Risk Factors in Cause and Course of Multiple Sclerosis

Naghmeh Jafari

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Risk Factors in Cause and Course of Multiple Sclerosis

Risicofactoren betreffend het ontstaan en beloop van multiple sclerose

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In memory of my beloved Maman Sona
who always whispered in my ear
'from cradle to grave, seek knowledge'

تقدیم به مامان سونا عزیزم

که همیشه در گوشم زمزمه میکرد

`ز گهواره تا گور دانش بجوی`

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Part I

Introduction

Chapter 1

General introduction and scope of this thesis

Multiple sclerosis (MS) is a leading non-traumatic cause of disability in young adults. It is a chronic neurological disorder characterized primarily by central nervous system (CNS) inflammation, myelin loss, and axonal pathology, resulting in progressive neurological dysfunction. Initially, inflammation is transient and partial remyelination occurs. MS is presumed to be an autoimmune disorder arising from complex interactions of both environmental and genetic factors.

Clinical disease course of MS

MS is a common disease, affecting over 2 million people worldwide. The population prevalence in North America and Northern Europe is about 0.1%.[1] The age at onset is relatively constant across different regions in the world. The incidence is low in childhood (2% of patients with MS present before the age of 10 years and 5% before the age of 16 years), rapidly increases after adolescence and reaches a peak between ages 25 and 35 years and then declines. Onset of MS at an age older than 50 is rare.[1-3] Life expectancy is reduced, and the median time to death is around 30 years from disease onset with a large range.[4] As MS afflicts individuals in their 20s and 30s, the uncertainty of disease progression and prognosis strongly influences their personal and professional decisions. More than 50% of MS patients experience depression,[5] and suicide is 7 to 8 times more prevalent in individuals with MS when compared with age-matched controls.[6]

MS is characterised by the latitude gradient and a female excess. The traditional view based on many early studies is that MS is particularly prevalent in Caucasian people of Nordic origin living in temperate zones and in high income countries. However, recent evidence suggests that the latitude gradient of MS incidence may be decreasing[7,8] or even never existed in certain areas.[9] Furthermore, recent studies indicate that the female to male ratio now exceeds 3.2:1.[10,11] These changes in MS incidence suggest influence of one or more environmental factors next to genetic factors.[12,13]

The majority of affected individuals (80-90%) present an acute episode involving one or several localisations, which is known as the clinically isolated syndrome (CIS). This is an acute or subacute demyelinating event that often involves the motor, sensory, visual and/or autonomic systems, but many other symptoms and signs like fatigue and cognitive impairment can be the first signs of MS onset.[14] The chance that CIS patients will have a second episode of (sub) acute demyelination and consequently convert to clinically definite MS (CDMS) varies from 50-80% in a 2 year follow-up. The clinical disease course for CDMS is variable. Approximately 80-90% of CDMS patients begin with a course of recurrent and reversible neurological deficits termed relapsing-remitting MS (RRMS). After a relapse, recovery can be complete or partial. During the course of the disease, most RRMS patients will develop progressive deficits, with continuous and irreversible neurological decline, indicated as secondary progressive

MS (SPMS). In around 10-20% of the patients, the illness is progressive from onset without clinical remissions, designated as primary progressive MS (PPMS).[15] The relapsing phase of the disease is mediated by focal bursts of inflammation in the white matter in the brain and spinal cord, whereas axonal and neuronal loss predominate during the progressive phase as a consequence of chronic demyelination, suggesting that MS is not only an immunological disease but also a neurodegenerative disorder.[16]

As discussed above, the development of CDMS after a CIS has a great social impact for these young adults in their most productive and fruitful period of life. Therefore, it is essential to improve prediction of the disease onset and course, given their precarious future.

MRI as a clinical tool for diagnosis of MS

The diagnostic criteria for MS have been refined over the past half century driven by the desire to shorten the diagnostic delay. Starting in 1965 with Schumacher and colleagues,[17] the diagnostic criteria for MS have been based on the demonstration of dissemination in time and space (table 1). This dictum requires that the symptoms or signs have to reflect disease activity at multiple time points (generally separated by more than one month and with a duration of at least 24 hours), and involvement of at least two distinct areas of the CNS typical of an acute inflammatory demyelinating event. To meet the criteria, other diagnoses have to be excluded. The early diagnostic criteria used clinical and paraclinical data such as visual evoked potentials (VEP) and cerebrospinal fluid (CSF) with limited prognostic information (table 1).[18] Following the technical improvements of magnetic resonance imaging (MRI) and with the expansion of the prognostic tools, new diagnostic criteria have been developed.[19,20] In 1997, Barkhof and colleagues introduced a cumulative chance model of 4 MRI parameters to improve the prediction of conversion from CIS to CDMS, and these criteria were modified further by Tintoré et al.[21] These MRI based criteria are called the Barkhof criteria (figure 1). The 4 parameters included (a) at least 1 gadolinium enhancing lesion or 9 T2 hyperintense lesions, (b) at least 1 juxtacortical lesion, (c) 3 periventriculair lesions, and (d) 1 infratentorial lesion. Fulfilling 3 out of these 4 criteria predicts a higher risk of conversion from CIS to CDMS.

In 2001 McDonald et al.[19] included the Barkhof criteria in the new diagnostic guidelines which have been used and repeatedly revised in the last decades with the aim to shorten the diagnostic delay at a lower cost and enabling to start treatment at an earlier stage of the disease.[22-24] Recently, new evidence and consensus has led to further revision of the McDonald criteria for the diagnosis of MS.[25] The latest revision simplifies the criteria and allows accelerated diagnostic procedures while preserving the sensitivity and specificity. Table 1 reviews diagnostic criteria and their revisions.

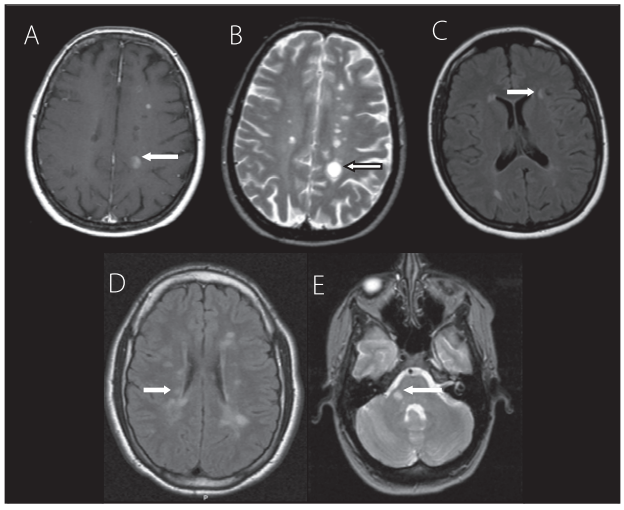
Table 1: Diagnostic criteria from 1983 to 2010.

	Poser 1983	McDonald 2001	McDonald 2005	McDonald 2010
DIS	a) ≥2 lesions with objective clinical evidence, involving different parts of CNS or clinical evidence of one lesion and para-clinical evidence of another, separate lesion.	a) Same b) ≥3 of the 4 Barkhof criteria fulfilled: - ≥9 T2 lesions or 1 Gd enhanced lesion - ≥3 periventricular lesions - ≥1 juxtacortical lesion - ≥1 infratentorial lesion (1 spinal lesion can replace 1 brain lesion) c) ≥T2 lesions plus positive CSF*	a) Same b) Same (1 spinal lesion can replace 1 infratentorial lesion. And any number of spinal lesions can be included in the total T2 lesion count)	a) Same b) ≥1 T2 lesion in at least 2 of 4 areas of the CNS: - Periventricular - Juxtacortical - Infratentorial - Spinal cord (symptomatic lesions in patients with brainstem or spinal cord syndrome are excluded)
DIT	a) ≥2 attacks separated by a period of at least a month	a) Same b) 1 Gd enhanced lesion ≥3 months after CIS c) A new T2 lesion compared with previous scan ≥3 months after CIS	a) Same b) Same c) A new T2 lesion compared with a previous scan obtained ≥ 30 days after CIS	a) Same b) new T2 and/or Gd enhanced lesion(s) on follow-up MRI, with reference to a baseline scan irrespective of the timing c) Simultaneous presence of asymptomatic Gd enhanced and non-enhanced lesions at any time
PPMS		Positive CSF* **AND** DIS demonstrated by ≥1 of following: - ≥9 T2 brain lesions - ≥2 spinal cord lesions - ≥ 4 T2 brain lesion plus 1 spinal cord lesion **AND** DIT demonstrated by 1 Gd enhanced lesion or a new T2 lesion compared with previous scan at least 3 months after CIS or continued progression for ≥1 year.	≥1 year disease progression and ≥2 of the following: - positive brain MRI# - positive spinal MRI$ - positive CSF*	≥1 year disease progression and ≥2 of the following: - ≥1 T2 lesion in at least 1 area characteristic for MS (periventricular, juxtacortical or infratentorial) - positive spinal MRI$ - positive CSF*

Based on Poser criteria,[18] McDonald criteria 2001,[19] 2005 revisions to the McDonald criteria,[20] 2010 revisions to the McDonald criteria.[25]
* Positive CIS defined as isoelectric focusing evidence of oligoclonal bands and/or elevated IgG index.
# Positive MRI defined as nine T2 lesions or ≥ 4 T2 lesions with abnormal VEP of MS type.
$ Positive spinal MRI defined as two focal T2 lesions.
DIS= dissemination in space; DIT= dissemination in time; CNS= central nervous system; CSF= cerebrospinal fluid; CIS= clinically isolated syndrome; Gd= gadolinium; MRI= magnetic resonance imaging; PPMS= primary progressive MS; VEP= visual evoked potentials.

In addition to the diagnostic practice, MRI has an important role as a prognostic tool in CIS patients. Studies have shown that the chance of fulfilling the diagnostic criteria for MS increases from 21% at 20 years for CIS patients without white matter lesions to 82% for patients with white matter lession on the baseline MRI.[26] Fulfilment of the Barkhof criteria[22] at baseline showed a conversion rate of 45% within 2 years versus 10% in those with no asymptomatic lesions at baseline.[27] Although introducing the Barkhof criteria improved the prediction of conversion to CDMS in near future, the sensitivity of the diagnostic criteria is relatively low (varying from 47 to 72%).[28] False positive predictions result in uncertain future perspectives in this young cohort. Therefore, identifying new and additional parameters to improve the predictive sensitivity of the tests in CIS patients is indispensable.

Figure 1: Barkhof criteria based on the MRI lesions.



Barkhof criteria are fulfilled when ≥3 of following parameters are present. 1 gadolinium enhancing lesion (A) or ≥ 9 T2 hyperintense lesions (B), ≥ 1 juxtacortical lesion (C), ≥ 3 periventricular lesions (D), and ≥ 1 infratentorial lesion (E).

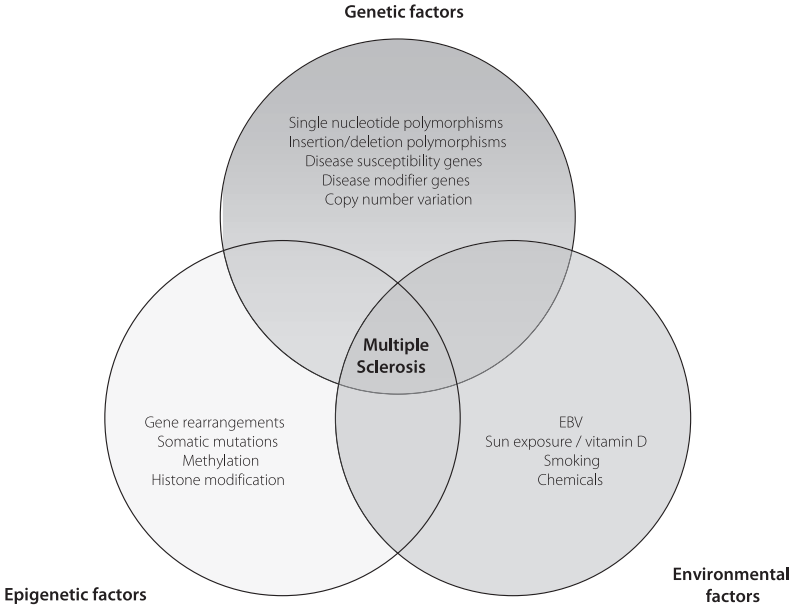
Genetic determinants of cause and course in MS

MS aggregation in families

Although familial aggregation of MS has long been recognised, systematic age-adjusted recurrence risks for relatives of persons with MS were first published in 1988.[29] Subsequent studies used standard genetic epidemiological methodology and age-correction, showing that first-, second- and third-degree relatives of patients with MS were more likely to have the disease than the general population.[30,31] The recurrence risk varies with the relatedness. The risk for a first degree relative of an MS proband (2-5%) is about 20 to 50 times increased

compared to the general population (0.1%).[30,32] Studies in twins showed a significant excess of concordance in monozygotic (about 30% concordance) compared to dizygotic twins (about 5% concordance).[33-36] These findings suggest underlying genetic determinants for MS susceptibility to be present, although until today no clear mode of inheritance can be inferred. Further, the role of genes has also been supported by adoption studies showing a higher risk of MS in genetically related family in contrast to a lower risk in the adopted family-member.[37] For half-siblings the risk is approximately half of the risk for full-siblings regardless of whether they were raised together or apart.[38,39] Genetic-epidemiological studies showed that maternal half-siblings, connected through an unaffected mother, were at a significantly higher risk of developing MS as compared with paternal half-siblings connected through an unaffected father (2.35% versus 1.31%, p = 0.048).[39] However, when patients with affected parents were studied, fathers with MS transmitted the disease to their offspring significantly more often than the mothers with MS.[40] A strong maternal influence on the determination of the sex ratio of MS offspring has been suggested.[41] The parent-of-origin effect in MS could be the result of a threshold effect due to an increased number of penetrant susceptibility genes in a given parental lineage. Another possibility is the involvement of epigenetic mechanisms in disease transmission by the affected parent (figure 2).

Figure 2: MS is a complex disease.



There is no simple cause for multiple sclerosis. The disease arises through a complex interplay between genetic, epigenetic, and environmental factors.

The term epigenetic refers to changes in gene expression that may or may not be heritable and do not involve changes in DNA sequence.[42] Epigenetic mechanisms are responsible for tissue-specific expression and X-inactivation in female cells.[43,44] Two major mechanisms of epigenetic gene regulation are DNA methylation and histone modification.[45] DNA methylation refers to the covalent addition of a methyl group to cytosine on the DNA strand.[46] Histone modification is the post-translational covalent addition of molecules to the histone subunit of nucleosomes.[47] These two mechanisms interact with each other in modulating chromatin architecture, which can lead to activation of gene transcription or to gene silencing.
Epigenetics can be modulated by the environment, providing a link between the external environment and internal genetic systems. Thus environmental effects on immune responses can be mediated by changes in epigenetic regulation. As an example, the environmental influences in utero ranging from radioactivity, stress, sunlight (vitamine D), toxic elements or viral infections can have an impact on epigenetics. This may be an explaination for the maternal parent-of-origin effect in MS.[48]

Gene discovery in MS

In 1972, MS was shown to be associated with alleles of the HLA class II region, which is part of the major histocompatibility complex (MHC).[49] The success in identifying this association was mainly due to the large effect size of this region (odds ratio (OR) >2). Later large international consortia were established to identify more genetic determinants of MS[50] and these have been able to narrow down the class II gene *HLA-DRB1* to a specific allele, the *HLA-DRB1*15* allele.[51-54] Although only the *HLA-DRB1* locus has so far been firmly linked to MS risk, other approaches have been used to identify other genetic risk factors.
First, linkage or association studies of candidate genes based on a priori knowledge of the pathogenesis or on some other selection process were performed. Later, genome wide association (GWA) studies[51] were used to screen for a link between a chromosomal region of interest or an association between markers and susceptibility genes resulting from linkage disequilibrium. However, it has proved to be very difficult to identify other risk genes than the HLA risk gene. One reason is that the attributable risk of non- HLA risk alleles for MS is very small. Another reason is the heterogeneity of the studied populations.
To overcome these problems the studies have often been performed in genetic isolates or other informative populations like multiplex families to increase the chance of finding associations. But it was not until the large international collaboration that the statistical power could be reached and various non-HLA MS risk genes were identified. In 2007 the International MS Genetics Consortium (IMSGC) performed a GWA study testing more than 300,000 single nucleotide polymorphisms (SNPs) in 931 families in the screening phase.[51] For the replication phase 110 SNPs were selected and genotyped in another set of more than 2,500 MS patients

and controls. This approach identified 17 SNPs located in 14 regions with an association with susceptibility for MS, however, only two regions, *HLA-DR* and *IL2RA*, achieved genome-wide significance ($p<5*10^{-08}$).

From 2007 until today several GWA studies have been performed,[55-63] but the most recent and largest published study was a collaboration between IMSCG and the Wellcome Trust Case Control Consortium 2 (WTCCC2).[64] This collaborative GWA study, involving 9,772 cases and 17,376 controls, did not only replicate almost all of the previously suggested non-MHC associations but also identified 29 novel susceptibility loci. In the near future, we expect that promising new techniques, such as next generation sequencing will help to discover genetic variants with higher risk alleles, though rare and possibly population specific.[65,66]

Susceptibility to MS: genes or environment?

The aggregation of MS in families and discovery of MS susceptibility genes support a genetic association. However, there are several arguments why genetics cannot be the only factor playing a role in the cause and course of MS. The large discordance observed within monozygotic twins (70%)[33,36] demonstrates that environmental factors influence MS risk. In 2010, Baranzini and colleagues performed a study to estimate sequence variation among monozygotic twins. They did not found any evidence for genetic, epigenetic or transcriptome differences to explain the disease discordance.[67] This has also been implied by the geographical spread of MS and by migration studies. The geographical spread of MS can be generalized as increasing with distance North or South of the equator, which suggests a relationship between latitude and disease prevalence. MS is more common in regions populated by Northern Europeans. However, this distribution is modified by the country of residency at young age, as shown by migration studies. Migrants who emigrate from low risk areas to the UK (a high MS risk area) maintained the low risk of their area of origin.[68] Migrants from the high risk areas to low risk area in the USA, had a risk intermediate compared to that of the areas of origin and destination.[69] Children of immigrants to the UK had risks of MS similar to those of other UK born children, which is a higher risk than their parents.[70]

Such rapid changes in risk over the course of a single generation implicate environmental factors in MS aetiology. Other evidence for environmental effects on MS risk comes from the observation that MS rates among UK migrants who live in Tasmania or the South Island of New Zealand resemble those in the UK, and are fivefold higher than the MS rates among UK migrants to sunny Queensland or the Northern Territory, even though their gene pools are broadly comparable.[71]

The interest in environmental factors influencing the MS risk has been further fuelled by the increase in prevalence of MS, particularly among women which leads to higher female to male sex ratios of MS.[10] The change in latitudinal gradient not only in Europe and North

America but also around equator further focuses our interest on the environment.[72] It is of noted that prevalence may also be influenced by other factors than the true incidence of MS. The diagnostic accuracy and ascertainment probability, both of which are related to the level of medical services in the country can play a role in the prevalence. Moreover, the level of medical services also influences the survival time that affects the prevalence.

Furthermore, the observation that birth month and risk for MS are associated, suggests a role of the environment in MS susceptibility during gestation or shortly after birth. A pooled analysis of data from Canada, the UK, Denmark, and Sweden, including more than 42.000 MS patients, showed that significantly more MS patients are born in May and significantly fewer in November.[73]

However, the incomplete disease concordance in monozygotic twins cannot be completely attributed to environmental triggers and might reflect the gene expression modification by epigenetic mechanisms.

Environmental risk factors of MS

Support for environmental risk factors rests on several grounds as discussed above. The identity of environmental factors involved in MS is not known yet, but accumulating evidence lends strong support to several candidates. Three main environmental risk factors for the development of adult onset MS have been identified, i.e. vitamin D exposure, Epstein-Barr virus (EBV) infection and cigarette smoking.

Sun exposure and vitamin D

Latitudinal differences in the prevalence of MS, with the disease being much more common in populations living farther away from the equator, have long been recognized. There are many factors associated with latitude, but one of the strongest correlates is the duration and intensity of sunlight exposure. Early ecological studies[74,75] noted an inverse correlation between sunlight radiation and MS prevalence. This was further supported by later studies showing that mortality from MS was inversely associated with residential and occupational exposure to sunlight,[76] high levels of sun exposure, and greater levels of actinic skin damage[77] and that individuals with MS were less likely to develop skin cancer.[78]

Biological mechanisms have been suggested to explain these findings. Ultraviolet light may have immunosuppressive effects, and it is important for the cutaneous synthesis of vitamin D3, which is subsequently hydroxylated in the liver to 25-hydroxy-vitamin D (25-OH-vitD).[79] The latter is a measurable precursor form of vitamin D in serum, and is converted in the kidneys and some other tissues to the active hormone 1,25-dihydroxy-vitamin D (1,25 $(OH)_2$ vitD).[80] Another less important source for vitamin D is food and the increasing use of supplements.

A possible role of vitamin D in MS was proposed more than 30 years ago.[81] Experimental

and epidemiological data supported the assumption that increasing vitamin D levels from diet or ultraviolet radiation were protective. A prospective nested case-control study found that high serum 25-OH-vitamin D levels were associated with lower MS risk.[82] Fukazawa and colleagues[83] reported an association between vitamin D receptor polymorphisms and MS in the Japanese population. Various studies showed that vitamin D has beneficial effects on autoimmune encephalomyelitis[84-86] and affects T-cell function.

The serum level of vitamin D fluctuates with seasonal changes in exposure to ultraviolet light, but levels are also influenced by cultural or individual variations in exposure and nutrition. Given the variability in exposure and intake, investigating sunlight and/or vitamin D is challenging, but it is plausible that interactions between genetic factors regulating the effects of vitamin D and environmental exposure to sunlight may explain some of the geographic differences in MS risk. A trend towards an inverse correlation between relapse rate and number of active lesions on MRI with vitamin D levels has been shown.[87] Providing further evidence might become important in altering the clinical course of the disease, especially in CIS patients. Recently we started an intervention study with vitamin D supplements in patients with optic neuritis (VIDEO). The immunomodulatory and neuroprotective effects of vitamin D will be studied by measuring the thickness and macular volume of the retinal nerve fibre layer.

The hygiene hypothesis and infectious agents in MS

In 1963, Poskanzer provided the first evidence of the so-called 'hygiene hypothesis' as explanation of the apparent increased incidence in all autoimmune diseases, including MS.[88] He concluded that the risk of MS, as is true for poliomyelitis, would increase with increasing age at infection, and would thus be more common in populations with high levels of hygiene. The hygiene hypothesis was supported by findings of increasing MS incidence with increasing sanitation in Israel[89] and with increasing socioeconomic status in the U.S.[90] and the U.K.[91] Ponsonby and colleagues reported in 2005 that increasing duration of contact during the first 6 years of life with a younger sibling was associated with reduced risk for MS.[92] This has not been confirmed by others, which is challenging the hygiene hypothesis within the familial microenvironment.[93,94] The Canadian Collaborative study on MS which investigated more than 10,000 individuals found that birth order had no effect on MS risk in most families.[94] Moreover, the observation, that spontaneous experimental autoimmune encephalomyelitis (EAE) may develop in transgenic mice housed in a non-sterile facility, but not in those maintained in sterile pathogen free conditions, also questions the hygiene hypothesis.[95]

The 'hygiene hypothesis' is based on the theory that the immature immune system needs to be challenged in early life to develop normally by modulating the immune response toward T-helper cells and regulatory T cells, and by reducing the proinflammatory cellular immunity.[96] Increased standards of hygiene, widespread use of antibiotics and vaccines reduce exposure,

frequency and variety of early childhood infections. Infections in late childhood or adolescence in individuals with a poorly developed immune system can lead to autoimmunity. The 'hygiene hypothesis' could also partly explain the geographic distribution of MS, with MS frequency being low around the equator where most people live in conditions with high infectious pressure in early childhood with viruses, bacteria and parasites.

Various potential causal agents for MS have been considered. Measles, rubella and mumps are common childhood infections associated with MS risk.[97] Although reports implicating specific risk agents for MS continue to appear,[98] human herpesvirus (HHV) type 6, *Chlamydia pneumoniae*, and EBV are the most leading candidates in the MS research.[99-101]

Epstein-Barr virus

Various epidemiological[102] and serological[103,104] studies have shown an association between MS and EBV. One of the few clear clinical risk factors for MS is infectious mononucleosis (IM),[102,105,106] a benign lymphoproliferative disease manifested in adolescence and adulthood. It is characterized by fever, lymphadenopathy and pharyngitis caused by the EBV. Handel and colleagues[107] showed in a meta-analysis that a history of infectious mononucleosis increases the risk of MS more than 2 times. Compared to EBV negative individuals, there is at least a 20-fold increase in risk among individuals with a history of mononucleosis.[8] This can be seen as the EBV paradox in the hygiene hypothesis. According to this hypothesis, individuals who have escaped infection in childhood and are EBV seronegative should have a higher MS risk. However, given the increased risk after mononucleosis, the age at infection with EBV seems to play a role in the pathogenesis of MS.

Following primary infection, which is usually clinically silent, the virus establishes a reservoir in memory B cells escaping immune detection by down-regulation of viral antigens.[108] Activation of replicative infection and outgrowth of latently infected cells is kept under tight control by human leukocyte antigen (HLA) class I-restricted CD8+ cytotoxic T lymphoctes.[109] At a certain point an equilibrium is established and the number of EBV infected B cells within an individual will stay stable over time.[110] In older patients, the primary infection manifests as IM in 25%-70% of individuals with a striking expansion of EBV specific cytotoxic T lymphocytes[111] resulting in EBV antibody titre increase and higher viral load.

Next to IM, there is a consistent finding that almost all patients with MS (>99%) are infected with EBV, as compared with about 90% of controls.[8] An European paediatric study reported comparable seropositivity for EBV infection in children with MS (99%) in contrast to 72% of age-matched control individuals.[112] Several studies reported elevated titres in MS patients against the EBV viral capsid antigen (VCA) expressed in the viral replicative cycle, the Epstein-Barr nuclear antigen (EBNA) in latently infected B lymphocytes and EBV early antigen (EA) representing active peripheral EBV replication.[113-117] However, some of these studies had

methodological problems.[118] Serum levels of IgG antibodies to EBNA complex or EBNA-1 were the strongest predictors. The risk of MS increased with the EBNA-1 antibody titres and the antibody level increased earlier than the onset of MS and remained significantly elevated at a plateau from the age of 25 years onward. The increase was independent of antibody titres to cytomegalovirus (CMV), a related herpes virus, indicating the specificity of these findings.[114] The seropositivity for both VCA and EBNA is >90% in MS cases and controls. However, several studies showed significantly higher seropositivity for EA in MS cases compared to healthy controls[119,120] indicating higher EBV reactivity in MS. Further evidence that EBV has an active role in MS has been provided by the observation that active viral replication occurs more commonly in MS patients with exacerbations than in patients with stable disease.[119,121] However, other investigators have not been able to replicate this result.[120,122]

The association of EBV infection with MS may be causative or EBV infection may simply be a prerequisite for the subsequent development of MS. This is also supported by associations to other putative autoimmune diseases.[123,124] The elevations in antibody titres and higher EBV reactivity are consistent with an association between infection with EBV and risk of MS[125] but could also simply reflect the immune dysregulation in MS patients[126] causing replication which might partly be due to shared genetic susceptibility. However, De Jager and colleagues[127] showed that the *HLA-DRB1*1501* allele acts independently but synergistically with high levels of EBV antibodies to increase the risk of MS.

Cigarette smoking

Cigarette smoking is one of the most often postulated environmental risk factors linked to onset and progression of MS in genetically susceptible individuals.[128,129] The history of the suggested association between MS and cigarette smoking goes back to the 1960's when a few studies were performed, although these studies analysed a large number of variables simultaneously and did not reach significant results.[130-132]

Since that time different studies were performed investigating the association of cigarette smoking with MS onset and MS progression. A recent meta-analysis demonstrated that cigarette smoking is important in determining MS susceptibility, but the effect on disease progression is less certain.[133] Although the odds ratios and relative risks are relatively low, the dose-response gradient observed in several studies[134-137] is a strong argument for causality. A French case-control study showed a positive association between parental smoking at home and early onset MS in their children,[138] suggesting a role for passive smoking. However, another study demonstrated no effect of maternal smoking during pregnancy on early onset MS in offspring.[139] Hedström and colleagues observed that never-smokers, who reported that they had been exposed to passive cigarette smoking, had an increased risk of developing MS compared to those who reported never having been exposed (OR 1.3: 95% CI 1.1-1.5). The risk

increased significantly with longer duration of exposure.[140] These findings support a direct role of tobacco components.

Several hypotheses have been proposed to explain the increased risk of MS among cigarette smokers. These include effects on the immune system, vascular effects, increased production of nitric oxide, neurotoxic effects of cyanides and other components of cigarette smoke and increased frequency of respiratory infections.[141-143]

Scope of the thesis

Despite extensive research in the past years, our understanding of the exact causal pathways in MS pathogenesis falls short and useful prediction is not feasible yet. Decades of research have shed some light on the prognostic factors for MS. However, predicting the clinical future in these young adults, who are in their productive years of life, is still very limited.

The aim of the studies described in this thesis is to demonstrate the prognostic value of some of the risk factors involved in the cause and clinical course of MS. And further, to show interaction between some of these risk factors. A second aim is to enhance the knowledge of MS disease pathways, which can direct strategies for prevention, diagnosis, and therapy.

Part II (chapter 2) investigates whether corpus callosum lesions can help predict the course of MS after a first demyelinating event. Although corpus callosum lesions have been mentioned as MS specific in some of the oldest studies, they were never included in the diagnostic criteria. We assessed the predictive value of corpus callosum lesions separately and in conjunction with the Barkhof criteria.

With the expansion in genetic techniques, risk genes have come into the spotlight of MS research. However, the relative risk of most new risk alleles has shown to be small. **Part III (chapter 3)** describes the perspectives on the use of multiple risk genes for prediction of MS today and in the future. In 2008 for the first time, we reported that a 'neurodegenerative gene' *KIF1B* (rs10492972) variant was associated with MS susceptibility. Although others could not replicate our finding, we studied whether *KIF1B* was associated with clinical markers of neurodegeneration **(chapter 4)**.

Environmental risk factors critically contribute to risk of MS and the disease course. **Part IV** addresses this issue by focusing on two main factors; Epstein-Barr virus **(chapter 5)** and cigarette smoking **(chapter 6)**.
In **Chapter 5.1** we demonstrated there is no evidence of intrathecal IgG synthesis against EBV nuclear antigen. This evidence argues against the presence of EBV infected B cells in the CNS. **Chapter 5.2** describes the association of HLA class I polymorphism with MS, which already has been linked to IM. **Chapter 5.3** assessed EBV reactivity and its interaction with the HLA class I polymorphism in patients with MS. Given the growing interest in cigarette smoking as a risk factor, **chapter 6.1** investigates the association with MS within multiplex families. Furthermore, **chapter 6.2** presents a review on the association between cigarette smoking and MS.

Finally, in the general discussion **Part V (chapter 7)**, the main findings of this thesis are addressed and suggestions are made for further research.

References

1. Compston A, Coles A. Multiple sclerosis. Lancet 2002;359:1221-1231.
2. Koch-Henriksen N. The Danish multiple sclerosis registry: a 50-year follow-up. Mult Scler 1999;5:293-296.
3. Mayr WT, Pittock SJ, McClelland RL, Jorgensen NW, Noseworthy JH, Rodriguez M. Incidence and prevalence of multiple sclerosis in Olmsted County, Minnesota, 1985-2000. Neurology 2003;61:1373-1377.
4. Bronnum-Hansen H, Koch-Henriksen N, Stenager E. Trends in survival and cause of death in Danish patients with multiple sclerosis. Brain 2004;127:844-850.
5. Feinstein A. The neuropsychiatry of multiple sclerosis. Can J Psychiatry 2004;49:157-163.
6. Sadovnick AD, Eisen K, Ebers GC, Paty DW. Cause of death in patients attending multiple sclerosis clinics. Neurology 1991;41:1193-1196.
7. Hernan MA, Olek MJ, Ascherio A. Geographic variation of MS incidence in two prospective studies of US women. Neurology 1999;53:1711-1718.
8. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part I: the role of infection. Ann Neurol 2007;61:288-299.
9. Zivadinov R, Iona L, Monti-Bragadin L, et al. The use of standardized incidence and prevalence rates in epidemiological studies on multiple sclerosis. A meta-analysis study. Neuroepidemiology 2003;22:65-74.
10. Orton SM, Herrera BM, Yee IM, et al. Sex ratio of multiple sclerosis in Canada: a longitudinal study. Lancet Neurol 2006;5:932-936.
11. Palacios N, Alonso A, Bronnum-Hansen H, Ascherio A. Smoking and increased risk of multiple sclerosis: parallel trends in the sex ratio reinforce the evidence. Ann Epidemiol 2011;21:536-542.
12. Pugliatti M, Sotgiu S, Solinas G, et al. Multiple sclerosis epidemiology in Sardinia: evidence for a true increasing risk. Acta Neurol Scand 2001;103:20-26.
13. Alonso A, Hernan MA. Temporal trends in the incidence of multiple sclerosis: a systematic review. Neurology 2008;71:129-135.
14. Feuillet L, Reuter F, Audoin B, et al. Early cognitive impairment in patients with clinically isolated syndrome suggestive of multiple sclerosis. Mult Scler 2007;13:124-127.
15. Compston A, Coles A. Multiple sclerosis. Lancet 2008;372:1502-1517.
16. Lassmann H. Recent neuropathological findings in MS-implications for diagnosis and therapy. J Neurol 2004;251:IV2-5.
17. Schumacher GA, Beebe G, Kibler RF, et al. Problems of experimental trials of therapy in multiple sclerosis: report by the panel on the evaluation of experimental trials of therapy in multiple sclerosis. Ann N Y Acad Sci 1965;122:552-568.
18. Poser CM, Paty DW, Scheinberg L, et al. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. Ann Neurol 1983;13:227-231.
19. McDonald WI, Compston A, Edan G, et al. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. Ann Neurol 2001;50:121-127.
20. Polman CH, Reingold SC, Edan G, et al. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". Ann Neurol 2005;58:840-846.
21. Tintore M, Rovira A, Martinez MJ, et al. Isolated demyelinating syndromes: comparison of different MR imaging criteria to predict conversion to clinically definite multiple sclerosis. AJNR Am J Neuroradiol 2000;21:702-706.
22. Barkhof F, Filippi M, Miller DH, et al. Comparison of MRI criteria at first presentation to predict conversion to clinically definite multiple sclerosis. Brain 1997;120:2059-2069.

23. Dalton CM, Brex PA, Miszkiel KA, et al. Application of the new McDonald criteria to patients with clinically isolated syndromes suggestive of multiple sclerosis. Ann Neurol 2002;52:47-53.
24. Poser CM. Revisions to the 2001 McDonald diagnostic criteria. Ann Neurol 2006;59:727-728.
25. Polman CH, Reingold SC, Banwell B, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. Ann Neurol 2011;69:292-302.
26. Fisniku LK, Brex PA, Altmann DR, et al. Disability and T2 MRI lesions: a 20-year follow-up of patients with relapse onset of multiple sclerosis. Brain 2008;131:808-817.
27. Korteweg T, Tintore M, Uitdehaag B, et al. MRI criteria for dissemination in space in patients with clinically isolated syndromes: a multicentre follow-up study. Lancet Neurol 2006;5:221-227.
28. Swanton JK, Fernando K, Dalton CM, et al. Modification of MRI criteria for multiple sclerosis in patients with clinically isolated syndromes. J Neurol Neurosurg Psychiatry 2006;77:830-833.
29. Sadovnick AD, Baird PA, Ward RH. Multiple sclerosis: updated risks for relatives. Am J Med Genet 1988;29:533-541.
30. Sadovnick AD. Familial recurrence risks and inheritance of multiple sclerosis. Curr Opin Neurol Neurosurg 1993;6:189-194.
31. Carton H, Vlietinck R, Debruyne J, et al. Risks of multiple sclerosis in relatives of patients in Flanders, Belgium. J Neurol Neurosurg Psychiatry 1997;62:329-333.
32. Robertson NP, Fraser M, Deans J, Clayton D, Walker N, Compston DA. Age-adjusted recurrence risks for relatives of patients with multiple sclerosis. Brain 1996;119:449-455.
33. Willer CJ, Dyment DA, Risch NJ, Sadovnick AD, Ebers GC. Twin concordance and sibling recurrence rates in multiple sclerosis. Proc Natl Acad Sci U S A 2003;100:12877-12882.
34. Ebers GC, Bulman DE, Sadovnick AD, et al. A population-based study of multiple sclerosis in twins. N Engl J Med 1986;315:1638-1642.
35. Hansen T, Skytthe A, Stenager E, Petersen HC, Bronnum-Hansen H, Kyvik KO. Concordance for multiple sclerosis in Danish twins: an update of a nationwide study. Mult Scler 2005;11:504-510.
36. Sadovnick AD, Armstrong H, Rice GP, et al. A population-based study of multiple sclerosis in twins: update. Ann Neurol 1993;33:281-285.
37. Ebers GC, Sadovnick AD, Risch NJ. A genetic basis for familial aggregation in multiple sclerosis. Canadian Collaborative Study Group. Nature 1995;377:150-151.
38. Sadovnick AD, Ebers GC, Dyment DA, Risch NJ. Evidence for genetic basis of multiple sclerosis. The Canadian Collaborative Study Group. Lancet 1996;347:1728-1730.
39. Ebers GC, Sadovnick AD, Dyment DA, Yee IM, Willer CJ, Risch N. Parent-of-origin effect in multiple sclerosis: observations in half-siblings. Lancet 2004;363:1773-1774.
40. Kantarci OH, Barcellos LF, Atkinson EJ, et al. Men transmit MS more often to their children vs women: the Carter effect. Neurology 2006;67:305-310.
41. Herrera BM, Ramagopalan SV, Orton S, et al. Parental transmission of MS in a population-based Canadian cohort. Neurology 2007;69:1208-1212.
42. Urdinguio RG, Sanchez-Mut JV, Esteller M. Epigenetic mechanisms in neurological diseases: genes, syndromes, and therapies. Lancet Neurol 2009;8:1056-1072.
43. Christensen BC, Houseman EA, Marsit CJ, et al. Aging and environmental exposures alter tissue-specific DNA methylation dependent upon CpG island context. PLoS Genet 2009;5:e1000602.
44. Brooks WH. X chromosome inactivation and autoimmunity. Clin Rev Allergy Immunol 2010;39:20-29.
45. Reik W, Dean W, Walter J. Epigenetic reprogramming in mammalian development. Science 2001;293:1089-1093.
46. Handel AE, Ebers GC, Ramagopalan SV. Epigenetics: molecular mechanisms and implications for disease. Trends Mol Med 2010;16:7-16.

47. Probst AV, Dunleavy E, Almouzni G. Epigenetic inheritance during the cell cycle. Nat Rev Mol Cell Biol 2009;10:192-206.
48. Curley JP, Mashoodh R, Champagne FA. Epigenetics and the origins of paternal effects. Horm Behav 2011;59:306-314.
49. Jersild C, Svejgaard A, Fog T. HL-A antigens and multiple sclerosis. Lancet 1972;1:1240-1241.
50. Sawcer S, Compston A. Multiple sclerosis: light at the end of the tunnel. Eur J Hum Genet 2006;14:257-258.
51. Hafler DA, Compston A, Sawcer S, et al. Risk alleles for multiple sclerosis identified by a genomewide study. N Engl J Med 2007;357:851-862.
52. Fogdell A, Hillert J, Sachs C, Olerup O. The multiple sclerosis- and narcolepsy-associated HLA class II haplotype includes the DRB5*0101 allele. Tissue Antigens 1995;46:333-336.
53. Lincoln MR, Montpetit A, Cader MZ, et al. A predominant role for the HLA class II region in the association of the MHC region with multiple sclerosis. Nat Genet 2005;37:1108-1112.
54. Oksenberg JR, Barcellos LF, Cree BA, et al. Mapping multiple sclerosis susceptibility to the HLA-DR locus in African Americans. Am J Hum Genet 2004;74:160-167.
55. International Multiple Sclerosis Genetics Consortium. Refining genetic associations in multiple sclerosis. Lancet Neurol 2008;7:567-569.
56. International Multiple Sclerosis Genetics Consortium. The expanding genetic overlap between multiple sclerosis and type I diabetes. Genes Immun 2009;10:11-14.
57. Rubio JP, Stankovich J, Field J, et al. Replication of KIAA0350, IL2RA, RPL5 and CD58 as multiple sclerosis susceptibility genes in Australians. Genes Immun 2008;9:624-630.
58. Hoppenbrouwers IA, Aulchenko YS, Janssens AC, et al. Replication of CD58 and CLEC16A as genome-wide significant risk genes for multiple sclerosis. J Hum Genet 2009;54:676-680.
59. De Jager PL, Jia X, Wang J, et al. Meta-analysis of genome scans and replication identify CD6, IRF8 and TNFRSF1A as new multiple sclerosis susceptibility loci. Nat Genet 2009;41:776-782.
60. Australia and New Zealand Multiple Sclerosis Genetics Consortium. Genome-wide association study identifies new multiple sclerosis susceptibility loci on chromosomes 12 and 20. Nat Genet 2009;41:824-828.
61. Sundqvist E, Baarnhielm M, Alfredsson L, Hillert J, Olsson T, Kockum I. Confirmation of association between multiple sclerosis and CYP27B1. Eur J Hum Genet 2010;18:1349-1352.
62. Jakkula E, Leppa V, Sulonen AM, et al. Genome-wide association study in a high-risk isolate for multiple sclerosis reveals associated variants in STAT3 gene. Am J Hum Genet 2010;86:285-291.
63. Aulchenko YS, Hoppenbrouwers IA, Ramagopalan SV, et al. Genetic variation in the KIF1B locus influences susceptibility to multiple sclerosis. Nat Genet 2008;40:1402-1403.
64. International Multiple Sclerosis Genetics Consortium, Wellcome Trust Case Control Consortium 2, Sawcer S, et al. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. Nature 2011;476:214-219.
65. Holm H, Gudbjartsson DF, Sulem P, et al. A rare variant in MYH6 is associated with high risk of sick sinus syndrome. Nat Genet 2011;43:316-320.
66. Zeggini E. Next-generation association studies for complex traits. Nat Genet 2011;43:287-288.
67. Baranzini SE, Mudge J, van Velkinburgh JC, et al. Genome, epigenome and RNA sequences of monozygotic twins discordant for multiple sclerosis. Nature 2010;464:1351-1356.
68. Dean G, McLoughlin H, Brady R, Adelstein AM, Tallett-Williams J. Multiple sclerosis among immigrants in Greater London. Br Med J 1976;1:861-864.
69. Visscher BR, Detels R, Coulson AH, Malmgren RM, Dudley JP. Latitude, migration, and the prevalence of multiple sclerosis. Am J Epidemiol 1977;106:470-475.

70. Elian M, Nightingale S, Dean G. Multiple sclerosis among United Kingdom-born children of immigrants from the Indian subcontinent, Africa and the West Indies. J Neurol Neurosurg Psychiatry 1990;53:906-911.
71. Pugliatti M, Sotgiu S, Rosati G. The worldwide prevalence of multiple sclerosis. Clinical neurology and neurosurgery 2002;104:182-191.
72. Koch-Henriksen N, Sorensen PS. The changing demographic pattern of multiple sclerosis epidemiology. Lancet Neurol 2010;9:520-532.
73. Willer CJ, Dyment DA, Sadovnick AD, Rothwell PM, Murray TJ, Ebers GC. Timing of birth and risk of multiple sclerosis: population based study. BMJ 2005;330:120.
74. Van der Mei IA, Ponsonby AL, Blizzard L, Dwyer T. Regional variation in multiple sclerosis prevalence in Australia and its association with ambient ultraviolet radiation. Neuroepidemiology 2001;20:168-174.
75. Acheson ED, Bachrach CA, Wright FM. Some comments on the relationship of the distribution of multiple sclerosis to latitude, solar radiation, and other variables. Acta Psychiatr Scand Suppl 1960;35:132-147.
76. Freedman DM, Dosemeci M, Alavanja MC. Mortality from multiple sclerosis and exposure to residential and occupational solar radiation: a case-control study based on death certificates. Occup Environ Med 2000;57:418-421.
77. Van der Mei IA, Ponsonby AL, Dwyer T, et al. Past exposure to sun, skin phenotype, and risk of multiple sclerosis: case-control study. BMJ 2003;327:316.
78. Goldacre MJ, Seagroatt V, Yeates D, Acheson ED. Skin cancer in people with multiple sclerosis: a record linkage study. J Epidemiol Community Health 2004;58:142-144.
79. Duthie MS, Kimber I, Norval M. The effects of ultraviolet radiation on the human immune system. Br J Dermatol 1999;140:995-1009.
80. Deluca HF, Cantorna MT. Vitamin D: its role and uses in immunology. FASEB J 2001;15:2579-2585.
81. Goldberg P. Multiple sclerosis: vitamin D and calcium as environmental determinants of prevalence. Part 1: Sunlight, dietary factors and epidemiology. Intern J Environmental Studies 1974;6:19-27.
82. Munger KL, Levin LI, Hollis BW, Howard NS, Ascherio A. Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. JAMA 2006;296:2832-2838.
83. Fukazawa T, Yabe I, Kikuchi S, et al. Association of vitamin D receptor gene polymorphism with multiple sclerosis in Japanese. J Neurol Sci 1999;166:47-52.
84. Brown SJ. The role of vitamin D in multiple sclerosis. Ann Pharmacother 2006;40:1158-1161.
85. Spach KM, Nashold FE, Dittel BN, Hayes CE. IL-10 signaling is essential for 1,25-dihydroxyvitamin D3-mediated inhibition of experimental autoimmune encephalomyelitis. J Immunol 2006;177:6030-6037.
86. Cantorna MT, Hayes CE, DeLuca HF. 1,25-Dihydroxyvitamin D3 reversibly blocks the progression of relapsing encephalomyelitis, a model of multiple sclerosis. Proc Natl Acad Sci U S A 1996;93:7861-7864.
87. Embry AF, Snowdon LR, Vieth R. Vitamin D and seasonal fluctuations of gadolinium-enhancing magnetic resonance imaging lesions in multiple sclerosis. Ann Neurol 2000;48:271-272.
88. Poskanzer DC, Schapira K, Miller H. Multiple sclerosis and poliomyelitis. Lancet 1963;2:917-921.
89. Leibowitz U, Antonovsky A, Medalie JM, Smith HA, Halpern L, Alter M. Epidemiological study of multiple sclerosis in Israel. II. Multiple sclerosis and level of sanitation. J Neurol Neurosurg Psychiatry 1966;29:60-68.
90. Beebe GW, Kurtzke JF, Kurland LT, Auth TL, Nagler B. Studies on the natural history of multiple sclerosis. 3. Epidemiologic analysis of the army experience in World War II. Neurology 1967;17:1-17.
91. Russell WR. Multiple sclerosis: occupation and social group at onset. Lancet 1971;2:832-834.
92. Ponsonby AL, van der Mei I, Dwyer T, Blizzard L, Taylor B, Kemp A. Birth order, infection in early life, and multiple sclerosis. Lancet Neurol 2005;4:793-794.
93. Bager P, Nielsen NM, Bihrmann K, et al. Sibship characteristics and risk of multiple sclerosis: a nationwide cohort study in Denmark. Am J Epidemiol 2006;163:1112-1117.

94. Sadovnick AD, Yee IM, Ebers GC. Multiple sclerosis and birth order: a longitudinal cohort study. Lancet Neurol 2005;4:611-617.
95. Goverman J, Woods A, Larson L, Weiner LP, Hood L, Zaller DM. Transgenic mice that express a myelin basic protein-specific T cell receptor develop spontaneous autoimmunity. Cell 1993;72:551-560.
96. Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. N Engl J Med 2002;347:911-920.
97. Granieri E, Casetta I. Selected reviews common childhood and adolescent infections and multiple sclerosis. Neurology 1997;49:S42-54.
98. Gilden DH. Infectious causes of multiple sclerosis. Lancet Neurol 2005;4:195-202.
99. Martyn CN, Cruddas M, Compston DA. Symptomatic Epstein-Barr virus infection and multiple sclerosis. J Neurol Neurosurg Psychiatry 1993;56:167-168.
100. Giovannoni G, Cutter GR, Lunemann J, et al. Infectious causes of multiple sclerosis. Lancet Neurol 2006;5:887-894.
101. Stratton CW, Wheldon DB. Multiple sclerosis: an infectious syndrome involving Chlamydophila pneumoniae. Trends Microbiol 2006;14:474-479.
102. Thacker EL, Mirzaei F, Ascherio A. Infectious mononucleosis and risk for multiple sclerosis: a meta-analysis. Ann Neurol 2006;59:499-503.
103. Ascherio A, Munger KL, Lennette ET, et al. Epstein-Barr virus antibodies and risk of multiple sclerosis: a prospective study. JAMA 2001;286:3083-3088.
104. Lünemann JD, Munz C. Epstein-Barr virus and multiple sclerosis. Curr Neurol Neurosci Rep 2007;7:253-258.
105. Ramagopalan SV, Valdar W, Dyment DA, et al. Association of infectious mononucleosis with multiple sclerosis. A population-based study. Neuroepidemiology 2009;32:257-262.
106. Nielsen TR, Rostgaard K, Nielsen NM, et al. Multiple sclerosis after infectious mononucleosis. Arch Neurol 2007;64:72-75.
107. Handel AE, Williamson AJ, Disanto G, Handunnetthi L, Giovannoni G, Ramagopalan SV. An updated meta-analysis of risk of multiple sclerosis following infectious mononucleosis. PloS One 2010;5:e12496.
108. Rickinson AB, Kieff E. Epstein-Barr virus. Fields Virology, vol.II. In: P.M.H. David, M. Knipe, Editors Lippincott-Raven Publishers, Philadelphia, 2007;2397-2446.
109. Khanna R, Burrows SR. Role of cytotoxic T lymphocytes in Epstein-Barr virus-associated diseases. Annu Rev Microbiol 2000;54:19-48.
110. Khan G, Miyashita EM, Yang B, Babcock GJ, Thorley-Lawson DA. Is EBV persistence in vivo a model for B cell homeostasis? Immunity 1996;5:173-179.
111. Crawford DH, Macsween KF, Higgins CD, et al. A cohort study among university students: identification of risk factors for Epstein-Barr virus seroconversion and infectious mononucleosis. Clin Infect Dis 2006;43:276-282.
112. Pohl D, Krone B, Rostasy K, et al. High seroprevalence of Epstein-Barr virus in children with multiple sclerosis. Neurology 2006;67:2063-2065.
113. Sundström P, Juto P, Wadell G, et al. An altered immune response to Epstein-Barr virus in multiple sclerosis: a prospective study. Neurology 2004;62:2277-2282.
114. Levin LI, Munger KL, Rubertone MV, et al. Temporal relationship between elevation of epstein-barr virus antibody titers and initial onset of neurological symptoms in multiple sclerosis. JAMA 2005;293:2496-2500.
115. Shirodaria PV, Haire M, Fleming E, Merrett JD, Hawkins SA, Roberts SD. Viral antibody titers. Comparison in patients with multiple sclerosis and rheumatoid arthritis. Arch Neurol 1987;44:1237-1241.
116. Compston DA, Vakarelis BN, Paul E, McDonald WI, Batchelor JR, Mims CA. Viral infection in patients with multiple sclerosis and HLA-DR matched controls. Brain 1986;109:325-344.

117. Alotaibi S, Kennedy J, Tellier R, Stephens D, Banwell B. Epstein-Barr virus in pediatric multiple sclerosis. JAMA 2004;291:1875-1879.

118. Marrie RA, Wolfson C. Multiple sclerosis and Epstein-Barr virus. Can J Infect Dis 2002;13:111-118.

119. Wandinger K, Jabs W, Siekhaus A, et al. Association between clinical disease activity and Epstein-Barr virus reactivation in MS. Neurology 2000;55:178-184.

120. Buljevac D, van Doornum GJ, Flach HZ, et al. Epstein-Barr virus and disease activity in multiple sclerosis. J Neurol Neurosurg Psychiatry 2005;76:1377-1381.

121. Lindsey JW, Hatfield LM, Crawford MP, Patel S. Quantitative PCR for Epstein-Barr virus DNA and RNA in multiple sclerosis. Mult Scler 2009;15:153-158.

122. Torkildsen O, Nyland H, Myrmel H, Myhr KM. Epstein-Barr virus reactivation and multiple sclerosis. Eur J Neurol 2008;15:106-108.

123. James JA, Kaufman KM, Farris AD, Taylor-Albert E, Lehman TJ, Harley JB. An increased prevalence of Epstein-Barr virus infection in young patients suggests a possible etiology for systemic lupus erythematosus. J Clin Invest 1997;100:3019-3026.

124. Cooke SP, Rigby SP, Griffiths DJ, Venables PJ. Viral studies in rheumatic disease. Ann Med Interne (Paris) 1998;149:30-33.

125. Ascherio A, Munch M. Epstein-Barr virus and multiple sclerosis. Epidemiology 2000;11:220-224.

126. Hunter SF, Hafler DA. Ubiquitous pathogens: links between infection and autoimmunity in MS? Neurology 2000;55:164-165.

127. De Jager PL, Simon KC, Munger KL, Rioux JD, Hafler DA, Ascherio A. Integrating risk factors: HLA-DRB1*1501 and Epstein-Barr virus in multiple sclerosis. Neurology 2008;70:1113-1118.

128. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part II: Noninfectious factors. Ann Neurol 2007;61:504-513.

129. Giovannoni G, Ebers G. Multiple sclerosis: the environment and causation. Curr Opin Neurol 2007;20:261-268.

130. Courville CB, Maschmeyer JE, Delay CP. Effects of smoking on the acute exacerbations of multiple sclerosis. Bull Los Angel Neuro Soc 1964;29:1-6.

131. Antonovsky A, Leibowitz U, Smith HA, et al. Epidemiologic Study of Multiple Sclerosis in Israel. I. an Overall Review of Methods and Findings. Arch Neurol 1965;13:183-193.

132. Simpson CA, Newell DJ, Schapira K. Smoking and multiple sclerosis. Neurology 1966;16:1041-1043.

133. Handel AE, Williamson AJ, Disanto G, Dobson R, Giovannoni G, Ramagopalan SV. Smoking and multiple sclerosis: an updated meta-analysis. PloS One 2011;6:e16149.

134. Ghadirian P, Dadgostar B, Azani R, Maisonneuve P. A case-control study of the association between socio-demographic, lifestyle and medical history factors and multiple sclerosis. Can J Public Health 2001;92:281-285.

135. Sundström P, Nyström L, Hallmans G. Smoke exposure increases the risk for multiple sclerosis. Eur J Neurol 2008;15:579-583.

136. Hernan MA, Olek MJ, Ascherio A. Cigarette smoking and incidence of multiple sclerosis. American journal of epidemiology 2001;154:69-74.

137. Villard-Mackintosh L, Vessey MP. Oral contraceptives and reproductive factors in multiple sclerosis incidence. Contraception 1993;47:161-168.

138. Mikaeloff Y, Caridade G, Tardieu M, Suissa S. Parental smoking at home and the risk of childhood-onset multiple sclerosis in children. Brain 2007;130:2589-2595.

139. Montgomery SM, Bahmanyar S, Hillert J, Ekbom A, Olsson T. Maternal smoking during pregnancy and multiple sclerosis amongst offspring. Eur J Neurol 2008;15:1395-1399.

140. Hedström A, Baarnhielm M, Olsson T, Alfredsson L. Exposure to environmental tobacco smoke is associated with increased risk for multiple sclerosis. Mult Scler 2011;17:788-793.

141. Pace E, Ferraro M, Siena L, et al. Cigarette smoke increases Toll-like receptor 4 and modifies lipopolysaccharide-mediated responses in airway epithelial cells. Immunology 2008;1244:401-411.

142. Hughes DA, Haslam PL, Townsend PJ, Turner-Warwick M. Numerical and functional alterations in circulatory lymphocytes in cigarette smokers. Clin Exp Immunol 1985;61:459-466.

143. Hans FJ, Wei L, Bereczki D, et al. Nicotine increases microvascular blood flow and flow velocity in three groups of brain areas. Am J Physiol 1993;265:2142-2150.

Part II

MRI as a clinical tool for prediction of MS

Chapter 2

Callosal lesion predicts future attacks after clinically isolated syndrome

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Abstract

Background Current MRI criteria can help predict a second attack after a clinically isolated syndrome (CIS). Given the known association between corpus callosum lesions (CC) and multiple sclerosis (MS), such lesions on MRI could provide additional predictive information. This study assessed whether the presence of CC lesion on MRI could, next to the modified Barkhof criteria, further enhance prediction of conversion from CIS to MS.

Methods Follow-up study of 158 patients with CIS who underwent MRI after CIS was performed. MRI were scored for the Barkhof criteria and CC lesion. Patients were classified as having MS according to Poser criteria. Cox regression models were used for the time to conversion from CIS to MS.

Results The Barkhof criteria and CC lesion were strongly associated with conversion to MS with hazard ratios (HR) respectively, of 2.6 (95% confidence interval [CI] 1.5-4.3) and 2.7 (95% CI 1.6-4.5). The HRs of CC lesion adjusted for the Barkhof criteria and the Barkhof criteria adjusted for CC lesion were similar (HRs 1.8, not significant). The combined prediction of the Barkhof criteria and CC lesion was 3.3 (95% CI 1.9-5.7). Patients not fulfilling the Barkhof criteria had a fourfold increased risk of MS (HR 3.8, 95% CI 1.5-9.3) when they had a lesion in the CC.

Conclusions Corpus callosum (CC) lesion and the Barkhof criteria both predicted conversion to multiple sclerosis (MS). When both variables were combined, the association was stronger. The assessment of CC lesion may be a useful additional tool for predicting conversion to MS in patients with clinically isolated syndrome.

Introduction

Most patients with multiple sclerosis (MS) present with a clinically isolated syndrome (CIS). CIS is defined as (sub)acute episode of neurological disturbance probably caused by an inflammatory demyelinating event in the CNS.[1]

Current diagnostic criteria for MS are based on clinical criteria, MRI assessment according to Barkhof criteria (BC) and CSF analysis. The BC as a central part of McDonald criteria[2] have an acceptable specificity, but rather low sensitivity in general clinical practice.[3] At time of the initial attack, fewer than 50% fulfill the BC.[4] Almost 40% of the patients fulfilling the BC did not develop MS within the follow-up time.[1] Because of the rather low sensitivity and given that half of the patients fulfill these criteria at baseline, additional tests are needed to make a better risk estimation for conversion from CIS to MS.

Corpus callosum (CC) lesions are associated with MS as determined in both MRI and postmortem studies.[5, 6] Previous studies implicated that CC lesions are a sensitive and specific marker for MS,[6] as well as an early marker.[6, 7] However, in the development of the diagnostic MRI criteria, CC lesions have been relatively neglected. After the landmark article that formed the basis of the BC[2], the value of assessing these lesions has not been included in most follow-up articles.[8, 9]

The present study aimed to assess the additional predictive value of CC lesion independently of the BC and the combined predictive value of the BC and CC lesion.

Methods

Patients and procedures

Within the Rotterdam MS Center, 170 patients were included who fulfilled the following criteria: 1) patients who experienced a first episode of symptoms suggestive of CNS demyelination. Neurologic symptoms lasting more than 24 hours were taken into consideration, only when confirmed by a physician; 2) age at onset of symptoms between 16 and 55 years; and 3) without life-threatening comorbidity (e.g., cancer, HIV). Patients were initially inquired about a previous history of neurologic signs or symptoms and seen on a regular basis. Patients who had experienced an earlier episode suggestive of CNS demyelination were not eligible to participate in this study.

A clinical diagnosis of MS was made when a newly confirmed neurologic abnormality occurred after an interval of at least 1 month after complete recovery and after other diagnoses had been excluded.[10]

Standard protocol approvals, registrations, and patient consents

This study was approved by the Medical Ethical Committee of the Erasmus MC and informed consent was obtained from patients.

MRI acquisition

MRI of the brain was performed on a 1.5 Tesla scanner (Philips, Best, the Netherlands, or General Electric, Milwaukee, WI) with a standard head coil and consisted of an axial spin echo proton density-weighted (PDW) and T2-weighted sequence with 5-mm slices, an axial fluid-attenuated inversion recovery (FLAIR), and a T1-weighted sequence with slice thicknesses of 2 or 5 mm. Postgadolinium T1-weighted sequences were added on indication (43%) in patients with T2 lesions that could be related to demyelination. To avoid reader bias, available MRI scans at baseline were rescored independently by one experienced MRI assessor (N.J.) and one neuroradiologist (Z.F.) blinded to clinical symptoms, disease evolution, and initial MRI analysis of the reporting neuroradiologist. When the assessors disagreed, an experienced blinded MS- neurologist provided consensus (R.Q.H.). MRI scans were scored using a standardized MRI record form. We applied the modified BC[8] in which the BC are fulfilled when ≥3 parameters are present. As an additional variable, white matter lesions located in the CC were scored. Lesions of at least 2 mm were scored only when present on at least 2 different T2 sequences (T2W, PDW or FLAIR). CC lesion was defined as a lesion visible on an axial slice, located medial, directly frontal, or posterior of the lateral ventricles or located directly on the first slice above the lateral ventricles in the paramedian region. These lesions were also scored on sagittal images, when available, within the anatomically well-defined CC region. After scoring infratentorial lesions, we assessed if these lesions were symptomatic or asymptomatic. These 2 criteria were analyzed separately.[11] The results of the analysis of the symptomatic infratentorial lesions were comparable to nonsymptomatic infratentorial lesions (data not shown).

Statistical analysis

The main outcome in this study was the conversion from CIS to MS during follow-up. Survival time was defined as the interval between the date of onset of first symptoms and the date of the confirmed second neurologic episode. For patients who were not diagnosed with MS, the follow-up time was defined as the time between the date of onset of the first symptoms and the last visit to our hospital. Cox proportional hazard regression analysis was performed with time to development of MS as a dependent variable and the dichotomized BC and the presence of CC lesion as predictor variables. All analyses were performed using SPSS 14.0.

Results

Clinical and demographic characteristics

Of the total 170 patients with a first attack suggestive for demyelination, 8 patients developed a definitive diagnosis other than MS (two Leber hereditary optic neuropathy, 3 neuromyelitis optica, 1 recurrent optic neuritis, 1 dissection of the vertebrobasilar artery, and 1 hereditary downbeat nystagmus) and were excluded from the analyses during the study. Four other patients were excluded because they declined MRI scan.

A total of 158 patients were included in the analyses. This group consisted of 110 women and 48 men with a median follow-up time of 39 months (interquartile range [IQR] 23- 70). The mean age at onset was 33 years (SD 9.0) ranging from 16 to 54 years. The patients presented with different clinical symptoms (table 1), mainly located in the optical nerve (40%). Sixty-four (41%) patients converted to MS during the follow-up, with a median conversion time of 23 months (IQR 8- 33). For all analyses, we assessed whether age at onset and sex were confounders, but no significant association was found (data not shown) and thus not included in the final analyses.

MRI

We were able to analyze 155 MRI scans (98%). The median time from CIS to MRI was 1 month (IQR 0- 5). In 67% of the patients, the baseline MRI was performed within 3 months after onset of symptoms and in 81% it was performed within 6 months. The arbitrary limit of 3 months was described by other studies using MRI within 3 months of onset.[12] In all hazard analyses, we used a dichotomized variable (MRI ≤3 months and MRI >3 months) as a covariate. To test whether this adjustment was justified, we did all analyses also only in patients who had their MRI within 3 months of onset and all hazard ratios were similar (data not shown).

Prediction of conversion by the BC and CC lesion

Twenty-four patients (15%) had a normal MRI scan showing no lesion and 74 patients (48%) showed an abnormal baseline MRI with <3 BC (table 1). Of the 57 patients (37%) who fulfilled the BC (≥3 BC) at baseline, 32 (56%) converted to MS compared to 31 of the 98 patients (32%) who did not fulfill the BC (figure 1). At baseline, 51 patients (33%) had at least 1 lesion in the CC. Of these patients, 29 developed MS (57%). A total of 104 patients did not have a lesion in the CC at baseline (67%), of which 34 (33%) developed MS during follow-up. CIS patients who met the BC, had significantly higher conversion to MS than those who did not meet the criteria (HR 2.6, 95% CI 1.5- 4.3) (table 2). Also patients who had a CC lesion had a higher conversion risk (HR 2.7, 95% CI 1.6- 4.5). When additionally adjusted for each other, the hazard ratios of the BC and the CC lesion were, respectively, 1.8 (95% CI 0.9- 3.5) and 1.8 (95% CI 0.9- 3.6). Patients with a callosal lesion or fulfilling the BC or both, had a more than threefold increased risk to convert from CIS to MS, compared to patients who were negative

for both criteria (table 2). Assessment of CC lesion within patients fulfilling the BC did not contribute to a better estimation of the conversion rate (HR 1.1, 95% CI 0.6- 2.4) as shown in figure 1. However, patients not fulfilling the BC (63%) had almost a fourfold increased risk for developing MS (HR 3.8, 95% CI 1.5-9.3) when they had at least 1 lesion in the CC (figure 1). Figure 2A shows the survival curves for the BC. Interestingly, when we stratified the BC negative group by CC lesion, the group with a callosal lesion had almost the same risk for conversion to MS as patients who fulfilled the BC (figure 2B).

Table 1: Clinical and MRI characteristics in the patients.

	No conversion to MS (n=94)	Converted to MS (n=64)	Total (n=158)
Females, n (%)	65 (69)	45 (70)	110 (70)
Age at CIS, y, mean (SD)	33 (SD 8.7)	32 (SD 9.6)	33 (SD 9.0)
Symptoms at CIS (%)			
Optic neuritis	41 (44)	22 (34)	63 (40)
Spinal cord syndromes	16 (17)	14 (22)	30 (19)
Brainstem/ cerebellar symptoms	15 (16)	7 (11)	22 (14)
Remaining (multiregional)	22 (23)	21 (33)	43 (27)
Follow-up time, y	30 (18-45) [a]	23 (8-33) [b]	NA
MRI [c] Barkhof Criteria (%)			
Normal MRI	18 (20)	6 (9)	24 (15)
Abnormal MRI, but 0/4 BC	10 (11)	7 (11)	17 (11)
1-2/4 BC	39 (42)	18 (29)	57 (37)
3-4/4 BC	25 (27)	32 (51)	57 (37)
MRI [c] corpus callosum (%)			
CC lesion present	22 (24)	29 (46)	51 (33)

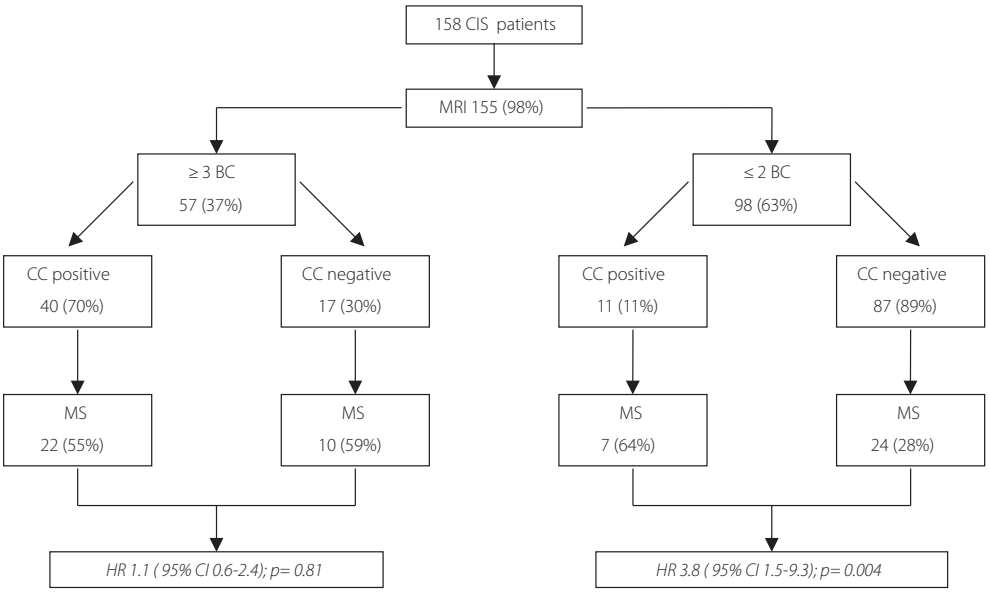
[a] Follow up time and [b] survival time both depicted as median (interquartile range).
[c] MRI was not available for 2 nonconverters and 1 converter.
MS=multiple sclerosis; CIS=clinically isolated syndrome; BC=Barkhof criteria; CC= corpus callosum.

Table 2: Hazard ratios for the 2 variables tested separately, next to each other or combined.

	Hazard ratio[#]	95% CI	p-value
Barkhof criteria [a]	2.6	1.5-4.3	<0.0001
Corpus callosum [b]	2.7	1.6-4.5	<0.0001
Barkhof criteria [c]	1.8	0.9-3.5	0.09
Corpus callosum	1.8	0.9-3.6	0.09
Either Barkhof or Corpus callosum [d]	3.3	1.9-5.7	<0.001

[#] All hazard ratios are adjusted for dichotomous variable for time from clinically isolated syndrome to MRI within 3 months or more than 3 months. Cox models were based on [a] fulfilment of Barkhof criteria, [b] presence of a corpus callosum lesion, [c] Barkhof criteria adjusted for a corpus callosum lesion and vice versa, [d] either fulfilment of the Barkhof criteria or the presence of a callosal lesion or both.
CI=confidence interval.

Figure 1: Flow chart specifying the fulfilment of Barkhof criteria (BC) and the presence of corpus callosum (CC) lesion.



Totals, percentages, and hazard ratios (HR) in the different subgroups are indicated.

Discussion

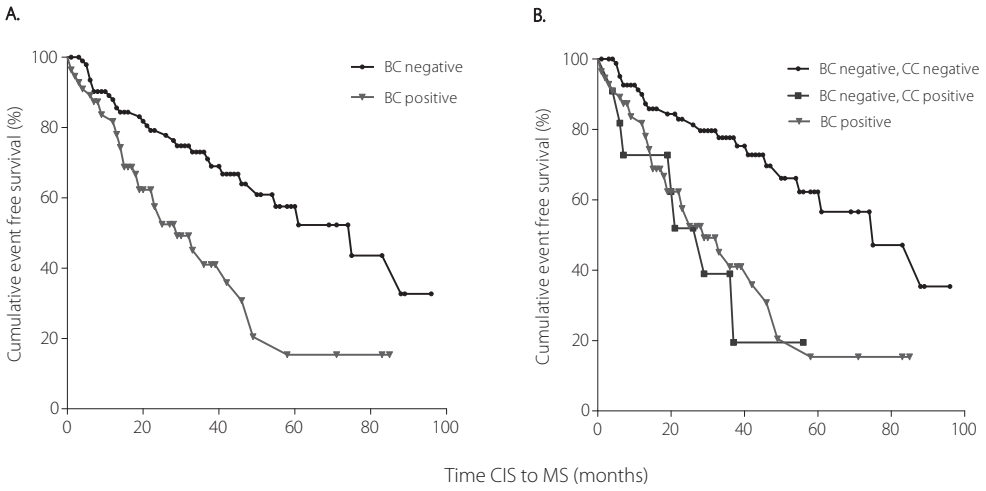
The present study assessed the predictive value of the BC and CC lesions and showed that the presence of a CC lesion on MRI is an important risk factor for the development of MS after CIS, independent of the BC. Not only does the combination of the 2 factors show a stronger association, but also CC lesion alone is associated strongly with conversion from CIS to MS especially in patients not fulfilling the BC.

At baseline, only 37% of all patients fulfilled the BC and 33% had at least 1 lesion in the CC. The HR of both variables was comparable. When we combined both variables, the HR increased to 3.3. Importantly, patients who did not fulfill the BC but had a CC lesion had an almost fourfold increased risk to develop MS. Thus, the additional assessment of CC lesions improved the estimation of the risk of conversion to MS.

This study evaluated CC lesion as a prognostic factor for the conversion from CIS to MS in a well defined cohort of CIS patients. CC lesions have been implicated in several previous studies as a marker for MS on MRI,[6, 7, 13] as well as in postmortem studies.[5] Although one of the studies included only MS patients with rather long disease duration, CC lesions have been identified as a sensitive and specific marker for MS.[6] Another study[7] included patients with clinically

suspected MS and concluded that subcallosal striations are probably an earlier manifestation of MS than the inner callosal-subcallosal and callosal-septal interface lesions described by others.[6, 13] However, their included patients had atypical CIS complaints.[7] Another study with 40 patients with MS and a control group, showed that 30% of all patients with MS had at least 1 focal lesion in the CC,[13] which is comparable to our study. Additional studies were performed analyzing atrophy of the CC as a marker for disability in MS,[14] but none of these studies have investigated the predictive value of CC lesions for the conversion from CIS to MS. The original article about the development of the BC calculated the sensitivity, specificity, positive predictive and negative predictive value of CC lesions.[2] In retrospect, all test criteria of CC lesions performed well; only the specificity was somewhat lower than other MRI parameters. However, the independent contribution of CC lesions in the logistic regression model has not been reported.[2]

Figure 2: Survival curves for the different subgroups of patients according to the Barkhof criteria (BC) and the presence of callosal lesion.



A) Survival curves according to the BC. B) Survival curves categorized by the BC and for patients not fulfilling the BC stratified according to the Corpus callosum (CC) lesion.

Some methodological issues used in our study should be noted. First, this cohort of 158 CIS patients with a first confirmed demyelinating attack in the CNS is somewhat smaller than those in other studies.[9, 12] However, there are various similarities between the studies. In our cohort, the conversion rate from CIS to MS was 41%, with a median conversion time of 23 months (IQR 8- 33), which is shorter than the follow-up time (median 30 months, IQR 18- 45) of our patients who did not convert. We are confident that our follow-up time in the CIS patients who did not convert to MS was sufficiently long to draw reliable conclusions and that our results were not

influenced by a too short follow-up time. Other characteristics of our study (e.g., women: men ratio, localization of CIS) were also similar to those characteristics in other studies.[3, 9]

Secondly, some of the MRIs were performed at the referral hospital, but all were centrally scored in our hospital. While this might be seen as a limitation of the study, the same method has been used by others, who found a HR of 2.64 (95% CI 1.57- 4.44) for the BC[9], which is similar to the HR in our study of 2.6 (95% CI 1.5- 4.3). Importantly, not all patients underwent a sagittal FLAIR image of the CC. Some studies have found that a sagittal image of the CC is a more sensitive marker for detecting MS lesions.[6, 7] Because it is reasonable to argue that the HR would have increased if all the patients had undergone a sagittal FLAIR of the CC, our findings might even be an underestimation of the true predictive value of CC lesions.

Because our study did not include trial patients, it may be more representative for general clinical practice than other studies. This implicates that patients were able to start with immunomodulating therapy during the study. Four of our patients received interferon beta treatment after their first attack, 3 of whom developed MS during follow-up. Since this number of patients is very low, we do not believe that this affected the results. Neither adjustment for disease-modifying therapy produced any significant effect (data not shown).

Currently, the gold standard for the conversion from CIS to MS is the McDonald criteria for MS.[15] Of course it is important to develop the most sensitive predictive model, since these criteria underlie the decision to start immune-modulating therapy. Besides the therapeutic considerations, patients want to be informed about their risk of developing MS. The development of a better predictive model based on MRI is therefore warranted.

Lesions in the CC are not exclusive to MS and can be seen in other diseases such as CNS infections, other inflammatory CNS diseases, or vascular diseases.[16, 17] Thus, a CC lesion needs to be assessed in the context of the whole clinical and MRI spectrum of a given patient. Its predictive value after CIS deserves to be part of future studies next to the established BC.

References

1. Dalton CM, Brex PA, Miszkiel KA, et al. Application of the new McDonald criteria to patients with clinically isolated syndromes suggestive of multiple sclerosis. Ann Neurol 2002;52:47-53.
2. Barkhof F, Filippi M, Miller DH, et al. Comparison of MRI criteria at first presentation to predict conversion to clinically definite multiple sclerosis. Brain 1997;120:2059-2069.
3. Korteweg T, Tintore M, Uitdehaag B, et al. MRI criteria for dissemination in space in patients with clinically isolated syndromes: a multicentre follow-up study. Lancet Neurol 2006;5:221-227.
4. Hintzen RQ, Giovannoni G. CSF analysis in suspected MS: do bands aid? Neurology 2008;70:1059-1060.
5. Barnard RO, Triggs M. Corpus callosum in multiple sclerosis. J Neurol Neurosurg Psychiatry 1974;37:1259-1264.
6. Gean-Marton AD, Vezina LG, Marton KI, et al. Abnormal corpus callosum: a sensitive and specific indicator of multiple sclerosis. Radiology 1991;180:215-221.
7. Palmer S, Bradley WG, Chen DY, Patel S. Subcallosal striations: early findings of multiple sclerosis on sagittal, thin-section, fast FLAIR MR images. Radiology 1999;210:149-153.
8. Tintore M, Rovira A, Martinez MJ, et al. Isolated demyelinating syndromes: comparison of different MR imaging criteria to predict conversion to clinically definite multiple sclerosis. AJNR Am J Neuroradiol 2000;21:702-706.
9. Swanton JK, Rovira A, Tintore M, et al. MRI criteria for multiple sclerosis in patients presenting with clinically isolated syndromes: a multicentre retrospective study. Lancet Neurol 2007;6:677-686.
10. Poser CM, Brinar VV. Diagnostic criteria for multiple sclerosis. Clin Neurol Neurosurg 2001;103:1-11.
11. Sastre-Garriga J, Tintore M, Rovira A, et al. Specificity of Barkhof criteria in predicting conversion to multiple sclerosis when applied to clinically isolated brainstem syndromes. Arch Neurol 2004;61:222-224.
12. Tintore M, Rovira A, Rio J, et al. Do oligoclonal bands add information to MRI in first attacks of multiple sclerosis? Neurology 2008;70:1079-1083.
13. Simon JH, Holtas SL, Schiffer RB, et al. Corpus callosum and subcallosal-periventricular lesions in multiple sclerosis: detection with MR. Radiology 1986;160:363-367.
14. Martola J, Stawiarz L, Fredrikson S, et al. Progression of non-age-related callosal brain atrophy in multiple sclerosis: a 9-year longitudinal MRI study representing four decades of disease development. J Neurol Neurosurg Psychiatry 2007;78:375-380.
15. Polman CH, Reingold SC, Edan G, et al. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". Ann Neurol 2005;58:840-846.
16. Uchino A, Takase Y, Nomiyama K, Egashira R, Kudo S. Acquired lesions of the corpus callosum: MR imaging. Eur Radiol 2006;16:905-914.
17. Correale J, de los Milagros Bassani Molinas M. Oligoclonal bands and antibody responses in multiple sclerosis. J Neurol 2002;249:375-389.

Part III

Genetic determinants of cause and course of MS

Chapter 3

Perspectives on the use of multiple sclerosis risk genes for prediction

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Abstract

Objective A recent collaborative genome-wide association study replicated a large number of susceptibility loci and identified novel loci. This increase in known multiple sclerosis (MS) risk genes raises questions about clinical applicability of genotyping. In an empirical set we assessed the predictive power of typing multiple genes. Next, in a modelling study we explored current and potential predictive performance of genetic MS risk models.

Methods Genotype data on 6 MS risk genes in 591 MS patients and 600 controls were used to investigate the predictive value of combining risk alleles. Next, the replicated and novel MS risk loci from the recent and largest international genome-wide association study were used to construct genetic risk models simulating a population of 100,000 individuals. Finally, we assessed the required numbers, frequencies, and ORs of risk SNPs for higher discriminative accuracy in the future.

Results Individuals with 10 to 12 risk alleles had a significantly increased risk compared to individuals with the average population risk for developing MS (OR 2.76 (95% CI 2.02-3.77)). In the simulation study we showed that the area under the receiver operating characteristic curve (AUC) for a risk score based on the 6 SNPs was 0.64. The AUC increases to 0.66 using the well replicated 24 SNPs and to 0.69 when including all replicated and novel SNPs (n=53) in the risk model. An additional 20 SNPs with allele frequency 0.30 and ORs 1.1 would be needed to increase the AUC to a slightly higher level of 0.70, and at least 50 novel variants with allele frequency 0.30 and ORs 1.4 would be needed to obtain an AUC of 0.85.

Conclusion Although new MS risk SNPs emerge rapidly, the discriminatory ability in a clinical setting will be limited.

Introduction

Multiple sclerosis (MS) is caused by an interplay of multiple genetic variants and environmental factors. The genetic influence on MS is substantial, as evidenced by the 20-fold risk increase for siblings of MS patients.[1] Part of the genetic risk is explained by the MHC class II locus (*HLA-DR15*).[2] In 2007 several novel risk alleles for MS were identified by a genome-wide association (GWA) study[3] and others confirmed the susceptibility loci by meta-analyses and replication.[4] Since GWA the progress has been rapid and more new risk loci have been identified and confirmed.[5-9] A recent study in 9,722 cases and 17,376 controls identified 53 associated variants.[9]

Given the gene-environmental and multi-genetic causes of MS, these susceptibility variants mainly have weak effects and are likely to contribute to a small increase in MS risk individually. It is commonly agreed that testing single susceptibility genes is not useful for prediction of MS risk, but the question remains whether combining susceptibility loci in risk models could have an added value on MS prediction in individuals. The predictive performance of genetic risk models has been investigated for other diseases in simulation studies.[10, 11] These studies suggest that the predictive value improves by combining multiple common low-risk loci.

We investigated the extent to which MS risk can be predicted using genetic risk models. First of all we tested in our empirical data the predictive performance of 6 combined genotyped SNPs, using risk scores compared to a prior chance of someone in our population having MS. However whether genetic risk models will potentially be used in clinical or public health practices depends on the accuracy of the test to discriminate between individuals who will develop MS and who will not. The discriminative accuracy is generally expressed as the area under the receiver operating characteristic curve (AUC). Therefore, secondly we tested the potential performance of SNP genotyping in a simulation study by adding risk genes into the model. For this, we constructed a risk model based on 1) the 6 genotyped SNPs, 2) the 24 recently well replicated genome-wide associated polymorphisms[9] and 3) the 53 replicated genome-wide associated polymorphisms including the 29 newly identified polymorphisms.[9] Finally, we included hypothetical variants in the risk model, in order to investigate the future potential.

Methods

Empirical study

Ethics Statement

This study was approved by the Ethics Committee of the Erasmus University Medical Centre, METC Erasmus MC Rotterdam. All participants were recruited in Erasmus University Medical Centre and written informed consent was obtained.

Study population

A total of 591 MS patients and 600 controls were included in this study. The MS patients were recruited and ascertained as part of an ongoing nationwide study on genetic susceptibility in MS and fulfilled McDonald criteria for MS.[12] Details on ascertainment are given elsewhere.[13]

Genotyping

The *HLA-DRB* rs3135388, *EVI5* rs10735781, *CLEC16A* rs64981169, *CD58* rs12044852, *IL7R* rs6897932, and *IL2RA* rs2104286 SNPs were genotyped using the MassARRAY system/ Homogeneous MassExtend assay, following the protocol provided by Sequenom. PCR extension primers were designed using the Assay Design 3.0 program (Sequenom). ThermoSequenase (Sequenom) was used for the base extension reactions. Analysis and scoring were performed using the program Typer 3.3 (Sequenom).

Risk score analysis

All statistical analyses on empirical data were performed using SPSS version 15. Associations of individual SNPs were investigated using logistic regression. We also applied logistic regression analyses to investigate the combined predictive value of the risk allele score based on all SNPs with and without *HLA-DRB* (rs3135388) using the *a priori* probability of an individual in our population developing MS as reference. As we tested a total of 6 SNPs in our empirical study, the Bonferoni-corrected p-value for significance was 0.008. The weighted risk allele score was calculated by multiplying the number of risk alleles with the effect size for each SNP obtained from the literature and summing this up for each participant with complete genotype information, with risk alleles being the alleles associated with increased risk of MS. All analyses were adjusted for age and sex.

Simulation study

Modelling strategy

We used a modelling procedure that has been developed and published previously,[14] and which has also been used by others.[15] Briefly, the procedure creates a dataset with information on genotypes and disease status for a population of 100,000 individuals. The dataset is constructed in such a way that the odds ratios and frequencies of the genotypes and the disease risk match the specified values, which are obtained from the literature. Predicted MS risks are calculated using Bayes' theorem, which states that the posterior odds of MS for each individual is obtained by multiplying the prior odds by the likelihood ratio (LR) of their genotype status on all polymorphisms. The prior odds is calculated from the baseline population MS risk (p) using the formula p/ (1-p). Under the assumption of independent genetic effects i.e., no linkage disequilibrium between the genetic variants, the LR is obtained by multiplying the

LRs of all individual genotypes that are included in the risk model.[16] The LRs of the genotypes of each single genetic variant are calculated from a genotype by disease status contingency table.[14] This table is constructed from the frequency and ORs of the genotypes and the population MS risk. The table can also be constructed from allele frequencies and per allele ORs when Hardy-Weinberg Equilibrium is assumed for the distribution of the genotypes. The frequencies and ORs all are specified as study parameters and varied between the simulation scenarios. The posterior odds are converted into MS risks using the formula odds/ (1+odds).

Discriminative accuracy

The discriminative accuracy is the extent to which the test results can discriminate between individuals who will develop MS and those who will not.[17] The AUC gives an assessment of the discriminative accuracy of a prediction model and ranges from 0.5 (equal to tossing a coin) to 1.0 (perfect prediction). All simulations were repeated 100 times to obtain robust estimates of the AUC. All results are presented as averages of the repeated simulations. The obtained confidence intervals were extremely small, often equal to the point estimate, and therefore not presented in this paper. Analyses were performed using R software (version 2.12.1).[18]

Simulation scenarios

Recently, a large GWA study was presented as part of the collaboration between Wellcome Trust Case Control Consortium 2 (WTCCC2) and the International Multiple Sclerosis Genetics Consortium (IMSGC).[9] Twenty-three MS associated non-major histocompability complex (MHC) loci were replicated in the primary GWAS involving 9,772 cases and 7,296 controls with $P_{GWAS} < 1*10^{-3}$. Table 2 provides the 23 replicated non-MHC SNPs with the combined ORs and p-value. The risk allele frequency represents the allele frequency in control population of UK, as being the largest sample. Table 2 also includes the *HLA-DRB1*15:01* MHC SNP, which have been shown to significantly increase the risk for MS. These 24 risk SNPs also include the 6 polymorphisms of our empirical data. The collaboration also presented the identification of 29 novel susceptibility loci as shown in table 3. This leads to a total of 53 risk SNPs.

Three different simulation scenarios were considered. In each scenario genotypes and MS status were simulated for 100,000 individuals, assuming a lifetime MS risk of 0.1%. The first scenario calculated the AUC within the empirical data weighted on literature frequency. The second scenario assessed the increase in AUC by adding additional risk alleles, starting with the 6 genotyped risk loci given the replicated ORs. We compared this to the calculated AUC for validation of the simulation model. Next, the AUC was calculated with the 24 replicated SNPs in the recent Nature paper including the 6 genotyped SNPs. And finally, the AUC was assessed on a risk model including the 29 novel susceptibility loci on top of the replicated SNPs, leading to a total of 53 SNPs. The third scenario investigated the magnitude of the allele ORs of 1 to

100 polymorphisms that need to be added to the risk model to increase the discriminative accuracy. Since there are no models known in the literature for predicting MS risk we pursued AUCs known to be used for other diseases in the literature.[19, 20] We investigated AUC thresholds of 0.70, 0.75, 0.80 and 0.85. The ORs were obtained for different frequencies of the risk alleles.

Results

Empirical study

A total of 588 cases and 599 controls were successfully genotyped for at least one polymorphism, while complete genotype information on all polymorphisms was available for 564 cases and 581 controls. The mean age (SD) within the cases and controls was 45 (12) and 49 (17) years, respectively. The cases included 71% female and the controls 55%. None of the polymorphisms deviated significantly from Hardy Weinberg Equilibrium (lowest Hardy Weinberg p-value = 0.15 for *IL2RA*: rs2104286).

Table 1 shows the individual effects of each SNP on MS risk in our genotyped population. Increased risk for MS was confirmed for the minor alleles of *EVI5*, *HLA-DRB* and *CLEC16A*, and for the major alleles of *CD58* and *IL7R*. For *IL2RA* the association was not statistically significant (OR 1.14, 95% CI 0.95-1.38). When adjusting for multiple testing only *HLA-DRB*, *CLEC16A* and *CD58* remained statistically significant.

Table 1: Individual association of 6 genotyped SNPs in the empirical study.

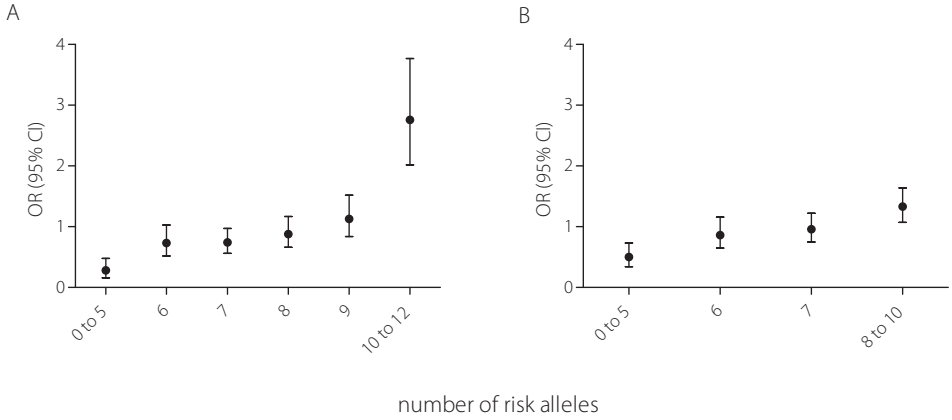
			Controls		*Cases*			
Gene	Variant	Risk Allele	n genotyped	RAF	n genotyped	RAF	OR (95% CI)	p-value
HLA-DRB	rs3135388	T	599	0.14	588	0.28	2.53 (2.02-3.17)	$8.14*10^{-16}$
EVI5	rs10735781	G	597	0.33	586	0.38	1.19 (1.01-1.42)	0.044
CLEC16A	rs64981169	G	593	0.33	583	0.39	1.27 (1.07-1.51)	0.006
CD58	rs12044852	C	599	0.88	587	0.91	1.50 (1.14-1.97)	0.004
IL7R	rs6897932	C	599	0.72	588	0.76	1.21 (1.00-1.46)	0.045
IL2RA	rs2104286	A	595	0.73	581	0.76	1.14 (0.95-1.38)	0.157

RAF: risk allele frequency, OR: odds ratio, 95% CI: 95% confidence interval.

Figure 1A shows the risk score when including all SNPs into the model. The reference category is based on the *a priori* risk for developing MS, which in our population was 49% (= 564 cases divided by 581 controls). Individuals with 0 to 5 risk alleles have a significantly decreased risk for developing MS of 0.28 (95% CI 0.16-0.48) compared to the *a priori* risk for developing MS. On the other end of the spectrum, individuals with 10 to 12 risk alleles have a significantly increased risk of 2.76 (95% CI 2.02-3.77). Figure 1B shows that, when excluding the variant

with the strongest risk effect (*HLA-DRB*) from the risk score, individuals with 0 to 5 risk alleles have a decreased risk of 0.50 (95% CI 0.34-0.73) and individuals with 8 to 10 risk alleles have an increased risk of 1.33 (95% CI 1.07-1.64) in comparison with the a priori risk for developing MS.

Figure 1: Weighted Risk scores for the genotyped SNPs.



The odds ratios for MS are shown according to the number of risk alleles carried. The reference value is based on the a priori probability of someone in the general population to carry MS risk alleles. A) Weighted risk scores for the 6 genotyped SNPs including *HLA-DRB*. B) Weighted risk scores for the 5 genotyped SNPs.

Simulation study

Table 2 provides the 24 replicated SNPs from the recent Nature paper,[9] which have been shown to significantly increase the risk for MS. These 24 risk SNPs include also the 6 polymorphisms of our empirical data. Table 3 shows the 29 newly identified polymorphisms in this Nature paper, leading to a total of 53 risk SNPs.

Table 2: Summary of the 24 replicated multiple sclerosis associated risk loci.

Gene	*Variant*	*Chromosome*	*Risk allele*	*RAF*	*OR* (95% CI)	*P-value*
MMEL1	rs4648356	1	C	0.67	1.14 (1.12-1.16)	$1.00*10^{-14}$
EVI5	rs11810217	1	A	0.25	1.15 (1.13-1.16)	$5.80*10^{-15}$
CD58	rs1335532	1	A	0.87	1.22 (1.19-1.24)	$3.20*10^{-16}$
RGS1	rs1323292	1	A	0.83	1.12 (1.10-1.14)	$2.30*10^{-8}$
KIF21B	rs7522462	1	G	0.70	1.11 (1.10-1.13)	$1.90*10^{-9}$
CBLB	rs2028597	3	G	0.91	1.13 (1.06-1.21)	$2.10*10^{-4}$
TMEM39A	rs2293370	3	G	0.80	1.13 (1.11-1.15)	$2.70*10^{-9}$
IL12A	rs2243123	3	G	0.29	1.08 (1.06-1.10)	$7.20*10^{-6}$
IL7R	rs6897932	5	G	0.73	1.11 (1.09-1.13)	$1.70*10^{-8}$
PTGER4	rs4613763	5	G	0.13	1.20 (1.18-1.22)	$2.50*10^{-16}$
HLA-DRB	rs3135388	6	A	0.13	3.08 (not shown)	$<1.0*10^{-320}$
OLIG3	rs13192841	6	A	0.27	1.10 (1.09-1.12)	$1.30*10^{-8}$
IL7	rs1520333	8	G	0.25	1.10 (1.08-1.11)	$1.60*10^{-7}$
IL2RA	rs3118470	10	G	0.32	1.12 (1.10-1.13)	$3.20*10^{-11}$
ZMIZ1	rs1250550	10	A	0.35	1.10 (1.09-1.12)	$6.30*10^{-9}$
CD6	rs650258	11	G	0.63	1.12 (1.10-1.13)	$2.00*10^{-11}$
TNFRSF1A	rs1800693	12	G	0.40	1.12 (1.11-1.14)	$4.10*10^{-14}$
CYP27B1	rs12368653	12	A	0.47	1.10 (1.09-1.12)	$1.70*10^{-9}$
MPHOSPH9	rs949143	12	G	0.28	1.08 (1.04-1.12)	$1.50*10^{-4}$
CLEC16A	rs7200786	16	A	0.46	1.15 (1.13-1.16)	$8.50*10^{-17}$
IRF8	rs13333054	16	A	0.23	1.11 (1.10-1.13)	$1.30*10^{-8}$
STAT3	rs9891119	17	C	0.36	1.11 (1.09-1.12)	$1.80*10^{-10}$
TYK2	rs8112449	19	G	0.67	1.08 (1.07-1.10)	$1.20*10^{-6}$
CD40	rs2425752	20	A	0.25	1.11 (1.10-1.13)	$5.10*10^{-10}$

RAF: risk allele frequency, OR: odds ratio, 95% CI: 95% confidence interval.
OR and p-value represent the combined discovery and replication study results.[9] Risk allele frequency refers to allele frequency in control population of UK samples. For CBLB is the discovery OR and p-value given.

Table 3: The 29 novel associated MS risk genes.

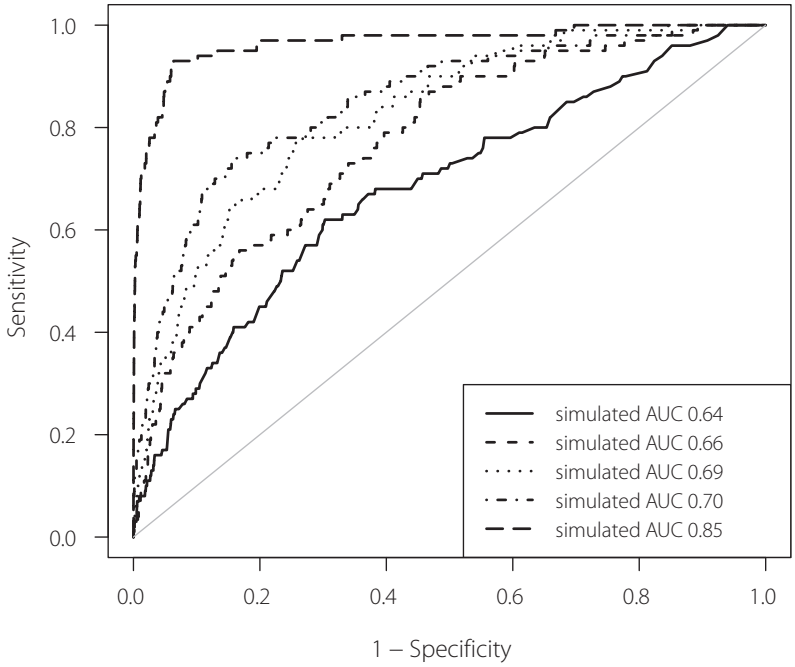
Gene	*Variant*	*Chromosome*	*Risk allele*	*RAF*	OR OR (95% CI)	*P-value*
VCAM1	rs11581062	1	G	0.29	1.12 (1.10-1.13)	$2.50*10^{-10}$
No gene	rs12466022	2	C	0.73	1.11 (1.10-1.13)	$6.20*10^{-10}$
PLEK	rs7595037	2	A	0.55	1.11 (1.10-1.12)	$5.10*10^{-11}$
MERTK	rs17174870	2	G	0.75	1.11 (1.09-1.13)	$1.30*10^{-8}$
SP140	rs10201872	2	A	0.18	1.14 (1.12-1.16)	$1.80*10^{-10}$
No gene	rs669607	3	C	0.48	1.13 (1.12-1.15)	$1.90*10^{-15}$
EOMES	rs11129295	3	A	0.36	1.11 (1.09-1.12)	$1.20*10^{-9}$
CD86	rs9282641	3	G	0.91	1.21 (1.18-1.24)	$1.00*10^{-11}$
IL12B	rs2546890	5	A	0.52	1.11 (1.10-1.13)	$1.20*10^{-11}$
BACH2	rs12212193	6	G	0.47	1.09 (1.08-1.10)	$3.80*10^{-8}$
THEMIS	rs802734	6	A	0.69	1.10 (1.09-1.12)	$5.50*10^{-9}$
MYB	rs11154801	6	A	0.36	1.13 (1.11-1.15)	$1.00*10^{-13}$
IL22RA2	rs17066096	6	G	0.24	1.14 (1.12-1.15)	$6.00*10^{-13}$
TAGAP	rs1738074	6	G	0.57	1.13 (1.12-1.15)	$6.80*10^{-15}$
ZNF746	rs354033	7	G	0.74	1.11 (1.10-1.13)	$4.70*10^{-9}$
MYC	rs4410871	8	G	0.72	1.11 (1.09-1.12)	$7.70*10^{-9}$
PVT1	rs2019960	8	G	0.23	1.12 (1.10-1.13)	$5.20*10^{-9}$
HHEX	rs7923837	10	G	0.62	1.10 (1.08-1.11)	$4.90*10^{-9}$
CLECL1	rs10466829	12	A	0.50	1.09 (1.08-1.11)	$1.40*10^{-8}$
ZFP36L1	rs4902647	14	G	0.53	1.11 (1.10-1.13)	$9.30*10^{-12}$
BATF	rs2300603	14	A	0.74	1.11 (1.09-1.12)	$2.00*10^{-8}$
GALC	rs2119704	14	C	0.92	1.22 (1.19-1.25)	$2.20*10^{-10}$
MALT1	rs7238078	18	A	0.77	1.12 (1.10-1.14)	$2.50*10^{-9}$
TNFSF14	rs1077667	19	G	0.79	1.16 (1.14-1.18)	$9.40*10^{-14}$
MPV17L2	rs874628	19	A	0.72	1.11 (1.09-1.12)	$1.30*10^{-8}$
DKKL1	rs2303759	19	C	0.25	1.11 (1.09-1.13)	$5.20*10^{-9}$
CYP24A1	rs2248359	20	G	0.61	1.12 (1.10-1.13)	$2.50*10^{-11}$
MAPK1	rs2283792	22	C	0.52	1.10 (1.08-1.11)	$4.70*10^{-9}$
ODF3B	rs140522	22	A	0.33	1.10 (1.09-1.12)	$1.70*10^{-8}$

RAF: risk allele frequency, OR: odds ratio, 95% CI: 95% confidence interval.
OR and p-value represent the combined discovery and replication study results.[9] Risk allele frequency refers to allele frequency in control population of UK samples.

First, we calculated that the AUC for the genotyped 6 SNPs within the empirical data weighted on literature frequency was 0.64. Second, in the simulation study we assessed the AUC increase

by including additional risk alleles. The AUC for the recently replicated ORs of the 6 SNP's used in the empirical study was 0.64. This showed to be the same as the calculated AUC from the empirical study. Next, including the 24 known polymorphisms in the model the AUC rised to 0.66, and slightly increased to 0.69 after including all 53 SNPs in the model (figure 2).

Figure 2: ROC curves for simulation models predicting MS. Four situations are depicted.



Solid line (—) represents ROC curve for simulation model based on 6 genotyped SNPs (AUC 0.64). Dashed line (----) ROC curve for simulation model based on 24 well replicated SNPs (AUC 0.66). Dotted line (·····) ROC curve for simulation model based on a total of 53 replicated and novel SNPs (AUC 0.69). Dash-dotted line (-·-·) ROC curve for simulation model based on 20 extra variants with an arbitrarily set allele frequency of 0.30 and OR 1.1 (AUC 0.70). Long- dashed line (— - —) ROC curve for simulation model based on 50 extra variants with an arbitrarily set allele frequency of 0.30 and OR 1.4 (AUC 0.85).

Finally, we explored the possibilities in the future with new risk alleles to be discovered. Table 4 shows the number of new risk genes with specific allele frequencies in combination with different ORs that would be needed in addition to the original 53 risk variants to obtain AUCs of 0.70, 0.75, 0.80 and 0.85. For example to increase the AUC just slightly to 0.70 we have to add to our model 20 new variants, with a realistic OR of 1.1 and an allele frequency of 0.30. However if we want to increase the AUC to 0.85 we have to add 50 new variants with an OR of 1.4 and an allele frequency of 0.30. For more realistic ORs this would mean we would have to add even more polymorphisms to the model.

Table 4: Odds ratios and related allele frequencies needed to obtain AUCs of 0.70-0.85 in addition to the 53 statistically significant genetic susceptibility variants (AUC=0.69).

Risk allele Frequency	Number of extra genetic variants	AUC 0.70	AUC 0.75	AUC 0.80	AUC 0.85
0.05	1	1.2	2.3	5.1	9.0
	5	1.2	1.7	2.6	3.6
	20	1.1	1.4	1.7	2.1
	50	1.1	1.2	1.4	1.6
	100	1.1	1.2	1.3	1.4
0.30	1	1.2	1.9	3.0	4.9
	5	1.2	1.4	1.8	2.2
	20	1.1	1.3	1.4	1.6
	50	1.1	1.2	1.3	1.4
	100	1.1	1.2	1.3	1.4
0.50	1	1.2	1.8	3.2	5.3
	5	1.2	1.4	1.8	2.1
	20	1.1	1.3	1.4	1.5
	50	1.1	1.2	1.3	1.4
	100	1.1	1.2	1.3	1.4

NOTE: odds ratios are presented as mean of 20 simulations each.
AUC: area under the receiver operating characteristic curve.

Discussion

This study investigated the extent of MS prediction by genetic risk models, using empirical and simulation data on the most updated genetic information for MS. First, we showed that the predictive performance of testing multiple genes can be enhanced by using a combination of individual MS risk alleles. As expected, *HLA-DR* influences the ability to predict MS considerably due to its high OR. However, even without *HLA-DR* there was an increased, but small, risk for developing MS in people with 8 to 10 risk alleles. This underlines the current insight that multiple genes exert a small effect on developing MS on top of the major influence of *HLA-DR*.[21, 22]

Next, after validating the genetic risk models with simulated genotype and MS status in a population of 100,000 individuals, we estimated that the predictive value as reflected in AUCs would be 0.66 when all 24 well replicated GWA derived polymorphisms were considered.

Moreover, we showed that including the 29 novel risk genes increased the AUC only slightly to 0.69, illustrating that even more than doubling the number of risk SNPs does not increase the AUC sufficiently to make it useful in clinical practice. The AUC of 0.69 is comparable to other risk prediction models in MS.[23-25] In 2009, De Jager and colleagues investigated the prediction of 16 MS susceptibility loci using weighted genetic risk scores in three cohorts.[23] They demonstrated a consistent discriminatory ability in three independent samples (AUC varying 0.64-0.70). Gourraud and colleagues also investigated the aggregation of genetic MS risk markers in individuals by comparing multiple and single case families.[24] They showed that a greater genetic burden in siblings of MS patients was associated with an increased MS risk (OR 2.1, p=0.001). However, the AUC for genetic burden differences between probands and siblings was only 0.57, indicating that the available genetic data is not sufficient to achieve case-control prediction of MS. They also used 16 MS susceptibility loci, partly matching with those of De Jager et al.

Before interpreting the clinical relevance of our findings, a methodological issue needs to be disclosed. We assumed that genetic variants inherited independently and that the combined effect of the genetic variants on disease risk followed a multiplicative risk model of independent effects (i.e., no statistical interaction terms were included in the model). Although so far no studies have demonstrated gene-gene interactions with MS risk, it is still possible that these will be discovered in future studies in larger populations. However, gene-gene interactions only improve the MS risk predictions if their effect sizes are substantially high (e.g., OR>5). When interaction effects are smaller, their effects on the predictive accuracy will be comparable with that of single gene effects, because by definition their frequencies are lower.

With the current model including 53 variants, we are still not able to differentiate with reasonable accuracy between individuals who will develop MS and those who will not (AUC 0.69). This makes our model not clinically useful. So the question is raised how to improve MS prediction.

We demonstrated in the simulation study that in order to obtain higher AUCs, a considerable number of additional common genetic variants or stronger associated variants with high ORs (table 4) need to be identified. The per-allele OR of the polymorphisms identified in GWA studies ranges from 1.08 to 2.1. When future GWA studies will identify polymorphisms with per-allele ORs around 1.1, the predictive ability of the genetic risk model can theoretically be improved beyond that of the existing models. Yet, even small improvements to 0.70 still require the discovery of 20 new statistically significant variants. Despite the increase, it is still not clinically applicable. Because even in a disease that is readily treatable and even preventable like coronary heart disease (as presented in the Framingham Risk score) an AUC of about 0.80 is used.[26] For MS there is still no cure or preventive treatment available, and so a higher

predictive accuracy is desirable to prevent false positives. We have shown that to pursue an AUC of 0.85, we have to include 50 new variants with ORs of 1.4 or a few common variants (minor allele frequency >30%) with high ORs (table 4). This may prove to be difficult, because the common genetic variants with high ORs may already have been identified, which would imply that even higher numbers of common genetic variants with relatively smaller ORs or many exceedingly rare variants (minor allele frequency <1%) with high ORs, will be needed. This seems not feasible. To note, unlike *HLA-DR* most of the genetic risk factors identified so far have only a slight effect on susceptibility to MS (with ORs that range from 1.1 to 1.2).[23] However, more high risk genetic MS risk variants can be expected in near future.[27] With novel techniques such as next generation sequencing we can expect new rare variants with high ORs to be discovered.[28] This approach has already been proven successful in rare Mendelian disorders and can potentially also identify rare variants explaining the high recurrence rate of MS within families.[29] Also, this technique potentially allows us to find the causal variants for MS which will most likely have higher ORs than those found in GWA studies.

Another approach to improve MS prediction could be combining genetic with nongenetic risk factors such as infection with Epstein-Barr virus, smoking, and serum vitamin D concentrations.[30] It is likely that risk prediction models combined with nongenetic factors will perform better, as ORs for SNPs tend to be smaller than ORs based on nongenetic factors (e.g. infectious mononucleosis[31]). De Jager and colleagues showed an enhanced discriminatory ability of 16 susceptibility genes by the inclusion of sex (AUC increasing from 0.70 to 0.74) and smoking and immune response to EBV (AUC increasing from 0.64 to 0.68). Others have performed studies combining the effects of *HLA-DR* and non-genetic factors like smoking and anti EBV serum levels.[32, 33] Also, integration of transcriptional, proteomics, and clinical factors will probably improve the prediction model and with that our understanding of MS genetics.[34] However, the added value of the SNPs might then be questioned. For other diseases it has been shown that the AUC does not improve a lot when adding SNPs to clinical risk factors. It should be noted though, that in these studies only small numbers of SNPs were added to the clinical risk factors.

Even if we can improve the prediction of MS in the future the question remains what the clinical implications of such predictive risk models would be. The discriminative accuracy that is required in preventive or clinical care depends on the goal of testing, the availability of (preventive) treatment, and the adverse effects of false-positive and false-negative test results. Although the early results from GWA studies have not yet been used clinically, at least a partial goal of understanding the genetic basis of MS is to investigate the use of these variants to predict disease risk, so that environmental changes or therapeutic interventions can be initiated before the inflammatory demyelinating process progresses or even starts.

Also, by better mapping the genetic of MS, we hope to improve our understanding of the pathofysiology of MS. This could help us finding better and new therapeutic drugs. By combining family history with a quantitative measure of genetic risk, a screening method might eventually be implemented that could identify clinically silent evidence of disease among first-degree relatives of MS patients, who have 20-50 times higher risk of developing MS.[35] However, the absolute risk is only 2-5% and therefore the models could be more useful in high risk populations with individuals who have had clinically isolated syndrome suggesting MS. These patients present with a neurological disability during their productive years of life and face the possibility of a chronic disease. Thus, they yearn for more clarity about their future. But also improving the risk prediction would enable us to distinguish individuals at risk to start early treatment for reducing the accumulation of neurological disability.[36]

Given the possible clinical consequences of false-positivity within these patients, the required prediction AUCs for the pre-symptomatic diagnosis is considerably higher than an AUC intended for clinically isolated syndrome. It has been suggested that identified genetic variants have stronger effects in multiplex families.[37] It is of note that the ORs assessed up to now in GWA studies and validation studies are generally derived from datasets on sporadic cases. In a multiplex family setting, with potential stronger effects for individual risk variants, our estimates may prove to be conservative.

In conclusion, our analyses show that prediction of MS risk based on low susceptibility variants theoretically can improve prediction of disease when more variants are being discovered. However, the discriminatory ability in a clinical setting will be limited.

Acknowledgment

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References

1. Sadovnick AD, Baird PA, Ward RH. Multiple sclerosis: updated risks for relatives. Am J Med Genet 1988;29:533-541.
2. Ebers GC. Environmental factors and multiple sclerosis. Lancet Neurol 2008;7:268-277.
3. Hafler DA, Compston A, Sawcer S, et al. Risk alleles for multiple sclerosis identified by a genomewide study. N Engl J Med 2007;357:851-862.
4. Hoppenbrouwers IA, Aulchenko YS, Janssens AC, et al. Replication of CD58 and CLEC16A as genome-wide significant risk genes for multiple sclerosis. J Hum Genet 2009;54:676-680.
5. Hoppenbrouwers IA, Aulchenko YS, Ebers GC, et al. EVI5 is a risk gene for multiple sclerosis. Genes Immun 2008;9:334-337.
6. De Jager PL, Jia X, Wang J, et al. Meta-analysis of genome scans and replication identify CD6, IRF8 and TNFRSF1A as new multiple sclerosis susceptibility loci. Nat Genet 2009;41:776-782.
7. Australia and New Zealand Multiple Sclerosis Genetics Consortium. Genome-wide association study identifies new multiple sclerosis susceptibility loci on chromosomes 12 and 20. Nat Genet 2009;41:824-828.
8. Hafler JP, Maier LM, Cooper JD, et al. CD226 Gly307Ser association with multiple autoimmune diseases. Genes Immun 2009;10:5-10.
9. The International Multiple Sclerosis Genetics Consortium and The Wellcome Trust Case Control Consortium. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. Nature 2011;476:214-219.
10. Janssens AC, Moonesinghe R, Yang Q, Steyerberg EW, van Duijn CM, Khoury MJ. The impact of genotype frequencies on the clinical validity of genomic profiling for predicting common chronic diseases. Genet Med 2007;9:528-535.
11. Van Zitteren M, van der Net JB, Kundu S, Freedman AN, van Duijn CM, Janssens AC. Genome-based prediction of breast cancer risk in the general population: a modeling study based on meta-analyses of genetic associations. Cancer Epidemiol Biomarkers Prev 2011;20:9-22.
12. Polman CH, Reingold SC, Edan G, et al. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". Ann Neurol 2005;58:840-846.
13. Aulchenko YS, Hoppenbrouwers IA, Ramagopalan SV, et al. Genetic variation in the KIF1B locus influences susceptibility to multiple sclerosis. Nat Genet 2008;40:1402-1403.
14. Janssens AC, Aulchenko YS, Elefante S, Borsboom GJ, Steyerberg EW, van Duijn CM. Predictive testing for complex diseases using multiple genes: fact or fiction? Genet Med 2006;8:395-400.
15. Pepe MS, Gu JW, Morris DE. The potential of genes and other markers to inform about risk. Cancer Epidemiol Biomarkers Prev 2010;19:655-665.
16. Sackett DL HR, Tugwell P. Clinical epidemiology: a basic science for clinical medicine. Boston/Toronto: Little, Brown and Company 1985.
17. Hanley JA, McNeil BJ. The meaning and use of the area under a receiver operating characteristic (ROC) curve. Radiology 1982;143:29-36.
18. Ihaka R GR. R: A language for data analysis and graphics. J Comput Graph Stat 1996;5:299-314.
19. Maller J, George S, Purcell S, et al. Common variation in three genes, including a noncoding variant in CFH, strongly influences risk of age-related macular degeneration. Nat Genet 2006;38:1055-1059.
20. Van Hoek M, Dehghan A, Witteman JC, et al. Predicting type 2 diabetes based on polymorphisms from genome-wide association studies: a population-based study. Diabetes 2008;57:3122-3128.
21. Oksenberg JR, Barcellos LF, Cree BA, et al. Mapping multiple sclerosis susceptibility to the HLA-DR locus in African Americans. Am J Hum Genet 2004;74:160-167.

22. Lincoln MR, Montpetit A, Cader MZ, et al. A predominant role for the HLA class II region in the association of the MHC region with multiple sclerosis. Nat Genet 2005;37:1108-1112.
23. De Jager PL, Chibnik LB, Cui J, et al. Integration of genetic risk factors into a clinical algorithm for multiple sclerosis susceptibility: a weighted genetic risk score. Lancet Neurol 2009;8:1111-1119.
24. Gourraud PA, McElroy JP, Caillier SJ, et al. Aggregation of multiple sclerosis genetic risk variants in multiple and single case families. Ann Neurol 2011;69:65-74.
25. Sawcer S, Ban M, Wason J, Dudbridge F. What role for genetics in the prediction of multiple sclerosis? Ann Neurol 2010;67:3-10.
26. Wilson PW, D'Agostino RB, Levy D, Belanger AM, Silbershatz H, Kannel WB. Prediction of coronary heart disease using risk factor categories. Circulation 1998;97:1837-1847.
27. Holm H, Gudbjartsson DF, Sulem P, et al. A rare variant in MYH6 is associated with high risk of sick sinus syndrome. Nat Genet 2011;43:316-320.
28. Zeggini E. Next-generation association studies for complex traits. Nat Genet 2011;43:287-288.
29. Ng SB, Buckingham KJ, Lee C, et al. Exome sequencing identifies the cause of a mendelian disorder. Nat Genet 2010;42:30-35.
30. Ascherio A, Munger K. Epidemiology of multiple sclerosis: from risk factors to prevention. Seminars in neurology 2008;28:17-28.
31. Ramagopalan SV, Valdar W, Dyment DA, et al. Association of infectious mononucleosis with multiple sclerosis. A population-based study. Neuroepidemiology 2009;32:257-262.
32. Simon KC, van der Mei IA, Munger KL, et al. Combined effects of smoking, anti-EBNA antibodies, and HLA-DRB1*1501 on multiple sclerosis risk. Neurology 2010;74:1365-1371.
33. Sundström P, Nyström L, Jidell E, Hallmans G. EBNA-1 reactivity and HLA DRB1*1501 as statistically independent risk factors for multiple sclerosis: a case-control study. Mult Scler 2008;14:1120-1122.
34. Oksenberg JR, Baranzini SE, Sawcer S, Hauser SL. The genetics of multiple sclerosis: SNPs to pathways to pathogenesis. Nat Rev Genet 2008;9:516-526.
35. Compston A, Coles A. Multiple sclerosis. Lancet 2008;372:1502-1517.
36. Kieseier BC, Wiendl H, Leussink VI, Stuve O. Immunomodulatory treatment strategies in multiple sclerosis. J Neurol 2008;255:15-21.
37. D'Netto MJ, Ward H, Morrison KM, et al. Risk alleles for multiple sclerosis in multiplex families. Neurology 2009;72:1984-1988.

Chapter 4

No influence of KIF1B on neurodegenerative markers in multiple sclerosis

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Introduction

In search of genetic causes of multiple sclerosis (MS), a number of genes have consistently shown association with MS susceptibility in the past couple of years.[1] All of these identified genes are directly or indirectly involved with the inflammatory process. However, it has become increasingly clear that MS consists of both an inflammatory and a progressive neurodegenerative process,[2] which is illustrated by the fact that new and potent anti-inflammatory drugs have been unable to halt neurodegeneration. The relation between episodes of inflammation and the neurodegenerative component characterized by irreversible axonal loss are far from clear at this point. Some authors have argued that neurodegeneration is independent of inflammation, while others argue that the 2 components are closely associated and are actually interdependent.[2,3] The neurodegenerative component is clinically highly relevant since it is held predominantly responsible for disability accumulation, although several questions regarding this issue remain.[2] Until recently, no genetic marker for neurodegeneration in MS was identified. However, in 2008, it was reported for the first time that a "neurodegenerative gene," i.e., the *KIF1B* rs10492972 [C] variant, was associated with MS susceptibility.[4] *KIF1B* is involved in axonal transport of mitochondria and synaptic vesicle precursors. Dysregulation of axonal transport plays a role in several neurodegenerative diseases.[4] The authors suggested that *KIF1B* could be the first gene involved in MS susceptibility with a possible neurodegenerative effect.[4] Unfortunately, this finding could later not be confirmed in other samples,[5] but perhaps this SNP explains some of the neurodegenerative phenotypic differences between patients with MS.

Methods

To assess the effect of this gene polymorphism on phenotype, the current study related genotype and carriership of the C allele of rs10492972 to neurodegenerative markers in 214 patients with MS. These patients with MS were selected from ongoing natural history studies in our MS center based on the availability of DNA and precise clinical characterization of the disease course and disease severity. First we assessed this in terms of clinical measures, using Multiple Sclerosis Severity Scores and Multiple Sclerosis Functional Composite Scores to assess disability; secondly, by use of MRI measures such as T1 hypointense lesion volume, T2 lesion volume, T1/T2 ratio, and atrophy measures (normalized brain volume and percent brain volume change), which were available for 164 of the 214 patients. The progression of both clinical and MRI measures was also analyzed at 2 years follow-up. Significance was tested using the Kruskal-Wallis test for genotype comparisons and Mann-Whitney U test for carriership comparisons ($p < 0.05$). Written informed consent was obtained from all participants, and the study was approved by the local ethics committee.

Results

In our group of patients with MS, 36.9% were male, 10.7% had a primary progressive disease course, 66.4% had a relapsing-remitting disease course, 19.2% had a secondary progressive disease course, and 3.7% had a clinically isolated syndrome course, and the mean disease duration of our total group was 12 years. Median Expanded Disability Status Scale score was 3.5. The C (risk) allele frequency in our cohort was 30.1%, which is comparable to the allele frequencies described by others.[4,5] The genotype distribution was in Hardy-Weinberg equilibrium. No association was found between carriership of the risk allele or genotype of rs10492972 and the described neurodegenerative markers, either on the clinical level or on MRI (for details, see table 1).

Table 1: Overview of clinical and paraclinical markers possibly related to neurodegeneration.[a]

	CC genotype (n=20)	CT genotype (n=89)	TT genotype (n=105)	Carriers of C risk allele	Noncarriers of C risk allele
Mean disease duration, y	11.9	12.8	11.4	12.7	11.38
Mean MSSS	3.83	4.45	4.87	4.33	4.87
MSFC					
Median TWT, s	4.25	4.95	4.70	4.70	4.70
Median 9-HPT (dominant hand), s	17.7	18.8	19.7	18.6	19.7
Median 9-HPT (non-dominant hand), s	19.5	20.0	20.7	20.0	20.7
Median PASAT (correct number)	56.5	54.5	53	55	53
Imaging parameters					
Median T1 lesion (mLx10-^{3}) (n=164)	592.6	583.6	388.5	568.6	388.5
Median T2 lesion (mLx10-^{3}) (n=162)	3370.9	2050.2	2539.2	2305.0	2539.2
Mean PBVC after 2 years, % (n=159)	-1.09	-0.90	-1.06	-0.91	-1.06

Abbreviations: 9-HPT = 9-Hole Peg Test; MSFC = Multiple Sclerosis Functional Composite; MSSS = Multiple Sclerosis Severity Score; PASAT = Paced Auditory Serial Addition Test; PBVC = percent brain volume change; TWT = timed walk test.

[a] Values are given per genotype and carriership of C-allele (risk-allele) of rs10492972. No significant differences were found.

Discussion

Based on this dataset, we conclude that no evidence could be found for a determining influence of carriership of the risk allele or genotype of the *KIF1B* gene on any of the neurodegenerative phenotypic markers. This finding should be confirmed in a larger cohort to more definitively exclude an association. Furthermore, it would be highly interesting to test the role of *KIF1B* in other diseases with neurodegenerative components. In *KIF1B* knockout mice, more atrophy was observed when compared to wild-type mice.[6] In humans, however, no effect of carriership

of the rs10492972 [C] variant was observed in susceptibility to and disability accumulation in patients with primary progressive MS,[7] nor in our study of a more general MS population. Although genetic susceptibility studies have consistently pointed toward the importance of inflammation in MS, the determining influence of genes on the neurodegenerative part of MS remains enigmatic. Different genetic markers within neurodegenerative pathways and their relationship to the MS phenotype should be investigated in future studies.

References

1. Hafler DA, Compston A, Sawcer S, et al. Risk alleles for multiple sclerosis identified by a genomewide study. N Engl J Med 2007;357:851– 862.
2. Frohman EM, Filippi M, Stüve O, et al. Characterizing the mechanisms of progression in multiple sclerosis: evidence and new hypotheses for future directions. Arch Neurol 2005;62:1345–1356.
3. Trapp BD, Nave KA. Multiple sclerosis: an immune or neurodegenerative disorder? Annu Rev Neurosci 2008;31: 247–269.
4. Aulchenko YS, Hoppenbrouwers IA, Ramagopalan SV, et al. Genetic variation in the KIF1B locus influences susceptibility to multiple sclerosis. Nat Genet 2008;40:1402–1403.
5. Booth DR, Heard RN, Stewart GJ, et al. Lack of support for association between the KIF1B rs10492972 [C] variant and multiple sclerosis. Nat Genet 2010;42:469 – 470.
6. Zhao C, Takita J, Tanaka Y, et al. Charcot-Marie-Tooth disease type 2A caused by mutation in a microtubule motor KIF1B beta. Cell 2001;105:587–597.
7. Martinelli-Boneschi F, Esposito F, Scalabrini D, et al. Lack of replication of KIF1B gene in an Italian primary progressive multiple sclerosis cohort. Eur J Neurol 2010;17:740 –745.

Part IV

Environmental risk factors in MS

Chapter 5

Epstein-Barr virus

Chapter 5.1

No evidence for intrathecal IgG synthesis to Epstein-Barr virus nuclear antigen-1 in multiple sclerosis

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Abstract

Background Recent studies suggest an intrathecal IgG response against Epstein-Barr virus (EBV) in multiple sclerosis (MS), implicating a pathogenic role for the virus in MS.

Objectives To determine the spectrum of anti-EBV antibodies and B-cell epitopes within EBV nuclear antigen-1 (EBNA-1). Furthermore, to determine whether EBNA-1-specific IgG is produced intrathecally.

Study design Immunoblot analysis was used to study the anti-EBV IgG response in serum and cerebral spinal fluid (CSF) in MS and controls. EBNA-1 B-cell epitopes were identified by immunoscreening of 12 residue long peptides, with 11 residue overlap, spanning EBNA-1. Thirteen peptides containing all immunoreactive regions were constructed and used in paired serum and CSF of MS patients (n = 17) and controls (n = 18). Subsequently, reactivity to the identified immunodominant peptide was analysed in a large cohort of serum and CSF of MS patients (n = 114) and disease controls (n = 62).

Results No difference was observed in the overall anti-EBV antibody diversity, but EBNA-1 reactivity was increased in MS patients versus controls for immunoblot and ELISA ($p<0.0001$). Epitope analysis on EBNA-1 revealed one immunodominant region covering residues 394-451: EBNA-$1^{394\text{-}451}$. Anti-EBNA-$1^{394\text{-}451}$ IgG levels in serum and CSF were significantly higher in MS patients compared to controls. However, normalization for total IgG content of paired serum and CSF samples abrogated this disease association.

Conclusions MS patients have normal overall anti-EBV antibody responses with increased reactivity to EBNA-$1^{394\text{-}451}$. No evidence was found for intrathecal EBNA-1-specific IgG synthesis in MS.

Background

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) resulting in demyelination and neurodegeneration. The disease develops in genetically predisposed individuals in response to environmental factors, most likely viral infections.[1] Although many viruses have been postulated to be implicated in MS pathology, including varicella zoster virus,[2] human herpesvirus 6[3] and measles virus,[4] none of these were irrefutably linked. However, recent studies advocate the role of Epstein-Barr virus (EBV) in MS.[5] EBV seroprevalence is higher in MS patients compared to controls (99% versus 90-95%) and MS is shown to have a clear and reproducible clinical relation with infectious mononucleosis.[6] Serum and intrathecal IgG levels to the latency-associated EBV nuclear antigen-1 (EBNA-1) are elevated before onset of MS and correlates with disease activity and prognosis.[5-10] Contrastingly, IgG to lytic EBV proteins including the viral capsid antigen (VCA) are not changed or only marginally increased, suggesting that EBV abnormalities in MS are associated with B-cell responses to latent EBV antigens.[5,8] Recently, Serafini et al. reported the presence of EBV-infected B-cells in meninges and perivascular regions of MS lesions.[11] However, this observation as well as the involvement of a local EBV-specific B- and T-cell response is still under debate.[5,12-14]

Objectives

The aim of our study was twofold. To substantiate the postulated EBV-MS association, we first identified the overall anti-EBV antibody reactivity and defined EBNA-1 B-cell epitopes recognized by serum and CSF IgG of MS patients and controls. Second, we determined whether EBNA-1-specific IgG is produced intrathecally.

Study design

Study population

The study group included 114 MS patients and 62 patients with non-inflammatory neurological diseases (NIND) recruited at the Erasmus Medical Centre (Rotterdam, Netherlands) and the diagnosis were controlled by an experienced neurologist (RQH) based on diagnostic McDonald criteria for MS.[15] As for the controls, the patient files and follow-up have been thoroughly checked to exclude possible MS in the NIND cohort. Serum and CSF samples were obtained for diagnostic purposes. For detailed EBNA-1 epitope analyses we obtained sera from 18 healthy EBV seropositive individuals from the USA (n = 7), The Netherlands (n = 6) and Hong Kong (n = 5). Sera from 4 EBV seronegative individuals were used for background measurements. Informed consent was obtained from all patients and the study was approved by the local ethical committee.

Routine serology

Overall IgG antibody responses to EBV proteins and defined EBNA and VCA markers were determined by standardized immunoblot and ELISA assays as described before.[16-18] Before analysis, all serum samples were diluted one-hunderd times and all CSF ten-times. Antibody responses to human cytomegalo virus (HCMV) antigens were determined by ELISA using a purified glycine-extracted antigen preparation.[19,20]

Epitope mapping of EBNA-1

To specify EBNA-1 B-cell epitopes from healthy EBV seropositive individuals, the IgG reactivity was determined to 12-mer synthetic peptides (n = 630), with 11 residue overlap, spanning the entire 641 residue long EBNA-1 sequence of the B95-8 EBV strain.[21] Peptide synthesis and immunoscreening were performed as described elsewhere from 18 EBV seropositive healthy individuals from globally distinct regions.[21,22] Mean OD_{450} values of four healthy EBV seronegative individuals was used to determine individual background value for each peptide.

EBNA-1 combipeptide reactivity

EBNA-1 peptide-specific IgG for the thirteen high affinity epitopes (table 1) were determined by ELISA and validated by western blotting using recombinant full-length EBNA-1 as described previously.[16-18] Sera from peptide-immunized and pre-immune rabbits were used as positive and negative controls, respectively. Monoclonal antibodies OT1x and 2B4, reacting with an alphahelical epitope located at residues 430-442 and a linear epitope at 446-451, respectively were used as positive controls.[23-24] Additionally, sera from healthy EBV-seropositive and seronegative donors were included in each ELISA assay for further standardization. Background levels of the serum ELISAs were determined using four EBV seronegative controls. Serum cut-off values (COV) were defined as the mean OD_{450} values plus 2-times standard deviation (SD) of these EBV seronegative controls. CSF ELISA COV was defined as the mean of all NIND CSF plus 2-times the SD. CSF ELISA OD_{450} values were standardized by dividing the mean of duplicate measurements for the clinical sample by the COV. Total IgG was determined with the PeliClass human IgG kit (Sanquin Reagents, Amsterdam, The Netherlands). EBNA-1-specific IgG was normalized for total IgG levels according to the following formula: (OD_{450} EBNA-1 IgG CSF/ OD_{450} EBNA-1 IgG serum)/ (IgG_{total} CSF/IgG_{total} serum). Results were statistically analysed with Mann-Whitney U and Spearman correlation test. P values <0.05 were considered statistically significant.

Table 1: Epstein-Barr nuclear antigen-1 (EBNA-1) synthetic peptides used in ELISA.

Antigen	aa Position	aa Sequence
EBNA-1	1-44	MSDEGPGTGPGNGLGEKGDTSGPEGSGGSGPQRRGGDNHGRGRG
Gly-Ala	147-168 174-195 268-289	AGAGGGAGGAGAGGGAGGAGG
Gly-Arg	348-369	GGSGGRRGRGRERARGGSRERA
EBNA-1	368-387	RERARGGSRERARGRGRGRG
EBNA-1	394-420	PPRRPPPGRRPFFHPVGEADYFEYHQE
EBNA-1	424-451	DGEPDVPPGAIEQGPADDPGEGPSTGPR
EBNA-1	436-461	QGPADDPGEGPSTGPRGQGDGGRRKK
EBNA-1	450-477	PRGQGDGGRRKKGGWFGKHRGQGGSNPK
EBNA-1	460-486	KKGGWFGKHRGQGGSNPKFENIAEGLR
EBNA-1	477-502	KFENIAEGLRALLARSHVERTTDEGT
EBNA-1	506-531	GVFVYGGSKTSLYNLRRGTALAIPQC
EBNA-1	459-607	RKKGGWFGKHRGQGGSNPKFENIAEGLRALLARSHVERTTDEGTWVAGVFVYGGSKT-SLYNLRRGTALAIPQCRLTPLSRLPFGMAPGPGPQPGPLRESIVCYFMVFLQTHIFAEVLK-DAIKDLVMTKPAPTCNIRVTVCSFDDGVDLP
EBNA-1	614-641	VEGAAAEGDDGDDGDEGGDGDEGEEGQE

EBNA-1; Epstein-Barr nuclear antigen-1; *aa:* amino acid; *Gly-Ala:* glycine-alanine repeat; *Gly-Arg:* glycine-arginine rich domain.

Results

Clinical characteristics

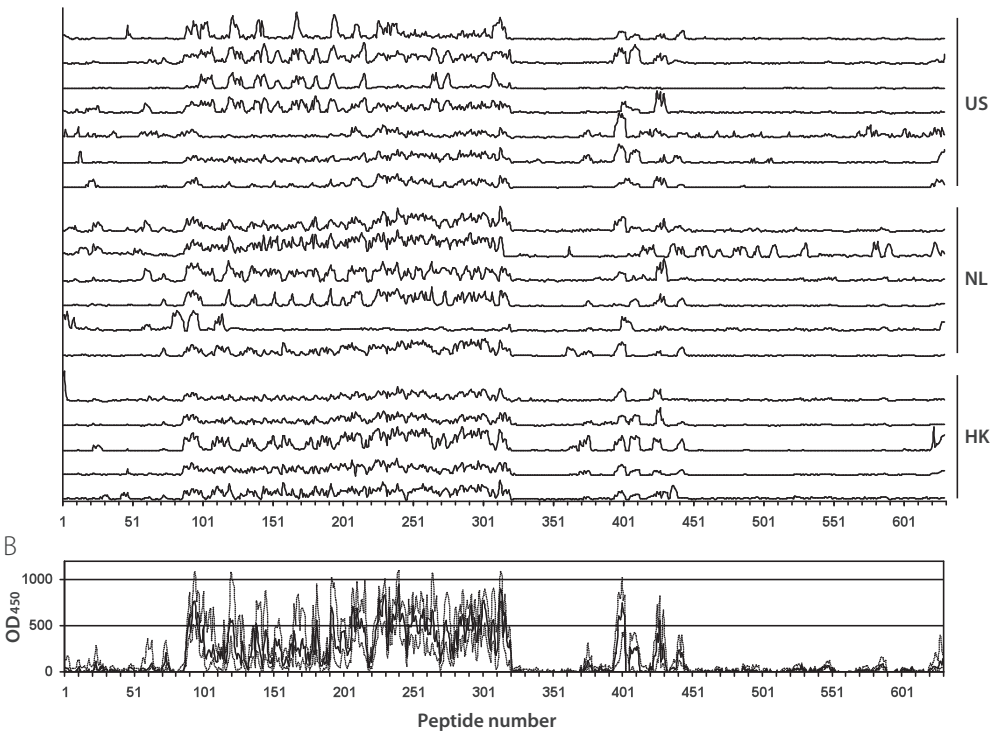
Patients analysed included 114 MS and 62 NIND cases. The mean (±SD) age of MS and NIND patients was 38 (±11.5) and 44 (±18.6) years, respectively. Among MS patients, clinical definite MS was confirmed in 61 of 114 (54%) and clinically isolated syndrome (CIS) in 53 of 114 patients (46%). The mean (±SD) age at MS onset was 34.2 (±10.1) years. Within definitive MS group, 35 and 26 patients had a relapsing remitting (RR-MS) and primary progressive (PPMS) course, respectively. The control group consisted of 62 NIND patients.

Similar repertoire of anti-EBV IgG response in serum and CSF of MS and NIND patients

Immunoblot analysis revealed a normal diversity pattern for anti-EBV IgG response in serum and CSF from both MS and NIND patients. Serum and CSF showed the characteristic limited diversity of anti-EBV IgG, involving antibodies directed to the recombinant antigens EBNA-1 (BKRF1), VCA-p18 (BFRF3), VCA-p40 (BDRF1) and the EBV transactivator protein Zebra (BZLF1), as observed for healthy EBV carriers (data not shown).[16,18,22] MS cases frequently showed

more intense EBNA-1 IgG reactivity compared to NIND (data not shown),[25,26] which led us to investigate the anti-EBNA-1 response in more detail.
Relevance of EBNA-1 for intrathecal anti-EBV response in MS patients was substantiated by testing all samples for VCA-p18 IgG using ELISA. Only serum, not CSF, showed significantly elevated VCA reactivity for MS compared to NIND. Parallel analysis of anti-HCMV IgG responses, revealed no differences between any of the study groups (data not shown).

Figure 1: OD_{450} values of EBV seropositive healthy individuals.



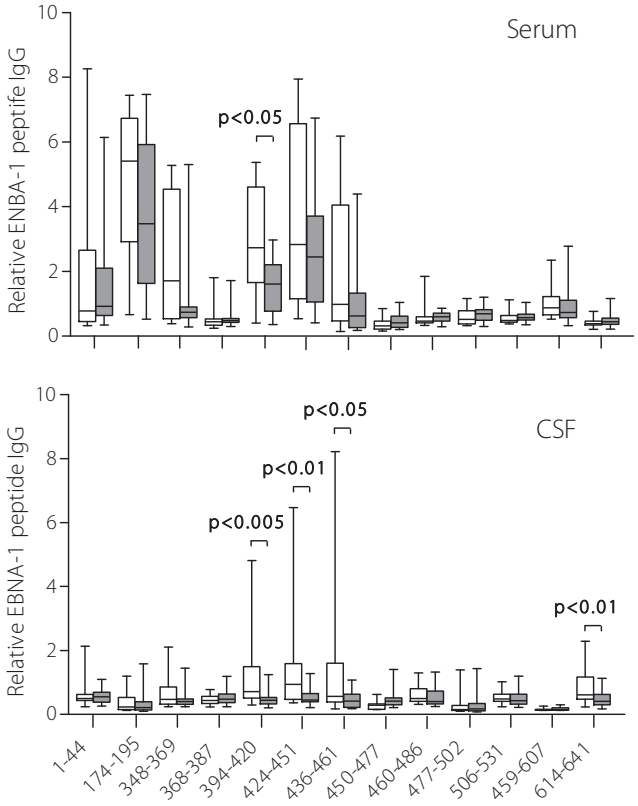
Plot of enzyme-linked immunosorbent assay OD_{450} values of 18 Epstein Barr virus (EBV) seropositive healthy individuals from United States (US), The Netherlands (NL) and Hong Kong (HK) for 12-mer peptides with an 11 residue overlap spanning the entire EBV nuclear antigen-1 (EBNA-1) protein (A). Horizontal axis shows peptide number: peptide 1 to 630. Average of all sera (B) shows that high reactive B-cell epitopes are confined to the Glycine-Alanine repeat (EBNA-$1^{90\text{-}325}$) and EBNA-$1^{395\text{-}451}$.

Identifying EBNA-1 B-cell epitopes

Epitope mapping by immunoscreening of 630 twelve residue long peptides spanning the entire EBNA-1 antigen showed that the B-cell epitopes are largely confined to the N-terminal

part of the protein (figure 1). Here, mostly the glycine-alanine repeat consisting of residue 90-325 (Gly-Ala, EBNA-1[90-325]) and to a lesser extent, the glycine-arginine rich domain (Gly-Arg, EBNA-1[348-369]) contained many B-cell epitopes. However, numerous proteins contain similar repeat sequences gainsaying that the IgG responses measured for these Gly-Ala and Gly-Arg repeats are EBNA-1-specific.[27,28] Thus, EBNA-1-specific IgG responses in healthy EBV seropositive individuals are predominantly directed to EBNA-1[394-451].

Figure 2: Epstein-Barr nuclear antigen-1 specific IgG responses in serum and cerebrospinal fluid of patients with MS and controls.



Epstein Barr nuclear antigen-1 (EBNA-1)-specific IgG responses in serum and cerebrospinal fluid (CSF) samples of multiple sclerosis patients (MS; n = 17; white bars) and non-inflammatory neurological disease controls (NIND; n = 22; grey bars). Serologic responses are presented as the relative IgG levels, calculated as described in the study design section, towards synthetic peptides covering the indicated residues of EBNA-1. Median values (centre line), interquartile ranges (boxes) and minimal and maximum values (whiskers) are indicated. Results were statistically analyzed with the Mann-Whitney U test.

Subsequent analyses of EBNA-1$^{394-451}$ in a larger set of MS patients (n = 114) and controls (n = 62) substantiated the significantly elevated IgG levels to EBNA-1$^{394-451}$ in MS patients (figure 3A). Western blotting using recombinant EBNA-1$^{394-451}$ confirmed ELISA data, showing that 95% of the MS sera and only 82% of NIND were EBNA-1$^{394-451}$ positive.

Increased IgG levels to EBNA-1$^{394-451}$ in serum and CSF of MS patients

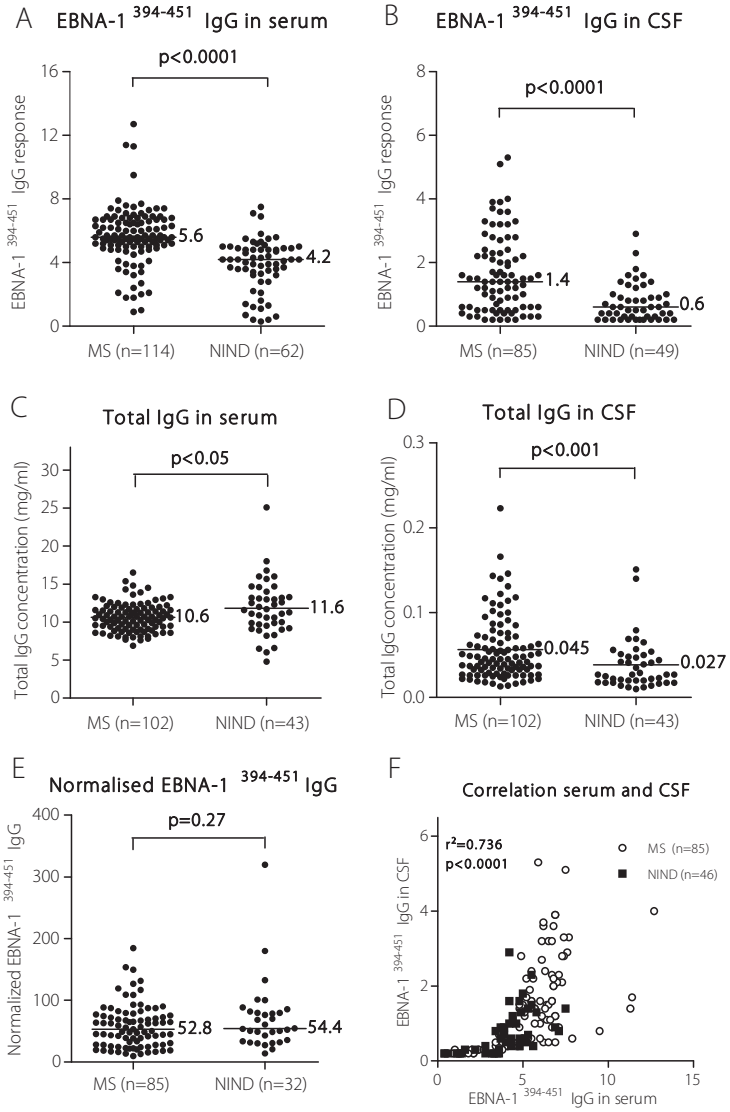
Elevated levels of EBNA-1 IgG have been reported in serum and CSF of MS patients.[7-10] To confirm this finding and to delineate recognized EBNA-1 B-cell epitopes, 13 partly overlapping immunoreactive EBNA-1-specific peptides (table 1) were synthesized based on the preceding EBNA-1 B-cell epitope mapping (figure 1). By computer aided minimal energy calculations, these longer peptides are predicted to acquire their normal structural conformation, as proven for EBNA-1$^{394-451}$ by OT1x and 2B4 monoclonal antibody reactivity, and thereby include epitopes having secondary structures (data not shown).[29]

IgG reactivity to these larger EBNA-1 peptides was determined in paired serum and CSF samples of 17 MS and 22 NIND patients. The overall pattern in serum and CSF was comparable between both patient groups (figure 2). In contrast to CSF, serum IgG responses were mainly directed to Gly-Ala and Gly-Arg repeat domains of EBNA-1 (figure 2). Interestingly, in serum and particularly CSF of MS patients significantly increased IgG responses to EBNA-1$^{394-461}$ and EBNA-1$^{614-641}$ were detected (figure 2). Low reactivity to EBNA-1$^{450-477}$ advocates EBNA-1$^{394-451}$ as the immunodominant region.

Blood-brain barrier dysfunction attributes to increased anti-EBNA-1$^{394-451}$ IgG levels in CSF of MS patients

The elevated CSF EBNA-1$^{394-451}$ IgG levels may be due to intrathecal synthesis or leakage of serum IgG into the CSF compartment. To differentiate between both options, anti-EBNA-1$^{394-451}$ IgG levels in paired serum and CSF of 85 MS and 46 NIND patients were normalized for total IgG of the respective samples. Total IgG in serum of MS patients were significantly lower compared to controls (figure 3C) and were significantly elevated in CSF of MS patients (figure 3D). Contradictory, normalized anti-EBNA-1$^{394-451}$ responses were similar between MS and NIND (figure 3E) and anti-EBNA-1$^{394-451}$ IgG levels in paired serum and CSF samples correlated significantly (figure 3F). Thus, the data suggest that elevated anti-EBNA-1$^{394-451}$ IgG responses in CSF of MS patients is not due to intrathecal IgG synthesis, but more likely associated with blood-brain barrier dysfunction. This conclusion is also supported by correlation of the Q albumin (albumin CSF/albumin serum) with Q EBNA-1 (EBNA-1$^{394-451}$ CSF/ EBNA-1$^{394-451}$ serum). Albumin was tested by routine diagnostic assays and the data were available for 77 MS cases and 31 NIND patients. Spearman correlation was statistical significant for the Q albumin and Q EBNA-1$^{394-451}$ with $r^2 = 0.35$ (95% CI 0.17-0.51; p = 0.0002).

Figure 3: Elevated IgG levels towards Epstein Barr nuclear antigen-1 (EBNA-1) protein region (residues 394-451; EBNA-1$^{394-451}$) in cerebrospinal fluid (CSF) of multiple sclerosis (MS) patients is not attributable to intrathecal IgG synthesis.



EBNA-1$^{394-451}$-specific IgG levels in serum (n =114) (A) and CSF samples (n =85) (B) of MS patients are significantly higher compared to patients with non-inflammatory neurological diseases (NIND; serum (n = 62) and CSF (n = 49)). Total IgG levels in serum (C) and CSF samples (D) of MS (n =102) and NIND patients (n = 43) differ significantly. Normalized EBNA-1$^{394-451}$ IgG levels (E), calculated as described in the study design section and the correlation of EBNA-1$^{394-451}$ IgG levels (F) in paired serum and CSF samples of MS (n = 85) and NIND (n = 32 in E; n = 49 in F) argues against intrathecal synthesis. (A-E) Horizontal lines represent median IgG levels. Results were statistically analyzed with the Mann-Whitney U (A-E) and Spearman correlation test (F).

Discussion

During the past three decades, several viruses including measles virus and human herpes viruses have been suggested to play a role in initiation and perpetuation of MS pathology.[1,8] Whereas most MS-associated viruses have not withstood scrutiny in time, the long-held assumption of EBV as causative agent in MS has recently been reinforced by the demonstration of latently EBV-infected B-cells in MS lesions and increased IgG responses to latency-associated EBV protein EBNA-1 in both serum and CSF of MS patients.[5,9-11] Confirming previous findings, we observed that all MS patients were EBV seropositive, either by IgG immunoblot or ELISA. Immunoblot analysis revealed that the recognized spectrum of EBV proteins was similar in serum and CSF from MS and NIND patients. Moreover, both patient groups showed similar patterns of limited EBV antigen diversity as observed in healthy EBV carriers. This indicates that aberrant lytic replication is unlikely to play a role in MS and is in agreement with the non-elevated EBV-DNA levels in both CSF and circulation of MS patients.[11,12]

The EBNA-1 immunoblot analyses confirmed increased aberrant EBNA-1 IgG reactivity in MS serum and CSF, suggestive of a role for latent EBV antigens in MS. Therefore, the main aim of our study was to delineate the EBNA-1 B-cell epitopes and to determine whether anti-EBNA-1 IgG is produced intrathecally in MS patients. We first identified EBNA-1 B-cell epitopes recognized by serum IgG in healthy EBV carriers and then compared it to the response in serum and CSF of MS and NIND patients. We identified one immunodominant EBNA-1 protein region (EBNA-1$^{394-451}$) in serum and CSF samples of MS (figure 2). The data are in line with a recent study by Sundström et al.,[25] describing significantly elevated serum IgG titers to EBNA-1$^{385-420}$ in MS patients. Notably, our data extend this association by also demonstrating significantly increased anti-EBNA-1$^{394-451}$ IgG levels in CSF next to serum (figure 3). Sundström et al. also suggests that antibodies to EBNA-1-specific domains *and HLA DRB1*1501* interact as risk factors.[25] Although this has not been tested in our study, we do not expect this would influence the message of our study since the higher prevalence of *HLA-DRB* found in MS would only contribute to a positive result.

To compare distinct disease courses of MS (RRMS, PPMS and CIS), we included a relatively high number of PPMS cases (43% of MS samples; normal 10-15% of MS population). However, no significant differences between these subgroups were observed (data not shown).

We demonstrated that EBNA-1-specific IgG responses are elevated both in serum and CSF of MS patients compared to controls. Confirming earlier studies,[8-10,12] this was not shown for other EBV proteins, including the VCA-p18 marker (data not shown). To determine whether increased EBNA-1-specific IgG levels in MS were due to intrathecal synthesis, we corrected for the possible dysfunction of the blood-brain barrier by normalizing for total IgG (figure 3C and D). This revealed comparable normalized anti-EBNA-1$^{394-451}$ IgG levels in MS and NIND patients, arguing against intrathecal IgG synthesis (figure 3E). Significant correlation between

EBNA-1$^{394-451}$ IgG levels in paired serum and CSF samples in both MS patients and controls strengthens this conclusion (figure 3F).

In conclusion, our data showed no evidence for intrathecal anti-EBV IgG synthesis, as also supported by others.[30] Whether peripheral infection or immune responses play a pathogenic role remains to be determined. Notably, the MS-associated EBNA-1$^{394-451}$ region identified encompasses several immunodominant *HLA-DR*-, including potential *HLA-DRB1*1501*-restricted CD4$^+$ T-cell epitopes.[31,32] Moreover, MS patients have elevated frequencies and broader epitope reactivity of EBNA-1-specific CD4$^+$T-cells,[32] including specific T-cells that cross-reacted with MS-associated myelin proteins.[26-33] We hypothesize that in genetically predisposed individuals,[1,34] EBNA-1 expression evokes a neuroantigen cross-reactive anti-EBNA-1 T-cell response, that upon entry into the CNS recognizes and target cells expressing the cognate neuroantigen.

References

1. Hauser SL, Oksenberg JR. The neurobiology of multiple sclerosis: genes, inflammation, and neurodegeneration. Neuron 2006;52:61-76.
2. Burgoon MP, Cohrs RJ, Bennett JL, Anderson SW, Ritchie AM, Cepok S, et al. Varicella zoster virus is not a disease-relevant antigen in multiple sclerosis. Ann Neurol 2009;65:474-479.
3. Riverol M, Sepulcre J, Fernandez-Diez B, Villoslada P, Fernandez-Alonso M, Rubio M, et al. Antibodies against Epstein-Barr virus and herpesvirus type 6 are associated with the early phases of multiple sclerosis. J Neuroimmunol 2007;192:184-185.
4. Sundström P, Juto P, Wadell G, Hallmans G, Svenningsson A, Nyström L, et al. An altered immune response to Epstein-Barr virus in multiple sclerosis: a prospective study. Neurology 2004;62:2277-2282.
5. Lünemann JD, Münz C. EBV in MS: guilty by association? Trends Immunol 2009;30:243-248.
6. Ramagopalan SV, Valdar W, Dyment DA, DeLuca GC, Yee IM, Giovannoni G, et al. Association of infectious mononucleosis with multiple sclerosis. A population-based study. Neuroepidemiology 2009;32:257-262.
7. Levin LI, Munger KL, Rubertone MV, Peck CA, Lennette ET, Spiegelman D, et al. Temporal relationship between elevation of Epstein-Barr virus antibody titers and initial onset of neurological symptoms in multiple sclerosis. JAMA 2005;293:2496-2500.
8. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part I: the role of infection. Ann Neurol 2007;61:288-299.
9. Farrell RA, Antony D, Wall GR, Clark DA, Fisniku L, Swanton J, et al. Humoral immune response to EBV in multiple sclerosis is associated with disease activity on MRI. Neurology 2009;73:32-38.
10. Cepok S, Zhou D, Srivastava R, Nessler S, Stei S, Bussow K, et al. Identification of Epstein-Barr virus proteins as putative targets of the immune response in multiple sclerosis. J Clin Invest 2005;115:1352-1360.
11. Serafini B, Rosicarelli B, Franciotta D, Magliozzi R, Reynolds R, Cinque P, et al. Dysregulated Epstein-Barr virus infection in the multiple sclerosis brain. J Exp Med 2007;204:2899-2912.
12. Willis SN, Stadelmann C, Rodig SJ, Caron T, Gattenloehner S, Mallozzi SS, et al. Epstein-Barr virus infection is not a characteristic feature of multiple sclerosis brain. Brain 2009;132:3318-3328.
13. Peferoen LA, Lamers F, Lodder LN, Gerritsen WH, Huitinga I, Melief J, et al. Epstein Barr virus is not a characteristic feature in the central nervous system in established multiple sclerosis. Brain 2010;133:e137.
14. Rand KH, Houck H, Denslow ND, Heilman KM. Epstein-Barr virus nuclear antigen-1 (EBNA-1) associated oligoclonal bands in patients with multiple sclerosis. J Neurol Sci 2000;173:32-39.
15. Poser CM. Revisions to the 2001 McDonald diagnostic criteria. Ann Neurol 2006;59:727-728.
16. Fachiroh J, Paramita DK, Hariwiyanto B, Harijadi A, Dahlia HL, Indrasari SR, et al. Single-assay combination of Epstein-Barr Virus (EBV) EBNA1- and viral capsid antigen-p18-derived synthetic peptides for measuring anti-EBV immunoglobulin G (IgG) and IgA antibody levels in sera from nasopharyngeal carcinoma patients: options for field screening. J Clin Microbiol 2006;44:1459-1467.
17. Van Grunsven WM, Spaan WJ, Middeldorp JM. Localization and diagnostic application of immunodominant domains of the BFRF3-encoded Epstein-Barr virus capsid protein. J Infect Dis 1994;170:13-19.
18. De Sanjosé S, Bosch R, Schouten T, Verkuijlen S, Nieters A, Foretova L, et al. Epstein-Barr virus infection and risk of lymphoma: immunoblot analysis of antibody responses against EBV-related proteins in a large series of lymphoma subjects and matched controls. Int J Cancer 2007;121:1806-1812.
19. Greijer AE, van de Crommert JM, Stevens SJ, Middeldorp JM. Molecular fine-specificity analysis of antibody responses to human cytomegalovirus and design of novel synthetic-peptide-based serodiagnostic assays. J Clin Microbiol 1999;37:179-188.
20. Middeldorp JM, Jongsma J, ter Haar A, Schirm J. The detection of immunoglobulin M and G antibodies against cytomegalovirus early and late antigens by enzyme-linked immunosorbent assay. J Clin Microbiol 1984;20:763-771.

21. Middeldorp JM, Meloen RH. Epitope-mapping on the Epstein-Barr virus major capsid protein using systematic synthesis of overlapping oligopeptides. J Virol Methods 1988;21:147-159.
22. Van Grunsven WM, Nabbe A, Middeldorp JM. Identification and molecular characterization of two diagnostically relevant marker proteins of the Epstein-Barr virus capsid antigen complex. J Med Virol 1993;40:161-169.
23. Chen MR, Middeldorp JM, Hayward SD. Separation of the complex DNA binding domain of EBNA-1 into DNA recognition and dimerization subdomains of novel structure. J Virol 1993;67:4875-4885.
24. Hennard C, Pfuhl T, Buettner M, Becker KF, Knöfel T, Middeldorp J, et al. The antibody 2B4 directed against the Epstein-Barr virus (EBV)-encoded nuclear antigen 1 (EBNA1) detects MAGE-4: implications for studies on the EBV association of human cancers. J Pathol 2006;209:430-435.
25. Sundström P, Nyström M, Ruuth K, Lundgren E. Antibodies to specific EBNA-1 domains and HLA DRB11501 interact as risk factors for multiple sclerosis. J Neuroimmunol 2009;215:102-107.
26. Lünemann JD, Edwards N, Muraro PA, Hayashi S, Cohen JI, Munz C, et al. Increased frequency and broadened specificity of latent EBV nuclear antigen-1-specific T cells in multiple sclerosis. Brain 2006;129:1493-1506.
27. Lang D, Vornhagen R, Rothe M, Hinderer W, Sonneborn HH, Plachter B. Cross-reactivity of Epstein-Barr virus-specific immunoglobulin M antibodies with cytomegalovirus antigens containing glycine homopolymers. Clin Diagn Lab Immunol 2001;8:747-756.
28. Fox R, Sportsman R, Rhodes G, Luka J, Pearson G, Vaughan J. Rheumatoid arthritis synovial membrane contains a 62,000-molecular-weight protein that shares an antigenic epitope with the Epstein-Barr virus-encoded associated nuclear antigen. J Clin Invest 1986;77:1539-1547.
29. Hennard C, Pfuhl T, Buettner M, Becker KF, Knöfel T, Middeldorp J, Kremmer E, Niedobitek G, Grässer F. The antibody 2B4 directed against the Epstein-Barr virus (EBV)-encoded nuclear antigen 1 (EBNA1) detects MAGE-4: implications for studies on the EBV association of human cancers. J Pathol 2006;209:430-435.
30. Sargsyan SA, Shearer AJ, Ritchie AM, Burgoon MP, Anderson S, Hemmer B, Stadelmann C, Gattenlöhner S, Owens GP, Gilden D, Bennett JL. Absence of Epstein-Barr virus in the brain and CSF of patients with multiple sclerosis. Neurology 2010;74:1127-1135.
31. Kusunoki Y, Huang H, Fukuda Y, Ozaki K, Saito M, Hirai Y, et al. A positive correlation between the precursor frequency of cytotoxic lymphocytes to autologous Epstein-Barr virus-transformed B cells and antibody titer level against Epstein-Barr virus-associated nuclear antigen in healthy seropositive individuals. Microbiol Immunol 1993;37:461-469.
32. Heller KN, Upshaw J, Seyoum B, Zebroski H, Munz C. Distinct memory CD4+ T-cell subsets mediate immune recognition of Epstein Barr virus nuclear antigen 1 in healthy virus carriers. Blood 2007;109:1138-1146.
33. Lünemann JD, Jelcić I, Roberts S, Lutterotti A, Tackenberg B, Martin R, et al. EBNA1-specific T cells from patients with multiple sclerosis cross react with myelin antigens and co-produce IFN-gamma and IL-2. J Exp Med 2008; 205:1763-1773.
34. Leen A, Meij P, Redchenko I, Middeldorp JM, Bloemena E, Rickinson A, et al. Differential immunogenicity of Epstein-Barr virus latent-cycle proteins for human CD4(+) T-helper 1 responses. J Virol 2001;75:8649-8659.

Chapter 5.2

Infectious mononucleosis-linked HLA class I single nucleotide polymorphism is associated with multiple sclerosis

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Abstract

Background Multiple sclerosis is a presumed autoimmune disease associated with genetic and environmental risk factors such as infectious mononucleosis. Recent research has shown infectious mononucleosis to be associated with a specific HLA class I polymorphism.

Objectives Our aim was to test if the infectious mononucleosis linked HLA class I single nucleotide polymorphism (rs6457110) is also associated with multiple sclerosis.

Methods Genotyping of the *HLA-A* single nucleotide polymorphism rs6457110 using TaqMan was performed in 591 multiple sclerosis cases and 600 controls. The association of multiple sclerosis with the *HLA-A* single nucleotide polymorphism was tested using logistic regression adjusted for age, sex and *HLA-DRB1*1501*.

Results *HLA-A* minor allele (A) is associated with multiple sclerosis (OR = 0.68; $p = 4.08 \times 10^{-5}$). After stratification for *HLA-DRB1*1501* risk allele (T) carrier we showed a significant OR of 0.70 (p=0.003) for *HLA-A*.

Conclusions HLA class I single nucleotide polymorphism rs6457110 is associated with infectious mononucleosis and multiple sclerosis, independent of the major class II allele, supporting the hypothesis that shared genetics may contribute to the association between infectious mononucleosis and multiple sclerosis.

Introduction

Multiple sclerosis (MS) is a complex autoimmune demyelinating disease in which genes play a critical role, either by themselves or in interaction with environmental factors. One of the established clinical risk factors for MS is infectious mononucleosis (IM), a benign lymphoproliferative disease characterized by fever, lymphadenopathy and pharyngitis, caused by the Epstein-Barr virus (EBV). This ubiquitous gamma herpesvirus infects over 90% of the world's population.[1] Primary infection generally occurs in early childhood and is usually subclinical; however at higher age it manifests as IM in 25-70% of cases.[2]

Various epidemiological[3] and serological studies[4, 5] have shown an association between MS and EBV. Patients with MS more often have a history of IM[6] and the EBV antibody titres are higher in the serum of MS patients compared with controls.[4, 7] The factors that determine the development of IM remain largely unknown, as does the relationship between MS and EBV infection.

One common relevant factor in the relation of EBV infection with MS occurrence as well as with development of IM appears to be the age of primary infection. Both diseases are common amongst young adults where the primary EBV infection is delayed until adolescence or adulthood, presumably due to a different cellular immune response in young children versus adults.[8] A disturbed immune response reflected by immunopathological symptoms of IM could be instrumental in triggering onset of MS.

An alternative explanation however could be that IM is not only a precursor of MS, but that IM and MS have a common genetic basis which makes certain individuals susceptible to EBV. HLA class I and II are important risk factors for MS.[9-12] An association between HLA class I polymorphisms and development of IM upon primary EBV infection was shown in a recent study.[13] Based on the established association between IM and MS, we aimed to test if the HLA class I single nucleotide polymorphism (SNP) (rs6457110) of interest is also associated with MS.

Methods

Study population

A total of 591 patients with MS and 600 controls were included in this study. Fifty patients with MS and 40 unrelated spouses were ascertained from the Genetic Research in Isolated Populations (GRIP) program. Details on ascertainment are given elsewhere.[14] Further we recruited and ascertained patients with MS as part of an ongoing nationwide study on genetic susceptibility in MS. This included 359 sporadic MS patients and 182 cases, plus 160 unrelated spouses from 120 multiplex MS families (i.e. families with two or more affected individuals). The 600 healthy controls consisted of the 200 unrelated spouses and 400 healthy

blood donors. At the time of enrolment 8% of the patients (n = 47) had a clinically isolated syndrome. The rest of the patients fulfilled the Poser's criteria[15] for definite MS.

Single nucleotide polymorphism analysis

Genotyping of the *HLA-A* SNP rs6457110 was performed using a standard Taqman PCR protocol.[16, 17] The genotyping for the *HLA-DRB1*1501* associated SNP rs3135388 was done for a previous study[18] using the MassARRAY system/Homogeneous MassExtend assay, following the protocol provided by Sequenom. PCR extension primers were designed using the Assay Design 3.0 program (Sequenom). ThermoSequenase (Sequenom) was used for the base extension reactions. Analysis and scoring were performed using the program Typer 3.3 (Sequenom).

Statistics

Logistic regression was used to test the association of SNP rs6457110 with MS, adjusting for age, sex and rs3135388. To test the independent effect we also tested the association of rs6457110 in *HLA-DRB1*1501* negative individuals. In addition, we examined a risk score composed of both SNPs in association with MS in order to see the additional effect of the *HLA-A* SNP on top of *HLA-DRB1*1501*. All statistical analyses were performed using SPSS version 15.

Results

The clinical characteristics of the study group are given in table 1. Rs6457110 was missing in 0.8% of the persons tested and the analyses were done with 583 cases and 599 controls. The SNP was in Hardy-Weinberg equilibrium (p = 0.86).

A significant association for the minor allele (A) with MS was observed, with odd ratio (OR) of 0.68 (95% CI 0.57-0.82; $p = 4.08 \times 10^{-5}$) corrected for sex, age and *HLA-DRB1*1501*. When looking at the genotypes, a significant, more than twofold, decrease of AA carriers in MS patients was observed, with an OR of 0.48 (95% CI 0.32-0.73; p = 0.001). A significant decrease of AT carriers was also observed (OR = 0.65; 95% CI 0.50-0.85; p = 0.001). No significant interaction was observed between the HLA class I and HLA class II SNPs (p = 0.78) (table 2).

To test if the association of class I SNP is independent of *HLA-DRB1*1501* (T allele), we checked the association for the minor allele (A allele) with MS within a *HLA-DRB1*1501* negative group. We found a significant OR of 0.70 (95% CI 0.56-0.89; p = 0.003). Allele A of class I was protective for MS, independent of positive or negative *HLA-DRB1*1501* (table 3).

Finally we tested the association of the joint effect of *HLA-A* and *HLA-DRB* with MS in a risk score (table 4). There was a strong significant association between this risk score and MS ($p = 5.08 \times 10^{-16}$). Using the persons with no risk alleles as a reference the risk of MS for those carrying three or four risk alleles increases to 5.18 (95% CI 2.94-9.12; $p = 1.18 \times 10^{-8}$).

Table 1: Clinical characteristics and genotyping of MS cases and healthy controls.

	Controls (n=599)	Cases (n=583)
Age: mean in years (SE)	49 (16.8)	45 (12.3)
Female %	55 %	71%
HLA class I SNP (rs6457110) n (%)		
TT	237 (40)	294 (51)
AT	284 (47)	235 (40)
AA	78 (13)	54 (9)
*HLA-DRB1*1501* (rs315388) n (%)		
CC	447 (75)	296 (50)
CT	140 (23)	258 (44)
TT	12 (2)	34 (6)

Table 2: Association of HLA class I with MS using logistic regression.

	OR	95% CI	p-value
Minor Allele A	0.68	0.57-0.82	$4.08x10^{-5}$
Genotype TT	1.00		reference
Genotype TA	0.65	0.50-0.85	0.001
Genotype AA	0.48	0.32-0.73	0.001

OR = Odds Ratio; CI = Confidence Interval; all analysis adjusted for age, sex and *HLA-DRB1*1501*.

Table 3: Association of HLA class I with MS using logistic stratified for *HLA-DRB1*1501* risk allele (T).

		Controls n (%)	Cases n (%)	OR[a]	95% CI	p-value
HLA-DRB-	Minor Allele			0.70	0.56-0.89	0.003
	Genotype TT	181 (40)	153 (52)	1.00		reference
	Genotype TA	212 (48)	113 (39)	0.63	0.45-0.87	0.005
	Genotype AA	53 (12)	27 (9)	0.57	0.33-0.96	0.034
HLA-DRB+	Minor Allele			0.64	0.47-0.87	0.004
	Genotype TT	56 (37)	140 (49)	1.00		reference
	Genotype TA	71 (47)	121 (42)	0.71	0.46-1.10	0.127
	Genotype AA	25 (16)	26 (9)	0.38	0.20-0.73	0.004

[a]logistic regression adjusted for age and sex; OR = Odds Ratio; CI = Confidence Interval.

Table 4: Risk score of HLA class I (reversed) and class II.

number of risk alleles	Controls n (%)	Cases n (%)	OR[a]	95% CI	p-value
0	53 (9)	27 (5)			reference
1	236 (40)	136 (23)	1.19	0.71-2.01	0.512
2	247 (41)	260 (45)	2.24	1.35-3.74	0.002
3 or 4 Trend	62 (10)	157 (27)	5.18	2.94-9.12	$1.18x10^{-8}$ $5.08x10^{-16}$

[a]logistic regression adjusted for age and sex; OR = Odds Ratio; CI = Confidence Interval.

Discussion

The genetic influence on MS susceptibility is substantial, as evidenced by the 20-fold increase in risk for siblings of patients with MS. Part of the high recurrence risk is explained by the HLA class I and II locus.[19]

Several studies have indicated that MS is associated with and linked to the HLA region of chromosome 6p21.3, with the strongest effect originating from the *HLA-DRB1*15* gene in the class II region.[20, 21] HLA class I associations have been demonstrated,[11, 22] but these were often credited to linkage disequilibrium (LD) with class II type.[23] However, independent effects of class I region genes have now been noted, also after adjusting for LD. Three reports have suggested a primary role for HLA class I independent of class II,[11, 22, 24] with *HLA-A03* increasing MS risk, and *A02* and *C05* having a protective role.

Given the role of HLA class I genes in MS[22] and IM,[13] and the well-known association of IM and MS,[3, 25] we tested the hypothesis of a shared genetic basis in these diseases using the SNP rs6457110 associated with IM,[13] which is localized on *HLA-A02* (6p21.3). In this study we showed a significant decrease of MS cases within the carriers of the minor allele A for this SNP (OR 0.68, $p = 4.08x10^{-5}$). This same allele also shows a protective effect in IM.[13]

This result supports the idea that *HLA-A02* is associated with changes in immune response, causing IM and MS. Thus, IM history may reflect in part genetic risk, in addition to reflecting an independent aetiology of EBV infection in MS. However we cannot rule out the possibility that the low MS risk among carriers of the minor *HLA-A02* allele is due to lower risk of IM among carriers. This is an important research question that should be investigated in future studies combining risk factors.

De Jager et al. showed in 2008 that an increase in anti-EBNA-1 antibody titre was associated with an increased risk of MS even after stratification for *HLA-DR 15*. This was not shown in healthy controls.[26] Given the long range and discontinuous LD between HLA class I and II,[27] the association we found could be due to LD. However, correcting for HLA-class II by using the SNP rs3135388, as a tagging SNP for the *DRB1*1501* allele[21, 28] we adjusted for LD in the

analysis. Also, the fact that after stratification for HLA class II the association remains intact, supporting the independent association of this SNP in MS.

This study supports a shared genetic background in the pathogenesis of both IM and MS, shown here by the common risk SNP rs6457110. Furthermore it appears relevant to further compare the contribution of the different HLA class I areas to MS risk. Similar to the class II region,[29] there may be substantial epigenetic interactions within this area itself, and also between the class I and II regions. In this study there was no evidence for interaction between HLA class I and II regions, but our study sample may be too small to detect this. Finally, interactions between anti-EBV responses and this class I region deserve to be further analysed, in analogy with the interaction demonstrated for EBV anti-EBNA responses and *HLA-DRB1*1501*.[26]

References

1. Henle G, Henle W. Immunofluorescence in cells derived from Burkitt's lymphoma. J Bacteriol 1966;91:1248-1256.
2. Crawford DH, Macsween KF, Higgins CD, et al. A cohort study among university students: identification of risk factors for Epstein-Barr virus seroconversion and infectious mononucleosis. Clin Infect Dis 2006;43:276-282.
3. Thacker EL, Mirzaei F, Ascherio A. Infectious mononucleosis and risk for multiple sclerosis: a meta-analysis. Ann Neurol 2006;59:499-503.
4. Ascherio A, Munger KL, Lennette ET, et al. Epstein-Barr virus antibodies and risk of multiple sclerosis: a prospective study. JAMA 2001;286:3083-3088.
5. Lünemann JD, Munz C. Epstein-Barr virus and multiple sclerosis. Curr Neurol Neurosci Rep 2007;7:253-258.
6. Ramagopalan SV, Valdar W, Dyment DA, et al. Association of infectious mononucleosis with multiple sclerosis. A population-based study. Neuroepidemiology 2009;32:257-262.
7. Levin LI, Munger KL, Rubertone MV, et al. Temporal relationship between elevation of epstein-barr virus antibody titers and initial onset of neurological symptoms in multiple sclerosis. JAMA 2005;293:2496-2500.
8. Faint JM, Annels NE, Curnow SJ, et al. Memory T cells constitute a subset of the human CD8+CD45RA+ pool with distinct phenotypic and migratory characteristics. J Immunol 2001;167:212-220.
9. Consortium TIMSG, Hafler DA, Compston A, et al. Risk alleles for multiple sclerosis identified by a genomewide study. N Engl J Med 2007;357:851-862.
10. De Jager PL, Jia X, Wang J, et al. Meta-analysis of genome scans and replication identify CD6, IRF8 and TNFRSF1A as new multiple sclerosis susceptibility loci. Nat Genet 2009;41:776-782.
11. Fogdell-Hahn A, Ligers A, Gronning M, Hillert J, Olerup O. Multiple sclerosis: a modifying influence of HLA class I genes in an HLA class II associated autoimmune disease. Tissue Antigens 2000;55:140-148.
12. Bergamaschi L, Leone MA, Fasano ME, et al. HLA-class I markers and multiple sclerosis susceptibility in the Italian population. Genes Immun 2010:11:173-180.
13. McAulay KA, Higgins CD, Macsween KF, et al. HLA class I polymorphisms are associated with development of infectious mononucleosis upon primary EBV infection. J Clin Invest 2007;117:3042-3048.
14. Hoppenbrouwers IA, Cortes LM, Aulchenko YS, et al. Familial clustering of multiple sclerosis in a Dutch genetic isolate. Mult Scler 2007;13:17-24.
15. Poser CM. Revisions to the 2001 McDonald diagnostic criteria. Ann Neurol 2006;59:727-728.
16. McGuigan FE, Ralston SH. Single nucleotide polymorphism detection: allelic discrimination using TaqMan. Psychiatr Genet 2002;12:133-136.
17. Livak KJ. Allelic discrimination using fluorogenic probes and the 5' nuclease assay. Genet Anal 1999;14:143-149.
18. Hoppenbrouwers IA, Aulchenko YS, Janssens AC, et al. Replication of CD58 and CLEC16A as genome-wide significant risk genes for multiple sclerosis. J Hum Genet 2009;54:676-680.
19. Ebers GC. Environmental factors and multiple sclerosis. Lancet Neurol 2008;7:268-277.
20. Oksenberg JR, Baranzini SE, Sawcer S, Hauser SL. The genetics of multiple sclerosis: SNPs to pathways to pathogenesis. Nat Rev Genet 2008;9:516-526.
21. Zivkovic M, Stankovic A, Dincic E, et al. The tag SNP for HLA-DRB1*1501, rs3135388, is significantly associated with multiple sclerosis susceptibility: cost-effective high-throughput detection by real-time PCR. Clin Chim Acta 2009;406:27-30.
22. Yeo TW, De Jager PL, Gregory SG, et al. A second major histocompatibility complex susceptibility locus for multiple sclerosis. Ann Neurol 2007;61:228-236.

23. Chao MJ, Barnardo MC, Lincoln MR, et al. HLA class I alleles tag HLA-DRB1*1501 haplotypes for differential risk in multiple sclerosis susceptibility. Proc Natl Acad Sci U S A 2008;105:13069-13074.
24. Harbo HF, Lie BA, Sawcer S, et al. Genes in the HLA class I region may contribute to the HLA class II-associated genetic susceptibility to multiple sclerosis. Tissue Antigens 2004;63:237-247.
25. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part I: the role of infection. Ann Neurol 2007;61:288-299.
26. De Jager PL, Simon KC, Munger KL, Rioux JD, Hafler DA, Ascherio A. Integrating risk factors: HLA-DRB1*1501 and Epstein-Barr virus in multiple sclerosis. Neurology 2008;70:1113-1118.
27. Chao MJ, Barnardo MC, Lui GZ, et al. Transmission of class I/II multi-locus MHC haplotypes and multiple sclerosis susceptibility: accounting for linkage disequilibrium. Hum Mol Genet 2007;16:1951-1958.
28. de Bakker PI, Burtt NP, Graham RR, et al. Transferability of tag SNPs in genetic association studies in multiple populations. Nat Genet 2006;38:1298-1303.
29. Handel AE, Ebers GC, Ramagopalan SV. Epigenetics: molecular mechanisms and implications for disease. Trends Mol Med 2010;16:7-16.

Chapter 5.3

Serological signs of EBV reactivation and interaction with HLA class I in MS patients of a genetic isolate

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Chapter 6

Cigarette smoking

Chapter 6.1

The association between cigarette smoking and multiple sclerosis

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Abstract

Genetic factors partially explain the susceptibility of multiple sclerosis (MS) and might even relate to the clinical course. Still, many epidemiological studies point at an important role for environmental factors in MS. Smoking is one of the major candidates. In this review we provide an overview of the epidemiological studies on cigarette smoking and the association on MS risk and MS clinical course. In addition, we discuss the possible biological pathways that may influence neurological damage in MS. Moreover, the relation of smoking with other environmental MS risk factors will be addressed.

Introduction

Multiple sclerosis (MS) is regarded as a disease with a multifactorial aetiology, comprising genetic as well as environmental influence. Migration studies, geographical gradients, and high rates of discordance in identical twins point to the influence of environmental factors interacting with genetics in determining disease susceptibility.[1] The landmark work of Kurtzke[2] showed that the MS risk declined twofold with migration from high- to low-risk areas which indicate that genetic factors can account for only a small proportion of geographical MS variety. This was also supported by others,[3] suggesting a role for a range of physical, chemical, biological, and social environmental factors. Moreover, the evidence on the rising worldwide prevalence and increasing female to male ratio focuses the interest on environmental factors.[4] The environmental risk factors implicated include sun exposure, vitamin D status, Epstein-Barr virus (EBV) infections and smoking. These factors combined can interact at different time points prior to and following the clinical onset of MS.[5] Cigarette smoking is emerging as one of the most postulated environmental risk factors linked to onset and clinical course of MS in genetically susceptible individuals.[6, 7] The history of the suggested association between MS and smoking goes back to the 1960s when few studies were performed, although these studies did not reach significance and analysed a large number of variables simultaneously.[8, 9] In this review we aim to give an overview of the studies conducted on the association between smoking and MS susceptibility, and clinical course. In addition, we discuss the possible pathogenic role of smoking in MS and the related underlying mechanisms. Finally, we provide some arguments supporting, but also some arguments challenging this association.

Cigarette smoking and risk of MS

Several retrospective and prospective studies have investigated the association between smoking and MS susceptibility. Table 1 gives a chronological summary of the key studies. One of the earliest papers to include smoking habit was an exploratory case-control study from Israel in 1965,[8] where 241 MS patients were questioned about ever smoking prior to disease onset. The control group included 964 subjects individually matched to patients by age, sex and region of birth. They found significantly more previous smokers in the patient group (44% vs. 36%, p=0.02). However, they did not correct for multiple comparisons.

It was not until the 1990s that two longitudinal studies among women in the United Kingdom[10,11] showed that women who regularly smoked were found to have a higher risk of MS, although these findings were not significant. In the Oxford Family Planning Association Study, the incidence of MS in women who smoked ≥15 cigarettes per day was 1.8 (95% CI 0.8-3.6) times higher than in women who never smoked.[10] The Royal College of General Practitioners' Oral Contraception Study demonstrated comparable findings with the incidence of MS in who smoked ≥15 cigarettes per day 1.4 (95% CI 0.9-2.2) times higher than never-smokers.[11]

Table 1: Chronological summary of fourteen main studies examining cigarette smoking and MS onset.

First author Publication year	Number of cases	Number of controls[a]	Female/ male ratio	Quantity smoked (Number of cases)[b]	OR/ RR (95% CI)	Adjustments and/or matching	Study type and comment (Country)
Antonovsky et al.[8]	241	964	0.8	Ever-smoker (106)	OR 1.4 (1.1-1.9)	Age, sex, region of birth	Case-control study. (Israel)
Villard-Mackintosh and Vessey[10]	63	17,032	All females	Ex-smoker (9) Current smoker (26) - 1-14/day (14) - ≥15/day (12)	RR 1.5 (0.6- 3.3) RR 1.6 (0.8- 3.1) RR 1.8 (0.8- 3.6)	Age, parity, clinic, date of admission to study	Prospective cohort study. (UK)
Thorogood and Hannaford[11]	114	46,000	All females	Ever-smoker (58) - 1-14/day (33) - ≥15/day (25)	 RR 1.2 (0.8- 1.8) RR 1.4 (0.9- 2.2)	Age, parity, social class	Prospective cohort study. Incident cases. All smokers started before disease onset. (UK)
Ghadirian et al.[15]	200	202	2.2	Ever-smoker (138) - 0-<10/day (15) - 10-20/day (34) - 20-40/day (71) - ≥ 40/day (16)	OR 1.6 (1.0- 2.4) OR 0.7 (0.3-1.5) OR 1.4 (0.8-2.4) OR 1.9 (1.2- 3.2) OR 5.5 (1.7- 17.8)	Age, sex, education	Incident case-control. Smoking in year prior to diagnosis. (Canada)
Hernan et al.[12]	315	237,264	All females	Ex-smoker (79) Current smoker (96) Ever-smokers (175) - 1-9 pack years (43) - 10-24 pack years (75) - >25 pack years (57)	RR 1.2 (0.9- 1.6) RR 1.6 (1.2- 2.1) RR 1.1 (0.8-1.6) RR 1.5 (1.2-2.1) RR 1.7 (1.2- 2.4)	Age, latitude, ancestry	Prospective study in NHS and NHS II[c] cohorts. Smoking 4 years prior to MS diagnosis. (USA)
Riise et al.[21]	86	22,312	NR	Ever-smoker (65) - Female (NR) - Male (NR)	RR 1.8 (1.1- 2.9) RR 1.6 (ns) RR 2.8 (ns)	Age, sex	Population based cross-sectional study. (Norway)
Zorzon et al.[16]	140	131	1.8	Ever-smoker (79) Current smoker (58)	OR 1.5 (0.9- 2.4) OR 1.9 (1.1- 3.2)	Age, sex	Case-control study. (Italy)
Hernan et al.[13]	201	1,913	2.4	Ex-smoker (NR) Current smoker (NR) Ever-smoker (92)	OR 1.0 (0.6-1.8) OR 1.4 (1.0- 1.9) OR 1.3 (1.0- 1.7)	Age, sex, family practise, date of joining practise and availability of smoking information	Prospective, nested case-control study. (USA)

First author Publication year	Number of cases	Number of controls[a]	Female/ male ratio	Quantity smoked (Number of cases)[b]	OR/ RR (95% CI)	Adjustments and/or matching	Study type and comment (Country)
Pekmezovic et al.[17]	196	210	2.8	Ever-smoker (114) - ≤15/day (67) - ≥16/day (47)	OR 1.6 *(p=0.02)* OR 1.5 OR 1.7 (*p-trend=0.02*)	Age, sex, residence	Case-control study. (Serbia)
Hedström et al.[22]	902	1,855	2.6	Ex-smoker (195) Current smoker (322) Ever-smoker (517) - Female (362) - Male (155)	OR 1.4 (1.1-1.8) OR 1.6 (1.3- 1.9) OR 1.5 (1.3- 1.8) OR 1.4 (1.2- 1.7) OR 1.8 (1.3- 2.5)	Age, sex, ancestry, residence	Population based case-control study. (Sweden)
Rodriguez Regal et al.[18]	138	138	2.1	Ex-smoker (21) Current smoker (66) Ever-smoker (87) - Female (NR) - Male (NR)	OR 3.3 (1.4-7.8) OR 2.0 (1.2-3.3) OR 2.2 (1.3- 3.6) OR 2.6 (NR) OR 1.3 (NR)	Age, sex, residency	Case-control study. (Spain)
Jafari et al.[24]	136	204	1.7	Ex-smoker (21) Current smoker (43) Ever-smoker (64) - < 7.9 pack years (32) - ≥ 7.9 pack years (32)	OR 1.2 (0.7- 2.2) OR 1.0 (0.6- 1.7) OR 1.1 (0.7- 1.7) OR 1.0 (0.6-1.8) OR 1.2 (0.7-2.1)	Age, sex	Case-control study in multiplex families. (The Netherlands)
Da Silva et al.[19]	81	81	2.1	Current smoker[d] (28)	OR 2.0 (0.9- 4.3)	Controls paired for sex, age, place of birth	Case-control study. (Brazil)
Carlens et al.[14]	214	277,777	All males	Ever-smoker (150)	RR 1.9 (1.4- 2.6)	Age, region of residence, Swedish moist snuff	Prospective cohort study. (Sweden)
Simon et al.[20]	441	865	4.2	Ever-smoker (264)	OR 1.4 (1.1- 1.8)	Age, sex	Case-control study including NHS and NHS II* (USA), Tasmanian (Tasmania) and Swedish MS study (Sweden).

Odds ratio (OR); rate ratio (RR); confidence interval (CI); not reported (NR).
[a]For cohorts total number followed up are given.
[b]Reference category classified as never-smoker.
[c]NHS= Nurses' Health Study (1976-1994) and Nurses' Health Study II (1989-1995).
[d]Reference category classified as never-smoker and ex-smoker.

It should be noticed that both studies included only females and small number of incident cases of MS (63 and 114 respectively). Furthermore, assessing smoking history was a secondary question since both studies were conducted to investigate the possible relation of oral contraceptives and MS risk.

A few years later a third prospective investigation was performed, which comprised more than 200,000 women participating in the Nurses' Health Study and Nurses' Health Study II and included 315 MS cases. This study showed a significant increase in MS risk among current smokers (OR 1.6, 95% CI 1.2-2.1). They also showed that the incidence of MS increased with the cumulative exposure to smoking.[12] The outcome was adjusted for age, latitude, and ancestry. To be noted, they studied smoking behaviour 4 years prior to MS diagnosis. Next, the same study group investigated the association between MS and smoking in a nested case-control study based on the General Practice Research Database including 201 cases. They confirmed the higher MS risk for ever-smokers and current smokers (respectively OR 1.3, 95% CI 1.0-1.7 and OR 1.4, 95% CI 1.0-1.9) compared to never-smokers.[13] Another prospective study was presented by Carlens and colleagues[14] who showed in a large cohort with 214 incident cases that the relative risk associated with ever-smoking was 1.9 (95% CI 1.4-2.6). Interestingly, this cohort included only males.

These results are consistent with several retrospective case-control studies,[15-20] and population based surveys[21, 22] (table 1). Ghadirian et al.[15] showed in a case-control study of 200 incident MS cases from Montreal that ever-smoking was significantly associated with a higher MS risk (OR 1.6, 95% CI 1.0-2.4) and concurred with the stronger association for heavy smokers. Their analysis was based on reported cigarette smoking in the year prior to MS diagnosis and was adjusted for age, sex, and education.

In a survey of the general population of Hordaland, Norway, including 22,312 individuals of which 86 had MS,[21] the risk of incident MS among current and ex-smokers was 1.8 (95% CI 1.1-2.9) times higher than the risk in subjects who had never smoked. Males had a higher risk ratio (RR 2.8) compared to females (RR 1.6), 95% CI were not given. The sex difference has also been demonstrated in a population based case-control study in Sweden. Hedström and colleagues[22] showed a higher OR for males compared to females (OR 1.8 vs. 1.4), next to a significant higher risk of MS for ever-smokers (OR 1.5, 95% CI 1.3-1.8). In 2009 Rodriguez Regal et al. confirmed in a Spanish case-control study the association of cigarette smoking and MS. Surprisingly, in contrast to other studies they showed stronger effect among ex-smokers (OR 3.3, 95% CI 1.4-7.8) compared to current smokers (OR 2.0, 95% CI 1.2-3.3).

There are few studies, which did not show an association between MS and smoking. Some of these studies were subject to methodological limitations. Casetta et al.[23] performed a community-based case-control study in Ferrara, Italy. The MS population including 104 subjects was compared to 150 controls. Their study demonstrated no association between

MS risk and smoking (results not shown). It is to be noted that they collected data on smoking and drinking in adolescence. In a recent case-control study in multiplex families,[24] we were not able to show an association of smoking and MS. This was possibly due to the higher genetic susceptibility in the multiplex families, which could obfuscate the role of smoking.
Recently it was demonstrated that exposure to environmental tobacco smoke is associated with an increased risk of MS. A French case-control study showed a positive association between parental smoking at home and early onset MS in their children,[25] suggesting a role for passive smoking. However, another study demonstrated no effect of maternal smoking during pregnancy on early onset MS in offspring.[26] Hedström and colleagues observed that never-smokers, who reported that they had been exposed to passive smoking, had an increased risk of developing MS compared to those who reported never to have been exposed (OR 1.3, 95% CI 1.1-1.5). The risk increased significantly with longer duration of exposure.[27]
Complementary to the studies investigating the association between cigarette smoking and MS susceptibility, a group in Austria studied the role of smoking after a first demyelinating event.[28] The clinically isolated syndrome (CIS) is the most common and recognised onset of MS. Most of the CIS patients with disseminated white-matter lesions on brain magnetic resonance imaging, and/or positive oligoclonal bands in the cerebrospinal fluid will develop clinically definite MS (CDMS) after a second relapse.[29] Di Pauli and colleagues[28] suggested that cigarette smoking was a risk factor for early conversion. They observed that a patient who smoked at onset of MS had a 1.8 fold increased risk of conversion to CDMS over 3 years compared with non-smokers (p=0.008). It should be noted that there was a higher proportion of smokers in their CIS patients (46%) relative to the general population of Austria (29%), however this reference group was not age and sex matched.

Cigarette smoking and the clinical course of MS

Aggravation of MS symptoms shortly after starting to smoke has been reported in several early studies.[9, 30-32] The relationship between smoking and disease progression is still arguable. Some studies have suggested increased risk and others showed no effect. The variability in outcome of these studies can partly be explained by different measurements used for progression. Some studies investigated symptom aggravation or relapse rate; others used Kurtzke Expanded Disability Status Scale (EDSS) or MS Severity Scales (MSSS) to study disease disability in MS, but most of the studies focused on conversion to secondary progressive MS (SPMS).
An acute worsening of MS symptoms immediately following smoking was reported in a few clinical studies conducted more than 40 years ago,[9] and it was confirmed in later investigations.[32] Emre and De Decker[32] showed that cigarette smoking causes a transient worsening of motor functioning. They used a battery of tests in 21 MS patients compared to

healthy controls. In a prospective cohort study including 142 relapsing remitting MS (RRMS) patients, Pittas and colleagues showed no statistically significant associations between relapse rate and smoking behaviour. The hazard ratio (HR) for ever-smokers was 0.9 (95% CI 0.7-1.3) and for current smokers 1.0 (95% CI 0.6-1.6).[33] There was also no dose related association. These analyses were adjusted for entry MSSS, follow-up time, gender, age at entry review, immunomodulatory treatment use, education level, and month of review.

Interestingly, they demonstrated in the same study that increasing smoking pack years were positively associated with progression of clinical disability within the observation period, depicted in the MSSS and EDSS ($p<0.001$). These effects persisted after further adjustment for factors like, time outside or in the sun in previous 6 months, serum 25(OH)D, total alcohol and fish intake.[33] A Swedish study estimated the effects of smoking in MS also using self-reported data on smoking habits in 122 incident cases. They assessed the MS disability using EDSS after a median disease duration of 6 years and showed that progressive disease disability was significantly more likely to occur in ever-smokers when compared with never-smokers ($p=0.006$). Individuals with an early smoking start (≤15 years of age) scored higher compared with those starting smoking later (>15 years of age) ($p=0.005$) or never-smokers ($p<0.001$).[34] Also the measurements by MSSS showed that ever-smokers have in average a significantly higher disability than never-smokers (median MSSS 5.2 versus 3.2, $p=0.042$).[34] And ever-smokers with early debut had in average a significantly higher disability than late starting smokers or never-smokers (median MSSS 6.5, 4.6 and 3.2; $p=0.011$ and $p=0.002$, respectively). However, another study assessing the impact of smoking on disability could not confirm these findings.[35] They compared prospectively collected clinical information with retrospectively collected data on smoking and reported that smoking was not related to time to reach the disability milestones of EDSS 4.0 or 6.0. Age at disease onset was the only variable associated with a shorter time to both EDSS milestones ($p<0.001$). Though, they demonstrated that pack-years smoked after the onset of MS were significantly correlated with MSSS increase in the whole patient group (rho=-0.11, $p=0.03$) and in women (rho=-0.15, $p=0.02$).

In 2009, Healy and colleagues did also not found any association between smoking status and EDSS progression at the end of 2 and 5 years.[36] The percentage in which disease progressed after 2 years was 23.3% in ever-smokers, 30.8% in ex-smokers, and 26.0% in never-smokers ($p=0.57$, adjusted for baseline age, sex, disease duration, and treatment). They also studied progression in terms of brain MRI changes. They showed a significantly greater increase in T2-weighted lesion volume in current smokers compared with never-smokers ($p=0.02$, adjusted for baseline age, sex, disease duration, and disease course). In 2009 Zivadinov et al.[37] confirmed the association with MS clinical course, measuring disease progression by EDSS increase but also by MRI lesion volume and atrophy. They showed that ever-smokers had higher EDSS scores compared to never-smokers (median EDSS scores 3.0 and 2.5 respectively, $p\leq0.001$).

Table 2: Studies on the association between cigarette smoking and secondary progression of MS.

First author	Number of RRMS cases	Female/ male ratio	Quantity smoked (Number of cases)	Regression analyses HR (95% CI)[a]	Adjustments and/or matching	Study type and comment (Country)
Hernan et al.[13]	179	2.4[b]	Ever-smoker (81)	3.6 (1.3- 9.9)	Age, sex, motor clinical onset	Prospective cohort study. (UK)
Koch et al.[35]	271	2.4	Ever-smoker (192)	0.9 (0.6- 1.3)	Age at disease onset, sex	Prospective cohort study with retrospective smoking data collection. (The Netherlands)
Sundström and Nystrom[34]	96	2.1	Ever-smoker (56)	2.4 (1.0- 6.0)	Age at disease onset, sex	Retrospective cohort study. (Sweden)
Pittas et al.[33]	148	2.2*	Total pack years smoked at cohort entry	OR 1.0 (1.0-1.1)	Age at cohort entry, sex, disease duration	Prospective cohort study. (Tasmania)
Healy et al.[36]	891	3.0*	Current smoker (154) Ex-smoker (237)	2.5 (1.4- 4.4) 1.1 (0.6- 1.8)	Age, sex, disease duration from symptom onset	Prospective cohort study. (USA)

Hazard ratio (HR); adjusted odds ratio (AOR); confidence interval (CI); relapsing remitting MS (RRMS); not reported (NR).
[a]Cox Proportional Hazard Regression was performed by the studies.
[b]Female/male ratio shown for the total cohort in the study, not limited to RRMS.

They also demonstrated that mean number of contrast enhanced lesions on MRI for ever-smokers was significantly higher compared to never-smokers (mean 1.2 vs. 0.72 respectively, p<0.001). Moreover, they showed that smoking was associated with increased lesion burden and greater atrophy (p<0.001).

The major focus of studies investigating association between cigarette smoking and MS clinical course has been on the conversion to SPMS (table 2). In 2005 a prospective cohort study using data from the General Practice Research Database showed that RRMS patients who smoked had >3 times higher risk of progression to SPMS.[13] This was studied in a small sample of 179 RRMS cases of which only 20 converted to SPMS during a mean follow-up time of 5.3 years. There were no data on duration or intensity of smoking.[13] Sundström and Nyström showed in 96 RRMS patients that the increased risk for secondary progression for ever-smokers compared with never-smokers is not due to differences in sex or age at disease onset (HR 2.4, 95% CI 1.0-6.0). The hazard ratio was borderline significant probably caused by the small numbers.[34] Also in another study was smoking associated with an increased risk of converting to a secondary progressive course within the cohort follow-up period, but this finding appeared partly due to smokers being of older age with longer disease durations.[33] In 2009 Healy and colleagues demonstrated in a much larger group of 891 patients an adverse influence of cigarette smoking for conversion of RRMS to SPMS.[36] During a mean (SD) follow-up of 3.3 (1.7) years, conversion to SPMS occurred in 72 patients. Current smokers converted faster from RRMS to SPMS compared to never-smokers (HR 2.5, 95% CI 1.4-4.4). This was not shown for ex-smokers (HR 1.1, 95% CI 0.6-1.8). Unlike previous mentioned positive studies, in 2007 Koch and his group found no association between cigarette smoking and progression to SPMS.[35] They compared prospectively collected clinical information with retrospectively collected data on smoking. The collection of data on smoking almost 20 years after recruitment of the patients could be a possible bias.

Taking all these studies into consideration, it is reasonable to conclude that smoking is a significant, but not powerful risk factor for MS onset and clinical course. A recently updated meta-analysis showed that smoking is associated with MS susceptibility in a conservative model with an RR of 1.5 (95% CI 1.4-1.6). However, the effect of smoking behaviour on the secondary progression of MS is less certain (RR 1.9, 95% CI 1.0-3.6). [38]

Cigarette smoking and biological pathways

As discussed previously, various studies have been performed investigating cigarette smoke and MS; however, they only indicated an association. There is no evidence for a possible causal link between cigarette smoke and MS onset or clinical course. Nevertheless, if we assume that there is a possible link, then there are several hypotheses to be considered.

Cigarette smoke contains over 4,500 of potentially toxic components, including tars, nicotine,

carbon monoxide, and other particles.[39, 40] Some components of cigarette smoke may have direct toxic effects on the central nervous system. Cigarette smoke exists of two phases: a particulate phase and a gaseous phase, both containing extremely high concentrations of free radicals,[40] which may cause axonal degeneration. The association between smoking and the risk of MS might be explained by the vulnerability of oligodendroglia, compared with astrocytes and microglia, to nitric oxide.[41, 42] Serum concentrations of cyanide, a component of cigarette smoke, and its main metabolite thiocyanate has long been known to cause demyelination in the CNS of animals.[43] As discussed earlier, smoking is suggested to be associated with MS clinical course. Progressive disease is characterised by the permanent neurological deficit, which is a result of axonal loss.[44] Exposure to nitric oxide has been shown to cause axonal degeneration or to block axonal conduction, especially in demyelinated axons,[45] suggesting a role for nitric oxide in secondary progressive MS. Moreover, studies suggesting increased risk for developing MS[25, 27] support a direct role of tobacco components in the pathway.

Interestingly, a recent study in Sweden showed that tobacco smoking, but not the Swedish snuff, is associated with elevated risk for MS.[22] This suggests that the critical effects of smoking may be caused by irritation in the lungs or by increased incidence of respiratory infections leading to higher MS risk, which has already been suggested.[46] This pro-inflammatory effect of smoking is most likely triggered via toll-like receptors.[47, 48]

The pro-inflammatory effects of cigarette smoke have been studied in relation to the risk of cardiovascular disease and emphysema.[49] Cigarette smoke stimulates the influx and activation of neutrophils, monocytes, and macrophages.[50] Both current and past smokers have higher fibrinogen levels, as a marker of inflammation, than non-smokers, and these levels correlate with the number of cigarettes smoked per day.[51, 52] Cigarette smoke elevates peripheral blood leukocyte counts,[53, 54] and is associated with important markers of inflammation like the C-reactive protein and IL-6.[55, 56] Abnormalities in T-cell function,[57, 58] reduction in natural killer cells[59] and impairment of both humoral and cell-mediated immunity[59, 60] have been observed in smokers.

Several hypotheses based on biological mechanisms link cigarette smoking to MS and to other autoimmune diseases such as rheumatoid arthritis,[61, 62] systemic lupus erythematosus,[63] Graves' disease,[61, 64] and inflammatory bowel disease.[65] This suggests either an immunomodulation or a pro-inflammatory influence on the immune system, including an increased pro-inflammatory cell activation in the lungs[66] or post-translational modifications of proteins which may break self-tolerance,[67] resulting in hypothetical autoimmune responses against antigens of the nervous system.

Another hypothesis could involve a direct effect of smoke components on microvascular blood flow and on the blood-brain barrier. Nicotine, a major component of cigarettes, has been shown to affect the integrity and function of the blood-brain barrier (BBB).[68] This is important

because leakage of BBB has been suggested as a factor in initiating the development of MS. This may be relevant to the recent report that cigarette smoking increases the risk of converting from CIS to CDMS.[28]

Additionally, cigarette smoking has anti-estrogenic effects as a result of producing inactive 2-hydroxy catechol estrogens.[69, 70] Women who are smokers undergo menopause earlier than non-smokers.[71] Estrogens can affect the Th1/Th2 immune balance and also have either pro- or anti-inflammatory actions depending on their concentration.[72]

Discussion

The picture of how environmental exposures lead to autoimmune diseases in genetically predisposed individuals is becoming more comprehensive because of the numerous studies in different fields. We have discussed different studies investigating the association of smoking on MS onset and on MS clinical course. A recent meta-analysis demonstrated that cigarette smoking is important in determining MS susceptibility but the effect on disease progression is less certain.[38]

There are several limitations to be noted in these studies varying from recall, response rate, difference between responders and non-responders, and possible misclassification in self-reported data. One of the most concerning disadvantages of all retrospective and questionnaire-based studies is that there is a possible recall bias. However, a recent study showed that participants reliably report smoking status over time.[73] But even when smoking habit is explored before MS onset, patients regularly confuse the time of MS onset with the time of diagnosis. This is sometimes difficult to determine even for the physicians. As a result, some outcome may relate to smoking after onset of MS. This issue is prevented in the prospective studies with data collection, in some studies at least 4 years prior to the first symptoms.[12, 13]

Another potential methodological problem in case-control studies is the possible selection bias due to recruitment. Introducing prospective studies enables measuring cigarette smoke exposure more accurately without recall or report bias. Preferably, population based studies should be used. However, these surveys are expensive, and collecting a statistically useful group size of incident cases is time consuming.

The heterogeneity in study design and outcome measures makes it difficult to combine the study results. The time of measurement differed in the studies; some studies examined the current smoking behaviour as a reflection of smoking prior to disease onset. There is evidence that smoking behaviour does not change significantly after diagnosis,[20] which implies that perhaps the difference in timing is not of special concern. Different studies showed a higher MS incidence with the cumulative exposure to smoking, however only few studies included the duration and quantity of smoking. It should also be noticed that the definition of the ever-smoker group is disputable. The composition of cigarettes over the years has changed and the

quantities of inhaled toxins in low-tar and filtered cigarettes are unknown. Most of the studies focused on smoking did not consider passive exposure to tobacco smoke, which recently has been linked to MS.[27] Moreover, this exposure may vary considerably by geographic location given the local smoking laws. In addition, studying MS clinical course the different studies use a variety of parameters for assessment of MS progression, such as relapse rate[33], EDSS or MSSS increase[35] and secondary progression.[13, 34, 36] These variations in measurements could account for some of the variability noted in the results.

Cigarette smoking cannot fully explain the latitude gradient of MS, the changes in risk with migration or the global variation in prevalence and sex differences.[74] However, it may partly explain the recently demonstrated increased female/male ratio in MS incidence.[4, 75] Moreover, investigating the association between smoking and MS can also be complicated by known and unknown confounders. There has been little attention for other health behaviours such as alcohol intake, body mass index or physical activity, although it has been suggested that MS patients are risk takers.[76, 77] These factors can influence the association between smoking cigarettes and MS. A well-known environmental risk factor for MS is infectious mononucleosis and the related anti-EBV antibodies. A recent study in healthy individuals showed that female gender, *HLA-DR2* and cigarette smoking are each positively associated with EBV antibody levels.[78] This raises questions on the role of EBV in a possible common pathway associated with these factors.[79-81] Simon and colleagues examined whether the effect of smoking is independent from that of anti-EBNA titres, by comparing the association between ever-smoking and MS risk with and without adjustment for EBV-titres.[20] They demonstrated that the increased MS risk associated with ever smoking (OR 1.4, 95% CI 1.1-1.8) was no longer evident upon adjustment for anti-EBNA antibody titres (OR 1.1; 95% CI 0.8-1.4). In cases that had high anti-EBNA titres the risk of MS associated with ever smoking was increased (OR 1.7, 95% CI 1.1-2.6). Interestingly, the association between increasing anti-EBNA antibody titres and increased MS risk appeared to be approximately twofold greater among ever smokers compared to never smokers (OR 3.9, 95% CI 2.7-5.7 vs. OR 1.8, 95% CI 1.4-2.3), suggesting interaction between smoking and anti-EBNA titres (p for interaction= 0.001). They also showed that the increased risk of MS associated with smoking was independent of *HLA-DR15* status, although there were implications of between-study differences. Another study[82] investigating the interaction of smoking and two human leukocyte antigens (*HLA-DR15* and *HLA-A02*) associated with MS, showed that smoking increased the MS risk by a factor of 2.8 in cases with both genetic risk factors in comparison with a factor of 1.4 in those without both genetic risk factors.

Next to EBV, vitamin D and exposure to sunlight are also important environmental risk factors associated with MS onset and perhaps even clinical course.[83-86] More than 30 years ago, vitamin D deficiency was first proposed as a risk factor for MS. After discovery of the immunomodulatory

effects of vitamin D,[87, 88] a role in MS susceptibility was warranted. Interestingly, lower serum levels of vitamin D and dietary intake of vitamin D in smokers have been reported in earlier studies.[89] An interaction between smoking and vitamin D has been suggested for rheumatoid arthritis,[90] however to our best knowledge this has not been demonstrated for MS.[86]

There is no lack of candidate mechanisms by which smoking could exert effects on susceptibility to MS as well as on the further clinical course. Different components of tobacco smoke have been discussed here. The suggestion that components of smoke play a role is also supported by a recent finding that even passive smoke exposure increases the MS risk.[27] Next to nitric oxide, which has putative roles in demyelination and axonal loss,[42, 91] some animal studies have suggested that smoke exposure affects several parts of the immune system, including innate immunity, B- and T-lymphocytes and natural killer cells.[92, 93] Other possible mechanisms have been suggested such as smoke induced (local) immunosuppression, with an associated increase in upper respiratory tract infections.[94]

In conclusion, the growing epidemiological evidence for an association between cigarette smoking and MS warrants further investigation with well-designed prospective studies. Animal models and basic research as well as ongoing large cohort studies will advance our understanding of mechanisms. This is important because understanding the role of smoking in the MS pathogenesis may enable us on one hand to temper disease onset and perhaps also control the clinical course in high risk individuals by putting a brake on exposure. On the other hand it may provide fruitful insight for understanding MS pathogenesis and further development of therapeutical targets.

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Table1 is partly derived from C.H. Hawkes (QJM 2005)

References

1. Kurland LT. The evolution of multiple sclerosis epidemiology. Ann Neurol 1994;36:S2-5.
2. Kurtzke JF, Beebe GW, Norman JE, Jr. Epidemiology of multiple sclerosis in US veterans: III. Migration and the risk of MS. Neurology 1985;35:672-678.
3. Miller DH, Hammond SR, McLeod JG, Purdie G, Skegg DC. Multiple sclerosis in Australia and New Zealand: are the determinants genetic or environmental? J Neurol Neurosurg Psychiatry 1990;53:903-905.
4. Alonso A, Hernan MA. Temporal trends in the incidence of multiple sclerosis: a systematic review. Neurology 2008;71:129-135.
5. Handel AE, Giovannoni G, Ebers GC, Ramagopalan SV. Environmental factors and their timing in adult-onset multiple sclerosis. Nat Rev 2010;6:156-166.
6. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part II: Noninfectious factors. Ann Neurol 2007;61:504-513.
7. Giovannoni G, Ebers G. Multiple sclerosis: the environment and causation. Curr Opin Neurol 2007;20:261-268.
8. Antonovsky A, Leibowitz U, Smith HA, et al. Epidemiologic Study of Multiple Sclerosis in Israel. I. an Overall Review of Methods and Findings. Arch Neurol 1965;13:183-193.
9. Courville CB, Maschmeyer JE, Delay CP. Effects of Smoking on the Acute Exacerbations of Multiple Sclerosis. Bull Los Angel Neuro Soc 1964;29:1-6.
10. Villard-Mackintosh L, Vessey MP. Oral contraceptives and reproductive factors in multiple sclerosis incidence. Contraception 1993;47:161-168.
11. Thorogood M, Hannaford PC. The influence of oral contraceptives on the risk of multiple sclerosis. Br J Obstet Gynaecol 1998;105:1296-1299.
12. Hernan MA, Olek MJ, Ascherio A. Cigarette smoking and incidence of multiple sclerosis. Am J Epidemiol 2001;154:69-74.
13. Hernan MA, Jick SS, Logroscino G, Olek MJ, Ascherio A, Jick H. Cigarette smoking and the progression of multiple sclerosis. Brain 2005;128:1461-1465.
14. Carlens C, Hergens MP, Grunewald J, et al. Smoking, use of moist snuff, and risk of chronic inflammatory diseases. Am J Respir Crit Care Med 2010;181:1217-1222.
15. Ghadirian P, Dadgostar B, Azani R, Maisonneuve P. A case-control study of the association between socio-demographic, lifestyle and medical history factors and multiple sclerosis. Can J Public Health 2001;92:281-285.
16. Zorzon M, Zivadinov R, Nasuelli D, et al. Risk factors of multiple sclerosis: a case-control study. Neurol Sci 2003;24:242-247.
17. Pekmezovic T, Drulovic J, Milenkovic M, et al. Lifestyle factors and multiple sclerosis: A case-control study in Belgrade. Neuroepidemiology 2006;27:212-216.
18. Rodriguez Regal A, del Campo Amigo M, Paz-Esquete J, et al. A case-control study of the influence of the smoking behaviour in multiple sclerosis. Neurologia 2009;24:177-180.
19. Da Silva KR, Alvarenga RM, Fernandez YFO, Alvarenga H, Thuler LC. Potential risk factors for multiple sclerosis in Rio de Janeiro: a case-control study. Arq Neuropsiquiatr 2009;67:229-234.
20. Simon KC, van der Mei IA, Munger KL, et al. Combined effects of smoking, anti-EBNA antibodies, and HLA-DRB1*1501 on multiple sclerosis risk. Neurology 2010;74:1365-1371.
21. Riise T, Nortvedt MW, Ascherio A. Smoking is a risk factor for multiple sclerosis. Neurology 2003;61:1122-1124.
22. Hedström AK, Baarnhielm M, Olsson T, Alfredsson L. Tobacco smoking, but not Swedish snuff use, increases the risk of multiple sclerosis. Neurology 2009;73:696-701.

23. Casetta I, Granieri E, Malagu S, et al. Environmental risk factors and multiple sclerosis: a community-based, case-control study in the province of Ferrara, Italy. Neuroepidemiology 1994;13:120-128.
24. Jafari N, Hoppenbrouwers IA, Hop WC, Breteler MM, Hintzen RQ. Cigarette smoking and risk of MS in multiplex families. Mult Scler 2009;15:1363-1367.
25. Mikaeloff Y, Caridade G, Tardieu M, Suissa S. Parental smoking at home and the risk of childhood-onset multiple sclerosis in children. Brain 2007;130:2589-2595.
26. Montgomery SM, Bahmanyar S, Hillert J, Ekbom A, Olsson T. Maternal smoking during pregnancy and multiple sclerosis amongst offspring. Eur J Neurol 2008;15:1395-1399.
27. Hedström AK, Baarnhielm M, Olsson T, Alfredsson L. Exposure to environmental tobacco smoke is associated with increased risk for multiple sclerosis. Mult Scler 2011;17:788-793.
28. Di Pauli F, Reindl M, Ehling R, et al. Smoking is a risk factor for early conversion to clinically definite multiple sclerosis. Mult Scler 2008;14:1026-1030.
29. O'Riordan JI, Thompson AJ, Kingsley DP, et al. The prognostic value of brain MRI in clinically isolated syndromes of the CNS. A 10-year follow-up. Brain 1998;121:495-503.
30. Perkin GD, Bowden P, Rose FC. Smoking and optic neuritis. Postgrad Med J 1975;51:382-385.
31. Franklin CR, Brickner RM. Vasospasm associated with multiple sclerosis. Arch Neurol Psychiatry 1947;58:125-162.
32. Emre M, de Decker C. Effects of cigarette smoking on motor functions in patients with multiple sclerosis. Arch Neurol 1992;49:1243-1247.
33. Pittas F, Ponsonby AL, van der Mei IA, et al. Smoking is associated with progressive disease course and increased progression in clinical disability in a prospective cohort of people with multiple sclerosis. J Neurol 2009;256:577-585.
34. Sundström P, Nyström L. Smoking worsens the prognosis in multiple sclerosis. Mult Scler 2008;14:1031-1035.
35. Koch M, van Harten A, Uyttenboogaart M, De Keyser J. Cigarette smoking and progression in multiple sclerosis. Neurology 2007;69:1515-1520.
36. Healy BC, Ali EN, Guttmann CR, et al. Smoking and disease progression in multiple sclerosis. Arch Neurol 2009;66:858-864.
37. Zivadinov R, Weinstock-Guttman B, Hashmi K, et al. Smoking is associated with increased lesion volumes and brain atrophy in multiple sclerosis. Neurology 2009;73:504-510.
38. Handel AE, Williamson AJ, Disanto G, Dobson R, Giovannoni G, Ramagopalan SV. Smoking and multiple sclerosis: an updated meta-analysis. PloS One 2011;6:e16149.
39. Costenbader KH, Karlson EW. Cigarette smoking and autoimmune disease: what can we learn from epidemiology? Lupus 2006;15:737-745.
40. Pryor WA, Stone K. Oxidants in cigarette smoke. Radicals, hydrogen peroxide, peroxynitrate, and peroxynitrite. Ann N Y Acad Sci 1993;686:12-27.
41. Mitrovic B, Parkinson J, Merrill JE. An in vitro model of oligodendrocyte destruction by nitric oxide and its relevance to multiple sclerosis. Methods 1996;10:501-513.
42. Smith KJ, Kapoor R, Felts PA. Demyelination: the role of reactive oxygen and nitrogen species. Brain Pathol 1999;9:69-92.
43. Philbrick DJ, Hopkins JB, Hill DC, Alexander JC, Thomson RG. Effects of prolonged cyanide and thiocyanate feeding in rats. J Toxicol Environ Health 1979;5:579-592.
44. Trapp BD, Ransohoff R, Rudick R. Axonal pathology in multiple sclerosis: relationship to neurologic disability. Curr Opin Neurol 1999;12:295-302.
45. Rejdak K, Eikelenboom MJ, Petzold A, et al. CSF nitric oxide metabolites are associated with activity and progression of multiple sclerosis. Neurology 2004;63:1439-1445.

46. Graham NM. The epidemiology of acute respiratory infections in children and adults: a global perspective. Epidemiol Rev 1990;12:149-178.
47. Pace E, Ferraro M, Siena L, et al. Cigarette smoke increases Toll-like receptor 4 and modifies lipopolysaccharide-mediated responses in airway epithelial cells. Immunology 2008;124:401-411.
48. Mortaz E, Lazar Z, Koenderman L, Kraneveld AD, Nijkamp FP, Folkerts G. Cigarette smoke attenuates the production of cytokines by human plasmacytoid dendritic cells and enhances the release of IL-8 in response to TLR-9 stimulation. Respir Res 2009;10:47.
49. Ambrose JA, Barua RS. The pathophysiology of cigarette smoking and cardiovascular disease: an update. J Am Coll Cardiol 2004;43:1731-1737.
50. Garey KW, Neuhauser MM, Robbins RA, Danziger LH, Rubinstein I. Markers of inflammation in exhaled breath condensate of young healthy smokers. Chest 2004;125:22-26.
51. Kannel WB, D'Agostino RB, Belanger AJ. Fibrinogen, cigarette smoking, and risk of cardiovascular disease: insights from the Framingham Study. Am Heart J 1987;113:1006-1010.
52. Smith FB, Lee AJ, Fowkes FG, Price JF, Rumley A, Lowe GD. Hemostatic factors as predictors of ischemic heart disease and stroke in the Edinburgh Artery Study. Arterioscler Thromb Vasc Biol 1997;17:3321-3325.
53. Smith CJ, Fischer TH. Particulate and vapor phase constituents of cigarette mainstream smoke and risk of myocardial infarction. Atherosclerosis 2001;158:257-267.
54. Petitti DB, Kipp H. The leukocyte count: associations with intensity of smoking and persistence of effect after quitting. Am J Epidemiol 1986;123:89-95.
55. Bermudez EA, Rifai N, Buring JE, Manson JE, Ridker PM. Relation between markers of systemic vascular inflammation and smoking in women. The Am J Cardiol 2002;89:1117-1119.
56. Tracy RP, Psaty BM, Macy E, et al. Lifetime smoking exposure affects the association of C-reactive protein with cardiovascular disease risk factors and subclinical disease in healthy elderly subjects. Arterioscler Thromb Vasc Biol 1997;17:2167-2176.
57. Hughes DA, Haslam PL, Townsend PJ, Turner-Warwick M. Numerical and functional alterations in circulatory lymphocytes in cigarette smokers. Clin Exp Immunol 1985;61:459-466.
58. Robbins CS, Dawe DE, Goncharova SI, et al. Cigarette smoke decreases pulmonary dendritic cells and impacts antiviral immune responsiveness. Am J Respir Cell Mol Biol 2004;30:202-211.
59. Moszczynski P, Zabinski Z, Moszczynski P, Jr., Rutowski J, Slowinski S, Tabarowski Z. Immunological findings in cigarette smokers. Toxicol Lett 2001;118:121-127.
60. Hersey P, Prendergast D, Edwards A. Effects of cigarette smoking on the immune system. Follow-up studies in normal subjects after cessation of smoking. Med J Aust 1983;2:425-429.
61. Silman AJ, Newman J, MacGregor AJ. Cigarette smoking increases the risk of rheumatoid arthritis. Results from a nationwide study of disease-discordant twins. Arthritis Rheum 1996;39:732-735.
62. Vessey MP, Villard-Mackintosh L, Yeates D. Oral contraceptives, cigarette smoking and other factors in relation to arthritis. Contraception 1987;35:457-464.
63. Prummel MF, Wiersinga WM. Smoking and risk of Graves' disease. JAMA 1993;269:479-482.
64. Bartalena L, Bogazzi F, Tanda ML, Manetti L, Dell'Unto E, Martino E. Cigarette smoking and the thyroid. Eur J Endocrinol 1995;133:507-512.
65. Calkins BM. A meta-analysis of the role of smoking in inflammatory bowel disease. Dig Dis Sci 1989;34:1841-1854.
66. Shan M, Cheng HF, Song LZ, et al. Lung myeloid dendritic cells coordinately induce TH1 and TH17 responses in human emphysema. Sci Transl Med 2009;1: 4ra10.
67. Makrygiannakis D, Hermansson M, Ulfgren AK, et al. Smoking increases peptidylarginine deiminase 2 enzyme expression in human lungs and increases citrullination in BAL cells. Ann Rheum Dis 2008;67:1488-1492.

68. Hans FJ, Wei L, Bereczki D, et al. Nicotine increases microvascular blood flow and flow velocity in three groups of brain areas. Am J Physiol 1993;265:H2142-2150.
69. Michnovicz JJ, Naganuma H, Hershcopf RJ, Bradlow HL, Fishman J. Increased urinary catechol estrogen excretion in female smokers. Steroids 1988;52:69-83.
70. Baron JA, La Vecchia C, Levi F. The antiestrogenic effect of cigarette smoking in women. Am J Obstet Gynecol 1990;162:502-514.
71. Hardy R, Kuh D, Wadsworth M. Smoking, body mass index, socioeconomic status and the menopausal transition in a British national cohort. Int J Epidemiol 2000;29:845-851.
72. Cutolo M, Sulli A, Capellino S, et al. Sex hormones influence on the immune system: basic and clinical aspects in autoimmunity. Lupus 2004;13:635-638.
73. Marrie RA, Cutter G, Tyry T, Campagnolo D, Vollmer T. Smoking status over two years in patients with multiple sclerosis. Neuroepidemiology 2009;32:72-79.
74. Handel AE, Handunnetthi L, Giovannoni G, Ebers GC, Ramagopalan SV. Genetic and environmental factors and the distribution of multiple sclerosis in Europe. Eur J Neurol 2010;17:1210-1214.
75. Palacios N, Alonso A, Bronnum-Hansen H, Ascherio A. Smoking and increased risk of multiple sclerosis: parallel trends in the sex ratio reinforce the evidence. Ann Epidemiol 2011;21:536-542.
76. Hawkes CH. Are multiple sclerosis patients risk-takers? Qjm 2005;98:895-911.
77. Marrie R, Horwitz R, Cutter G, Tyry T, Campagnolo D, Vollmer T. High frequency of adverse health behaviors in multiple sclerosis. Mult Scler 2009;15:105-113.
78. Nielsen TR, Pedersen M, Rostgaard K, Frisch M, Hjalgrim H. Correlations between Epstein-Barr virus antibody levels and risk factors for multiple sclerosis in healthy individuals. Mult Scler 2007;13:420-423.
79. Levin LI, Munger KL, Rubertone MV, et al. Temporal relationship between elevation of Epstein-Barr virus antibody titers and initial onset of neurological symptoms in multiple sclerosis. JAMA 2005;293:2496-2500.
80. Ascherio A, Munger KL, Lennette ET, et al. Epstein-Barr virus antibodies and risk of multiple sclerosis: a prospective study. JAMA 2001;286:3083-3088.
81. DeLorenze GN, Munger KL, Lennette ET, Orentreich N, Vogelman JH, Ascherio A. Epstein-Barr virus and multiple sclerosis: evidence of association from a prospective study with long-term follow-up. Arch Neurol 2006;63:839-844.
82. Hedström AK, Sundqvist E, Baarnhielm M, et al. Smoking and two human leukocyte antigen genes interact to increase the risk for multiple sclerosis. Brain 2011;134:653-664.
83. van der Mei IA, Ponsonby AL, Dwyer T, et al. Past exposure to sun, skin phenotype, and risk of multiple sclerosis: case-control study. BMJ 2003;327:316.
84. Embry AF, Snowdon LR, Vieth R. Vitamin D and seasonal fluctuations of gadolinium-enhancing magnetic resonance imaging lesions in multiple sclerosis. Ann Neurol 2000;48:271-272.
85. Munger KL, Levin LI, Hollis BW, Howard NS, Ascherio A. Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. JAMA 2006;296:2832-2838.
86. Ascherio A, Munger KL, Simon KC. Vitamin D and multiple sclerosis. Lancet Neurol 2010;9:599-612.
87. Spach KM, Nashold FE, Dittel BN, Hayes CE. IL-10 signaling is essential for 1,25-dihydroxyvitamin D3-mediated inhibition of experimental autoimmune encephalomyelitis. J Immunol 2006;177:6030-6037.
88. Brown SJ. The role of vitamin D in multiple sclerosis. Ann Pharmacother 2006;40:1158-1161.
89. Morabia A, Bernstein MS, Antonini S. Smoking, dietary calcium and vitamin D deficiency in women: a population-based study. Eur J Clin Nutr 2000;54:684-689.
90. Merlino LA, Curtis J, Mikuls TR, Cerhan JR, Criswell LA, Saag KG. Vitamin D intake is inversely associated with rheumatoid arthritis: results from the Iowa Women's Health Study. Arthritis Rheum 2004;50:72-77.
91. Scolding N, Franklin R. Axon loss in multiple sclerosis. Lancet 1998;352:340-341.

92. Motz GT, Eppert BL, Wortham BW, et al. Chronic cigarette smoke exposure primes NK cell activation in a mouse model of chronic obstructive pulmonary disease. J Immunol 2010;184:4460-4469.

93. Fusby JS, Kassmeier MD, Palmer VL, et al. Cigarette smoke-induced effects on bone marrow B-cell subsets and CD4+:CD8+ T-cell ratios are reversed by smoking cessation: influence of bone mass on immune cell response to and recovery from smoke exposure. Inhal Toxicol 2010;22:785-796.

94. Tremlett H, van der Mei IA, Pittas F, et al. Monthly ambient sunlight, infections and relapse rates in multiple sclerosis. Neuroepidemiology 2008;31:271-279.

Chapter 6.2

Cigarette smoking and risk of MS in multiplex families

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Abstract

Background Recent studies suggest that a history of cigarette smoking is a risk factor for multiple sclerosis (MS).

Objective We aimed to test the smoking effect in multiplex families, matching for both environmental and genetic factors.

Methods In a matched case-control study, 136 MS patients from 106 multiplex MS families were compared with their 204 healthy siblings as controls. Participants completed self-report questionnaires. Conditional logistic regression was used to analyse smoking and MS risk association while controlling for confounding by age and sex. Smoking history was classified in different variables.

Results Within our survey the smoking history of MS patients and the controls did not differ. The odds of MS were comparable for different smoking levels. However, more intense exposure and women showed higher odds ratios, although non-significant.

Conclusion Association studies in families with relatively high genetic loading are unlikely to be confounded by smoking history.

Introduction

While progress is being made in the identification of genetic risk factors in multiple sclerosis (MS), evidence is accumulating for the relevance of environmental risk factors in MS pathogenesis.[1] The incidence of MS is increasing, due to yet unknown non-genetic factors.[2] Smoking is one of the few consistent factors that have been shown to be associated with development of MS.[1] The possible role of smoking in MS pathogenesis has been linked to immunomodulatory effects of cigarette components (nicotine or tobacco glycoprotein).[3] A meta-analysis done by Hawkes in 2007 indicated an odds ratio (OR) of 1.34 ($p<0.001$) for the increased risk after smoking.[4] Most such studies have thus far been performed in sporadic MS patients and unrelated controls.[4-6] Studies testing gene-environment interactions in MS are scarce. A family-based case-control study using siblings as controls will better match for environmental and genetic factors. MS risk is higher in multiplex MS families (two or more affected children), indicating higher genetic loading of the MS risk genes. Therefore, we assessed whether smoking contributes to MS risk in a family-based case-control study with an enhanced genetic predisposition and whether smoking could be an important covariate for studies on genetic risk factors for MS.

Methods

Subjects in this study belong to multiplex families and were recruited from our outpatient ErasMS clinic or were referred to us by other neurological clinics in the Netherlands because of our ongoing study on gene-environment interaction in MS. Family and medical history were collected using a standard assessment and MS diagnosis was verified according to standard McDonald criteria for dissemination in time and space.[7] Age at onset was defined as the recorded age in medical reports at the time of the first signs of MS. Probands without verified diagnosis were excluded. Healthy siblings were included in the multiplex family study as controls and were questioned about neurological signs suggestive for MS.

Inclusion

A total of 150 MS patients and 265 unaffected siblings from the family ErasMS database were included. Self-report questionnaires, including questions on a detailed smoking history, were mailed to the sibships. The overall response rate was 87% (362 participants). For this analysis, we included 106 sibships containing at least one affected sibling (n = 136) and one unaffected sibling control (n = 204). Six patients not fulfilling the McDonald criteria[7] were excluded. Three controls were excluded because of incomplete questionnaires and two controls because they reported signs of possible MS. The other 11 participants were excluded from the analysis due to incomplete sibships as a result of previous exclusions.

Statistics

To assess the association between cigarette smoking and risk of MS, we used conditional logistic regression models, matching case and control subjects by sibship to account for familial correlations in the data. The outcome variable was the risk of MS, with MS patients (cases) compared with their unaffected siblings (controls). To better establish temporal sequence of disease and exposure, we measured cigarette smoking relative to a reference age within a sibship preceding MS diagnosis. This reference age was defined as the mean age of onset of MS in the affected siblings.

Smoking history

The questionnaire was similar to the one used by the Parkinson study group in Durham, NC, USA.[8] The enquiry form contained questions on ever/never smoking, current smoking, starting and quitting dates, dates of non-smoking periods (minimal duration of one year) and number of cigarettes smoked in different periods. Ever smokers were defined as individuals who smoked at least 100 cigarettes in their lifetime, smoked at least weekly and started smoking prior to the reference age. Ever smokers were classified as current smokers who smoked at reference age and otherwise as past smokers. The ever, current and past smokers were compared with a reference group of never smokers. For the ever smokers based on the smoking history prior to reference age, duration of smoking and packs smoked per day were calculated. To examine the combined effect of duration and dosage, the pack-years measure (defined as 20 cigarettes smoked per day for one year) was used. For each classification two indicator variables were constructed using the median value within the group, based on individuals who began smoking prior to the reference age (table 1). These two exposure levels were compared with the never smokers.[8]

Conditional logistic regression models were constructed for all variables, controlling for confounding by age and sex. Current age was included in the analyses as a continuous variable, and sex was included as a dichotomous variable, with female as the referent level. Because the association between MS and smoking can be influenced by sex and age of onset, further analyses stratified by these confounders were conducted. For analysis by age, we stratified sibships into two groups based on the median age at onset in the sibship; one group containing participants with age at onset prior to the median age of 32 years, and another with onset at or after 32 years. For gender specific analysis, we considered only same-sex siblings. Adjusted ORs (and 95% confidence interval (CI)) were calculated for each model, and p values < 0.05 were considered significant. All analyses were performed using SPSS version 14.0.

Results

This study included 106 sibships, with a mean of 1.3 (range: 1 to 3) affected and 1.9 (range: 1 to 6) of unaffected individuals in a sibship. Of the 136 patients, 86 (63%) were women, the mean age at study enrolment was 52.6 ± 11.4 years (range: 30 to 81 years), and the mean age at onset of clinical signs of MS was 32.9 ± 9.5 years (range: 13 to 56 years). Of the study patients 78% had a relapse onset and 22% had a primary progressive onset. Among the 204 controls, 92 (45%) were women, and the mean age at study enrolment was 52.8 ± 11.6 years (range: 22 to 79 years). Percentages of ever smokers were comparable in both groups (table 1). As expected, individuals with MS were more likely to be women (OR 2.0; 95% CI: 1.34-3.27; p = 0.002). Given the bilateral relation of gender with MS and with smoking,[9] gender was considered to be an important confounder.

Table 1 shows the odds of MS for different levels of smoking controlled for sex and current age. The various smoking measurements were unrelated to MS. However, the groups with more intense exposure dose or longer smoking duration showed slightly higher, but non-significant, risk of MS.

Table 1: Relation between cigarette smoking and multiple sclerosis in 106 multiplex families.

	% Exposed[a]		OR[c] (95%CI) with exposure measured at reference age
Model	Cases N=136	Controls N=204	
Ever smoked			
Yes	64%	65%	1.09 (0. 68-1.73)
No (referent)	36%	35%	1.0
Smoking history			
Current	43%	46%	1.03 (0.61-1.73)
Past	21%	19%	1.19 (0.65-2.20)
Never (referent)	36%	35%	1.0
p trend			*0.893*
Smoking duration[b]			
≥12 years	32%	36%	1.07 (0.61- 1.89)
<12 years	32%	30%	1.05 (0.62- 1.80)
Never (referent)	36%	34%	1.0
p trend			*0.803*
Packs smoked per day[b]			
≥ 0.7	35%	31%	1.30 (0.74- 2.31)
< 0.7	29%	34%	0.93 (0.54- 1.60)
Never (referent)	36%	35%	1.0
p trend			*0.416*
Pack-years[b]			
≥ 7.9	32%	32%	1.16 (0.65- 2.08)
< 7.9	32%	32%	1.03 (0.61- 1.75)
Never (referent)	36%	35%	1.0
p trend			*0.623*

[a]Analyses do not take matching by sibships into account. 1 patient and 3 controls have missing- data on "packs/day" and "pack-years".
[b]Categories formed by dichotomizing continuous variable at median values among individuals who ever smoked.
[c]Conditional logistic regression analyses adjusted for current age and sex.

We also studied the effect of smoking after stratification for either sex or age at time of first clinical signs suggestive of MS (table 2). Within siblings with earlier disease onset, the risk of MS is slightly higher for ever smokers and for longer smoking duration, compared with siblings with an older age of onset. The ORs for different smoking measurements were slightly higher in female patients. All results were not significant (table 2).

Table 2: The relation between cigarette smoking and multiple sclerosis in 106 sibships, after stratification by age at onset and sex.

	OR (95%CI) with exposure measured at reference age			
Stratification by:	Onset age <32 yr 54 sibships (cases=67, controls=104)[b]	Onset age ≥ 32 yr 52 sibships (cases=69, controls=100)[b]	Women 45 sibships (cases=53, controls=73)[c]	Men 32 sibships (cases=36, controls=49)[c]
Ever smoked				
Yes	1.19 (0.59- 2.38)	1.10 (0.57- 2.11)	1.26 (0.61- 2.61)	0.97 (0.37- 2.57)
No (referent)	1.0	1.0	1.0	1.0
Smoking history				
Current	1.19 (0.58- 2.45)	0.98 (0.44- 2.16)	1.35 (0.55- 3.33)	1.17 (0.41- 3.37)
Past	1.14 (0.38- 3.45)	1.23 (0.57- 2.64)	1.17 (0.46- 2.97)	0.62 (0.15- 2.50)
Never (referent)	1.0	1.0	1.0	1.0
p trend	*0.632*	*0.991*	*0. 499*	*0.751*
Smoking duration[a]				
≥12 years	1.29 (0.45- 3.73)	1.05 (0.52- 2.13)	1.24 (0.49- 3.15)	1.00 (0.32- 3.12)
<12 years	1.15 (0.56- 2.37)	1.21 (0.51- 2.86)	1.17 (0.51- 2.69)	0.78 (0.24- 2.60)
Never (referent)	1.0	1.0	1.0	1.0
p trend	*0.615*	*0.900*	*0.622*	*0.986*
Packs smoked per day[a]				
≥ 0.7	1.36 (0.61- 3.01)	1.48 (0.63- 3.49)	1.68 (0.63- 4.45)	0.94 (0.33- 2.71)
< 0.7	0.99 (0.42- 2.31)	0.93 (0.45- 1.92)	0.95 (0.39- 2.31)	1.02 (0.31- 3.32)
Never (referent)	1.0	1.0	1.0	1.0
p trend	*0.461*	*0.453*	*0.378*	*0.906*
Pack-years[a]				
≥ 7.8	0.98 (0.39- 2.47)	1.28 (0.59- 2.79)	1.44 (0.57- 3.67)	1.06 (0.36- 3.08)
< 7.8	1.28 (0.61- 2.72)	0.95 (0.44- 2.05)	1.08 (0.47- 2.51)	0.82 (0.22- 3.07)
Never (referent)	1.0	1.0	1.0	1.0
p trend	*0.931*	*0.558*	*0.466*	*0.922*

[a]Categories formed by dichotomizing continuous variable at median values among individuals who ever smoked.
[b]Stratification by median value of the age at onset in affected siblings, conditional logistic regression analyses adjusted for sex and current age.
[c]Only considering same-sex discordant sibships, conditional logistic regression analyses adjusted for current age.

Discussion

This study found no significant association of cigarette smoking and MS in multiplex families with shared genetic-environmental risk factors.

Our results differ from studies that showed significantly elevated odds ratios, indicating an increased risk of MS for those who smoked prior to the onset of disease.[4] A possible explanation for this dissimilarity can be found in our study design. Although matching of cases to their unaffected sibling reduced confounding by ethnic background and environmental factors, there are some side effects.

One of these unintended effects can be overmatching of the smoking history within siblings. This can be a result of similar smoking behaviour within families[10] as well as matching for unmeasured genetic factors which may predispose to smoking behaviour.[11] Furthermore, exposure to passive smoking in childhood and adolescence could act as a confounder.[12] However, in addition using multiplex families can possibly have blurred the association of smoking and MS by the relatively stronger genetic contribution in such families. This phenomenon has also been suggested in migraine studies,[13] where the effect of exogenous factors is predictive only in non-familial migraine cases.

In addition to the study design, our survey may also have been influenced by recall bias. However, since smoking is generally expected to be positively associated with disease, one would expect over-reporting of smoking with a false positive response.

Although our results are negative regarding the association of smoking and MS, it is to be noted that stratification by sex showed slightly higher (non-significant) ORs among women. This may be a clue in the direction of a possible gender effect. As this has not been mentioned in the meta-analysis, where two out of six studies included 100% women,[4] gender interaction needs to be considered. Moreover, recently smoking is mentioned as one of the candidates that may influence the increase of MS incidence in women.[2]

In conclusion, this study found no association for smoking and risk of MS, which is assessed for the first time in multiplex families. This implies that smoking is not to be considered as a confounder in future association studies performed in families. Also, the indication of a possible gender effect of smoking and MS deserves further attention.

References

1. Giovannoni G, Ebers G. Multiple sclerosis: the environment and causation. Curr Opin Neurol 2007;20:261-268.
2. Orton SM, Herrera BM, Yee IM, et al. Sex ratio of multiple sclerosis in Canada: a longitudinal study. Lancet Neurol 2006;5:932-936.
3. Sopori ML, Kozak W, Savage SM, Geng Y, Kluger MJ. Nicotine-induced modulation of T Cell function. Implications for inflammation and infection. Adv Exp Med Biol 1998;437:279-289.
4. Hawkes CH. Smoking is a risk factor for multiple sclerosis: a metanalysis. Mult Scler 2007;13:610-615.
5. Hernan MA, Jick SS, Logroscino G, Olek MJ, Ascherio A, Jick H. Cigarette smoking and the progression of multiple sclerosis. Brain 2005;128:1461-1465.
6. Villard-Mackintosh L, Vessey MP. Oral contraceptives and reproductive factors in multiple sclerosis incidence. Contraception 1993;47:161-168.
7. McDonald WI, Compston A, Edan G, et al. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. Ann Neurol 2001;50:121-127.
8. Scott WK, Zhang F, Stajich JM, Scott BL, Stacy MA, Vance JM. Family-based case-control study of cigarette smoking and Parkinson disease. Neurology 2005;64:442-447.
9. Morley KI, Hall WD. Explaining the convergence of male and female smoking prevalence in Australia. Addiction 2008;103:487-495.
10. Hamilton AS, Lessov-Schlaggar CN, Cockburn MG, Unger JB, Cozen W, Mack TM. Gender differences in determinants of smoking initiation and persistence in California twins. Cancer Epidemiol Biomarkers Prev 2006;15:1189-1197.
11. Cheng LS, Swan GE, Carmelli D. A genetic analysis of smoking behavior in family members of older adult males. Addiction 2000;95:427-435.
12. Mikaeloff Y, Caridade G, Tardieu M, Suissa S. Parental smoking at home and the risk of childhood-onset multiple sclerosis in children. Brain 2007;130:2589-2595.
13. Bigal ME, Lipton RB, Winner P, Reed ML, Diamond S, Stewart WF. Migraine in adolescents: association with socioeconomic status and family history. Neurology 2007;69:16-25.

Part V

Discussion

Chapter 7

General discussion and future perspectives

Multiple sclerosis (MS) is a complex disease, which results from several factors including genetics, epigenetics, and the environment. The fact that young adults in the most reproductive years of their lives are often facing a precarious future emphasizes the importance of better understanding and prediction of the disease.

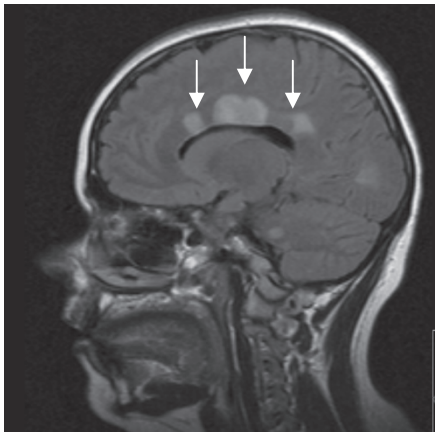
The primary aim of this thesis was to demonstrate the prognostic value of a few selected risk factors involved in MS and to describe their interactions. This chapter summarizes the main findings of the studies presented in this thesis and places them in a broader perspective. Finally, directions for future research are discussed.

MRI as a clinical tool for prediction of MS

One of the first, easy to study risk factors for diagnosing MS is the clinical presentation. Even the very first episode suggestive of demyelination, named clinically isolated syndrome (CIS), provides some prognostic information. Not only the neuro-anatomical localisation of the first attack, but also the ability to recover completely, has provided us with some predictive information. Patients whose attack involves the brainstem or motor function, and patients who do not recover completely after an attack, await a bleaker future.[1] MRI has been shown to be a useful clinical tool in predicting a second attack after CIS but is also used as a diagnostic tool. In 1997 the MRI was implemented in predicting and diagnosing MS using the Barkhof criteria,[2] and these criteria have been simplified over time.[3] In **chapter 2**, we showed that the presence of a corpus callosum lesion on MRI is an important risk factor for the development of MS after CIS, independent of the Barkhof criteria. Furthermore, we reported that the predictive value of a corpus callosum lesion increases when it is combined with the Barkhof criteria. Interestingly, we demonstrated that a single corpus callosum lesion in patients not fulfilling the Barkhof criteria increases the risk of developing MS four fold. In conclusion, corpus callosum lesions are easy to assess parameters with a predictive value comparable to the Barkhof criteria. Preferably, the corpus callosum lesion should be scored on a sagittal fluid-attenuated inversion recovery (FLAIR) image (figure 1).
However, corpus callosum lesions are not exclusive to MS and can also be seen in other diseases such as central nervous system (CNS) infection, other inflammatory CNS diseases, lymphoma, metabolic diseases, metastatic or vascular diseases.[4] Moreover, neuropathological and imaging studies have shown that MS pathology goes well beyond white matter lesions visible on conventional MRI sequences.[5,6] MS lesions are surrounded with subtle abnormalities presented in the normal appearing white matter. Surprisingly, studies have shown lesions in the grey matter, especially in the cerebral cortex.[6,7] These cortical lesions can be detected at the earliest clinical stages of MS, and the lesion burden positively correlates with the severity of physical and cognitive impairments.[7-9] However, the latest MRI technologies and expertise

to apply these criteria are not yet available in all clinical neurological settings around the world. Therefore, simplification of the diagnostic criteria is useful and in line with this, we argue that corpus callosum lesion is a valuable risk parameter independent of prevailing criteria and deserves further study.

Figure 1: Corpus callosum lesions.



Sagittal fluid-attenuated inversion recovery (FLAIR) image showing corpus callosum lesions (white arrows).

Another longstanding clinical tool for MS diagnosis is cerebrospinal fluid (CSF). This is used for investigation of neuroinflammatory diseases and was included in the diagnostic criteria to support the MRI requirement for dissemination in space in relapsing remitting MS (RRMS) but also in the diagnostic criteria for primary progressive MS. Moreover, it is included as one of the requisites for MS diagnosis namely exclusion of alternative causes by using additional paraclinical tests including CSF analysis.[10,11] Recent studies in patients with CIS demonstrated that a positive CSF finding (intrathecal oligoclonal immunoglobulin G (IgG) bands or higher IgG index) significantly increases the risk of conversion to MS independently of MRI results, and the predictive value increases by combining MRI and CSF findings.[12] In the 2010 revisions to the McDonald criteria by simplifying the diagnostic process for RRMS, the CSF findings were excluded from the criteria. This has raised discussion about the utility and correct interpretation of CSF analysis.[13,14] Further studies are needed to explore the value of CSF findings in the light of the new criteria. It is necessary to study whether the inclusion of oligoclonal IgG bands and/or IgG index in the recent published criteria can help increase the sensitivity and/or specificity of the criteria.

Genetic determinants of cause and course of MS

Next to aggregation of MS in families, the increasing number of risk genes associated with MS is an important determinant of MS susceptibility. Starting from alleles of the human leukocyte antigen (HLA) class II region, many new risk alleles of MS are identified over time. Genome-wide association (GWA) studies and international collaboration were mainly the basis for this rapid development. In **chapter 3**, we used weighted risk scores and showed that the predictive value of multiple genes is mainly driven by the *HLA-DR* locus, which is caused by the large effect size and the high frequency of *HLA-DRB1*1501*. Recently a large GWA study replicated 24 of the previously suggested GWA associations and identified a further 29 novel susceptibility loci.[15] Using the 53 well replicated and novel loci, we demonstrated that even more than a doubling of the known risk alleles gives only a marginal increase in the discriminating value between cases and controls. Furthermore, we performed a simulation study and demonstrated that in order to obtain higher discriminative value, a considerable number of additional common genetic variants or a few strong associated variants with high odds ratios need to be identified. We concluded that even in the future, a clinically useful predictive model only based on risk genes seems unfeasible. Probably, the attributable risk is limited because of the small effect size and the low frequency of the risk alleles. Individually, the non-HLA SNPs identified till today contribute a small proportion to overall MS risk, with allelic odds ratios between 1.1 - 1.3.[15]
In conclusion, genetic risk factors have only a small effect on susceptibility to MS as was already suggested by epidemiological studies showing 70% discordance in monozygotic twins. However, in the future the growing numbers of risk genes in combination with gene-gene interaction will bring us to a better understanding of the pathogenesis of MS. Perhaps the main inference of genetic studies so far has been the recognition that most of the identified risk loci are involved in immune system function. Although the Gene Ontology immune system genes only account for 7% of human genes, interestingly in 30% of the associated regions shown in the recent Nature study, the nearest gene to the lead SNP was an immune system gene. This information and studies on gene-environment interaction will lead to a further understanding of MS pathology.

Whereas genetic influence on the risk of MS is well established, there is little known about a possible association of genes with MS disease course and severity. Some studies have suggested an association of *HLA-DR* with MS disease course[16] or increased lesion load at MS onset.[17] Others did not find any evidence that MS severity or progression was altered by recent non-HLA risk alleles in MS patients.[18,19] Progression is considered to reflect chronic demyelination and axonal loss, manifesting as the neurodegenerative process.
It has been shown that MS consists of both an inflammatory and a progressive

neurodegenerative process,[20] which is illustrated by the fact that new and potent anti-inflammatory drugs have been unable to halt neurodegeneration. In 2008, we reported that the *KIF1B* variant rs10492972 was associated with MS. However, till today this association could not be replicated in other studies.[21] The kinesin super-family members are responsible for axonal transport of mitochondria and synaptic vesicle precursors.[22,23] Irreversible axonal loss is an important mechanism in the development of permanent neurological symptoms.[24,25] Therefore, we hypothesised that this SNP perhaps explains some of the neurodegenerative phenotypic differences between MS patients. In **chapter 4**, we concluded that there is no evidence for a determining influence of carriership of the risk allele or genotype of the *KIF1B* gene on any of the neurodegenerative phenotypic markers, such as clinical measures (Multiple Sclerosis Severity Scores and Multiple Sclerosis Functional Composite Scores) or MRI measures like lesion load and atrophy.
Recent studies identified *KIF21B* and *KIF5A*, which are also kinesin-like proteins involved in axonal transport, as MS risk factors.[26,27] Although *KIF21B* has not been functionally associated with neurodegeneration or inflammation, given the nature and role of its protein in neurons a possible role in MS is suggested. *KIF5A* has previously been associated with other auto-immuun diseases such as rheumatoid arthritis and type 1 diabetes.[28,29] Neurodegeneration in MS deserves further studies for better understanding of the disease.

Environmental risk factors in MS

The influence of migration,[30] latitude,[31] and month of birth[32] on MS prevalence strongly suggests a role for the environment. This has been further strengthened by the evidence on the rising worldwide incidence and increasing female to male ratio.[33] However, determining the role of environmental risk factors in aetiology of MS is difficult because several factors are capable of causing MS in a genetically susceptible individual, and it is not feasible to study only one factor by correcting others. In this thesis we elucidated in more detail the two most promising environmental risk factors; Epstein-Barr virus (EBV) **(chapter 5)** and cigarette smoking **(chapter 6)**.

Is EBV the missing link in MS?

There is accumulating evidence through epidemiological and serological studies for the role of EBV in MS, underlined by the association of infectious mononucleosis (IM) and MS.[34-37] The similarity between IM and MS in terms of age, geographical distribution, socioeconomic status, and ethnicity is striking.[38] Although we are learning more and more about EBV and its association with MS, the cause and pathogenic pathways involved remain enigmatic. A possible explanation for the association of higher MS risk and IM is that there is a common factor involved in both diseases (figure 2A). The correlation of the age of infection with EBV

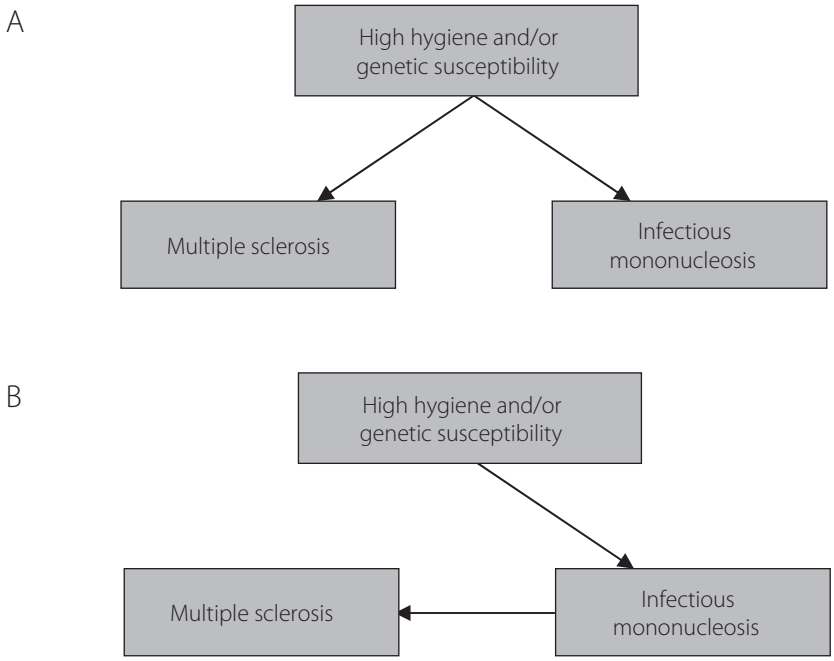
and MS might reflect the confounding effect of more general hygienic conditions.
Infection with EBV at older age in EBV seronegative individuals causes a severe immune reaction resulting in higher EBV antigen levels and hence IM. The late onset EBV infection can imbalance the immune system, which may explain the suggested higher EBV reactivity as shown by a marker of EA antibody levels in **chapter 5.3**. The suggested association between EBV and pediatric MS implies that there are more mechanisms linking EBV and MS than the delayed infection as involved in the hygiene hypothesis.[39,40] Another common factor involved in both diseases could be shared genetic susceptibility. The immune dysregulation in an individual with a certain genetic susceptibility can be the constitutional factor which plays a role in anti-EBV reaction and MS. This can also explain the low MS risk in EBV negative individuals as genetically resistant to both EBV infection and MS.[41] In **chapter 5.2** we demonstrated that HLA class I could be the shared genetic risk factor, as being associated with both IM and MS.

Another explanation for the association of higher MS risk and IM could be that the high hygiene in childhood or the genetic susceptibility increases the risk of late EBV infection leading to IM. This could disrupt the immune system and push it over the threshold inducing autoimmune diseases like MS (figure 2B). This hypothesis is supported by the fact that EBV predisposes to autoimmunity in general, as suggested by a positive association with systemic lupus erythematosus.[42] Possible mechanisms involved in the generation of autoimmunity by EBV include infection of auto-reactive B-cells and immortalization, which could produce auto-antibodies and act as professional antigen-presenting cells in the target organ. Other possible mechanisms are bystander activation, non-specific general up-regulation of the immune system, and molecular mimicry. Molecular mimicry is the cross-reactivity between self antigens and microbial antigens. Interestingly, cross-reactivity between EBV viral structures and myelin antigens inducing demyelination has been described.[43,44]

The role of EBV infection in MS is also demonstrated by enhanced systemic B- and T-cells responses to EBV in MS patients compared to controls. An elevation in antibody titres to specific EBV antigen (EBV nuclear antigen-1) many years before the onset of neurological symptoms is shown by others.[37,45-47] Anti-EBNA-1 is a protein consistently expressed in latently infected B lymphocytes, and the titres decline by immune suppression.[48] Increased EBV antibody levels in CSF[43] and EBV infected B-cells in MS lesions in the brain shown by Serafini et al[49] imply a causative role for EBV. However, our group and several others could not confirm the presence of the EBV virus in MS lesions.[50,51] In **chapter 5.1**, we demonstrated that EBNA-1-specific IgG responses are elevated both in serum and CSF of MS patients compared to controls. However, after correction for the possible dysfunction of the blood-brain barrier by normalizing for total IgG, we found no evidence for intrathecal IgG synthesis. This argues against the intrathecal presence of EBV in MS. Nonetheless, the possibility remains that EBV-specific T-cells generated

in the peripheral immune system play a role in MS pathogenesis. In that scenario, there is no need for intrathecal presence of EBV but merely an immunomodulating role of this virus in the periphery may be sufficient.

Figure 2: The hypothesis of (common) pathway for multiple sclerosis and infectious mononucleosis.



The association of multiple sclerosis and infectious mononucleosis could be explained by a possible common cause for both diseases (A) or a causal relation between them (B). The common cause could be high hygiene or genetic susceptibility. MS and IM can arise from the high hygiene (absence of EBV infection during childhood) and/or genetic factors (A). High hygiene in childhood and/or genetic factors can increase the likelihood of a late age at infection with EBV (IM) leading to an immune dysregulation and increased risk of MS (B).

Interestingly, searching for a common genetic factor for IM and MS, in **chapter 5.2**, we studied and argued that a *HLA-A02* polymorphism is possibly the common underlying factor for IM and MS. Moreover, in **chapter 5.3**, we suggested an interaction between this HLA-class I SNP and EBV reactivity. The association with HLA-class I genes provides ample evidence for the additional pathogenic role of CD8 T-cells and environmental factors like EBV to induce MS in genetically susceptible individuals.[52-54]

The question is how peripheral EBV specific T-cells may play a role in MS. Even though evidence for the presence of EBV in MS lesions is insufficient, EBV may evoke T-cell mediated MS immunopathology in different ways.[43,55-60] Virus-specific T-cells may recognize EBV-

infected cells in the brain or cross-react with (non-)myelin antigens (i.e. molecular mimicry). Alternatively, IM-associated systemic B-cell activation may activate peripheral blood T-cells, including neuroantigen-specific T-cells, in a non-specific manner. As EBV can affect trafficking of (self)peptide during antigen presentation, also here there may be a triggering contribution to autoimmunity. In all scenarios, activated T-cells may enter the brain upon recognition of locally expressed cognate antigens, and subsequently initiate and perpetuate MS immunopathology.[53,61,62]

Considering the strong association of MS with IM, vaccination with a recombinant protein neutralizing EBV infectivity could be a possible approach. As suggested by Lauer[63], for a possible future treatment, it is perhaps possible to eliminate EBV-infected B-cells by reducing more generally the number of B-cells for example, by the recombinant monoclonal antibody Rituximab, by vaccination with recombinant protein itself, by monoclonal antibodies to recombinant protein, or by transfer of autologous EBV-specific cytotoxic T-cells; these are all different ways to keep EBV reactivity low.

Cigarette smoking

The picture of how environmental exposures lead to autoimmune diseases in genetically predisposed individuals is becoming more comprehensive due to the numerous studies in different fields. Cigarette smoking as one of the promising environmental factors in cause and course of MS has been investigated in **chapter 6.1**. In this thesis we presented a review of studies performed on association of cigarette smoking and MS susceptibility and progression. There are a few studies which gave us more insight in the role of smoking in MS. Recently, passive exposure to tobacco smoke has been linked to MS.[64] It has been demonstrated that tobacco and not the use of sniff is associated with increased MS risk. A recent meta-analysis demonstrated that cigarette smoking is important in determining MS susceptibility and that there is a possible effect on disease progression.[65] It is to be noted that investigating smoking can be complicated by known and unknown confounders, such as alcohol intake and body mass index.[66,67] These possible risk factors can influence the observed association between smoking cigarettes and MS. Recently, it was demonstrated that the association between smoking and MS was dependent of anti-EBNA antibody titres.[68] Also, the association between increasing anti-EBNA antibody titres and MS risk was twofold greater among ever smokers compared to non-smokers. Next to an interaction between smoking and anti-EBNA titres, there was an interaction between smoking and *HLA-DR15* and *HLA-A02*. In light of other studies investigating environmental factors, it seems that the plausible immunomodulatory effect of smoking next to other environmental factors plays an important role in the MS onset and course.

Given the accumulating evidence for the association of cigarette smoking and MS risk, and our large number of genetic studies in MS multiplex families, we aimed to trace a possible confounding by smoking history in the association studies with relatively high genetic load. In **chapter 6.2**, we demonstrated that the smoking history of MS patients and related controls did not differ. Our results contrast earlier studies indicating an increased risk of MS for smokers.[65] An explanation for this may be found in our study design. We matched cases to their unaffected siblings to reduce confounding by ethnic background and environmental factors. However, this may have resulted in overmatching because of possible similar smoking behaviour within families.[69] Furthermore, exposure to passive smoking in childhood and adolescence could act as a confounder.[70]
In conclusion, although cigarette smoking is not shown to be a confounder in family studies with high genetic load, further study of the role of cigarette smoking in MS and its interaction with other environmental factors is warranted.

Recommendations for future research
Despite recent advances, the exact causal interplay of MS risk factors remains largely unknown. MS displays several characteristics that are common to numerous autoimmune diseases, including moderate polygenic heritability, environmental factors, clinical and genetic heterogeneity, increased frequency in women, and genetic susceptibility. Genes within the HLA region encoding antigen-presenting molecules account for the largest part of the genetic susceptibility.[71] However, the discriminating ability, even after including more than 50 current-known non-HLA genes[15] is small. Moreover, we showed that the predictive value of future models including more common allelic variants is limited. We also demonstrated the association of EBV and cigarette smoking with MS susceptibility, and the interaction between EBV and HLA class I. However the predictive value of all the above-mentioned features is small. An important conclusion of this thesis is that focussing on one risk factor at a time will not bring fundamental changes in our understanding of MS pathogenesis. It is essential that future studies will combine genetic, environmental, and clinical assessments, especially for a better prediction of conversion to clinically definite MS after CIS. A major challenge for the next years is to further unravel the pathophysiology of MS by investigating the gene-gene and gene-environmental interactions.

As MS is one of the most common non-traumatic neurological diseases among young adults, the prediction of conversion to clinically definite MS after CIS is indispensable. Furthermore, till today the therapies for MS are only able to reduce relapse and related disease progression. With the current knowledge of the complexity of the disease, curative therapy seems not feasible. Present knowledge seems to indicate that it is important to start immunomodulatory

therapy early in the disease to slow down further damage. Therefore, an early recognition and prediction of MS is essential. We have already started with a nationwide prospective observational follow-up study including CIS patient and collecting clinical information in combination with biological samples, called PROUD (Predicting the Outcome of a Demyelinating event).

References

1. Degenhardt A, Ramagopalan SV, Scalfari A, Ebers GC. Clinical prognostic factors in multiple sclerosis: a natural history review. Nat Rev Neurol 2009;5:672-682.
2. Barkhof F, Filippi M, Miller DH, et al. Comparison of MRI criteria at first presentation to predict conversion to clinically definite multiple sclerosis. Brain 1997;120:2059-2069.
3. Polman CH, Reingold SC, Banwell B, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. Ann Neurol 2011;69:292-302.
4. Uchino A, Takase Y, Nomiyama K, Egashira R, Kudo S. Acquired lesions of the corpus callosum: MR imaging. Eur Radiol 2006;16:905-914.
5. Allen IV, McQuaid S, Mirakhur M, Nevin G. Pathological abnormalities in the normal-appearing white matter in multiple sclerosis. Neurol Sci 2001;22:141-144.
6. Raz E, Cercignani M, Sbardella E, et al. Clinically isolated syndrome suggestive of multiple sclerosis: voxelwise regional investigation of white and gray matter. Radiology 2010;254:227-234.
7. Dalton CM, Chard DT, Davies GR, et al. Early development of multiple sclerosis is associated with progressive grey matter atrophy in patients presenting with clinically isolated syndromes. Brain 2004;127:1101-1107.
8. Chard DT, Griffin CM, Rashid W, et al. Progressive grey matter atrophy in clinically early relapsing-remitting multiple sclerosis. Mult Scler 2004;10:387-391.
9. Sanfilipo MP, Benedict RH, Weinstock-Guttman B, Bakshi R. Gray and white matter brain atrophy and neuropsychological impairment in multiple sclerosis. Neurology 2006;66:685-692.
10. Miller DH, Weinshenker BG, Filippi M, et al. Differential diagnosis of suspected multiple sclerosis: a consensus approach. Mult Scler 2008;14:1157-1174.
11. Freedman MS, Thompson EJ, Deisenhammer F, et al. Recommended standard of cerebrospinal fluid analysis in the diagnosis of multiple sclerosis: a consensus statement. Arch Neurol 2005;62:865-870.
12. Tintore M, Rovira A, Rio J, et al. Do oligoclonal bands add information to MRI in first attacks of multiple sclerosis? Neurology 2008;70:1079-1083.
13. Tumani H, Deisenhammer F, Giovannoni G, et al. Revised McDonald criteria: The persisting importance of cerebrospinal fluid analysis. Ann Neurol 2011;70:520.
14. Galea I, Freedman MS, Thompson EJ. Cerebrospinal fluid analysis in the 2010 revised McDonald's multiple sclerosis diagnostic criteria. Ann Neurol 2011;70:183.
15. International Multiple Sclerosis Genetics Consortium, Wellcome Trust Case Control Consortium 2, Sawcer S, et al. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. Nature 2011;476:214-219.
16. Barcellos LF, Oksenberg JR, Begovich AB, et al. HLA-DR2 dose effect on susceptibility to multiple sclerosis and influence on disease course. Am J Hum Genet 2003;72:710-716.
17. Hauser SL, Oksenberg JR, Lincoln R, et al. Interaction between HLA-DR2 and abnormal brain MRI in optic neuritis and early MS. Optic Neuritis Study Group. Neurology 2000;54:1859-1861.
18. Lundström W, Greiner E, Lundmark F, et al. No influence on disease progression of non-HLA susceptibility genes in MS. J Neuroimmunol 2011;237:98-100.
19. Jensen CJ, Stankovich J, Van der Walt A, et al. Multiple sclerosis susceptibility-associated SNPs do not influence disease severity measures in a cohort of Australian MS patients. PloS One 2010;5:e10003.
20. Frohman EM, Filippi M, Stüve O, et al. Characterizing the mechanisms of progression in multiple sclerosis: evidence and new hypotheses for future directions. Arch Neurol 2005;62:1345-1356.
21. Gourraud PA. When is the absence of evidence, evidence of absence? Use of equivalence-based analyses in genetic epidemiology and a conclusion for the KIF1B rs10492972*C allelic association in multiple sclerosis. Genet Epidemiol 2011;35:568-571.

22. Boldogh IR, Pon LA. Mitochondria on the move. Trends Cell Biol 2007;17:502-510.
23. Nangaku M, Sato-Yoshitake R, Okada Y, et al. KIF1B, a novel microtubule plus end-directed monomeric motor protein for transport of mitochondria. Cell 1994;79:1209-1220.
24. DeLuca GC, Williams K, Evangelou N, Ebers GC, Esiri MM. The contribution of demyelination to axonal loss in multiple sclerosis. Brain 2006;129:1507-1516.
25. Dutta R, Trapp BD. Pathogenesis of axonal and neuronal damage in multiple sclerosis. Neurology 2007;68:S22-31.
26. International Multiple Sclerosis Genetics Consortium. Comprehensive follow-up of the first genome-wide association study of multiple sclerosis identifies KIF21B and TMEM39A as susceptibility loci. Hum Mol Genet 2010;19:953-962.
27. Goris A, Boonen S, D'Hooghe M B, Dubois B. Replication of KIF21B as a susceptibility locus for multiple sclerosis. J Med Genet 2010;47:775-776.
28. Cooper JD, Walker NM, Healy BC, et al. Analysis of 55 autoimmune disease and type II diabetes loci: further confirmation of chromosomes 4q27, 12q13.2 and 12q24.13 as type I diabetes loci, and support for a new locus, 12q13.3-q14.1. Genes Immun 2009;10:S95-120.
29. Raychaudhuri S, Remmers EF, Lee AT, et al. Common variants at CD40 and other loci confer risk of rheumatoid arthritis. Nat Genet 2008;40:1216-1223.
30. Dean G, McLoughlin H, Brady R, Adelstein AM, Tallett-Williams J. Multiple sclerosis among immigrants in Greater London. Br Med J 1976;1:861-864.
31. Hernan MA, Olek MJ, Ascherio A. Geographic variation of MS incidence in two prospective studies of US women. Neurology 1999;53:1711-1718.
32. Willer CJ, Dyment DA, Sadovnick AD, Rothwell PM, Murray TJ, Ebers GC. Timing of birth and risk of multiple sclerosis: population based study. BMJ 2005;330:120.
33. Alonso A, Hernan MA. Temporal trends in the incidence of multiple sclerosis: a systematic review. Neurology 2008;71:129-135.
34. Lünemann JD, Münz C. Epstein-Barr virus and multiple sclerosis. Curr Neurol Neurosci Rep 2007;7:253-258.
35. Thacker EL, Mirzaei F, Ascherio A. Infectious mononucleosis and risk for multiple sclerosis: a meta-analysis. Ann Neurol 2006;59:499-503.
36. Nielsen TR, Rostgaard K, Nielsen NM, et al. Multiple sclerosis after infectious mononucleosis. Arch Neurol 2007;64:72-75.
37. Levin LI, Munger KL, Rubertone MV, et al. Temporal relationship between elevation of epstein-barr virus antibody titers and initial onset of neurological symptoms in multiple sclerosis. JAMA 2005;293:2496-2500.
38. Ascherio A, Munger KL. Epstein-barr virus infection and multiple sclerosis: a review. J Neuroimmune Pharmacol 2010;5:271-277.
39. Pohl D, Krone B, Rostasy K, et al. High seroprevalence of Epstein-Barr virus in children with multiple sclerosis. Neurology 2006;67:2063-2065.
40. Alotaibi S, Kennedy J, Tellier R, Stephens D, Banwell B. Epstein-Barr virus in pediatric multiple sclerosis. JAMA 2004;291:1875-1879.
41. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part I: the role of infection. Ann Neurol 2007;61:288-299.
42. James JA, Kaufman KM, Farris AD, Taylor-Albert E, Lehman TJ, Harley JB. An increased prevalence of Epstein-Barr virus infection in young patients suggests a possible etiology for systemic lupus erythematosus. J Clin Invest 1997;100:3019-3026.
43. Cepok S, Zhou D, Srivastava R, et al. Identification of Epstein-Barr virus proteins as putative targets of the immune response in multiple sclerosis. J Clin Invest 2005;115:1352-1360.

44. Lang D, Vornhagen R, Rothe M, Hinderer W, Sonneborn HH, Plachter B. Cross-reactivity of Epstein-Barr virus-specific immunoglobulin M antibodies with cytomegalovirus antigens containing glycine homopolymers. Clin Diagn Lab Immunol 2001;8:747-756.
45. Ascherio A, Munger KL, Lennette ET, et al. Epstein-Barr virus antibodies and risk of multiple sclerosis: a prospective study. JAMA 2001;286:3083-3088.
46. DeLorenze GN, Munger KL, Lennette ET, Orentreich N, Vogelman JH, Ascherio A. Epstein-Barr virus and multiple sclerosis: evidence of association from a prospective study with long-term follow-up. Arch Neurol 2006;63:839-844.
47. Sundström P, Juto P, Wadell G, et al. An altered immune response to Epstein-Barr virus in multiple sclerosis: a prospective study. Neurology 2004;62:2277-2282.
48. Lennette ET, Rymo L, Yadav M, et al. Disease-related differences in antibody patterns against EBV-encoded nuclear antigens EBNA 1, EBNA 2 and EBNA 6. Eur J Cancer 1993;29A:1584-1589.
49. Serafini B, Rosicarelli B, Franciotta D, et al. Dysregulated Epstein-Barr virus infection in the multiple sclerosis brain. J Exp Med 2007;204:2899-2912.
50. Peferoen LA, Lamers F, Lodder LN, et al. Epstein Barr virus is not a characteristic feature in the central nervous system in established multiple sclerosis. Brain 2010;133:e137
51. Willis SN, Stadelmann C, Rodig SJ, et al. Epstein-Barr virus infection is not a characteristic feature of multiple sclerosis brain. Brain 2009;132:3318-3328.
52. Ebers GC. Environmental factors and multiple sclerosis. Lancet Neurol 2008;7:268-277.
53. Lünemann JD, Münz C. EBV in MS: guilty by association? Trends Immunol 2009;30:243-248.
54. Friese MA, Fugger L. Pathogenic CD8(+) T cells in multiple sclerosis. Ann Neurol 2009;66:132-141.
55. Sundström P, Nyström M, Ruuth K, Lundgren E. Antibodies to specific EBNA-1 domains and HLA DRB1*1501 interact as risk factors for multiple sclerosis. J Neuroimmunol 2009;215:102-107.
56. Jilek S, Schluep M, Meylan P, et al. Strong EBV-specific CD8+ T-cell response in patients with early multiple sclerosis. Brain 2008;131:1712-1721.
57. Lünemann JD, Huppke P, Roberts S, Brück W, Gärtner J, Münz C. Broadened and elevated humoral immune response to EBNA1 in pediatric multiple sclerosis. Neurology 2008;71:1033-1035.
58. Lünemann JD, Jelcic I, Roberts S, et al. EBNA1-specific T cells from patients with multiple sclerosis cross react with myelin antigens and co-produce IFN-gamma and IL-2. J Exp Med 2008;205:1763-1773.
59. Rand KH, Houck H, Denslow ND, Heilman KM. Epstein-Barr virus nuclear antigen-1 (EBNA-1) associated oligoclonal bands in patients with multiple sclerosis. J Neurol Sci 2000;173:32-39.
60. Buljevac D, van Doornum GJ, Flach HZ, et al. Epstein-Barr virus and disease activity in multiple sclerosis. J Neurol Neurosurg Psychiatry 2005;76:1377-1381.
61. Goverman J. Autoimmune T cell responses in the central nervous system. Nat Rev Immunol 2009;9:393-407.
62. Münz C, Lünemann JD, Getts MT, Miller SD. Antiviral immune responses: triggers of or triggered by autoimmunity? Nat Rev Immunol 2009;9:246-258.
63. Lauer K. Environmental risk factors in multiple sclerosis. Expert Rev Neurother 2010;10:421-440.
64. Hedström AK, Bäärnhielm M, Olsson T, Alfredsson L. Exposure to environmental tobacco smoke is associated with increased risk for multiple sclerosis. Mult Scler 2011;17:788-793.
65. Handel AE, Williamson AJ, Disanto G, Dobson R, Giovannoni G, Ramagopalan SV. Smoking and multiple sclerosis: an updated meta-analysis. PloS One 2011;6:e16149.
66. Hawkes CH. Are multiple sclerosis patients risk-takers? Qjm 2005;98:895-911.
67. Marrie R, Horwitz R, Cutter G, Tyry T, Campagnolo D, Vollmer T. High frequency of adverse health behaviors in multiple sclerosis. Mult Scler 2009;15:105-113.

68. Simon KC, van der Mei IA, Munger KL, et al. Combined effects of smoking, anti-EBNA antibodies, and HLA-DRB1*1501 on multiple sclerosis risk. Neurology 2010;74:1365-1371.
69. Hamilton AS, Lessov-Schlaggar CN, Cockburn MG, Unger JB, Cozen W, Mack TM. Gender differences in determinants of smoking initiation and persistence in California twins. Cancer Epidemiol Biomarkers Prev 2006;15:1189-1197.
70. Mikaeloff Y, Caridade G, Tardieu M, Suissa S. Parental smoking at home and the risk of childhood-onset multiple sclerosis in children. Brain 2007;130:2589-2595.
71. Ramagopalan SV, Ebers GC. Genes for multiple sclerosis. Lancet 2008;371:283-285.

Part VI

Summary & Samenvatting

Summary

Multiple sclerosis (MS) is a common neurological disorder of the central nervous system that is characterised by inflammation, demyelination and axonal loss. It is an episodic disorder predominantly affecting females in their 20s or 30s and evolves over time into a progressive disease. Despite all research the cause and prediction of the course of MS has not been established yet. However, there is accumulating evidence that MS is a complex disease with an interaction between environmental and genetic factors. Given the precarious future of these young adults in their fertile years of life, further research is essential.

This thesis aimed to identify a few selected prognostic factors involved in cause and course of MS in detail and focused on their interactions. A secondary objective was to enhance our knowledge for a better understanding of MS pathophysiology, which may direct strategies for prevention, diagnosis and therapy.

Chapter 1 gives a general introduction of the epidemiology and the risk factors involved in susceptibility and course of MS.

Magnetic resonance imaging (MRI) is until today the only clinically used tool for prediction of MS. In 1997, the Barkhof criteria were implemented in predicting and diagnosing MS. Although the specificity of these criteria is acceptable, they have rather low sensitivity. In **chapter 2**, the predictive value of a single corpus callosum lesion in patients with a clinically isolated syndrome (CIS) was assessed next to and in addition with the Barkhof criteria. We demonstrated that a corpus callosum lesion and the Barkhof criteria both predicted conversion to clinically definite MS after CIS. When both variables were combined, the association was stronger. Interestingly, we showed that especially in patients not fulfilling the Barkhof criteria the corpus callosum lesion had a strong predictive value. Our findings suggest the importance of the assessment of corpus callosum lesion as a useful additional tool for prediction of conversion to MS in patients with CIS.

Next to aggregation of MS in families, the increasing number of risk genes associated with MS is an important determinant of MS susceptibility. Starting from alleles of the human leukocyte antigen (HLA) class II region, many new risk alleles of MS have been identified over time. These rapid developments were mainly due to genome-wide association (GWA) studies and international collaboration. **Chapter 3** focused on the predictive value of multiple genes today and in the future. First, we demonstrated in an empirical study using weighted risk scores that when combining multiple genes, the predictive values is mainly driven by *HLA-DR1*1501*. This is probably caused by the large effect size and the high frequency of this

allele. Recently, a large GWA study replicated 23 of the previously associated risk loci for MS and identified a further 29 novel susceptibility loci for MS. Including the well replicated and the novel loci, we demonstrated that even a doubling of the known risk alleles gives only a marginal increase in the discriminating value between cases and controls. Moreover, we performed a simulation study and showed that even in the future, a clinically useful predictive model based on risk genes is unfeasible.

In 2008, we reported that the *KIF1B* variant rs10492972 was associated with MS. *KIF1B* has shown to be involved in axonal transport of mitochondria and synaptic vesicle precursors. Irreversible axonal loss is an important mechanism in the development of permanent neurological symptoms and neurodegeneration. Although, other studies could not replicate this association, we investigated whether this single nucleotide polymorphism (SNP) can explain some of the neurodegenerative phenotypic differences between MS patients. In **chapter 4**, we concluded that there is no evidence could be found for determination of the influence of carrier ship of the risk allele or genotype of the *KIF1B* on any measured clinical or MRI based neurodegenerative markers.

Next to genetic risk factors, there is an increasing evidence for a role of environment involved in susceptibility and course of MS. In this thesis, we elucidated in more detail two promising environmental risk factors: Epstein-Barr virus (EBV) **(chapter 5)** and cigarette smoking **(chapter 6)**.

Epidemiological and serological studies have suggested a role of EBV in MS pathogenesis, underlined by the association of infectious mononucleosis and MS. The similarity between infectious mononucleosis and MS in terms of age, geographical distribution, socioeconomic status, and ethnicity is striking. The role of EBV infection in MS is suggested by an elevation in antibody titres of a specific EBV antibody, the EBV nuclear antigen-1 (EBNA-1). Increased EBV antibody levels in cerebrospinal fluid and EBV infected B-cells in MS lesions in the brain shown by others imply a causative role for EBV. In **chapter 5.1**, we demonstrated that EBNA-1 specific immunoglobulin G (IgG) responses are elevated both in serum and cerebrospinal fluid of MS patients compared with controls. However, after correction for the possible dysfunction of the blood-brain barrier by normalizing for total IgG, we found no evidence for intrathecal IgG synthesis. Interestingly, searching for a common genetic factor for infectious mononucleosis and MS, in **chapter 5.2**, we showed and argued that *HLA-A02* SNP is possibly the common underlying factor of infectious mononucleosis and MS. We demonstrated that the HLA class I SNP rs6457110 is associated with both diseases, independent of the major class II allele. This supports the hypothesis that shared genetics may contribute to the association between

infectious mononucleosis and MS. Moreover, in **chapter 5.3**, we suggested an interaction between this HLA-class I SNP and EBV reactivity. First, we observed a gradual higher EBV early antigen IgG levels as a marker for chronic EBV reactivation in MS patients compared with related and unrelated controls. Second, we demonstrated an interaction between EBV reactivity and *HLA-A02* SNP (rs6457110) suggesting a different control of EBV infection in individuals with genetic susceptibility.

In **chapter 6.1**, we give a review of cigarette smoking as one of the promising environmental factors in cause and course of MS. We also discuss the possible biological pathways playing a role in the association between cigarette smoking and MS. Moreover, the relation of smoking with other environmental MS risk factors is addressed.

Given the accumulating evidence for this association, we aimed to investigate whether the history of cigarette smoking influences our genetic studies in MS multiplex families using siblings as controls. In **chapter 6.2**, we demonstrated that smoking history of MS patients and related controls did not differ. An explanation may be found in overmatching in smoking behaviour within families or exposure to passive smoking by using unaffected siblings as controls. We concluded that cigarette smoking was not a confounder in family studies.

Finally, **chapter 7** provides a discussion of our main findings and their contribution to understanding disease susceptibility. Based on this thesis, we can confirm that various risk factors involved in cause and course of MS have only a small contribution in our understanding of MS when investigated separately. For better understanding and prediction, it is necessary that future research will combine genetic, environmental and clinically assessments, and will focus on their interactions.

Samenvatting

Multipele sclerose (MS) is een veel voorkomende neurologische aandoening van het centrale zenuwstelsel, die gekenmerkt wordt door ontsteking, demyelinisatie en axonaal verlies. Het is een aandoening die zich vaak in aanvallen presenteert en komt met name voor onder jong volwassenen, bij vrouwen meer dan mannen. Deze aandoening ontwikkelt zich na verloop van tijd tot een progressieve ziekte. Ondanks al het verrichte onderzoek is er tot op heden weinig bekend over de oorzaak van MS. Ook het beloop van de ziekte blijft erg onzeker. Er zijn steeds meer aanwijzingen dat MS een ingewikkelde ziekte is met een wisselwerking tussen genetische en omgevingsfactoren. Gezien de onzekere toekomst van deze groep jong volwassenen in hun meest vruchtbare levensjaren, is verder onderzoek van groot belang.

Dit proefschrift had als hoofddoelstelling om de voorspellende waarde van een aantal factoren betrokken bij het ontstaan en het beloop van MS aan te tonen. Ook werd de interactie tussen enkele van deze factoren nader onderzocht. Een tweede doelstelling was het vergroten van onze kennis van de pathosfysiologie van MS, dat kan leiden tot het ontwikkelen van de methoden ter preventie, diagnose en uiteindelijk zelfs behandeling.

Hoofdstuk 1 geeft een algemene inleiding over de epidemiologie en de risicofactoren betrokken bij het ontwikkelen en het beloop van MS.

Magnetic resonance imaging (MRI) is tot op heden het enige klinisch gebruikte instrument voor het voorspellen van MS. In 1997 werden de Barkhof criteria geïmplementeerd in het voorspellen en diagnosticeren van MS. Hoewel de specificiteit van deze criteria acceptabel is, is de gevoeligheid laag. **Hoofdstuk 2** toont de voorspellende waarde van de aanwezigheid van een laesie in het corpus callosum (hersenbalk) bij patiënten met een klinisch geïsoleerd syndroom (CIS) afzonderlijk van, en in combinatie met de Barkhof criteria. We hebben aangetoond dat een corpus callosum laesie en de Barkhof criteria beiden de overgang naar klinisch definitieve MS voorspellen na CIS. Na combinatie van beide variabelen, is de relatie met MS sterker. Bovendien laten we zien dat vooral bij patiënten die niet voldoen aan de Barkhof criteria, de aanwezigheid van een corpus callosum afwijking een sterke voorspellende waarde heeft. Onze bevindingen suggereren dat het scoren van een corpus callosum laesie een nuttig aanvullend instrument kan zijn bij het voorspellen van de overgang naar MS bij patiënten met CIS.

Naast de waarneming dat MS in bepaalde families vaker voorkomt, is het toenemend aantal risico genen geassocieerd met MS een belangrijk aanknopingspunt voor rol van genen in MS. Sinds de ontdekking van de risico allelen van de humaan leukocyten antigen (HLA) klasse II

regio, zijn er in de loop van de tijd veel nieuwe risico allelen van MS vastgesteld. Deze snelle ontwikkelingen zijn vooral te danken aan de 'genome wide association' (GWA) studies en de internationale samenwerking. **Hoofdstuk 3** richt zich op de voorspellende waarde van het combineren van meerdere genen nu en in de toekomst. Allereerst hebben we in een empirisch onderzoek met behulp van gewogen risicoscores aangetoond dat wanneer we meerdere genen combineren, de voorspellende waarde bepaald wordt door het risico allel op het HLA-DR locus (*HLA-DR1*1501*). Dit wordt waarschijnlijk veroorzaakt door de sterkte van de relatie en de hoge frequentie van dit locus. Onlangs heeft een grote GWA-studie 23 van eerder aangetoonde risico loci voor MS gerepliceerd. Bovendien hebben ze 29 nieuwe loci geïdentificeerd die gerelateerd zijn met MS. Door het includeren van deze gerepliceerde en nieuwe risico allelen hebben we laten zien dat zelfs een verdubbeling van de bekende risco loci slechts leidt tot een marginale toename van de discriminerende waarde tussen patiënten en controles. Bovendien hebben we een simulatiemodel opgezet en hebben we aangetoond dat zelfs in de toekomst een voorspellend model gebaseerd op alleen risico genen klinisch niet bruikbaar is.

In 2008 heeft onze studiegroep gemeld dat de *KIF1B* variant rs10492972 geassocieerd is met MS. Het *KIF1B* gen is betrokken bij het axonaal transport van de mitochondrieën en synaptische vesikels. Hoewel andere studies deze relatie niet konden repliceren, is een rol van *KIF1B* in MS niet uitgesloten. We hebben in **hoofdstuk 4** onderzocht of deze 'single nucleotide polymorphism' (SNP) een aantal van de neurodegeneratieve fenotypische verschillen tussen MS patiënten kan verklaren. We concludeerden dat het dragen van het risico allel of genotype van *KIF1B* geen invloed heeft op de klinische en/of MRI gebaseerde neurodegeneratieve markers.

Behoudens genetische risicofactoren zijn er steeds meer aanwijzingen dat de omgeving een rol speelt in het ontstaan en het beloop van MS. In dit proefschrift hebben we twee veelbelovende omgevingsrisicofactoren bestudeerd, namelijk het Epstein-Barr virus (EBV) **(hoofdstuk 5)** en het roken van sigaretten **(hoofdstuk 6)**.

Epidemiologische en serologische studies hebben al langer gesuggereerd dat EBV een rol speelt in de pathogenese van MS. Dit wordt verder onderstreept door de aangetoonde associatie van de ziekte van Pfeiffer met MS. De gelijkenis tussen de ziekte van Pfeiffer en MS ten aanzien van leeftijd, geografische spreiding, sociaaleconomische status en etniciteit is opvallend. Een verhoging van de titer van specifieke EBV-antilichamen, zoals het EBV nucleair antigeen-1 (EBNA-1), suggereert een rol voor EBV-infectie in MS. Verhoogde titers van EBV-antilichamen in hersenvocht en EBV geïnfecteerde B-cellen in de MS laesies in de hersenen impliceren een oorzakelijke rol voor EBV. In **hoofdstuk 5.1**, hebben we aangetoond dat MS patiënten, in

vergelijking met gezonde controles, zowel in het serum als in het hersenvocht een hogere EBNA-1 specifieke IgG reactie hebben. Echter, na correctie voor het mogelijk disfunctioneren van de bloed-hersenbarrière met behulp van normaliseren voor totaal IgG, vonden we geen bewijs voor intrathecale IgG synthese. Op zoek naar een gemeenschappelijke genetische factor voor de ziekte van Pfeiffer en MS, hebben we in **hoofdstuk 5.2** aangetoond dat een *HLA-A02* SNP mogelijk de onderliggende gemeenschappelijke factor is. We hebben laten zien dat de HLA klasse I SNP rs6457110 geassocieerd is met beide ziekten, onafhankelijk van het belangrijke klasse II allel. Dit ondersteunt de hypothese dat gedeelde genetica bijdraagt aan de gevonden relatie tussen de ziekte van Pfeiffer en MS. Bovendien is in **hoofdstuk 5.3** een interactie tussen een HLA klasse I SNP en EBV reactiviteit gesuggereerd. Allereerst hebben we een hoger 'early antigen' IgG waarde gemeten bij patiënten met MS in vergelijking met verwante en niet verwante controles. Het 'early antigen' is een marker voor chronische EBV reactiviteit. Ten tweede hebben we een interactie tussen EBV reactiviteit en een *HLA-A02* SNP (rs6457110) gevonden, die kan duiden op een verschillende controle van een EBV-infectie bij mensen met een onderliggende genetische aanleg.

In **hoofdstuk 6.1** geven we een overzicht van het roken van sigaretten als één van de veelbelovende omgevingsfactoren betrokken in het ontstaan en het beloop van MS. We bespreken ook de mogelijke biologische mechanismen die een rol spelen in de relatie tussen het roken van sigaretten en MS. Bovendien wordt ook de relatie van het roken van sigaretten met andere omgevingsfactoren als risico voor MS besproken.
Gezien het opeenstapelende bewijs voor de associatie tussen het roken van sigaretten en MS, hebben we onderzocht of rookgedrag onze genetische studies in families met meerdere MS patiënten beïnvloedt. In **hoofdstuk 6.2** hebben we aangetoond dat het rookgedrag van patiënten met MS in vergelijking met hun broers/zussen niet significant verschilt. Een mogelijke verklaring voor het ontbreken van een associatie tussen roken en MS kan overmatching in rookgedrag binnen de families zijn. Ook blootstelling aan passief roken kan van belang zijn aangezien we broers en zussen als controles gebruiken. We concludeerden dat het roken van sigaretten geen verstorende factor blijkt in onze familiestudies.

Tenslotte worden in **hoofdstuk 7** onze belangrijkste bevindingen en hun bijdrage tot het begrijpen van de ziekte weergegeven. Met de bevindingen beschreven in dit proefschrift kunnen we bevestigen dat de verschillende risicofactoren die betrokken zijn bij de oorzaak en het beloop van MS slechts een kleine bijdrage in ons begrip van MS hebben wanneer ze afzonderlijk worden onderzocht. Voor een beter begrip van de ziekte en voorspelling van het beloop van de ziekte is het noodzakelijk dat in toekomstig onderzoek zowel genetische en klinische gegevens als informatie over omgevingsfactoren zullen worden gecombineerd en dat ook hun interacties nader worden onderzocht.

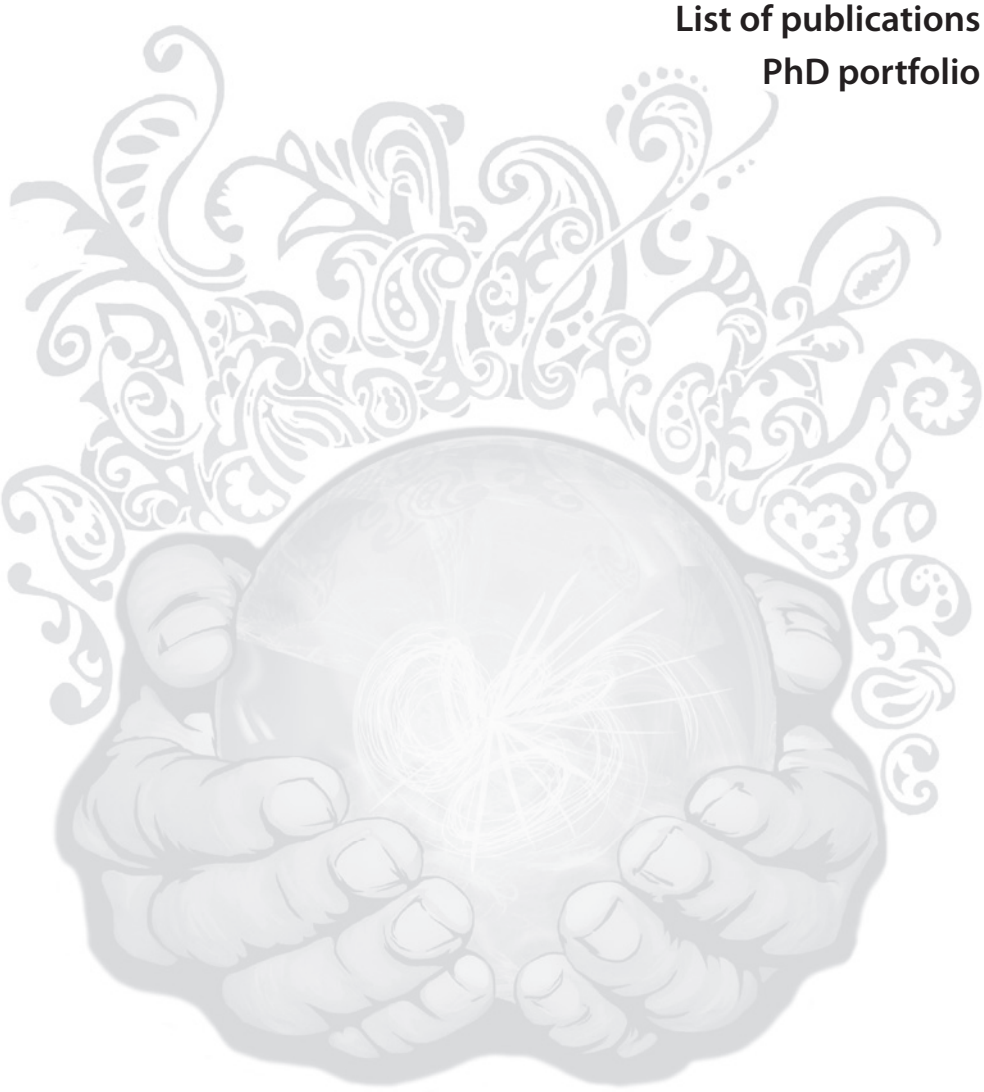
List of abbreviations

List of Abbreviations

AUC	area under the curve
BC	Barkhof criteria
CC	corpus callosum
CDMS	clinically definite multiple sclerosis
CI	confidence interval
CIS	clinically isolated syndrome
CMV	cytomegalovirus
CNS	central nervous system
CSF	cerebrospinal fluid
EA	EBV early antigen
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus
EDSS	expanded disability status scale
ELISA	enzyme-linked immunosorbent assay
FLAIR	fluid-attenuated inversion recovery
GWA	genome wide association
HLA	Human leukocyte antigen
HR	hazard ratio
IgG	immunoglobulin G
IM	infectious mononucleosis
IQR	interquartile range
MRI	magnetic resonance imaging
MS	multiple sclerosis
MSSS	MS severity scale
NIND	non-inflammatory neurological disease
OD	optical density
OR	odds ratio
PdW	proton density weighted
PPMS	primary progressive MS
RRMS	relapsing remitting MS
RR	risk ratio
SD	standard deviation
SNP	single nucleotide polymorphism
SPMS	secondary progressive MS
VCA	EBV viral capsid antigen

Epilogue

Dankwoord
About the author
List of publications
PhD portfolio



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Promotor
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About the author

Naghmeh Jafari was born on April 21st , 1977 in Tehran, Iran. In 1987 she left Iran with her parents to make the first and most important journey of her life. After two years joining the school of life, her family settled in 1989 in The Netherlands.

In 1990 she attended secondary school at the Trichter College in Maastricht, from which she graduated (Gymnasium) in 1996. The same year, she started Medical School in Utrecht and obtained her Medical degree in October 2003.

From November 2003 until April 2005 she worked at Department of Neurology at Sint Franciscus Gasthuis in Rotterdam and Albert Schweitzer Hospital in Dordrecht. In April 2005 she continued working as resident in Erasmus MC in Rotterdam and in September 2005 started her PhD research at the ErasMS under supervision of Prof. dr. R.Q. Hintzen. She established and coordinated the nationwide PROUD (Predicting the Outcome of demyelinating event) study. She also participated in the genetic family studies and performed part of her research in this cohort.

From January 2010 onwards, Naghmeh works as a resident in Neurology in Erasmus MC in Rotterdam (head: Prof. dr. P.A.E. Sillevis Smitt).

List of publications

Aulchenko YS, Hoppenbrouwers IA, Ramagopalan SV, Broer L, **Jafari N**, Hillert J, Link J, Lundstrom W, Greiner E, Dessa Sadovnick A, Goossens D, Van Broeckhoven C, Del-Favero J, Ebers GC, Oostra BA, van Duijn CM, and Hintzen RQ. Genetic variation in the KIF1B locus influences susceptibility to multiple sclerosis. Nat Genet 2008;40:1402-1403.

Jafari N, Hoppenbrouwers IA, Hop WC, Breteler MM, and Hintzen RQ. Cigarette smoking and risk of MS in multiplex families. Mult Scler 2009;15:1363-1367.

Jafari N, Kreft KL, Flach HZ, Janssens AC, and Hintzen RQ. Callosal lesion predicts future attacks after clinically isolated syndrome. Neurology 2009;73:1837-1841.

Petzold A, Altintas A, Andreoni L, Bartos A, Berthele A, Blankenstein MA, Buee L, Castellazzi M, Cepok S, Comabella M, Constantinescu CS, Deisenhammer F, Deniz G, Erten G, Espino M, Fainardi E, Franciotta D, Freedman MS, Giedraitis V, Gilhus NE, Giovannoni G, Glabinski A, Grieb P, Hartung HP, Hemmer B, Herukka SK, Hintzen R, Ingelsson M, Jackson S, Jacobsen S, **Jafari N**, Jalosinski M, Jarius S, Kapaki E, Kieseier BC, Koel-Simmelink MJ, Kornhuber J, Kuhle J, Kurzepa J, Lalive PH, Lannfelt L, Lehmensiek V, Lewczuk P, Livrea P, Marnetto F, Martino D, Menge T, Norgren N, Papuc E, Paraskevas GP, Pirttila T, Rajda C, Rejdak K, Ricny J, Ripova D, Rosengren L, Ruggieri M, Schraen S, Shaw G, Sindic C, Siva A, Stigbrand T, Stonebridge I, Topcular B, Trojano M, Tumani H, Twaalfhoven HA, Vecsei L, Van Pesch V, Vanderstichele H, Vedeler C, Verbeek MM, Villar LM, Weissert R, Wildemann B, Yang C, Yao K, and Teunissen CE. Neurofilament ELISA validation. J Immunol Methods 2010;352:23-31.

Jafari N, van Nierop GP, Verjans GM, Osterhaus AD, Middeldorp JM, and Hintzen RQ. No evidence for intrathecal IgG synthesis to Epstein Barr virus nuclear antigen-1 in multiple sclerosis. J Clin Virol 2010;49:26-31.

Jafari N, Broer L, Hoppenbrouwers IA, van Duijn CM, and Hintzen RQ. Infectious mononucleosis-linked HLA class I single nucleotide polymorphism is associated with multiple sclerosis. Mult Scler 2010;16:1303-1307.

Sombekke MH, **Jafari N**, Bendfeldt K, Mueller-Lenke N, Radue EW, Naegelin Y, Kappos L, Matthews PM, Polman CH, Barkhof F, Hintzen R, and Geurts JJ. No influence of KIF1B on neurodegenerative markers in multiple sclerosis. Neurology 2011;76:1843-1845.

Neuteboom RF, Janssens AC, Siepman TA, Hoppenbrouwers IA, Ketelslegers, IA, **Jafari N**, Steegers EA, de Groot CJ, and Hintzen RQ. Pregnancy in multiple sclerosis: clinical and self-report scales. J Neurol 2011;352:23-31.

Jafari N and Hintzen RQ. The association between cigarette smoking and multiple sclerosis. J Neurol Sci 2011;311:78-85.

Jafari N, Broer L, van Duijn CM, Janssens ACJW, and Hintzen RQ. Perspectives on the use of multiple sclerosis risk genes for prediction. PloS One 2011, accepted.

Jafari N, Melles DC, Broer L, van Duijn CM, van Doornum GJJ, and Hintzen RQ. Serological signs of EBV reactivation and interaction with HLA class I in MS patients of a genetic isolate. Submitted.

Jafari N, van Rooij LC, Runia TF, and Hintzen RQ. Assessing fatigue in clinically isolated syndrome. In preparation.

Runia TF, **Jafari N**, Kreft KL, and Hintzen RQ. Application of the 2010 revised McDonald criteria for the diagnosis of multiple sclerosis to patients with clinically isolated syndrome. In preparation.

PhD portfolio

1. PhD training	Year	Workload (ECTS)
Academic and research skills		
Classical Methods for Data Analysis, Nihes, Rotterdam, NL	2006	5.0
Biomedical English Writing and Communication, Rotterdam, NL	2007	2.0
Regression Analysis for Clinicians , Nihes, Rotterdam, NL	2009	1.9
Genetic Analysis in Clinical Research, Nihes, Rotterdam, NL	2009	1.0
Oral presentations		
Environment and MS, Neurology Department, Erasmus Rotterdam, NL	2006	1.0
Corpus Callosum Lesions Predict Future Attacks in CIS, MS Research, Leiden, NL	2008	1.0
Predicting the Outcome of a Demyelinating Event, Rotterdam, NL	2008	1.0
Epstein- Barr Virus Research in ErasMS, Rotterdam, NL	2009	1.0
Cigarette Smoking and Risk of Multiple Sclerosis, ECF, Fiuggi, Italy	2010	1.5
Poster presentations		
4x poster presentation at Ectrims, Madrid (Spain), Montreal (Canada), Dusseldorf (Germany), Amsterdam (NL)	2006-2011	0.8
4x Poster presentation MS Onderzoeksdagen, NL and Belgium	2007-2010	0.4
International conferences		
5x Conference of European Committee for Treatment and Research in Multiple Sclerosis (Ectrims), Madrid (Spain), Montreal (Canada), Dusseldorf (Germany), Gothenburg (Sweden), Amsterdam (NL)	2006-2011	4.0
3rd BioMS Meeting, London, UK	2007	0.5
NeuroproMiSe: Viral Triggers of Autoimmunity:Focus on Epstein-Barr and Multiple Sclerosis, Rome, Italy	2008	0.5
A Reappraisal of Nutrition and Environment in Multiple Sclerosis, ECF, Fiuggi, Italy	2010	0.5
Seminars/ Workshops/ Courses		
Basiscursus Multilple Sclerose, Garderen, NL	2005	0.4
6x Annual MS meeting of the Dutch MS Research Foundation, Hasselt (Belgium), Amsterdam, Rotterdam, Leiden, Groningen, Alphen aan den Rijn (NL)	2005-2010	1.8
The Role of DNA Polymorphism in Complex Traits and Diseases, Amsterdam, NL	2006	0.5
Inflammatory Mechanisms in Neurodegenerative Diseases, Molmed, Rotterdam, NL	2006	0.4
Methodologie van patiëntgebonden onderzoek en voorbereiding van subsidieaanvragen, CPO, Rotterdam, NL	2007	0.1

1. PhD training (Continued)	Year	Workload (ECTS)
Symposium Post-Infectious Diseases: Molecular Mimicry and Beyond, Rotterdam, NL	2007	0.3
Course Biomedical Research Techniques VII, Rotterdam, NL	2008	1.0
SNP Course V, Molmed, Rotterdam, NL	2009	1.5
Neuroimmunology, Department of immunology, Rotterdam, NL	2007-2008	0.3
The Photoshop and Illustrator CS5 Workshop, Rotterdam, NL	2011	0.3
9th ESNI Course, Istanbul, Turkey	2009	0.7
Other		
Design, organisation and coordination of the research study Predicting the Outcome of a Demyelinating Event (PROUD).	2007-2010	10
2. Teaching	**Year**	**Workload (ECTS)**
Supervising master and medical students		
Corpus Callosum Predicts Future Attacks after Clinically Isolated Syndrome	2008	6
Assessing Fatigue in Clinically Isolated Syndrome	2009	4.0
Other		
Review of various papers for international journals	2009-2011	0.6
Investigating Doctor in clinical MS trials (FTY-720, Tysabri)	2006-2009	5.0

Multiple sclerosis is a complex disorder of the central nervous system with multiple factors playing a role in the onset and course of the disease. Counselling a patient after a first clinical event sometimes feels like crystal ball prediction.

This thesis investigated different risk factors of multiple sclerosis with the aim to clarify the future of the patients at risk for multiple sclerosis.