gender differences. We further observed that subjects who combined smoking and snuff use had a significantly lower risk for MS than smokers who had never used moist snuff (table 3).

Table 3. OR with 95% CI of developing MS for subjects with different combinations of smoking and snuff use.

Tobacco use	ca/co*	OR (95% CI)	P
Never	3286/4679	1.0 (reference)	
Ever smoking (no snuff use)	3704/3600	1.49 (1.40–1.59)	<0.0001
Current smoking (no snuff use)	2313/2161	1.56 (1.45–1.67)	<0.0001
Past smoking (no snuff use)	1391/1439	1.35 (1.24–1.47)	<0.0001
Snuff use (no smoking)	223/392	0.75 (0.63–0.90)	0.002
Snuff use and ever smoking	668/765	1.19 (1.06–1.34)	<0.0001
Snuff use and current smoking	359/344	1.42 (1.21–1.65)	<0.0001
Snuff use and past smoking	309/421	1.03 (0.88–1.20)	0.7

* exposed number of cases and controls

adjusted for age, gender, residential area, educational level, and ancestry.

5.3 PAPER III

When exploring the possible influence of passive smoking on MS risk, we included only subjects who had never smoked (695 cases and 1635 controls). Compared with those who had never been exposed to passive smoking before the index year, the OR of developing MS was 1.3 (95% CI 1.1-1.6) for those who had been exposed. We observed a significant trend that showed higher risk of MS with longer duration of exposure (p value 0.003). When the duration of exposure was twenty years or longer, the OR of developing MS was 1.8 (95% CI 1.2-2.6) compared with subjects who had never been exposed (table 4). The analyses were adjusted for a broad range of potential confounding variables.

Table 4. OR with 95% CI of developing MS for subjects exposed to passive smoking, compared with those who have never been exposed, by duration of exposure. All subjects were never smokers.

Passive smoking	ca/co*	OR (95% CI)#	OR (95% CI)α
Never	423/1094	1.0 (reference)	1.0 (reference)
<10 years	89/182	1.3 (1.0-1.8)	1.4 (1.0-2.0)
10-20 years	133/295	1.3 (1.0-1.7)	1.4 (1.0-1.8)
≥20 years	50/84	1.8 (1.2-2.6)	1.8 (1.2-2.7)
		0.003	0.003

* exposed number of cases and controls

adjusted for age, gender, residential area, and ancestry.

α adjusted for age, gender, residential area, ancestry, EBNA1 titres, a history of infectious mononucleosis, vitamin D status, UVR exposure habits, heredity, educational level, socioeconomic status, and body mass index.

5.4 PAPER IV

Our analyses of smoking and MS risk, based on EIMS and GEMS, included 7883 cases and 9264 controls. The study period for EIMS was April 2005 to March 2012, whereas study participants in EIMS were recruited between November 2009 and November 2011. We aimed to investigate aspects of the association between smoking and MS risk that had previously been investigated only to a limited extent. Compared with subjects classified as never smokers, ever smokers had an increased risk of developing MS (OR 1.5, 95% CI 1.4-1.6, p<0.0001). A trend test for a dose response relationship between smoking and MS risk, using a continuous variable for cumulative dose of smoking, rendered a p value of <0.0001. When duration and intensity of smoking were mutually adjusted for one another in the same multivariate model, both factors contributed independently to the risk of MS (p values <0.0001).

Age at start of regular smoking did not affect the risk of developing MS (table 5; figure 4). We further observed that the detrimental effect of smoking abates a decade after smoking cessation regardless of age at exposure (table 5; figure 4) and regardless of the cumulative dose of smoking (table 6; figure 5).

Figure 4. OR of developing MS among smokers and past smokers, compared to never smokers, by age at starting smoking.

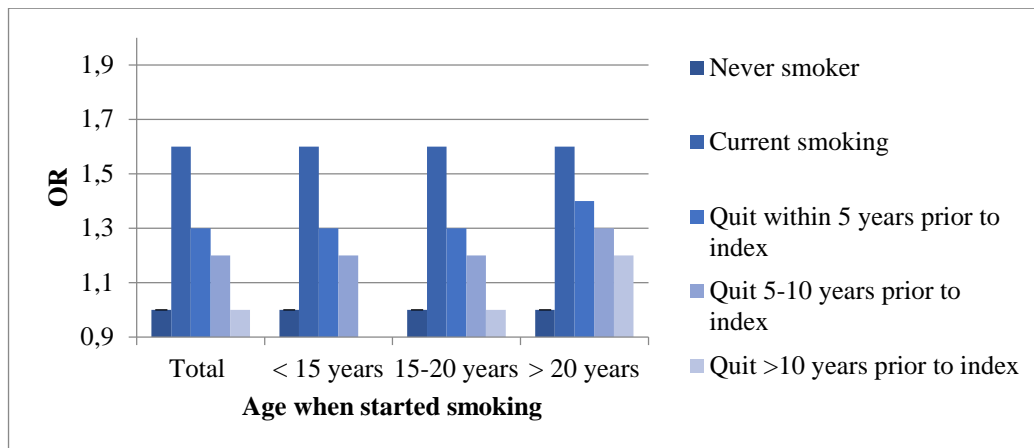


Figure 5. OR of developing MS among smokers and past smokers, compared to never smokers, by cumulative dose of smoking

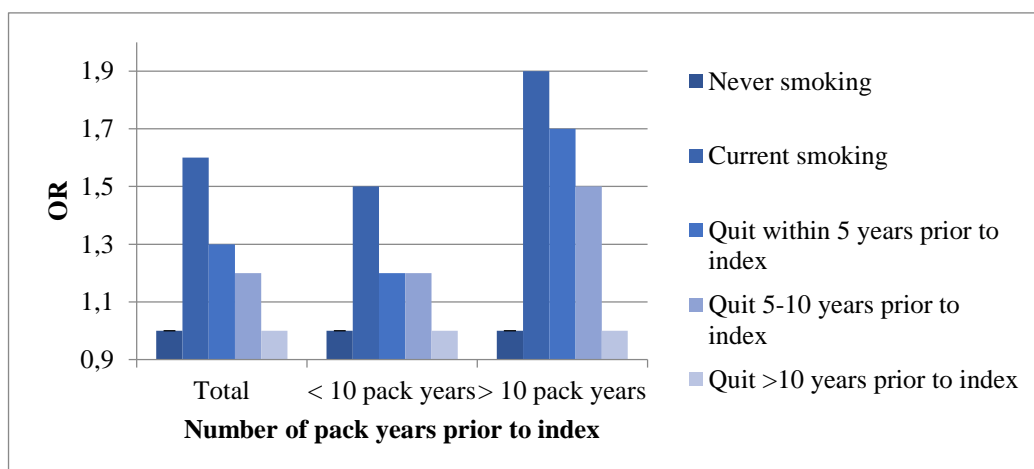


Table 5. OR with 95% CI of developing MS for current and past smokers compared with never smokers, by age at starting smoking.

Smoking habits	Age when started smoking							
	Total		<15 years		15-20 years		>20 years	
	ca/co	OR (95% CI)	ca/co	OR (95% CI)	ca/co	OR (95% CI)	ca/co	OR (95% CI)
Never smoking	3509/4964	1.0 (-)	3509/4964	1.0 (-)	3509/4964	1.0 (-)	3509/4964	1.0 (-)
Current smoking	2971/2574	1.6 (1.5-1.7)	979/852	1.6 (1.5-1.8)	1432/1257	1.6 (1.5-1.8)	402/358	1.6 (1.4-1.9)
Quit within 5 years prior to index	407/447	1.3 (1.1-1.5)	113/134	1.3 (1.0-1.7)	267/279	1.3 (1.1-1.6)	67/68	1.4 (1.0-2.0)
Quit 5-10 years prior to index	392/457	1.2 (1.1-1.4)	114/129	1.2 (0.9-1.6)	250/300	1.2 (1.0-1.4)	65/70	1.3 (0.9-1.9)
Quit >10 years prior to index	604/822	1.0 (0.9-1.2)	163/258	0.9 (0.8-1.1)	397/532	1.0 (0.9-1.2)	88/104	1.2 (0.9-1.6)
p for trend		<0.0001		<0.0001		<0.0001		0.06

* number of exposed cases and controls

‡ adjusted for age, gender, residential area, ancestry, passive smoking, and study.

Table 6. OR with 95% CI of developing MS for current and past smokers compared with never-smokers, by cumulative dose of smoking.

Smoking habits	Number of pack years prior to index					
	Total		<10 pack years		>10 pack years	
	ca/co	OR (95% CI)	ca/co	OR (95% CI)	ca/co	OR (95% CI)
Never smoking	3509/4964	1.0 (-)	3509/4964	1.0 (-)	3509/4964	1.0 (-)
Current smoking	2971/2574	1.6 (1.5-1.7)	1928/1768	1.5 (1.4-1.7)	1043/806	1.9 (1.7-2.1)
Quit within 5 years prior to index	407/447	1.3 (1.1-1.5)	283/339	1.2 (1.0-1.4)	124/108	1.7 (1.3-2.2)
Quit 5-10 years prior to index	392/457	1.2 (1.1-1.4)	305/372	1.2 (1.0-1.4)	87/85	1.5 (1.1-2.0)
Quit >10 years prior to index	604/822	1.0 (0.8-1.2)	505/678	1.0 (0.9-1.2)	99/144	1.0 (0.7-1.3)
p for trend		<0.0001		0.0001		0.001

* number of exposed cases and controls

‡ adjusted for age, gender, residential area, ancestry, passive smoking, and study.

5.5 PAPER V

Our analysis included 843 cases and 1209 controls, all of whom had provided blood samples. The strongest genetic associations with MS were those for the HLA-DRB1*15 allele and absence of the HLA-A*02 allele, and these were included in the interaction analysis. The observed LD between DRB1*15 and HLA-A*02 was low ($r^2 < 0.0005$). An interaction was observed between smoking and the genetic risk factors presence of HLA-DRB1*15 and absence of HLA-A*02, with regard to MS risk. Smokers with neither of the genetic risk factors had an OR of 1.4 (95% CI 0.9-2.1), compared to non-smokers without genetic risk. Both genetic risk factors among non-smokers rendered an odds ratio of 4.9 (95% CI 3.6-6.6) whereas the OR was 13.5 (95% CI 8.1-22.6) among current smokers with the same genotype (table 7; figure 6). All analyses were adjusted for age, gender, residential area, and ancestry.

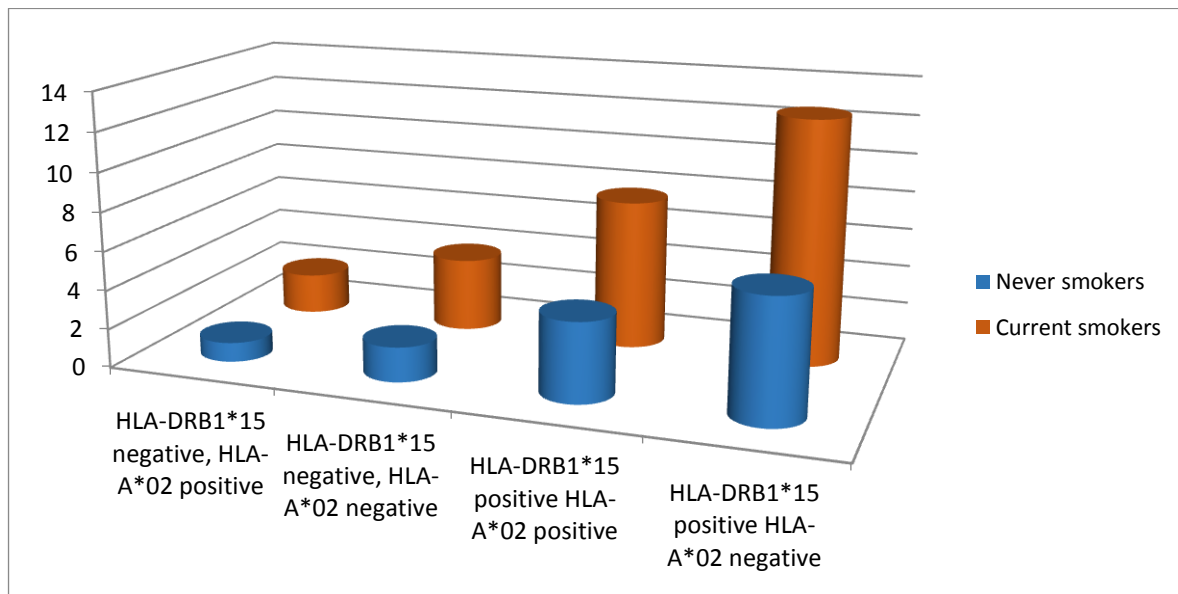
Table 7. OR with 95% CI of developing MS for subjects with different combinations of smoking habits and the genetic risk factors carriage of HLA-DRB1*15 and absence of HLA-A*02, compared with non smokers carrying none of the genetic risk factors.

HLA-DR15+	HLA-A*02-	Smoking	ca/co*	OR (95% CI)#	p-value
-	-	-	111/376	1.0 (reference)	
-	+	-	137/301	1.6 (1.2-2.1)	0.003
+	-	-	152/140	3.7 (2.7-5.1)	<0.0001
+	+	-	192/136	4.9 (3.6-6.6)	<0.0001
-	-	+	41/104	1.4 (0.9-2.1)	0.1
-	+	+	63/81	2.7 (1.8-4.0)	<0.0001
+	-	+	60/49	4.3 (2.8-6.6)	<0.0001
+	+	+	87/22	13.5 (8.1-22.6)	<0.0001

* number of exposed cases and controls.

analyses were adjusted for age, gender, residential area, and ancestry.

Figure 6. OR with 95% CI of developing MS for subjects with different combinations of the genetic risk factors carriage of HLA-DRB1*15 and absence of HLA-A*02, compared with non smokers carrying none of the genetic risk factors, among smokers and non smokers. Statistics are shown in table 7.



6 DISCUSSION

Our finding of an association between smoking and increased MS risk is consistent with previous findings⁵³⁻⁶⁰. However, our study is the largest to date, and we had the opportunity to investigate this association in detail. From a clinical point of view, our results support the view that abstinence from smoking should be recommended to those at risk of developing MS such as children of patients with MS. The results in paper IV show that even smoking a few cigarettes a day for a longer period confers an increased MS risk while stopping smoking at any age can lower the risk of MS regardless of previous smoking habits.

Of particular interest is also the observation that age at exposure does not affect the increased risk of MS associated with smoking. Both family studies and migration studies suggest that the influence of environmental factors contributes to MS at different age periods. The effects of vitamin D deficiency are likely to have an impact early in life, whereas EBV infection probably influences risk during adolescence or early adulthood⁸⁸. High BMI during adolescence has been associated with increased risk of developing MS later in life⁷²⁻⁷³. Certain aspects of adolescence thus seem to be critical regarding the impact of several environmental factors on MS risk. Smoking, on the contrary, affects MS risk regardless of age at exposure, and the detrimental effect abates a decade after smoking cessation.

Our finding of an interaction between smoking, HLA-DRB1*15 and the absence of HLA-A*02 in paper V may represent a breakthrough in the process of understanding the influence of smoking on MS risk. The molecular pathways responsible for the observed association between smoking and MS are not yet known, but a variety of mechanisms have been suggested to explain the association. We hypothesize that the mechanism linking smoking and passive smoking to MS susceptibility involves autoimmunity against proteins with post-translational modifications that are cross-reactive with CNS antigens. Since smoking, but not use of oral tobacco in the form of moist snuff, increases MS risk, the critical effects of smoking and passive smoking is likely to be caused by irritation in the lungs. Exposure to tobacco smoke results in increased pro-inflammatory cell activation in the lungs and post-translational modifications of proteins⁸⁹, which may break self-tolerance⁹⁰⁻⁹¹. Autoimmune memory cells are present and available for triggering in the lungs. In EAE studies, these cells strongly proliferate after local stimulation of the lungs and, after assuming migratory properties, reach the CNS, with inflammation as a consequence⁸⁰.

The main function of the HLA gene products is antigen presentation to T cells. The amino acid sequences of HLA class I and II alleles determine to a large extent the ability to respond to an antigen. Preferences in peptide binding by allelic variants of class I and II molecules are likely to be critical for the influence of HLA on autoimmune diseases. With regard to risk of rheumatoid arthritis, an interaction has been identified between smoking, HLA alleles, and autoimmunity to post-translationally modified proteins⁹². This is consistent with class II allele specific recognition of particular altered self peptides in the lungs, with ensuing organ-specific inflammatory disease depending on preferential peptide binding by allelic variants of class II molecules. We thus hypothesize that smoke-induced lung-irritation in the context of MS risk HLA genes may post-translationally modify

peptides cross-reactive with CNS antigens, promoting a CNS-directed autoaggressive immunity that results in MS.

Our study of interaction between smoking and genotype emphasizes the value, and sometimes need, to include data on environmental exposures in genetic analyses of complex diseases, since it has now been demonstrated that the strongest genetic risk factors for MS are significantly influenced by the presence of smoking in the population at risk, and vice versa.

Although the net effect of smoking is pro-inflammatory, there is increasing evidence that smokers have a lower incidence of several inflammatory diseases, such as ulcerative colitis and sarcoidosis⁹³. The protective effect has been attributed to the ability of nicotine to dampen inflammation⁹⁴. In paper II, the finding that snuff use is inversely associated with MS may have practical implications in counseling MS patients who smoke. Circumstantial evidence suggests that smoking not only increases the risk of developing MS, but also leads to an aggravated disease course⁹⁵⁻⁹⁶. Hence, stopping smoking through the use of alternative nicotine routes may be advised.

Moist snuff contains a number of different substances apart from nicotine and any of them could theoretically be involved in the protective effect. However, nicotine stands out as the main candidate in view of numerous studies on its immunomodulatory effects. Nicotine may exert systemic effects on the immune system by inhibiting the production of pro-inflammatory cytokines from immune cells, such as macrophages, via the alpha7 subunit of the acetylcholine nicotinic receptor⁹⁷. Since MS is most likely driven by systemic immune responses targeted at the CNS, nicotine may also be involved in this disease, consistent with the apparent lower incidence in long term snuff users.

The design of EIMS and GEMS offered several methodological advantages. Cases in both studies were identified using strict diagnostic criteria and the possibility of misclassification of MS diagnosis was negligible. Another strength was the large number of participants. Our studies also have some limitations. The recruitment of cases and controls may introduce selection bias. In EIMS, the proportion of responders with regard to participation in the study was 91% for cases and 69% for controls and in GEMS, the proportion of responders was 82% for cases and 66% for controls. In Sweden, there is equal access to publicly sponsored health care for all residents, and patients with MS are cared for by neurologists. In EIMS, there are 40 reporting neurology clinics, including all university hospitals in Sweden. However, due to shortage of personnel at the clinics, there may have been patients who never received an invitation to participate in the study. Some of the incident cases unidentified in EIMS would instead have been identified in GEMS, in which all prevalent cases registered in the Swedish National MS-Registry were asked to participate. We consider it unlikely that the unidentified cases would be related to the exposures studied in the present thesis. We thus believe that misclassification of disease will only bias the observed results to a minor or negligible extent.

A potential selection bias may result from the relatively high proportion of non-responders among the controls. The loss of controls was however less than stated above, since some non-responders among the invited controls were likely not part of the study base since the proportion of immigrants is high among the non-responders and an inclusion criteria of the study is knowledge of the Swedish language. However, this bias is probably modest since life

style habits such as prevalence of smoking and snuff use among the controls was consistent with that expected for the general population in similar ages⁹⁸.

Only controls who had responded to the questionnaire were asked to provide a blood sample, and 58% of these controls consented to do so. There were no differences with respect to age, gender, residential area or smoking habits between those who provided blood and those who did not, indicating that selection did not take place in this step. We consider it unlikely that our main finding of an interaction between smoking and HLA genotype would be affected by bias to a large extent, especially since such a bias would then depend on HLA types.

Both EIMS and GEMS were designed as population-based case-control studies, in which information regarding exposures and lifestyle factors was collected retrospectively. On account of this design, recall bias may have occurred, thereby introducing systematic error in the calculation of the association between tobacco and MS risk. In order to minimize recall bias, EIMS primarily included cases who had received the diagnosis within the past year. The rationale for only including cases of recent onset was that these subjects were considered unlikely, during the short duration of their illness, to have changed their habits on account of the illness, avoiding error introduced by the endorsement of current rather than former habits.

In GEMS, in which the mean disease duration is about 17 years, the probability of recall errors is higher. MS-related declines in memory and cognition may have resulted in a greater degree of error, in the form of incorrect classification of exposure.

In both studies, we took great care to obtain information in an identical way for the cases and the controls. The questionnaire contained a wide range of questions regarding many potential environmental risk factors and no section in the questionnaire was given prime focus.

Furthermore, when EIMS and GEMS were analyzed separately, the association between smoking and MS was similar in both studies. Consequently, the potential recall bias is likely to be small. When paper I was published, the relationship between snuff use and MS risk had not previously been investigated, and the quality of the reported information on snuff use does probably not differ between cases and controls. In paper III, almost all subjects who were classified as exposed to passive smoking reported that they had been exposed at home, which is relatively easy to remember. Recall bias is probably small in this study, and there is no reason to believe that potential recall problems should differ between cases and controls.

Our analyses were adjusted for a number of potential confounding factors. However, as with most epidemiological studies, we cannot completely rule out that our findings could be due to residual confounding or unknown confounding factors.

7 SUMMARY OF FINDINGS

- Smoking and exposure to passive smoking increase the risk of developing MS.
- A dose-response correlation exists between cumulative dose of smoking and the risk of developing the disease.
- Duration and intensity of smoking contribute independently to the risk of MS.
- The risk of MS increases with longer duration of exposure to passive smoking.
- Smoking affects MS risk regardless of age at exposure.
- The detrimental effect of smoking abates a decade after smoking cessation.
- A substantial interaction was observed involving smoking and the genetic risk factors: presence of HLA-DRB1*15 and absence of HLA-A*02.
- Snuff use is associated with decreased MS risk.

8 CONCLUDING REMARKS

In yet unknown ways, MS develops from a combination of a complex genetic predisposition and environmental factors. Interactions between different genetic loci as well as between genes and environmental factors may further add to the complexity of disease susceptibility. Over recent years, GWAS have identified a large number of risk loci that influence disease susceptibility¹⁸⁻¹⁹. The contribution of lifestyle and environmental factors is more difficult to study. However, it is important to identify these factors as they are potential targets for prevention and may also lead to hypothesis on critical pathogenic events.

The main aim of this thesis was to enhance the understanding of the impact of tobacco on MS risk. Although our findings will hopefully have made a contribution to the exploration of the relationship between tobacco and MS, much remains to be done. We hope that our finding of an interaction between smoking and HLA genotype can provide important guidance for further studies leading to an understanding of the mechanisms by which smoking and passive smoking increase the risk of developing MS. A replication of the gene-environment interaction in other populations, or an observation of an interaction between passive smoking and HLA genotype, would support our hypothesis that priming of the immune response in the lungs may subsequently lead to MS in people with a genetic susceptibility to the disease.

Susceptibility to MS results from the combined effects of many different combinations of risk alleles. Each individual locus is probably neither necessary nor sufficient for the development of MS, but an accumulation of risk alleles may generate a high level of disease risk. It would therefore be of interest to take other genetic loci into consideration when investigating the association between smoking and HLA genotype with regard to MS risk.

Paper III demonstrates that passive smoking is associated with an increased risk for MS, suggesting that also lower degrees of lung irritation may contribute to the triggering of MS. Further studies would be valuable in order to investigate the impact of other forms of lung irritation, such as air pollution, in the etiology of MS.

Recent studies show that the immune system can be significantly regulated by the vagus nerve via the peripheral release of acetylcholine⁹⁷. The neurotransmitter also functions as an immune cytokine that inhibits the release of pro-inflammatory cytokines from immune cells, such as macrophages, through a mechanism dependent on the alpha7 nicotinic receptor. Nicotine, a more selective cholinergic agonist, is more efficient than acetylcholine at attenuating the production of pro-inflammatory cytokines⁹⁹⁻¹⁰⁰. However, the development of nicotine as a therapeutic tool is not an option due to lack of pharmacological specificity, toxicity-related side effects and unknown long-term effects on human health. Nevertheless, selective agonists for the alpha7 nicotinic receptor could represent a pharmacological strategy to control a variety of pro-inflammatory cytokines, and may prove beneficial in several inflammatory and neurodegenerative disorders¹⁰⁰⁻¹⁰¹.

Advances in our understanding of environmental risk factors can lead to avenues of research exploring how these factors may play a role in the pathogenesis of the disease. It has become increasingly clear that the risk conveyed by an environmental factor may substantially differ depending on genetic background. Future studies must resist the temptation to investigate environmental risk factors for MS in isolation, since interactions with both other environmental influences and an individual's genetic background are likely to contribute to MS development.

9 ACKNOWLEDGEMENTS

I would like to express my gratitude to all the MS patients and healthy controls who have participated in the studies included in my thesis. Thanks also to all medical staff at the neurology units responsible for the recruitment of patients to the EIMS study.

I am grateful to my main supervisor Lars Alfredsson and my co-supervisor Tomas Olsson. Your expertise and continued support made it possible to generate new knowledge in the field of MS research. Thank you for listening to my ideas and for letting me explore them in any way I wanted. I had great fun.

My mentor and friend, Thomas Masterman, for fascinating discussions and great support.

I would also like to thank the following research colleagues for their cooperation and support:

Jan Hillert, for contributions to study design and review of manuscripts. Your efforts in developing the MS Registry have been crucial for this thesis.

Maria Bäärnhielm, my co-author at IMM. Best of luck in the future.

My co-authors at CMM, Emilie Sundqvist, Nina Nordin, Izaura Lima Bomfim and, in particular, Ingrid Kockum, for your contributions in the area of genetics.

Present and past administrative personnel at the IMM for your wonderful job in coordinating and organizing the EIMS and GEMS studies, including Charlotte Anderberg and Karin Lycke. Special thanks to Linda Forsberg for editing figures and tables in manuscripts and posters, as well as in my thesis.

Sheila Macdonald-Rannström for always finding the time to proof-read my texts.

Statisticians Ryan Ramanujam and Michalis Katsoulis, for fruitful collaboration and for introducing me to AFT models and helping me create a SAS code for three-way analyses.

My co-authors at the University of California at Berkeley, Lisa Barcellos, Ellen Mowry, Milena Gianfrancesco, Farren Briggs, and Xiarong Shao, for a rewarding collaboration.

My co-authors Malin Lundkvist Ryner, Anna Fogdell-Hahn, Jenny Link, and Katharina Fink, for collaboration in the area of smoking and NAb development.

All members of the Department of Neurology of Karolinska University Hospital Huddinge, especially Lise-Lotte Bengtsson, Marita Ingemarsson, Ewa Roos Waldebäck, Anna Aronsson and Hodayoun Roshanisefat for creating a wonderful atmosphere.

Leszek Stawiarz, for always finding a way to open unopenable files and render unreadable files readable.

My co-author at Stockholm University, Torbjörn Åkerstedt, for sharing your wisdom and knowledge in the area of sleep research.

And, finally, I extend my gratitude and affection to the following people from the world beyond the world of research:

Lotta, Håkan, Ann, Margareta och Göran, Knarik and family. Thank you for all the nice times.

My sisters, Marie and Josefin, and their wonderful families.

My mother.

Timo and my children, Zackarias, Ossian and Alison.

The studies in this thesis were supported by grants from the Swedish Medical Research Council; the Swedish Council for Working Life and Social Research; the FP6 EU program Neuropromise; the Knut and Alice Wallenberg Foundation; the AFA Foundation; the Bibbi and Niels Jensen Foundation; the Montel Williams Foundation; the Söderberg Foundation; the Swedish Brain Foundation; and the Swedish Association for Persons with Neurological Disabilities.

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