Genetic and Imaging Markers of Multiple Sclerosis Prognosis and Diagnosis

Madeleine Sombekke

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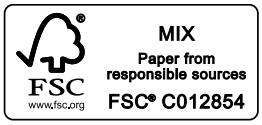

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Chapter 1

General Introduction

MULTIPLE SCLEROSIS: GENERAL INTRODUCTION

Multiple Sclerosis (MS) is the most common chronic (non-traumatic) disabling neurological disease in young adults[1]. It was first described in detail by Jean-Marie Charcot in 1868 as a rare disease, but nowadays MS has a prevalence of 0.1-0.2 percent in the Western European population[2]. Females are twice as often affected compared to males[1]. The average age at onset is 30 years. The majority of patients (85%) presents initially with an acute or subacute episode of focal neurological deficits (most frequently affecting the optic nerve, brainstem/cerebellum, spinal cord or cerebral hemispheres)[3]. The symptoms often diminish over several weeks (sometimes full recovery occurs). This first episode is called Clinically Isolated Syndrome (CIS). Of these CIS patients around 30-70 percent will develop MS with a relapsing remitting course (RRMS), with full or partial recovery from these relapses[4,5]. In a substantial part of the RRMS patients, the phase of relapses and remissions is followed by slowly progressive increase of neurological disability (Secondary Progressive MS (SPMS)[6]. A minority of MS patients (10-15%) will not experience any relapses, but show disease progression from onset (Primary Progressive MS (PPMS))[6,7]. See **figure 1** for schematic illustration of the course of the different types of MS.

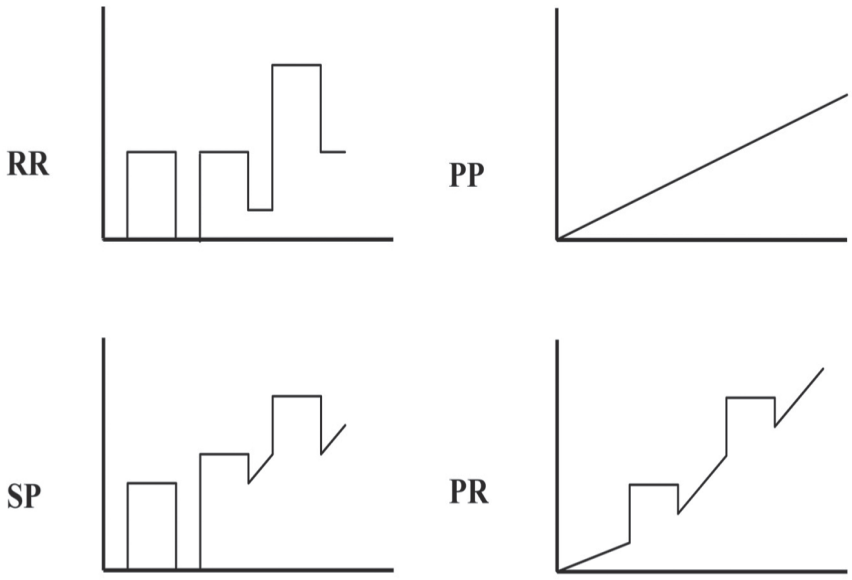

Figure 1: Schematic illustration of the course of the different types of MS. The X-axis represents time and the Y-axis represents neurological disability. RR = relapsing remitting, PP = primary progressive, SP = secondary progressive, PR = progressive relapsing.

The disease course is highly variable between MS patients: in addition to the differences between subtypes indicated above, timing and nature of the neurological complaints are heterogeneous between individual patients with the same disease subtype. Symptoms can vary from decreased vision to (temporary) blindness, paresis and/or sensory disturbances in the limbs, cognitive complaints and mood disorders, clumsiness in the limbs to diplopia. These symptoms are caused by lesions in respectively the optic nerve, brain or spinal cord, cerebellum and brainstem. Quantification of these symptoms is difficult due to this variety of neurological symptoms and lack of one outcome-parameter comprising the full scope of MS. In our center we use the Multiple Sclerosis Functional Composite (MSFC) (assessing ambulatory function, upper limb function and (parts of) cognitive function)[8] and the Expanded Disability Status Scale (EDSS). The EDSS uses information from neurological examination to evaluate the following seven neurological functional systems: visual, brainstem, pyramidal, sensory, cerebellar, bladder/bowel and cerebral function[9]. The EDSS is internationally the most commonly used outcome parameter, however, no single measure reflects the entire scope of disability (necessitating a combination of other, more functional, outcome parameters such as the MSFC)[10]. In general, disability accumulates during the disease course and patients with a longer disease duration experience more symptoms.
The degree and speed of disability is highly variable. Some MS patients will only have mild or no complaints during their lifetime, while others are in need of a wheelchair within a year after disease onset[4]. This uncertainty on prognosis due to this heterogeneity (unknown time and place of new complaints and the accompanied disability) places an enormous burden on often young MS patients. From observational studies we already established some clinical indicators for a fast disability accumulation: a primary progressive disease course, a rapid deterioration in the first years after disease onset, a higher initial relapse rate, a shorter interval to the second relapse and a higher EDSS score at 5 years[1,11-13]. In addition to these clinical prognostic markers, more and more evidence from long-follow-up studies indicates that MRI parameters early in the disease may have additional prognostic value for disability after 20 years: T2 lesion volume at disease onset and increases of this T2 lesion burden within the first 5 years are correlated with disability after 20 years[5].

Pathophysiology

Although the exact etiology of MS is unknown, it is generally thought that MS is an auto-immune complex trait, resulting from an interplay between environmental factors and genes[1]. It is characterized by focal lesions in the central nervous system (brain and spinal cord) that can be observed postmortem by histopathological examination and in vivo using Magnetic Resonance Imaging (MRI). These lesions are scattered throughout the brain and spinal cord involving the white and grey matter. Only a small proportion of the new inflammatory lesions gives rise to neurological symptoms[14], the remaining new lesions remain clinically silent.

The commonly accepted hypothesis is that these lesions are caused by an increase in auto-reactive T cells that generate an inappropriate inflammatory response against myelin or oligodendrocytes[1]. After the initial inflammatory response remyelination occurs, but also gliosis.
The pathology of MS is not restricted to lesions only. Also abnormalities in the normal appearing white matter (and grey matter) have been demonstrated, together with axonal loss. Axonal loss can be visualized by MRI by assessing the volume of the brain. In MS patients this volume declines faster than in normal aging people (a higher atrophy rate). This has been demonstrated in all stages of MS and it occurs not only during the inflammatory response, but also independent from the inflammation[14].

Therapy

Therapy in MS is aimed on reducing relapses and slowing disease progression, since no curative treatment for MS is available. These Disease Modifying Therapies (DMTs) are only effective in the relapsing phase of the disease. No DMTs are available for secondary or primary progressive MS patients. The available therapies for RRMS are Interferons (Interferon Beta 1a and Interferon Beta 1b), Glatiramer acetate and since a couple of years Natalizumab has been added. These DMTs modestly succeeded in decreasing the number of relapses and (partially) inhibit the formation of new T2 lesions (and T1 gadolinium enhancing lesions) on MRI-scans[15-20]. In CIS patients DMT can delay a second relapse[20]. In RRMS patients Interferon has been demonstrated to delay clinical disability, however, the true long-term effects need to be determined in future studies[20-22]. Interferons and glatiramer acetate show comparable effectiveness and the same route of administration (requires frequent subcutaneous or intramuscular injections). Natalizumab (a monoclonal antibody against alpha-4-integrin, administered as monthly intravenous infusions) is more effective, however it is used as a second line drug due to the occasional occurrence of progressive multifocal leucoencephalopathy[23]. Recently oral drugs have been studied. One of them (Fingolimod) showed a higher effectiveness of this drug on reducing relapses and new lesions on MRI, when compared to the current first line drugs (intramuscular interferon beta 1a)[24]. Fingolimod has become recently available in the Netherlands as a second line oral disease modifying therapy. Longterm effects of these DMTs are still lacking.

DIAGNOSIS OF MULTIPLE SCLEROSIS

Relapsing MS usually starts with an acute onset of neurological complaints, such as sensory disturbances, unilateral optic neuritis, diplopia, limb weakness, clumsiness, gait ataxia or bladder or bowel symptoms, but also cognitive impairment. This first episode in which a

patient presents neurological symptoms due to demyelination is called: clinically isolated syndrome (CIS). Only 30-70% of these patients will develop MS later in their lives[4,5]. No single diagnostic test for MS exists. The diagnosis of MS requires dissemination in time and place, as was described in 1983 by Poser[25] and other causes of the clinical condition should be excluded. Dissemination in time (DIT) using the Poser criteria is defined as the occurrence of new neurological complaints lasting at least 24 hours and starting at least 30 days after the onset of the first episode. Dissemination in space (DIS) is defined as two different areas of the central nervous system that are affected, as detected by clinical (and paraclinical) signs and symptoms. If patients fulfilled clinically dissemination in time and space (had a second relapse, on a new location) the diagnosis: clinically definite MS (CDMS) could be made. Since 2001 Magnetic Resonance imaging (MRI) criteria of brain and spinal cord are allowed as evidence for dissemination in space and dissemination in time, facilitating early diagnosis and early treatment with disease modifying therapy[26-30]. In 2010 the International Panel on Diagnosis of MS set out the most recent diagnostic criteria[30]. Compared to previous criteria, these criteria are easier to use and in some patients the diagnosis can be made on one brain MRI scan only. Please see **table 1** for a summary of the most recent diagnostic criteria.

GENETICS OF MULTIPLE SCLEROSIS

MS is considered to be a complex auto-immune disease, in which an interplay of genetic and environmental factors is thought to cause the disease. The prevalence of MS varies considerably over the world, with the highest prevalence of more than 30 per 100,000 in northern Europe, southern Australia and North America[2]. Increasing latitude (distance from equator) correlates with increasing prevalence and incidence. This is possibly due to a lower exposure to sunlight in these countries and lower Vitamin D3 levels[31-33]. Also several viruses have been implicated in the pathogenesis of MS (Epstein-Barr virus has the most reliable epidemiological and laboratory data related to MS susceptibility)[34,35].

Although it is hypothesized that environmental factors contribute significantly to MS susceptibility, the majority of these environmental factors are still unidentified. In this thesis we focus on genetic factors in MS.

Table 1: The revised McDonald 2010 criteria for diagnosis of MS[30].

Clinical Presentation	Additional Data Needed for MS diagnosis
≥2 attacks; objective clinical evidence of ≥2 lesions or objective clinical evidence of 1 lesion with reasonable historical evidence of a prior attack	None
≥2 attacks, objective clinical evidence of 1 lesion	Dissemination in space, demonstrated by ≥1 T2 lesion in at least 2 of 4 MS-typical regions of CNS (periventricular, juxtacortical, infratentorial or spinal cord); or await a further clinical attack implicating a different CNS site
1 attack; objective clinical evidence of ≥2 lesions	Dissemination in time, demonstrated by: Simultaneous presence of asymptomatic gadolinium enhancing and non-enhancing lesions at any time; or a new T2 and/ or gadolinium-enhancing lesion(s) on follow-up MRI, irrespective of its timing with reference to a baseline scan; or await a second clinical attack
1 attack; objective clinical evidence of 1 lesion (Clinically Isolated Syndrome (CIS))	Dissemination in space and time, demonstrated by: For DIS: ≥1 T2 lesion in at least 2 of 4 MS typical regions of the CNS (periventricular, juxtacortical, infratentorial or spinal cord); or await a seond clinical attack implicating a different CNS site; and for DIT: Simultaneous presence of asymptomatic gadolinium enhancing and nonenhancing lesions at any time; or a new T2 and/or gadolinium-enhancing lesion(s) on follow-up MRI, irrespective of its timing with reference to a baseline scan; or await a second clinical attack.
Insidious neurological progression suggestive of Primary Progressive MS (PPMS)	One year of disease progression (retrospectively or prospectively determined) plus 2 of 3 of the following criteria: 1. Evidence for DIS in the brain based on ≥1 T2 lesion in the MS characteristic regions (periventricular, juxtacortical or infratentorial). 2. Evidence for DIS in the spinal cord based on ≥2 T2 lesions in the cord. 3. Positive CSF (isoelectric focusing evidence of oligoclonal bands and/or elevated IgG index)

Genetic role in MS susceptibility

Familial clustering of MS cases has been demonstrated in several studies[36-40]. Recurrence in monozygotic twins is >20%[41]. First degree relatives have a higher risk (~2%) of developing MS than the general population (0.1%). The risk correlates with the amount of shared genes with the affected family member. MS is a complex genetic disease, that is characterized by modest disease risk heritability and additionally gene-environment interactions[35,42]. The strongest gene effect, involved in MS susceptibility and already discovered in the mid-1970s, stems from the HLA-DRB1*1501 allele[43,44].

After decennia using (often underpowered) linkage and candidate gene studies to determine the heritability of MS (with little further progress), the past five years several Genome

Wide Association Studies (GWAS) were performed. GWAS studies are able to assess large quantities of Single Nucleotide Polymorphisms (SNPs) (300.000 – 500.000) at once and when performed in large groups of MS patients, this high-throughput-technique is able to detect SNPs with a modest effect on MS susceptibility. SNPs are DNA sequence variations occurring when a single nucleotide in the genome differs between people, of which some variations are associated with a higher risk on developing MS. Several GWAS were performed in large sets of MS patients and identified several loci related to MS susceptibility with "genome-wide significance" (cut off p-value of $< 5 \times 10^{-7}$)[45-50]. All of these risk alleles are common, exert only modest individual effects on risk (odds ratios 1.1-1.3) and act seemingly independently[51]. Please see **Table 2** for a list of risk alleles associated with MS susceptibility.

Effect of genes on MS phenotype

Multiple Sclerosis is characterized by enormous variations in phenotype expression. Not only clinically: e.g. symptoms that can affect the entire body and varying rate of disability accumulation, but also paraclinically. When assessed by MRI: several areas of the brain and spinal cord are affected in varying degrees. The biological factors that underlie this heterogeneity are poorly understood. The current hypothesis is that disease progression and other phenotypic variability of MS are influenced by genetic and non-genetic factors[52].
Studies in families with multiple MS patients provided evidence for a familial effect on disease course, age at onset and disease progression[53-56]. Moreover, pathological studies on these lesions in MS show four distinct, but overlapping histological types[57]. The histopathological appearances are generally similar between lesions from each patient, but differ between patients, indicating heterogeneity in pathology between patients[57].
Before the "*GWAS-era*", several genes (APOE, IL1RN, MHC class II and IL1B) were implicated to be related to disease severity, however effect sizes and size of study populations were small and results were often ambiguous[58-60]. Most of these genotype-phenotype associations await confirmation in different datasets.

Table 2: Established non-MHC MS susceptibility genes.[86]

Susceptibility gene	rs-number	OR estimate
VCAM1	rs11581062	1.12
PLEK	rs7595037	1.11
MERTK	rs17174870	1.11
SP140	rs10201872	1.14
EOMES	rs11129295	1.11
CD86	rs9282641	1.21
IL12B	rs2546890	1.11
BACH2	rs12212193	1.09
THEMIS	rs802734	1.10
MYB	rs11154801	1.13
IL22RA2	rs17066096	1.14
TAGAP	rs1738074	1.13
ZNF746	rs354033	1.11
MYC	rs4410871	1.11
PVT1	rs2019960	1.12
HHEX	rs7923837	1.10
CLECL1	rs10466829	1.09
ZFP36L1	rs4902647	1.11
BATF	rs2300603	1.11
GALC	rs2119704	1.22
MALT1	rs7238078	1.12
TNFSF14	rs1077667	1.16
MPV17L2	rs874628	1.11
DKKL1	rs2303759	1.11
CYP24A1	rs2248359	1.12
MAPK1	rs2283792	1.10
SCO2	rs140522	1.10
MMEL1	rs4648356	1.14
EV15	rs11810217	1.15
RGS1	rs1323292	1.12
C1orf106	rs7522462	1.11
CBLB	rs2028597	1.13
IL7	rs1520333	1.10
IL2RA	rs3118470	1.12
TMEM39A	rs2293370	1.13
IL12A	rs2243123	1.08
NFKB1	rs228614	1.09
IL7RA	rs6897932	1.11
PTGER4	rs4613763	1.20
ZMIZ1	rs1250550	1.10
CD6	rs650258	1.12
CXCR5	rs6309023	1.12
TNFRSF1A	rs1800693	1.12
CYP27B1	rs12368653	1.10
ARL6IP4	rs949143	1.08
SOX8	rs2744148	1.12
CLEC16A	rs7200786	1.15
IRF8	rs13333054	1.11
STAT3	rs9891119	1.11
RPS6KB1	rs180515	1.09
TYK2	rs8112449	1.08
CD40	rs2425752	1.11
TNFRSF6B	rs6062314	1.16
Intergenic	rs13192841	1.10
Intergenic	rs669607	1.13
Intergenic	rs12466022	1.11

IMAGING MULTIPLE SCLEROSIS

Brain MRI

MS lesions can be visualized using Magnetic Resonance Imaging (MRI) and have greatly improved the sensitivity and specificity of the diagnostic MS criteria by detecting clinically silent lesions[28]. The typical MS lesions are especially apparent on T2-weighted images as hyperintense (bright) spots. However, T2 lesions do not have pathological specificity and are also present in normal ageing and other neurological diseases. The MS lesions may be pathologically characterized by inflammation, demyelination, gliosis, edema or axonal loss [61,62]. New T2 lesions are considered a subclinical marker for MS disease activity, and their numbers are moderately correlated with relapse frequency. To distinguish active and inactive inflammatory lesions, gadolinium-based contrast agents are administered. Lesions that appear bright on T1 weighted MR images after contrast administration (gadolinium enhancing lesions), reflect disruption of the blood brain barrier due to acute inflammation. On the same T1 weighted images without prior injection of contrast agent, dark spots are commonly found in MS patients. These spots are called hypo-intense lesions (also: black holes). These T1 hypo-intense lesions are indicative of extensive brain tissue damage, but may also arise as a result of inflammation related edema.

Although the entrance of MRI in the field of MS has increased the diagnostic possibilities, the relation between MRI abnormalities and disability remain limited: this has often been referred to as the "clinico-radiological paradox"[63]. The number and volume of lesions are frequently used in monitoring disease evolution and assess treatment efficacy in MS, even though an ambiguous relationship between the extent of lesions and clinical disability appears[64,65]. It has been shown that focal lesions only partially explain disability. Apart from focal lesions, a higher atrophy rate of the brain is present in MS patients in all stages of the disease, when compared to healthy controls[66]. This brain volume loss due to neurodegeneration is better correlated to disability change and can be calculated using standard T1 weighted images. However, still no full explanation for the clinico-radiological paradox has been demonstrated. Another possible explanation for this paradox could be the presence of diffuse changes in the white matter and grey matter lesions (difficult to visualize using the conventional MRI sequences). New MRI techniques such as DIR (detecting grey matter lesions in the cortex and basal ganglia)[67], MR spectroscopy, diffusion tensor, magnetization transfer and functional MRI are currently being studied to assess their ability to detect these less visible changes in the MS brain, that may explain the disease progression. Finally, the location of lesions within the brain have been found to be related to disease disability and might explain part of the clinico-radiological paradox (**box 1**)[68].

Box 1: Lesion Probability Mapping

Lesion location within the brain is highly variable between MS patients. Although there are some predeliction sites such as the periventricular, juxtacortical and infratentorial region, it has been demonstrated that the distribution is highly variable between patients. Within families, however, a higher concordance has been observed in lesion location[69]. It is hypothesized that lesion location determines symptomatology (and is able to explain part of the clinico-radiological paradox). To determine whether lesion location is genetically determined (see **chapter 3.2**) we used the Lesion Probability Mapping (LPM) method. This method enables us to assess relations between genotype and lesion location without a priori dividing the brain into a number of regions. For all patients T2 lesion-maps are generated. On these maps the lesions are outlined and groups with a certain genotype could be compared to the rest of the patients not carrying this genotype. All lesion masks of patients carrying a certain genotype were stacked and one mask was created indicating for every voxel the frequency of the presence of a lesion for these patients. For every voxel we assessed whether the patients carrying a certain genotype have a significantly higher probability of having a lesion in this voxel compared to patients not carrying the genotype.

Spinal cord MRI

Abnormalities in the spinal cord (focal lesions and diffuse abnormalities) are found in the majority of established MS patients (>90%)[70]. In MS patients, lesions are usually less than two vertebral segments in length, mostly located in the cervical cord[71]. These lesions can be visualized using MRI scan of the spinal cord, using conventional cardiac triggered sagittal PD and T2-weighted dual echo spin echo sequences. Spinal cord lesions may occur independently of MS brain abnormalities[72]. Moreover a small proportion of CDMS patients (less than 5%) does have abnormalities in the spinal cord, but does not exhibit abnormalities in the brain[73]. It has been suggested that a subgroup of MS patients might be more susceptible to a high amount of spinal cord lesions and few brain lesions, while others have extensive brain lesions and no spinal cord lesions. This heterogeneity is not well understood. Not only focal abnormalities are present in MS, but also spinal cord atrophy and diffuse abnormalities can be present[66].

The importance of spinal cord lesions on diagnosing MS has been accepted by MS experts, as is reflected in the most recent diagnostic criteria[30]. In CIS patients often asymptomatic spinal cord lesions are found (prevalence around 30-40%)[74,75] and spinal cord imaging is useful for several reasons. First, in spinal cord CIS, spinal cord MRI can rule out compressive spinal cord disease and characterize intrinsic spinal cord lesions. Second, in brain CIS, the finding of asymptomatic spinal cord lesions is of additional value in demonstrating dissemination

in space. Finally, CIS patients with asymptomatic spinal cord lesions more often convert to clinically definite MS than patients without spinal cord lesions[74]. Although spinal cord MRI can be useful in diagnosing MS, it was found less suitable for monitoring dissemination in time using previous diagnostic criteria, as brain MRI shows more new lesions over time than spinal cord MRI[73].

Spinal cord parameters have been found to be associated with disability. About 30% of new lesions in the spinal cord cause symptoms, compared to only 6% of new brain MRI lesions[73]. The number of focal lesions within the spinal cord correlates weakly with the degree of disability (mainly associated with bladder symptoms and walking disability)[72,76]. Mounting evidence points out that diffuse abnormalities may be more predictive of disability[77]. Diffuse abnormalities have been found to be associated with higher disability and a progressive disease course[72,78,79]. However, these diffuse abnormalities are only present in a minority of MS patients. In contrast, spinal cord atrophy is a more frequent finding in MS and has been found to be an important factor leading to irreversible disability[77,80,81]. No correlation was found between the extent of focal lesions (in brain and spinal cord) and cord atrophy, indicating that cord atrophy might be an independent feature of MS [82-85].

AIMS AND OUTLINE OF THIS THESIS

As has become clear from the previous paragraphs, the heterogeneity in MS is enormous, not only clinically (symptoms throughout the entire body and disability ranging from no complaints until death due to MS), but also paraclinically (a huge variety in lesion location throughout the brain and the spinal cord). Knowledge on factors involved in MS phenotype is important (especially early in the disease). This knowledge may enable us to identify patients susceptible for early disability accumulation. These patients might benefit more from early treatment with a more aggressive disease modifying therapy. While in patients with an expected benign disease course, disease modifying therapy could be postponed or maybe not started at all. This would increase the cost-effectiveness of disease modifying therapy and prevent “unnecessary” adverse events. Moreover, from a patient perspective, an earlier diagnosis of MS and a more accurate prediction on what to expect in the future, can be valuable for patients. This knowledge might help (often young) patients in planning their lives and may reduce the high degree of uncertainty that MS patients are faced with nowadays. Ultimately, knowledge of the processes underlying disease progression may lead to new treatment strategies.

So far, few clinical parameters have been found to have some prognostic value in patients: age at onset, disease subtype (progressive onset) and high relapse frequency early in the disease[1,11-13]. Additional markers that could be assessed early in the disease are needed.

Information on the genetic background of several phenotypic differences is still scarce, however several studies imply a genetic influence on these differences. Of these phenotypic differences, disease progression and the speed of disability accumulation are considered as (one of) the most important measures related to the prognosis of the patient. We hypothesize that genetic information and spinal cord parameters (lesions and atrophy of the spinal cord) can explain part of the phenotypic heterogeneity of MS, especially disease progression and speed of disability accumulation. Can we identify endophenotypes, using genetic information, spinal cord MRI variables in combination with clinical hallmarks?
Both biomarkers (genetic information and MRI hallmarks) may be proven valuable, based on their own characteristics. While genetic information possibly reflects a predisposition for a certain aspect of disease phenotype, it has the advantage of remaining constant over time and the possibility to assess this early in the disease. In addition, imaging variables (such as spinal MRI features) however changes over time and visualizes the regions of the central nervous system that are affected. These characteristics may provide crucial information on the evolving disease and the speed of disability accumulation.

In **chapter 2** we assessed the genetic effect on disease clinical phenotype. In **chapter 2.1** we test our hypothesis that disease severity is caused by a combination of genes in addition to several clinical factors. In a large group of well characterized MS patients, we genotyped around 80 SNPs. This genetic information was added to a statistical model to test whether the prediction of disease severity could be improved in addition to established clinical predictive variables.
In **chapter 2.2** we examined the role of the Interleukin 7 Receptor gene. This gene has been found to be associated with the risk of MS. The genetic variation (single nucleotide polymorphism (SNP)) that has the highest association with MS susceptibility is causing changes on mRNA level. We hypothesized that the function of Interleukin 7 Receptor in the inflammatory process might also lead to disease modifying capacities.
As mentioned above, disease severity is thought to be caused predominantly by the neurodegenerative part of MS, while the inflammatory process is more relevant in relapse occurrence and development of new lesions. However, only one MS susceptibility gene with a direct role in neurodegeneration has been found up till now: the KIF1B gene. This gene was found in three different populations associated with MS susceptibility. In **chapter 2.3** we examine whether this KIF1B-gene has a role in some of the neurodegenerative phenotypes of MS (atrophy rate, disability accumulation in the long run).
In **chapter 3** we tried to assess the genetic role in lesion distribution in MS. It is described that lesion location is one parameter that might indirectly affect disease severity, as some lesions in clinical relevant areas of the central nervous system, are more likely to cause more complaints. Several studies have suggested a genetic role in the lesion distribution. First in

chapter 3.1 we assessed the genetic role in distribution of lesions between the brain and the spinal cord.
Second, in **chapter 3.2** we tried to see whether lesion distribution within the brain could be explained by genotype of a group of selected genes that are thought to be related to MS.
In chapter 4 we assessed the clinical relevance of spinal cord lesions on diagnosing MS (**chapter 4.1**) using the new diagnostic criteria. Furthermore we assessed the relevance of upper cervical cord atrophy and other clinical and MRI features (brain and spinal cord) to predict disease severity (**chapter 4.2**).

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Chapter 2

Genetic markers of clinical Multiple Sclerosis phenotype

Chapter 2.1

Analysis of multiple candidate genes in association with phenotypes of Multiple Sclerosis

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Abstract

Background: Multiple sclerosis (MS) is a heterogeneous neurological disease with varying degrees of severity. The common hypothesis is that susceptibility to MS and its phenotype are caused by a combination of environmental and genetic factors. The genetic part exerts its effect through several genes each having modest effects.

Objectives: We evaluated whether disease severity could be predicted by a model based on clinical data and data from a DNA chip. The DNA chip was designed containing several single nucleotide polymorphisms (SNPs) in 44 genes, previously described to be associated with MS.

Methods: A total of 605 patients with Multiple Sclerosis were included in this analysis, using gender, onset type and age at onset as clinical covariates. We correlated 80 single nucleotide polymorphisms to the degree of disease severity using the following three outcome measures: linear Multiple Sclerosis Severity Score (MSSS), dichotomous MSSS (using a cut-off point of 2.5) and time to reach EDSS 6.

Results: Sixty-nine single nucleotide polymorphisms were included in the analysis. No individual single nucleotide polymorphism showed a significant association; however, a combination of single nucleotide polymorphisms significantly improved the prediction of disease severity in addition to the clinical variables. In all three models the Interleukin 2 gene was included, confirming a previously reported modest effect on disease severity. The highest power was obtained using the dichotomized MSSS as outcome.

Conclusions: Several single nucleotide polymorphisms showed their added predictive value over the clinical data in the predictive models. These results support our hypothesis that disease severity is determined by clinical variables and genetic influences (through several genes with small effects) in concert.

Introduction

Multiple Sclerosis (MS) is a presumed auto-immune disease affecting the central nervous system, characterized by demyelination and neurodegeneration. The clinical disease course is highly variable; some patients remain without significant functional loss for many years, while others become wheelchair-bound within a short period of time[1]. As different treatment options have become available (with varying efficacy and side-effects) the identification of patients prone to develop high disability within a short period, has become highly relevant. However, predictors for future disability are scarce.

Susceptibility and disease progression in MS is believed to be conferred by the interplay of genetic and environmental factors. The major histocompatibiliy complex (MHC) class II region on chromosome 6 was found to be highly associated with susceptibility to MS. In addition, a few single nucleotide polymorphisms (SNPs) showed genome-wide significance of association with small effect sizes to MS susceptibility[2-4]. The current opinion is that in addition to these genes, several other genes, each exerting a small effect on susceptibility to MS remain to be discovered[5]. Much effort has been put into unravelling the genetic and environmental influences on disease susceptibility, but less attention has been given to causes of disease variability and severity. The current hypothesis is that disease progression and other phenotypic variability of MS are influenced by genetic and non-genetic factors[6].

Earlier studies have reported intrafamilial concordance for certain phenotypes: disease course, disease severity and age at onset[7-11]. Several genes (APOE, IL1RN, MHC class II and IL1B) have been studied in relation to disease severity, however effect sizes were small and results were ambiguous[12-14]. Recently, alleles at the HLA-DRB1 locus have quite convincingly been shown to affect disease severity[15-16]. HLA-DRB1 alleles are also found associated with the development of antibodies against interferon-beta therapy and magnetic resonance imaging (MRI) parameters, possibly exerting an effect on disease course[17-19].

Determining the genetic influence on the disease process is important to gain insight into the pathophysiological mechanisms involved, and may suggest therapeutic approaches more readily than identifying genes involved in disease susceptibility[12]. Clinical predictors of disease severity were already identified, the most important being onset type (relapsing versus progressive), age at onset and gender.

We hypothesize that a combination of genes might improve the prediction of expected disease severity over purely clinical variables and designed a DNA chip to address this theory. In this study we evaluated the additional prognostic value of genetic information of a DNA chip, containing a set of candidate genes, previously correlated to MS (either susceptibility or phenotypes) over available demographics and clinical characteristics, aiming to improve the prediction of the expected disease severity for future patients.

Material and methods

Study participants:
A total of 605 unrelated Dutch Caucasian patients were selected retrospectively from natural history studies conducted at the MS Center at the VU University Medical Center (VUmc) in Amsterdam. The selection was based on the availability of DNA, clinical assessment of disability and the confirmed diagnosis of MS. No inclusion criteria for disability status, age, gender or onsettype were applied during selection of data for analysis. This study was carried out with the approval of the Medical Ethics Committee of the VUmc and informed consent was obtained from all participants. Patients were all diagnosed with MS according to Poser or McDonald criteria[20-21]. Clinical data were collected retrospectively including age, gender, onsettype, disease course, age at onset and duration of the disease. Disability status was determined for all subjects by using Kurtzke's Expanded Disability Status Scale (EDSS)[22]. These scores were not acquired during relapses.
Since no single golden standard for disease severity exists, we considered two outcomes for the patients: the Multiple Sclerosis Severity Score (MSSS) and time to reach EDSS 6, a clinically relevant endpoint, indicating that assistance for walking is required. We calculated the global MSSS from EDSS scores and disease duration at the time of patients' last visit as described earlier[23]. The global MSSS denotes the speed of disability accumulation of an individual patient compared with a large patient cohort.

Selection of SNPs:
SNPs were selected based on involvement in MS pathogenesis, prognosis or response to treatment, published in literature before July 2007. The polymorphisms were confirmed and associated to an identifier by using dbSNP database (www.ncbi.nlm.nih.gov/SNP). Nucleotide sequences for the design of allele-specific probes and PCR primers were retrieved from the SNPper database (http://snpper.chip.org/bio) and NCBI database (www.ncbi.nlm.nih.gov/SNP). Sequence specific probes and primers were designed by using the software Primer3, which is freely available at http://frodo.wi.mit.edu/. Gene names were applied according to the HUGO Gene Nomenclature Committee.

Genotyping:
Genomic DNA was isolated from anti-coagulated blood with DNAzol reagent (Molecular Research Center, Inc., Cincinnati, OH). Genotyping was carried out using a newly developed low-density DNA microarray based on allele-specific probes. The design, fabrication, validation and analysis of the arrays were performed following the procedure described earlier with minor modifications[24].

Variable selection:

We employed feature selection to identify the most important and predictive features in the models to be analyzed. This approach of variable filtering is based on the marginal association between each variable (SNP or clinical variable) and phenotype, as variables are typically filtered on the basis of a p-value cut-off from a univariate analysis.

The following outcome measures for disease severity were applied in our study: the time to reach EDSS 6 and MSSS. The MSSS outcome was employed in two ways: as a continuous outcome measure and after dichotomization, using a cut-off point of MSSS 2.5. The purpose of the latter approach was to compare relatively benign patients to more severely affected patients.

For the continuous MSSS we used the non-parametric Kruskal-Wallis (KW) test. For the dichotomized MSSS, the Chi-Square test was used. For time to reach EDSS 6 groups were compared by logrank tests.

To correct for multiple testing False Discovery Rate (FDR) according to Benjamini and Hockberg was applied[25]. The corrected number represents the expected proportion false discoveries for a given p-value cut-off. We used cut-off 0.05 after FDR correction.

Additionally, we tested the association of the SNPs included on the chip with onsettype (relapsing vs progressive onset) with a Chi-Square statistic on contingency tables. P-values were adjusted using the FDR as described above.

Predictive models:

HelixTree® software (Golden Helix, Inc., Bozeman, MT, USA) was used to calculate allelic association between different groups and deviations from Hardy-Weinberg Equilibrium (HWE). The same software calculated linkage disequilibrium among SNPs. In order to filter the SNPs included in the analyses, those SNPs that were monomorphic and those with minor allele frequency below 5% in our studied population were removed from the analysis. In addition, when a complete linkage disequilibrium among SNPs was observed (r^2 >0.8) only the one with the lowest p value for allelic association between SNPs and phenotypes was included in the regression model. Multivariate prognostic models were constructed for the following outcomes: continuous MSSS, dichotomous MSSS with the cut-off point of 2.5 and time to reach EDSS 6, using linear regression model, logistic regression model and Cox-regression model respectively, using SPSS version 15.0 (SPSS Inc. Headquarters, Chicago, IL, USA) and R packages Design (Harrell, 2001) and Stats (R Development Core Team, 2008).

First clinical variables (age at onset, gender and onsettype) were included as independent variables in all models. Secondly, backward selection was applied to select the SNPs that contributed to the model to increase the predictive power.

The goodness of fit of the model was evaluated using Hosmer-Lemeshow statistic and the accuracy was assessed by calculating the bootstrap area under the Receiver Operating

Characteristic (ROC) curve (AUC) with 95% confidence intervals. Bootstrapping on the model's AUC was conducted 500 times using 100% random sampling by replacement, using R package Boot. To measure the impact of the SNPs and variables included in the logistic regression model of the analyzed phenotypes, the sensitivity, specificity, and positive likelihood ratio (LR+ = sensitivity / (1-specificity)) were computed by means of ROC curves. AUC – ROC curves of the models based on clinical variables alone and clinical variables plus SNPs were compared by using the method by Delong et al.[26] implemented in the software Analyse-it (Analyse-it Software Ltd. Leeds, UK). The goodness of fit of the models based on clinical variables alone and clinical variables plus SNPs, was compared using a Likelihood Ratio Test and Akaike's Information Criteria (AIC)[27]. Both analyses were performed using R statistical software.

Results

Patient characteristics

Our patient group (n=605) reflects a representative MS population, with approximately 35% male and 17% primary progressive MS patients (see **Table 1**). Eighty-six out of 605 patients (14.2%) from the study population had MSSS values < 2.5 indicating a relatively benign disease course.

SNP selection

Eighty validated polymorphisms located in 44 different genes were studied on a DNA chip (see **supplementary Table 1** for the complete list). Five SNPs were monomorphic and 6 had a minor allele frequency below five percent. These eleven SNPs were excluded prior to univariate analysis. Finally, 69 SNPs were included in the analyses.

Univariate analysis on SNPs in relation to severity outcome-measures

We first determined the correlations of the individual SNPs to the outcome measures: MSSS, time to reach EDSS 6 and dichotomous MSSS using MSSS 2.5 as a cut-off point. The raw p-values and corrected p-values of the SNPs are noted in **supplementary Table 2**. No SNP remained significantly associated after correcting for multiple testing.

Effect of SNPs on onsettype

The analysis of the effect of the different SNPs on the onsettype did not reveal any significant association after correcting for multiple testing.

Table 1: Patient characteristics. Patient demographics and clinical characteristics divided in MS subtypes.

	All	RR	SP	PP
Total number of patients	605	310	190	105
Gender (n; % Male)	219 (36.2)	96 (31.0)	78 (41.1)	45 (42.9)
Mean age at onset (SD)	32.4 (9.5)	30.4 (8.0)	30.6 (8.8)	41.6 (9.5)
Mean disease duration (SD)	13.1 (8.3)	9.7 (5.9)	18.2 (9.3)	13.8 (7.6)
Median EDSS (Interquartile range)	4.0 (3.5)	3.0 (2.0)	6.5 (1.5)	6.5 (3.5)
Median MSSS (Interquartile range)	5.6 (4.7)	3.9 (3.7)	7.9 (3.7)	7.3 (3.3)
Number of patients that reached EDSS 6 (%)	234 (38.7)	27 (8.7)	147 (77.4)	60 (57.1)
Median time to reach EDSS 6 in months (Interquartile range)	103 (105)	102 (83)	114 (121)	84 (91)

EDSS: Expanded Disability Status Scale; MSSS: Multiple Sclerosis Severity Score; PP: primary progressive; RR: relapsing remitting; SD: standard deviation, SP: secondary progressive.

Multivariate prognostic models on disease severity:

The linear model (MSSS) returns two non-zero coefficients for the SNPs, both originating from the IL2 SNP rs2069763, in addition to the clinical variables (see **Table 2**). The Cox-regression analysis returns nine non-zero coefficients for the SNPs. However, all coefficients are close to zero or the 95%-confidence interval include 1 (see **Table 3**).

Table 2: Predictive model using MSSS as a continuous outcome variable (linear regression). Indicator variables have been created for the categorical predictors. The reference group for gender is “female”, the reference group for onsettype is “relapsing”, and the reference group for IL2 (rs2069763) is “GG”.

	Indicator variable	**B-coefficient (unstandardized)**	**β-coefficient (standardized)**	**95% Confidence interval for B-coefficient (unstandardized)**	**Significance**
Age at onset		0.05	0.17	0.02-0.07	0.0001
Gender	Male	0.51	0.09	0.09-0.93	0.0183
Onsettype	Progressive	0.94	0.13	0.34-1.53	0.0021
IL2 (rs2069763)	GT	-0.51	-0.10	-0.95- -0.08	0.02
	TT	-1.00	-0.13	-1.65- -0.08	0.0027

When using the model for the dichotomous outcome of the MSSS (with a cut-off point of 2.5) three clinical covariates and six SNPs were included in the model (see **Table 4**).

The linear regression corrected R-square is 0.088 and the Cox regression R-square is 0.02. The R-square for the logistic regression is 0.219, indicating a higher predictive power for the logistic regression model using the dichotomous outcome, although these are not directly comparable. Therefore, only for the dichotomous model ROC curves are obtained to test the additive value of the SNPs over the clinical data relevant to disability accumulation.

Table 3: Predictive model using survival analysis on time to reach EDSS = 6 (Cox-regression).

		β-coefficient	OR	95% Confidence interval for OR	Significance
Age at onset		0.03	1.033	1.02-1.05	<0.001
Gender					
	Male vs female	0.28	1.32	1.01-1.73	0.046
NDUFS7 (rs2074897)					0.030
	AG vs GG	-0.05	0.96	0.69-1.33	0.786
	AA vs GG	0.36	1.44	0.99-2.08	0.054
ADAMTS14 (rs4747075)					0.001
	AG vs GG	-0.54	0.58	0.42-0.80	0.001
	AA vs GG	-0.53	0.59	0.39-0.89	0.011
FAS (rs2234978)					0.016
	CT vs CC	0.25	1.28	0.97-1.70	0.086
	TT vs CC	0.60	1.83	1.19-2.82	0.006
IL2 (rs2069762)					0.008
	TG vs TT	0.28	1.33	1.00-1.75	0.044
	GG vs TT	0.70	2.02	1.23-3.31	0.005
SPP1 (rs2853744)					
	GT vsGG	0.58	1.78	1.18-2.67	0.006

Figure 1 shows ROC curves obtained with the "clinical model" and the "clinical-genotypic model". The clinical model includes age at onset, onsettype and gender. While the clinical-genotypic model includes the selected SNPs (see **table 4**) in addition to the clinical parameters. The curve obtained from the clinical-genotypic model shows a sensitivity of 37% with specificity of 95.3% and an LR+ of 7.9 (AUC=0.78), whilst the clinical model had a sensitivity of 27.4% with specificity of 95.3% and an LR+ of 5.8 (AUC=0.68).

By including genetic information (SNPs) in the model, a significant improvement in predictive power was obtained as calculated by means of the AIC and the Likelihood Ratio Test. The Likelihood Ratio Test showed that the model including SNPs fits the data significantly better than the model based on clinical variables only (X^2=46.89, df=10, p<0.0001). In addition to this, the model including SNPs showed a lower AIC than that based on clinical variables only (443.9 vs 470.7). The multivariate model combining clinical data and genotypic data significantly predicted the severity of the disease (model X^2=78.8, p< 0.001). The model discriminated well between patients who had mild and more severe forms of the disease (AUC = 0.78, bootstrap 95% CI 0.75-0.84).

Table 4: Predictive model using dichotomous MSSS as outcome measure (threshold is set on MSSS < 2.5) (logistic regression).

		β-coefficient	OR	95% Confidence interval for OR	Significance
Age at onset		0.05	1.05	1.02-1.08	0.004
Gender					
	Male vs female	0.70	2.02	1.14-3.57	0.015
Onsettype					
	Progressive vs relapsing	1.55	4.69	1.32-16.63	0.017
NOS2 (rs1137933)					0.005
	AG vs GG	-0.63	0.53	0.32-0.89	0.016
	AA vs GG	-1.41	0.24	0.09-0.67	0.006
PITPNC1 (rs1318)					0.009
	AG vs AA	-0.81	0.45	0.27-0.75	0.002
	GG vs AA	-0.53	0.59	0.18-1.95	0.387
IL2 (rs2069763)					0.004
	GT vs GG	-0.94	0.39	0.22-0.70	0.001
	TT vs GG	-0.98	0.38	0.17-0.84	0.016
CCL5 (rs2107538)					0.062
	CT vs CC	0.71	2.04	1.12-3.70	0.020
	TT vs CC	0.38	1.47	0.38-5.67	0.576
IL1RN (rs423904)					
	CT/TT vs CC	-0.52	0.60	0.36-0.99	0.047
PNMT (rs876493)					
	GG vs AA/AG	-0.65	0.52	0.29-0.92	0.025

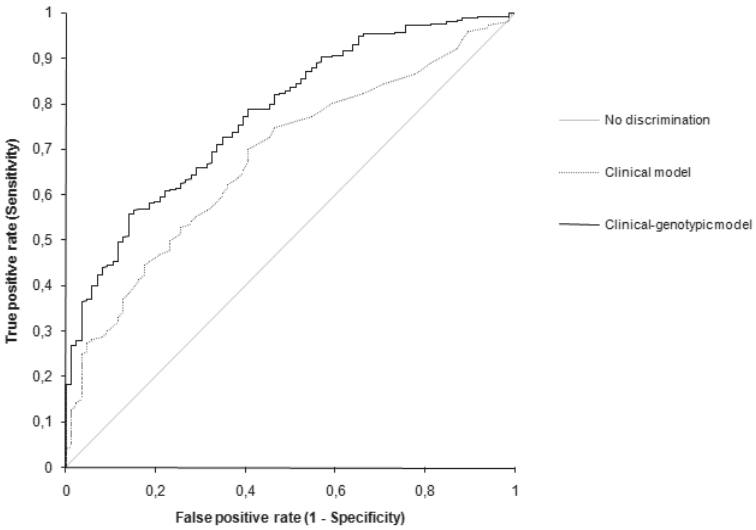

Figure 1: ROC curves for the "clinical model" and the "clinical-genotypic model". The clinical model includes the following variables: age at onset, gender and onsettype. The clinical-genotypic model includes the variables mentioned in Table 4. The "clinical model" had a AUC of 0.68 and the clinical-genotypic model had a AUC of 0.78. The Likelihood Ratio Test showed that the model including SNPs fits the data significantly better than the model based on clinical variables only (X^2=46.89, df=10, p<0.0001).

Discussion

As expected, we could not detect one major SNP / gene related to disease severity in our study. The current hypothesis concerning susceptibility genes and MS is based on the assumption that multiple genes exert a small effect on developing MS on top of the major influence of HLA-DRB1*1501. Our hypothesis was based on transferring this assumption to genes involved in disease progression. We therefore developed an instrument to detect these effects and built predictive models including several SNPs in addition to relevant clinical parameters.

We tested three different predictive models based on different outcome measures, as no single golden standard has been described in literature.

Our results show that a combination of SNPs increases the predictive value of the models.

Different genes were included in the different models, showing in general small individual effects on the outcome. The most prominent gene, included in all 3 models with moderately high coefficients encodes for interleukin-2 (IL-2). IL-2 (T cell growth factor) is an immunoregulatory cytokine important for the T cell homeostasis and is involved in the regulation of auto-immunity[28-30]. Previously this gene was found to be related to susceptibility to MS[31] and genetic differences in this gene were found between relapsing remitting MS patients and secondary progressive MS patients[31-32]. Interestingly, Daclizumab, a humanized monoclonal antibody (mAb) that blocks the binding of interleukin-2 to the interleukin-2 receptor alpha unit (IL-2R-alpha chain; CD25), has shown to be effective in most patients who experienced persistent MS disease activity with first-line therapy[33]. Moreover, in secondary progressive MS patients heightened levels of IL-2 were reported and CSF concentrations of IL-2 were correlated with the degree of disability in patients with clinically active patients[34]. Genetic variation within the IL-2 gene is likely to modify disease progression.

Interestingly, rs3135388 (a surrogate marker for HLA-DRB1*1501) did not show any effect on disease severity in any of the models. This contrasts other studies that have shown that carriers of the HLA-DRB1*1501 had a more severe disease course using an extreme-outcome-strategy[16]. When applying the same strategy and definitions to our patient group, sample sizes of the benign and severe MS patient groups were too small to test this hypothesis.

The most convincing model with the highest predictive power and the highest coefficients was the dichotomous model on the MSSS. The dichotomous model on the MSSS discriminates benign patients from the more severely disabled patients, by using a cut-off point of MSSS 2.5 (equivalent to an EDSS of three or lower at 15 years of disease duration; the common definition of benign MS). A dichotomous model might be more sensitive in detecting the small effects of the SNPs, however the survival model (time to reach EDSS 6) and the linear MSSS model provide more detailed information on the speed of disability

accumulation and on the severity of the disability. Unfortunately in our study only 38.7% of the patients reached the EDSS of 6, thereby limiting the power of survival analysis. Although we tried to lower the chance of overfitting of our predictive model, our findings should be further explored and confirmed in a different cohort of MS patients, preferably with a longer disease duration.

To improve the prediction on disability, selection of the right SNPs is essential. Our selection of SNPs was based on a heterogeneously reported association between this gene and multiple sclerosis susceptibility or phenotypes. The selection of SNPs was performed in the "*pre-Genome-Wide Association Study (GWAS)- era*". Using information from recently published data of genes influencing disease phenotype[35] could substantially improve our SNP selection in search of predictive models. Baranzini et al showed that MSSS was associated with genes, relatively new to MS literature. These genes are involved in cellular mechanisms such as protein amino acid N-linked glycosylation, cellular respiration and embryonic development. This study illustrates that disease-modifying genes are not necessarily identical to disease susceptibility genes[35].

More and more evidence points towards a predictive value of MRI parameters early in the disease for disability later on[36]. Including these early MRI parameters in the predictive model could improve the predictive value on future disability accumulation. Moreover inclusion of yet to be discovered genes and maybe environmental factors might increase the explained variance in disease severity.

In summary, we showed in a relatively large sample of well characterised patients that in addition to clinical variables, genetic information is valuable to improve the prediction of disease severity in MS. However, to more precisely estimate the true genetic influence on disability accumulation in MS, replication of our results is key.

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Supplementary table 1: SNPs included on the DNA chip. MAF=minor allele frequency in our sample.

Gene	rs-number	Chromosome	Polymorphism	MAF
ADAMTS14 *	rs4747075	10q22	A/G	0.29
ADAMTS14	rs7081273	10q22	C/G	0.35
ADAMTS14	rs4746060	10q22	C/T	0.09
BTNL2 *	rs2076530	6p21.3	A/G	0.23
CACNG4	rs4790896	17q24	C/T	0.41
CCDC46	rs987931	17q24	G/T	0.34
CCL5	rs2280788	17q11.2-q12	C/G	0.03**
CCL5	rs2107538	17q11.2-q12	C/T	0.19
CCR5	rs333	3p21	-/+	0.10
CD24	rs8734	6q21	C/T	0.00**
CIITA	rs3087456	16p13	A/G	0.26
CNTF	rs1800169	11q12	A/G	0.13
CRYAB	rs14133	11q21-q23	C/G	0.26
CRYAB	rs762550	11q21-q23	A/G	0.37
CTLA4	rs231775	2q33	A/G	0.37
CTLA4	rs5742909	2q33	C/T	0.08
EBF1	rs1368297	5q34	A/T	0.38
FAS	rs1800682	10q23	C/T	0.45
FAS	rs3781202	10q23	C/T	0.40
FAS	rs2234978	10q23	C/T	0.31
GABBR1	rs1805057	6p21.3	A/G	0.00**
HELZ	rs2363846	17q24	C/T	0.49
HLA *	rs2395166	6p21.3	C/T	0.47
HLA-DRA	rs2213584	6p21.3	C/T	0.41
HLA	rs2227139	6p21.3	C/T	0.41
HLA-DRA	rs3135388	6p21.3	C/T	0.31
HLA	rs9268458	6p21.3	A/C	0.18
HLA *	rs6457594	6p21.3	A/G	0.39
HLA-DRA	rs2395182	6p21.3	G/T	0.39
HLA-DRA	rs2239802	6p21.3	C/G	0.39
HSPB2	rs2234702	11q21-q23	C/G	0.00**
IFNAR1	rs2257167	21q22	C/G	0.11
IFNGR2	rs9808753	21q22	A/G	0.15
IL1B	rs1799916	2q14	G/T	0.00**
IL1B	rs1143627	2q14	C/T	0.34
IL1B	rs1143634	2q14	C/T	0.27
IL1RN	rs419598	2q12-q14	C/T	0.27
IL1RN	2073 C/T	2q12-q14	C/T	0.27
IL2	rs2069763	4q26-27	G/T	0.36
IL2	rs2069762	4q26-27	G/T	0.25
IL4R	rs1801275	16p12	A/G	0.23
IL7R	rs11567685	5p13	C/T	0.25
IL7R	rs7718919	5p13	G/T	0.14
IL7R	rs11567686	5p13	A/G	0.35
IL10 *	rs1800896	1q32	A/G	0.50

MC1R	rs1805009	16q24	C/G	0.02**
MC1R	rs1805006	16q24	A/C	0.01**
MEFV	rs28940577	16p13.3	A/G	0.00**
MOG	rs3130250	6p22	A/G	0.20
MOG	rs3130253	6p22	A/G	0.11
NDUFA7	rs2288414	19p13.2	C/G	0.03**
NDUFA7	rs561	19p13.2	A/G	0.18
NDUFS5	rs2889683	1p34.2	C/T	0.30
NDUFS5	rs6981	1p34.2	A/G	0.06
NDUFS7	rs2074897	19p13.3	A/G	0.49
NFKBIL1	rs3130062	6p21.3	C/T	0.15
NOS2	rs1137933	17q11.2	C/T	0.24
NOS2	rs2779248	17q11.2	C/T	0.40
NOTCH4	rs367398	6p21.3	A/G	0.21
PDCD1	rs11568821	2q37	A/G	0.11
PITPNC1	rs1318	17q24	C/T	0.20
PITPNC1	rs2365403	17q24	C/G	0.17
PNMT	rs876493	17q11-q23	C/T	0.42
PRKCA	rs7220007	17q24	A/G	0.48
PRKCA	rs887797	17q24	C/T	0.29
PRKCA	rs2078153	17q24	C/G	0.23
PRKCA *	rs3890137	17q24	A/G	0.36
PTPN22	rs2476601	1p13	A/G	0.10
PTPRC	rs17612648	1q31	C/G	0.02**
PTPRC	rs4915154	1q31	A/G	0.01**
SPP1	rs1126616	4q21	C/T	0.23
SPP1	rs1126772	4q21	A/G	0.19
SPP1	rs2853744	4q21	G/T	0.05
SPP1	rs9138	4q21	A/C	0.24
SPP1	rs4754	4q21	C/T	0.24
TNF	rs1800629	6p21.3	A/G	0.17
TNFSF10	rs1131568***	3q26	A/G	0.34
UCP2	rs659366	11q13	C/T	0.36
VDR	rs1544410	12q13	A/G	0.40
VDR	rs731236	12q13	C/T	0.39

* Deviation from Hardy Weinberg proportions with $p < 0.05$.
**Excluded from analyses due to minor allele frequency < 0.05
*** Previously rs-number: rs9880164.

See list of abbreviations on page 181

Supplementary Table 2: Results of univariate analysis before and after correction for multiple testing. Significant values are printed in bold.

Gene	rs-Number	p-value logrank test on time to reach EDDS6	Corrected p-value (FDR) logrank test on time to reach EDDS6	p-value (Kruskal Wallis) on linear MSSS	Corrected p-value (FDR) on linear MSSS	p-value (ChiSquare) on dichotomous MSSS	Corrected p-value on ChiSquare on dichotomous MSSS
ADAMTS14	rs4747075	**0.02**	0.52	**0.02**	0.60	0.09	0.86
ADAMTS14	rs4746060	0.11	0.64	0.38	0.84	0.16	0.87
ADAMTS14	rs7081273	**0.03**	0.55	0.19	0.68	0.30	0.87
BTNL2	rs2076530	0.58	0.97	0.50	0.88	0.30	0.87
CACNG4	rs4790896	0.78	0.97	0.61	0.91	0.55	0.97
CCDC46	rs987931	0.17	0.66	0.62	0.92	0.46	0.97
CIITA	rs3087456	0.87	0.97	0.87	0.93	0.94	0.97
CCL5	rs2107538	0.19	0.66	0.21	0.68	**0.04**	0.47
CCR5	rs333	0.33	0.82	0.42	0.84	0.61	0.97
CNTF	rs1800169	0.83	0.97	0.72	0.93	0.59	0.97
CRYAB	rs762550	0.30	0.80	0.83	0.93	0.66	0.97
CRYAB	rs14133	0.98	1.00	0.71	0.93	0.74	0.97
CTLA4	rs5742909	0.85	0.97	0.18	0.68	0.11	0.87
CTLA4	rs231775	0.27	0.80	0.44	0.85	0.64	0.97
EBF1	rs1368297	0.86	0.97	0.93	0.96	0.71	0.97
FAS	rs2234978	**0.01**	0.52	0.47	0.85	0.49	0.97
FAS	rs1800682	0.99	1.00	0.81	0.93	0.64	0.97
FAS	rs3781202	0.83	0.97	0.97	0.97	0.85	0.97
HELZ	rs2363846	0.52	0.97	0.21	0.68	0.55	0.97
HLA-DRA	rs3135388	0.32	0.82	0.12	0.65	0.25	0.87
HLA	rs2395166	0.12	0.64	0.20	0.68	0.96	0.97
HLA	rs9268458	0.29	0.80	0.36	0.84	0.51	0.97
HLA-DRA	rs2213584	0.66	0.97	0.38	0.84	0.54	0.97
HLA	rs2227139	0.70	0.97	0.43	0.84	0.65	0.97
HLA	rs6457594	0.69	0.97	0.47	0.85	0.71	0.97
HLA-DRA	rs2395182	0.30	0.80	0.06	0.65	0.16	0.87
HLA-DRA	rs2239802	0.24	0.76	0.06	0.65	0.16	0.87
IFNAR1	rs2257167	0.15	0.64	0.10	0.65	**<0.01**	0.10
IFNGR2	rs9808753	0.11	0.64	0.08	0.65	0.12	0.87
IKBL	rs3130062	0.71	0.97	0.41	0.84	0.30	0.87
IL1B	rs1143627	0.06	0.55	0.10	0.65	0.83	0.97
IL1B	rs1143634	0.94	1.00	0.76	0.93	0.64	0.97
IL1RN	2073 C/T	0.78	0.97	0.89	0.93	0.18	0.87
IL1RN	rs419598	0.72	0.97	0.96	0.97	0.20	0.87
IL2	rs2069763	**0.05**	0.55	**0.01**	0.49	**0.01**	0.12
IL2	rs2069762	0.07	0.55	0.17	0.68	0.97	0.97
IL4	rs1801275	0.06	0.55	0.42	0.84	0.67	0.97
IL7R	rs11567685	**0.05**	0.55	0.08	0.65	0.32	0.87
IL7R	rs7718919	0.61	0.97	0.25	0.69	0.75	0.97
IL7R	rs11567686	0.67	0.97	0.66	0.93	0.90	0.97

IL10	rs1800896	0.14	0.64	0.22	0.69	0.83	0.97
MOG	rs3130250	0.18	0.66	0.11	0.65	0.61	0.97
MOG	rs3130253	0.36	0.86	0.12	0.65	0.91	0.97
NDUFA7	rs561	0.80	0.97	0.86	0.93	0.26	0.87
NDUFS5	rs6981	0.47	0.97	0.20	0.68	0.26	0.87
NDUFS5	rs2889683	0.24	0.76	0.38	0.84	0.61	0.97
NDUFS7	rs2074897	0.07	0.55	0.19	0.68	0.81	0.97
NOS2	rs1137933	0.47	0.97	0.42	0.84	**<0.01**	0.12
NOS2	rs2779248	0.85	0.97	0.60	0.91	0.38	0.97
NOTCH4	rs367398	0.70	0.97	0.87	0.93	0.96	0.97
PDCD1	rs11568821	0.85	0.97	0.61	0.91	0.75	0.97
PITPNC1	rs1318	0.19	0.66	0.10	0.65	**0.01**	0.12
PITPNC1	rs2365403	0.84	0.97	0.83	0.93	0.24	0.87
PNMT	rs876493	0.38	0.87	0.30	0.80	**0.01**	0.19
PRKCA	rs887797	0.84	0.97	0.25	0.69	0.35	0.93
PRKCA	rs3890137	0.13	0.64	0.69	0.93	0.80	0.97
PRKCA	rs2078153	0.69	0.97	0.83	0.93	0.90	0.97
PRKCA	rs7220007	0.90	0.98	0.70	0.93	0.85	0.97
PTPN22	rs2476601	0.51	0.97	0.81	0.93	0.64	0.97
SPP1	rs2853744	0.09	0.64	0.10	0.65	0.59	0.97
SPP1	rs4754	0.76	0.97	0.51	0.88	0.65	0.97
SPP1	rs9138	0.74	0.97	0.56	0.90	0.67	0.97
SPP1	rs1126616	0.77	0.97	0.55	0.90	0.68	0.97
SPP1	rs1126772	0.99	1.00	0.78	0.93	0.94	0.97
TNF	rs1800629	1.00	1.00	0.54	0.90	0.27	0.87
TNFSF10	rs1131568	0.82	0.97	0.24	0.69	0.18	0.87
UCP2	rs659366	0.95	1.00	0.84	0.93	0.26	0.87
VDR	rs731236	0.65	0.97	0.87	0.93	0.84	0.97
VDR	rs1544410	0.85	0.97	0.79	0.93	0.95	0.97

See list of abbreviations on page 181

Chapter 2.2

Relevance of IL7R genotype and mRNA expression in Dutch patients with Multiple Sclerosis

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Abstract

Background: The Interleukin 7 Receptor (IL7R) has been recognized as a susceptibility gene for Multiple Sclerosis (MS). Analysis of rs6897932 (the most strongly MS-associated Single Nucleotide Polymorphism (SNP)), showed effects of genotype on the relative expression of membrane-bound to total amount of IL7R mRNA.

Objective: We assessed the relevance of IL7R on MS phenotype (including clinical and magnetic resonance imaging (MRI) parameters) at DNA and mRNA level in Dutch patients with MS.

Methods: Genotype of rs6897932 was analyzed in 697 MS patients and 174 healthy controls. The relevance of genotype and carriership of the C-allele on MS phenotype (disease activity and severity, using clinical and MRI parameters) was assessed. In addition, relative gene expression of membrane-bound to total IL7R mRNA, was analyzed with respect to disease phenotype in a subgroup of 95 patients with early relapsing MS.

Results: In particular, homozygosity for the risk-allele is a risk factor for MS in our population ($OR_{CC\ vs\ CT\ and\ TT}$=1.65 (95% CI: 1.18 – 2.30), two-sided p=0.004). However, no effect of genotype or the relative expression of membrane-bound IL7R (presence of exon 6-7) to total amount of IL7R mRNA (presence of exon 4-5) was found on MS phenotype.

Discussion: Homozygosity for the IL7R exon 6 rs6897932 C allele is associated with a higher risk for MS in our Dutch population. No effect was found of genotype or mRNA expression on disease phenotype.

Introduction

The demyelinating neurodegenerative disease Multiple Sclerosis (MS) is the most common cause of chronic neurological disease in young adults[1]. Evidence indicates a complex interplay of genetic and environmental factors in the predisposition to this disease[2-4]. The strongest and most consistent genetic component is the Major Histocompatibility Complex (MHC) HLA-DRB1*1501-DQB1*0602 haplotype[5]. More recently, several non-MHC Single Nucleotide Polymorphisms (SNPs) were found, showing a more modest effect on susceptibility[6-10]. One of the SNPs identified and confirmed in several cohorts is rs6897932, located in exon 6 of the Interleukin 7 Receptor (IL7R) gene on chromosome 5p13[8-15]. In linkage studies the chromosome 5p12-14 region was related to MS susceptibility. A role for the IL7R-gene in MS susceptibility was furthermore suggested because of its function in survival and proliferation of T and B cells[16]. For its action the cytokine IL-7 depends on the expression of its corresponding receptor on the cell surface (IL-7R)[17,18]. Two isoforms of the IL-7R receptor exist, a membrane-bound and a soluble isoform.

The SNP with the strongest association within the IL7R gene, identified by the whole genome association studies and haplotype analysis, was rs6897932 (a non-synonymous SNP, leading to a coding change (T244I))[9]. In vitro analysis shows that the 'C' allele augments an exonic silencer resulting in an approximately two-fold increase in the skipping of exon 6 when compared with the 'T' allele, leading to increased production of the soluble form of IL-7R[8].

In the family of cytokines and cytokine receptors, alternative splicing of pre-mRNA is a widespread regulatory mechanism that has been demonstrated to be involved in the control of gene expression influencing both cell development and cell activation[17,19].

Due to the functional consequences of this polymorphism it can be postulated that this SNP not only has an influence on disease susceptibility, but may also affect the disease course: patients carrying the risk allele might have a more active disease. To our knowledge no other studies have addressed the possible effect of IL7R polymorphisms and relative expression levels combined in one study on MS disease course.

In this study our aim is to assess the importance of this IL7R SNP (rs6897932) in MS susceptibility in a Dutch population. Secondly, we study the relevance of this SNP (on DNA and mRNA level) on the disease course (measured by clinical and imaging outcome parameters).

Material and Methods

Subjects

We sampled 697 patients from MS natural history studies at the MS Center of the VU University Medical Center, Amsterdam, the Netherlands. Dutch Caucasian patients only were included based on availability of at least one clinical assessment of disability using the Expanded Disability Status Scale (EDSS) and the availability of DNA material. Patients had a diagnosis of MS according to McDonald criteria[20] or Poser criteria[21], depending on the time of data acquisition. Patients that presented with a first relapse with symptoms suggestive for demyelination that lasted at least 24 hours, however that do not fulfill the McDonald criteria at their most recent follow-up were also included (Clinically Isolated Syndrome (CIS))[20]. We included 174 unrelated Dutch Caucasian healthy controls in this study. This group was also incorporated (and described) in the study by Schrijver et al.[22]
This study was carried out with the approval of the Medical Ethical Committee of the VUmc and informed consent was obtained from all participants.

MS-phenotype

For all patients the following clinical data on disease severity were collected: age at disease onset, most recent EDSS[23] scores, Multiple Sclerosis Severity Scales (MSSS)[24] and time to reach EDSS = 6 (a clinically relevant time-point indicating when a patient requires help in walking). When available Multiple Sclerosis Functional Composite (MSFC) scores[25] were collected and used as secondary outcomes of disease severity. The MSFC is a composite score consisting of three separate tests, the Timed 25-Foot Walk (T25FW), the 9-hole Peg Test (9HPT) and the Paced Auditory Serial Addition Test (PASAT). Testing was performed under standardized conditions and tests were practiced at least once before baseline assessment. In patients that were followed up annually from disease onset up to at least 2 years (n=210), the following disease activity measures were collected: occurrence of new relapses, use of disease modifying therapy (DMT) and the number of intravenously administered methylprednisolone treatment courses.

Patient selection sub study on expression analysis:

For this sub study we selected a group of 95 relapse-onset patients from our total MS (DNA) cohort (n=697). This selection was based on the availability of mRNA. These patients were all sampled from one of our ongoing prospective natural history studies in early MS patients. Patients eligible for this study presented with CIS or were diagnosed with MS within the previous 6 months. RNA was collected at baseline and an MRI scan of the brain was performed at baseline and after two years of follow-up. At annual follow-up visits the above described measures of disease severity and disease activity were collected.

Genotyping

Genomic DNA was isolated from anticoagulated blood using DNAzol reagent (Molecular Research Center, Inc, Cincinnati, Ohio). The single nucleotide polymorphism (SNP) in exon 6 of the interleukin 7 receptor gene (IL7R) (dbSNP rs ID: rs6897932; HGVS nomenclature version 2.0: NG_009567.1:g.22585C>T) was analyzed using the C___2025977_10 predesigned and validated Taqman Genotyping Assay on Demand from Applied Biosystems (Foster City, CA, USA).

Expression analysis

Blood samples were collected using PaxGene RNA tubes followed by standard processing according to the manufacturers instructions. Automated RNA isolation was performed, within 3 months after freezing on the BioRobot MDX (Qiagen). The cDNA synthesis was performed with the SuperScript III First-Strand Synthesis System (Invitrogen) according to the manufacturer's conditions. The total RNA input per reaction was 100 ng using a reverse IL7R primer (5'-TTC TTG GTT TCT TAC AAA GAT GTT CC-3') complementary to exon 7. Quantitative PCR was performed in duplicate on the ABI7300 with Platinum Quantitative PCR supermix-UDG w/Rox (Invitrogen). Quantitative analysis was done with the ABI7500 software.

The amounts of total (membrane-bound plus soluble) IL7R and membrane-bound form of IL7R mRNA were measured by assaying Hs00233682_m1 for exons 4–5 enabling the detection of total IL7R cDNA, and via Hs00904814_m1 spanning exons 6–7 reflecting only membrane-bound IL7R. Results are normalized to the expression level of 'housekeeping gene' glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to correct for experimental variations. Gene expression results were analyzed using the 2-ΔΔCt method for relative quantification[26]. The relative expression of membrane-bound IL7R to total amount of IL7R mRNA was expressed as a 'fold-change' value.

Magnetic Resonance Imaging (MRI)

Per protocol MRI scans were performed at baseline and at year 2 for patients from our ongoing natural history study in early MS. Scans were acquired either on 1.0 Tesla or 1.5 Tesla (Siemens AG, Erlangen, Germany) scanners with standard head coils, using standard 2D conventional or fast spin-echo Proton Density (PD) - and T2-weighted images (TR: 2200-3000 ms, TE: 20-30 & 80-100 ms) with slice thicknesses of 3-5 mm, a maximum gap between slices of 0.5 mm, and an in-plane resolution of 1x1 mm^2. The number and volume of T2 weighted lesions, T1 hypo-intense lesions and gadolinium-enhancing T1 weighted lesions on brain MRI were assessed and compared to baseline. The lesions were identified by an independent rater, blinded for IL7R expression results and clinical data.

Disease severity on MRI was assessed by measuring the T1 hypo-intense lesion volume and the T2 lesion volume at baseline and after two years of follow-up. We used the following *disease activity* MRI parameters: number of new T2 lesions at follow-up and volume of T1 gadolinium enhancing lesions.
Baseline scans were performed on the day of blood sampling for patients included in the expression analysis.

Statistical analysis

The annualized relapse rate and rate of intravenous methylprednisolone treatment courses were calculated from the most recent available data by dividing the total number of relapses and respectively the number of methylprednisolone-courses by the total follow-up duration. Clinically relevant changes in EDSS and MSFC were assessed according to generally accepted guidelines[27,28].
Hardy–Weinberg equilibrium (HWE) was evaluated using Pearson's goodness-of-fit $\chi 2$ test (degree of freedom= 1) for genotyping results. Genotype distribution and allele frequencies were compared between healthy controls and MS subjects using Pearson Chi-Square testing and Fisher's exact test respectively; odds ratios were calculated including 95% confidence intervals using the best fitted genetic model (recessive, dominant or additive).
To test for significant differences in MS phenotype, the parameters of disease severity and disease activity (clinically and using MRI parameters) were compared for every genotype using Kruskal Wallis Test; differences between carriers and non-carriers of the risk-allele were assessed using the Mann Whitney Test. In addition, multivariate regression analysis was performed to correct the results for relevant clinical parameters (disease duration, gender, use of disease modifying therapy, age and onset type).
The 2 -ΔΔCT method was used to calculate the relative change in gene expression of membrane-bound to total IL7R mRNA. We assessed whether there were differences in the relative expression of membrane-bound to total IL7R mRNA between genotypes using the Kruskal Wallis Test. Differences in membrane-bound to total IL7R expression between groups for baseline characteristics (clinically and using MRI parameters) were tested with Mann-Whitney, Kruskal-Wallis and Spearman test where appropriate. Moreover, multivariate regression analysis was performed to correct the results for relevant clinical parameters (disease duration, gender, age, use of disease modifying therapy and onset type).
For the time to first relapse and time to reach EDSS = 6, we constructed Kaplan-Meier curves and with log-rank we tested differences between dichotomized levels of membrane-bound to total IL7R expression and between genotypes. All reported p-values are based on two-tailed significance tests. The threshold for significance was set at 0.01. For all statistical procedures SPSS 15.0 for Windows was used.

Results

Patients

A total of 697 unrelated, Dutch Caucasian patients were included in the DNA analysis. The median disease duration was 10.8 years (IQR: 10.5 years). The median EDSS was 4.0 (IQR: 4.0) (see **Table 1** for more patient characteristics). MSFC results were available for 441 patients (data not shown).

Clinical data on disease activity was prospectively collected for 210 relapse-onset patients from disease onset (median follow-up 6 years). Some 57.1% of patients used Disease Modifying Therapy (DMT) during follow-up. The median relapse rate in this group was 0.47. The median number of treatment courses with methylprednisolone per year was 0.12 (for more details on this subgroup see **Supplementary table 1** for patient characteristics).

The control subjects (n=174) consisted of 97 females (55.7%) and had a mean age of 46.5 years.

Table 1: Patient characteristics.

	All (n=697)	RR (n=367)	SP (n=192)	PP (n=111)	CIS (n=27)
Gender (n; % Male)	250 (35.9%)	112 (30.5%)	79 (41.1%)	49 (44.1%)	10 (37.0%)
Median age at onset (IQR)	32.1 (13.6)	29.9 (11.4)	29.8 (11.2)	42.1 (13.6)	39.6 (11.0)
Median disease duration at most recent visit (IQR)	10.8 (10.5)	8.2 (7.8)	17.1 (10.8)	12.2 (10.3)	4.1 (3.3)
Median EDSS at most recent visit (IQR)	4.0 (4.0)	3.0 (2.0)	7.0 (1.5)	6.5 (3.0)	2.0 (2.0)
Number of patients that reached EDSS 6 (%)	249 (35.7%)	32 (8.7%)	151 (78.6%)	64 (57.7%)	2 (7.4%)
Median time to reach EDSS 6 in years (IQR)	8.5 (9.0)	8.8 (7.4)	9.4 (10.6)	6.6 (7.3)	0.3 (*)
Median T2 lesion volume at baseline-scan in mm^3 (IQR) (n=226)	1649 (3380)	1861 (3523)	3027 (5344)	2180 (3330)	607 (1259)
Median T1 hypo-intense lesion volume at baseline scan in mm^3 (IQR) (n=226)	101 (333)	94 (326)	140 (573)	183 (687)	86 (171)
Median number of new T2 lesions at follow-up two years after baseline scan (IQR) (n=182)	4 (7)	4 (9)	2 (14)	2 (5)	1 (4)

SD= standard deviation; MSSS= Multiple Sclerosis Severity Scale at final clinical follow-up; EDSS= Extended Disability Status Scale. IQR: Interquartile range.

* Only based on 2 patients (0 and 8 months, no interquartile range could be calculated).

Association of genotype of rs6897932 (IL7R) and susceptibility to MS

Genotype-frequencies in the healthy controls and in the total MS patient group did not deviate from Hardy Weinberg Equilibrium ($p>0.01$). Genotype frequencies in MS patients were distributed as follows: CC 58.4%, CT 35.0% and TT 6.6%. In our MS patient cohort the frequency of the risk-allele C is 75.8% and in our healthy control cohort 67.8%.

Genotype distribution was significantly different between healthy controls and MS patients (Pearson Chi-Square $p<0.01$). The recessive model fitted best (CC vs CT and TT) and conferred an increased risk of disease for homozygous carriers of the 'C' allele (0.58 vs 0.46, OR: 1.65 (95% confidence interval: 1.18 – 2.30), $p=0.004$). Homozygosity for the risk-allele is a risk factor for MS development in our population. Genotype frequencies, odds ratios and 95% confidence-intervals were calculated (**see Table 2**). Stratification for disease subtype revealed that the association of this SNP with MS is predominantly present in the secondary progressive (SP) MS patient group (**see Table 2**). Carriership of the risk-allele was significantly more frequent in SP MS patients, when compared to healthy controls. The pre-study power of this susceptibility study is approximately 20% to detect a OR of 1.2 when comparing carriers of the CC genotype compared to the CT and TT genotype.

Association of rs6897932 (IL7R) and MS phenotype

EDSS was obtained of all 697 patients. A brain MRI scan was available from 226 patients at baseline and from 182 (80%) patients at follow-up after two years (see **Table 1**). See **supplementary table 2** for patientcharacteristics of the subgroup of which an MRI was available. No association could be detected between genotype of rs6897932 and disease severity (clinically and using MRI parameters). There was also no association of genotype with disease activity measures (using clinical and MRI parameters) (see **Table 3**). After correction for disease duration, gender and onset type no significant differences were found.

Table 2: Genotype frequencies and allele frequencies of rs6897932 within IL7R gene in MS patients vs. healthy controls. Significant associations are printed in bold.

	Total number:	**Frequency risk allele (C) (%)**	**CC genotype n (%)**	**CT genotype n (%)**	**TT genotype n (%)**	**OR CC vs. CT and TT (95% CI) compared to healthy controls (= recessive model)**	**2-sided P-value (Fisher's exact test) CC vs. CT and TT compared to healthy controls**
MS patients total cohort	697	75.8%	407 (58.4%)	244 (35.0%)	46 (6.6%)	**1.65 (1.18 – 2.30)**	**0.004**
RR	367	74.5%	207 (56.4%)	133 (36.2%)	27 (7.4%)	**1.52 (1.06-2.18)**	**0.027**
SP	192	79.4%	121 (63%)	63 (32.8%)	8 (4.2%)	**2.00 (1.32 – 3.04)**	**0.001**
PP	111	75.9%	61 (55 %)	40 (36%)	10 (9%)	1.43 (0.89 – 2.31)	0.15
Healthy controls	174	67.8%	80 (46.0%)	76 (43.7%)	18 (10.3%)	NA	NA

OR: odds ratio, CI: confidence interval; RR: relapsing remitting, SP: secondary progressive, PP: primary progressive.

Table 3: Genotype-Phenotype comparisons for genotype of rs6897932 and clinical and MRI parameters of disease activity and severity.

	CC	CT	TT
Clinical parameters of disease severity:			
Median EDSS	4.0	4.0	3.5
Median MSSS	5.60	5.42	5.56
Median Time to reach EDSS=6	110 months	91 months	151 months
Number of patients that reached an edss of 6	151 / 407 (37.1%)	83 / 244 (34.0%)	15 / 46 (32.6%)
MSFC scores:			
Median Timed walk test (sec) (n=228)	4.0	4.0	3.7
Median 9-HPT (dominant hand) (sec) (n=227)	18.2	18.3	17.8
Median 9-HPT (non-dominant hand) (sec) (n=228)	19.3	19.2	18.7
Median PASAT 3 (number of correct answers) (n=222)	56	55	55.5
Clinical parameters of disease activity			
Median number of methylprednisolone treatments relapse rate per year of follow-up (IQR) (n=210)	0.12 (0.31)	0.12 (0.22)	0.14 (0.28)
Median number of relapses per year of follow-up (IQR) (n=210)	0.50 (0.49)	0.47 (0.45)	0.33 (0.40)
MRI markers of disease severity:			
Median volume of T2 lesions at baseline (IQR)	1578 (2998)	2226 (4403)	1631 (2287)
Median volume of hypo-intense T1 lesions (IQR)	93 (317)	120 (405)	111 (197)
MRI marker of disease activity:			
Number of new T2 lesion (IQR)	4 (7)	4 (7)	2 (6)

* No significant associations were found between genotype and the above mentioned parameters. Also after correction for disease duration, gender, age, use of disease modifying therapy and onset type no significant differences were found.

IL7R gene expression profiles and MS characteristics.

For 95 patients mRNA was available and IL7R mRNA expression profiles (the relative expression of membrane-bound IL7R (presence of exon 6-7) to total amount of IL7R mRNA (presence of exon 4-5)) were determined. These early MS patients had a median disease duration at baseline of 7 months (IQR: 10.3), a median EDSS of 2.5 (IQR: 1.5) and 51.6% of patients used DMT during follow-up. Baseline MRI scans were available for 93 patients.

Two patients were lost to follow-up before the second year and an additional 15 patients did not have a follow-up MRI scan of the brain after 2 years.

In this substudy 61.1% of the MS patients were homozygous for the 'C' risk allele, while 32.6% carried the CT genotype and 6.3% of our patients was homozygous for the T-allele. This distribution did not deviate from the original cohort. No association of genotypes of rs6897932 and the relative expression of membrane-bound IL7R to total amount of IL7R mRNA were found.

There were no differences observed in relative expression of membrane-bound IL7R to total amount of IL7R mRNA between patients that received an intravenous treatment course with methylprednisolone or experienced a relapse in the 3 months preceding the blood withdrawal compared to patients that did not experience such an event in the previous 3 months. Furthermore, no association was found between MS phenotype (clinically and using MRI scans of the brain) at the time of blood withdrawal and relative gene expression of membrane-bound to total IL-7R mRNA. Also, the membrane-bound to total IL-7R expression ratio neither predicted disease activity, nor did it predict disease severity using clinical parameters (after a median follow-up duration of 4 years and 4 months) and MRI parameters of the brain, also when controlled for use of disease modifying therapy, gender, age, onsettype and disease duration. The pre-study power of our study to detect a clinically relevant difference of 0.5 point on the MSSS is approximately 50%.

Discussion

We present here the results on the effect of IL7R SNP rs6897932 and expression of alternatively spliced IL7R mRNAs on clinical disease course in a large and well documented Dutch MS patient group. We confirm the relevance of the genotype of the exon 6 SNP: rs6897932 on MS susceptibility in our Dutch MS population. Especially the homozygote C (risk-allele) genotype is associated with MS susceptibility in our study, possibly indicating a dose-dependent-effect. Although an effect of heterozygotes can not be excluded due to the small OR (~1.2) as calculated in previous studies[8,9,13]. Although the power of our susceptibility study is relatively low (20%), due to the low number of healthy controls, the allele frequencies of the risk C allele in our MS population and healthy controls are however

similar to other MS studies[9,14]. Moreover, the genotype distribution in our healthy controls is similar to a study with Dutch controls (n=465)[29], strengthening the robustness of our positive association of the CC genotype and MS susceptibility.

In accordance with a previous study[11], the association of this SNP with MS suscepibility is predominantly driven by the genotype distribution in our SP MS patients. Because of this overrepresentation of the risk allele in the generally more disabled MS patients, we postulated that the same C-allele is not only involved in disease susceptibility, but may also be involved in MS disease progression and severity. However, we did not find a correlation between genotype and disease severity. Previous studies demonstrated no effect of this IL7R SNP (and other susceptibility SNPs) on markers of disease severity in large cohorts[8,30]. We confirm this observation in our study in Dutch MS patients, by using more extensive disease severity markers (including imaging parameters) in a well documented MS population. In addition, we show in our relapse-onset cohort that genotype is not related to disease activity parameters in early MS. More and more evidence points towards different genetic influence on MS severity compared to MS susceptibility[31]. Genes involved in MS severity are often involved in different processes than genes found related to MS susceptibility. However, modest effects of some genes might be present in both MS susceptibility and MS severity[32]. HLA-DR15, for example, was associated with a lower age of onset in MS[33] and with female sex[34]. Furthermore, carriers of DRB1*1501 presented with higher numbers of focal brain lesions at the time of initial presentation in the Optic Neuritis Treatment Trial[35] and were associated with disease severity inferred by HMR spectroscopy and MRI measures[36].

Because mRNA might be a more direct reflection of the time-dependent biological relevance of the IL7R receptor, we hypothesized that the membrane-bound to total IL-7R expression ratio would be associated with disease phenotype at the moment of blood withdrawal or influence the future disease course. However, no association was found between the expression profiles and MS phenotype (disease severity and disease activity) in our study. Moreover, surprisingly, no association was found between different genotypes for SNP rs6897932 and the membrane-bound to total IL7R expression. These expression results contradict previous results to some extent, as the association of the C major allele with alternative splicing of exon 6, leading to more soluble compared to membrane-bound IL7R was seen in a study of 94 healthy controls[8], but not in a study of 24 MS subjects[11]. This might be due to the fact that we used whole blood mRNA in stead of peripheral blood mononuclear cells (PBMCs). Differences in mRNA profiles have been described within different blood types[37]. Although we excluded that a recent relapse or prednisolone treatment (common in early MS) would influence the relative expression of membrane-bound to total IL7R mRNA, we cannot exclude other possible confounding factors. Also, the effect of disease modifying drugs on measures of disease-severity has not been taken into account in any of the reported studies.

Although we showed no influence of mRNA expression on disease phenotype in our homogenous group of early MS patients, this may change later on in the disease (since analysis showed evidence for a role in disease susceptibility in secondary progressive MS). Unfortunately no mRNA was collected from secondary progressive MS patients or healthy controls. Maybe mRNA expression would serve more as an indicator of disease severity as opposed to a predictor for disease phenotype. Future studies comparing early patients to patients in a more advanced stage of MS would be interesting and should preferably incorporate studies on protein level as well. Because knowledge on disease progression might be very important in developing future treatment in order to prevent disability accumulation, more studies (including genetic and environmental factors) are warranted to elucidate the mechanisms involved in disease progression.

In conclusion, we confirm the relevance of rs6897932 in MS susceptibility. Considering this and previous publications[8-15] IL7R is firmly established as a (modest) genetic contributor to MS susceptibility. No effect of this polymorphism was found on disease phenotype (disease severity and disease activity). Due to the described effect of this SNP on mRNA expression and the overrepresentation of the risk-allele in the more severely disabled MS patients (secondary progressive MS), we studied the effect on disease severity and activity. However, no effect was found of the genotype and the membrane-bound to total IL7R expression ratio on MS phenotype.

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Supplementary table 1: Characteristics of patients (n=210) included in substudy on relationship genotype of rs6897932 and MS disease activity.

MS subtype (n=210)	
CIS	27
RR	173
SP	10
Gender (% Male)	31.4%
Median disease duration in years at most recent visit (IQR)	5.9 (3.4)
Median EDSS at most recent visit (IQR)	2.5 (2.0)
% of patients ever used disease modifying treatment	57.1%
Most recent disease modifying treatment:	
Interferons	81 (38.5%)
Glatiramer acetate	19 (9%)
Natalizumab	15 (7.1%)
Other (Mitoxantrone, Fingolimod)	5 (2.5%)
Median annual number of relapses during follow-up (IQR)	0.47 (0.47)
Median annual number of intravenous methylprednisolone treatments during follow-up (IQR).	0.12 (0.31)
Genotype distribution	
% CC	57.1
% CT	35.2
% TT	7.6

Supplementary table 2: Characteristics of patients (n=226) with MRI variables available, included in study on relationship genotype of rs6897932 and MS disease activity and disease severity.

MS subtype (n=226)	
CIS	26
RR	165
SP	10
PP	25
Gender (% Male)	34.5%
Median disease duration in years at most recent visit (IQR)	6.0 (3.3)
Median EDSS at most recent visit (IQR)	2.5 (1.6)
% of patients ever used disease modifying treatment	52.2%
Most recent disease modifying treatment:	
Interferons	81 (35.8%)
Glatiramer acetate	18 (8.0%)
Natalizumab	14 (6.2%)
Other (Mitoxantrone, Fingolimod)	5 (2.2%)
Genotype distribution	
% CC	55.8%
% CT	36.7%
% TT	7.5%

Chapter 2.3

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 e.g. 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 two 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 still 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 MS patients.

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 MS patients. These MS patients 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 (MSSS) and Multiple Sclerosis Functional Composite Scores to assess disability. Secondly, by use of Magnetic Resonance Imaging (MRI) measures such as T1 hypo-intense 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 MS patients, 36.9% was male, 10.7% had a primary progressive disease course, 66.4% a relapsing remitting disease course, 19.2% a secondary progressive disease course and 3.7% a clinically isolated syndrome course, and the mean disease duration of our total group was 12 years. Median EDSS 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 H-W equilibrium. No association was found between carriership of the risk-allele or genotype of rs10492972 and the described neurodegenerative markers, neither on the clinical level, nor on MRI (for details see **Table 1**).

Discussion

Therefore, 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 primary progressive MS patients[7], nor in our study of a more general MS population.
Although genetic susceptibility studies have consistently pointed towards 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.

Table 1: Overview of clinical and paraclinical markers possibly related to neurodegeneration. Values are given per genotype and carriership of C-allele (risk-allele) of rs10492972.

	CC genotype (n=20)	CT genotype (n=89)	TT genotype (n=105)	Carriers of C-risk-allele	Non-carriers of C-risk allele
Mean disease duration (years)	11.85	12.83	11.38	12.65	11.38
Mean MSSS	3.83	4.45	4.87	4.33	4.87
MSFC					
Median TWT (seconds)	4.25	4.95	4.70	4.70	4.70
Median 9-HPT (dominant hand) (seconds)	17.7	18.8	19.7	18.6	19.7
Median 9-HPT (non-dominant hand) (seconds)	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 (ml x 10^{-3}) (n=164)	592.6	583.6	388.5	568.6	388.5
Median T2 lesion (ml x 10^{-3}) (n=162)	3370.9	2050.2	2539.2	2305.0	2539.2
Median PBVC after 2 years (%) (n=159)	-1.09	-0.90	-1.06	-0.91	-1.06

No significant differences were found. MSSS= Multiple Sclerosis Severity Scale at most recent follow-up; MSFC= Multiple Sclerosis Functional Composit, TWT= Timed Walk Test; 9-HPT= nine Hole Peg Test; PASAT= Paced Auditory Serial Addition Test; PBVC= Percentage Brain Volume Change.

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Chapter 3

Genetic markers of lesion distribution in Multiple Sclerosis

Chapter 3.1

HLA-DRB1*1501 and spinal cord Magnetic Resonance Imaging lesions in Multiple Sclerosis

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Abstract

Background: Multiple Sclerosis (MS) is a heterogeneous neurological disease with extensive variation with respect to the most affected central nervous system region (brain vs spinal cord).

Objective: To test the hypothesis that this variation in lesion location (brain vs spinal cord) might be (partially) genetically determined.

Design: Candidate gene study. **Setting**: Academic research.

Patients: Patients were selected for the availability of DNA material, clinical variables, and brain and spinal cord magnetic resonance images (evaluating T2-weighted lesion load in the brain and the number of spinal cord lesions).

Main Outcome Measures: For genotyping we used a DNA chip, containing a set of genes mentioned in previous publications noting their relation to different phenotypes of MS. We assessed the association between brain and spinal cord abnormalities and the genotypes of the patients.

Results: One hundred fifty patients were included in the analysis. Five single- nucleotide polymorphisms within the Major Histocompatibility Complex region were associated with the number of focal abnormalities in the spinal cord. The most significant was rs3135388 (surrogate marker for the HLA-DRB1*1501 allele). Carriers of HLA-DRB1*1501 had a median of 4 spinal cord lesions compared with 2 lesions for non-carriers ($p<0.001$). No significant association was noted between the single nucleotide polymorphisms and T2-weighted lesion load in the brain.

Conclusions: Carriership of HLA-DRB1*1501 (via rs3135388) was associated with the extent of focal abnormalities in the spinal cord. Spinal cord lesions might be an explanation for increased MS disease severity in patients carrying HLA-DRB1*1501.

Introduction

Multiple Sclerosis (MS) is an autoimmune disease of the central nervous system, characterized by inflammation, demyelination and axonal loss in the brain and spinal cord. The current hypothesis is that MS is caused by a complex interplay of genetic and environmental factors. The genetic influence is characterized by the interaction of multiple genes that exert modest effects. The most striking among these is the association of the major histocompatibility complex (MHC) with MS susceptibility, which has consistently been reported over the past decades[1].

Clinically, MS is a heterogeneous disease with a diverse spectrum of neurologic deficits and variable outcome. Some studies have focused on genetic predictors of disease phenotypes, such as disease severity, disease subtypes, magnetic resonance (MR) imaging characteristics and response to treatment, showing some effect of different genes[1-8].

Besides clinical heterogeneity, considerable variation exists between patients regarding type and anatomic location of the lesions[9,10]. Varying degrees of involvement of the cerebrum, brainstem, cerebellum, and spinal cord have been described in post-mortem and MR imaging studies [11,12]. This variability is unexplained, and much effort is being put in unravelling it. Several arguments point toward a genetic role in the regional distribution of lesions in the brain and spinal cord in MS. First, differences in pathologic manifestation of MS have been observed between Asian and Western populations. Asian-type MS is characterized by predominant involvement of the optic nerve and spinal cord, whereas Western type MS predominantly involves the brain. Genetic differences within the HLA region between these populations could underlie this variability[3,13-16]. Second, recent studies confirm a tendency of patients to develop relapses in the same locations, including spinal cord lesions[17,18]. This tendency for localized exacerbations could be genetically predetermined. Third, involvement of a genetic factor has already been demonstrated in lesion distribution in experimental autoimmune encephalomyelitis (an animal model that shows many similarities to MS). Butterfield et al. showed that in mouse experimental autoimmune encephalomyelitis the lesions in the brain and spinal cord were controlled by different quantitative trait loci[19].

Many researchers have demonstrated the clinical relevance of spinal cord lesions in diagnosing MS and disability accumulation. However, we are unaware of any studies that included spinal cord MR imaging variables in genetic analyses, searching for genotype-phenotype correlations.

In this study, we used a DNA-chip containing a set of single nucleotide polymorphisms (SNPs) in candidate genes to assess the genetic effect on regional lesion distribution in the brain and spinal cord as seen on MR imaging. The SNPs on this chip were selected based on published associations with MS pathogenesis, prognosis or response to treatment.

Material and methods

Study participants:
Unrelated Dutch Caucasian patients were selected retrospectively from natural history studies conducted at the MS Center at the VU University Medical Center (VUmc) in Amsterdam, the Netherlands. Patients were selected for the availability of DNA material, as well as brain and spinal cord MR images, that fulfilled certain standardized requirements (described herein) and were obtained less than two years apart. The study was performed with the approval of the medical ethics committee of the VU University Medical Center and informed consent was obtained from all participants. Patients were diagnosed as having MS as ascertained using Poser or revised McDonald criteria[20,21]. For patients included in the analyses, clinical data were collected, including age, sex, type of disease, age at onset, disease course and duration of disease. Disability status was determined for all subjects using Kurtzke's Expanded Disability Status Scale (EDSS)[22] and, whenever available, the Multiple Sclerosis Functional Composite Scale (MSFC)[23].

Selection of SNPs:
Single-nucleotide polymorphisms were selected based on involvement in MS pathogenesis, prognosis or response to treatment, according to the literature published before July 2007. The polymorphisms were confirmed and associated with an identifier using dbSNP database (http://www.ncbi.nlm.nih.gov/SNP). Nucleotide sequences for the design of allele-specific probes and polymerase chain reaction primers were retrieved from the SNPper database (http://snpper.chip.org/bio). Sequence-specific probes and primers were designed using freely available Primer3 software (http://frodo.wi.mit.edu/).
If a polymorphism was not present in the database, position and sequences were established by performing a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the data available in the literature.

Genotyping
Genomic DNA was isolated from anti-coagulated blood using DNAzol reagent (Molecular Research Center, Inc., Cincinnati, OH). Genotyping was performed using a newly developed low-density DNA microarray based on allele-specific probes. The design, fabrication, validation and analysis of the arrays were performed following the procedure described by Tejedor et al. with minor modifications[24].

Brain MR Imaging
Magnetic Resonance Images were acquired on 1.0 Tesla or 1.5 Tesla systems (Siemens AG, Erlangen, Germany) with standard head coils, using 2D conventional or fast spin-echo Proton

Density-weighted and T2-weighted images (TR: 2200-3000 ms, TE: 20-30 & 80-100 ms) with section thicknesses of 3-5 mm, a maximum gap between sections of 0.5 mm, and an in-plane resolution of 1x1 mm^2 . Lesions were identified by an expert reader and then outlined on the corresponding proton density-weighted image using home-developed semi-automated seed-growing software based on a local thresholding technique (Show_images)[25]. Lesion areas were multiplied by the intersection distance to obtain total T2-weighted brain lesion volume for each patient.

Spinal cord MR Imaging:

Spinal cord scanning included a cardiac-triggered sagittal proton density-weighted and T2-weighted dual-echo spin echo sequence with a section-thickness of 3mm covering the whole spinal cord (TR: 2500 – 3000 ms, TE: 20-30 & 80-100 ms), with a gap between sections of 0.3 mm, and an in-plane resolution of 1x1 mm. From this sequence the number of focal lesions and the presence of diffuse abnormalities were scored by one experienced reader (CL). Diffuse abnormalities were defined as poorly delineated areas with increased signal intensity compared with the signal intensity of spinal cerebrospinal fluid best seen on proton density-weighted images[26]. Lesion volume was assessed in the spinal cord as the total number of focal pathologic segments involved (total extension of all spinal cord lesions over several corresponding vertebral segments).

Statistical analysis:

First, the associations between the brain variable (T2-weighted lesion load) and the spinal cord variables (the number of focal lesions and the presence of diffuse abnormalities) were tested per SNP and per clinical variable. We used the non-parametric Spearman ρ rank correlation test, Kruskal-Wallis test, Mann Whitney test and χ^2 test as appropriate, applying the false discovery rate according to Benjamini and Hochberg[27] to correct for multiple testing. The corrected number represents the expected proportion of false discoveries for a given p-value cut-off. We used the cut-off point of $p<0.05$ after false discovery rate correction. Spearman rank correlation coefficient was used to test the correlations between two scaled variables.

Secondly, multivariate analysis by general linear models was performed on the associated SNPs ($p<0.10$ significance after correction for multiple testing). We used log-transformed brain T2-weighted lesion loads and the square-root-transformed number of focal abnormalities in the spinal cord to correct for significant clinical variables and the type of MR imaging system (1.0 T vs 1.5 T).

All analyses were performed using commercially available software packages. These included SPSS (version 15; SPSS Inc., Chicago, IL, USA), Excel 2003 and HelixTree (Golden Helix, Inc., Bozeman, MT).

Results

Patient characteristics:

One hundred and fifty patients were included in the analysis. Our patient group reflects a representative population with MS, with 36.7% being male and 20.0% having primary progressive MS (**Table 1**). Most patients (132/150) demonstrated abnormalities (mostly focal lesions) on spinal cord MR imaging, while all patients had abnormalities on brain MR imaging.

Table 1: Patient demographic, clinical and Magnetic Resonance (MR) Imaging characteristics according to Multiple Sclerosis subtype.

	All (n=150)	RR (n=88)	SP (n=32)	PP (n=30)
Male sex, No. (%)	55 (36.7%)	26 (29.5%)	17 (53.1%)	12 (40.0%)
Age at MRI, y*	41.4 (11.2)	36.1 (9.2)	46.5 (8.9)	51.2 (9.8)
Disease duration, y*	7.1 (7.4)	4.4 (6.2)	12.8 (7.0)	9.2 (7.1)
Median EDSS (Interquartile range)	3.5 (2.5)	2.0 (2.0)	5.5 (2.5)	4.0 (3.0)
Brain T2-weighted lesion load (ml)*	7.7 (10.3)	4.9 (6.6)	16.2 (14.6)	7.0 (9.2)
Focal lesions in the spinal cord*	3.4 (3.0)	3.3 (2.8)	4.5 (3.9)	2.8 (2.3)
patients with diffuse abnormalities, No. (%)	20 (13.3)	9 (10.2)	6 (18.8)	5 (16.7)

*Data are given as mean (SD).

Genotyping:

In total, 80 SNPs in 44 genes were studied on the DNA chip. Twelve SNPs were excluded from further analysis (5 SNPs were monomorphic and 7 SNPs had a minor allele frequency < 5% (details are provided in **supplementary Table 1**). Hardy-Weinberg equilibrium was calculated for all SNPs.

Correlation between lesion load in the brain and genotypes:

In the univariate analyses of T2 lesion load in the brain versus all SNPs on the DNA chip, the only suggestive correlation was rs2107538 (CCL5) (OMIM: 187011) (see **Table 2**). Two clinical covariates were significant and were included in the general linear model for brain T2-weighted lesion load namely, disease duration and MS subtype. The type of MR imaging system (1.0 T vs 1.5 T) was not associated with T2-weighted lesion load in the brain. After inclusion of rs2107538 (CCL5) in the model that contained the clinical covariates, this SNP showed significant association with brain T2-weighted lesion load (p=0.03).

Correlation between spinal cord abnormalities and genotypes:

Several MHC SNPs were found to be related to the number of focal spinal cord abnormalities (**Table 2**). The most significant is SNP rs3135388. Carrier status of the A-allele (surrogate marker for HLA-DRB1*1501) was associated with significant more lesions in the spinal cord (**Figure 1**). The median number of focal abnormalities in carriers of HLA-DRB1*1501 (n=80) was 4 lesions and in non-carriers (n=70) was 2 lesions (p<0.001 Mann Whitney test).

When corrected for multiple testing, five SNPs within the MHC class II region (rs3135388, rs2395182, rs2239802, rs2227139 and rs2213584), remained significant and one SNP within the C2TA gene (Class II TransActivator) showed a suggestive result. The five HLA SNPs are in strong linkage disequilibrium.

In addition, the aforementioned MHC class II SNPs were also found to be correlated with a higher lesion volume in the spinal cord. Specifically carriership of HLA-DRB1*1501 was associated with more segments of the spinal cord affected by focal lesions (p=0.01, Mann Whitney test).

No clinical covariate (disease duration, age of patients, or MS subtype) was significantly correlated with the number of focal lesions in the spinal cord. No general linear model that included the five significantly associated MHC class II SNPs could be produced, because of the high level of collinearity owing to the high linkage disequilibrium (r^2 ranging from 0.33-0.99). Only rs3087456 (C2TA) (OMIM: 600005) showed a marginally significant p-value (p=0.05) in the general linear model that included rs3135388 (p=0.002). No association was observed between the presence of diffuse abnormalities and the evaluated SNPs.

Table 2: Correlation by Kruskal Wallis test of Single-Nucleotide Polymorphisms (SNPs) with Magnetic Resonance Imaging variables.

SNP	Gene	P value		Uncorrected *P* Value for Association With Lesion Volume in the Spinal Cord**
		Uncorrected	False disco-very rate corrected	
No. of focal Lesions in the spinal cord				
rs3135388*	MHC2	<0.001	0.03	0.02
rs2395182 *	MHC2	0.001	0.03	0.003
rs2239802*	MHC2	0.001	0.03	0.003
rs2227139*	MHC2	0.002	0.03	0.005
rs2213584*	MHC2	0.003	0.05	0.009
rs3087456	C2TA	0.009	0.10	0.18
T2-Weighted Lesion Load in the Brain				
rs2107538	CCL5	0.001	0.07	Not applicable

* These SNPs reside on three haplotype blocks with pair wise r2 values ranging from 0.33-0.99.
** Defined as the total number of segments affected by focal lesions.

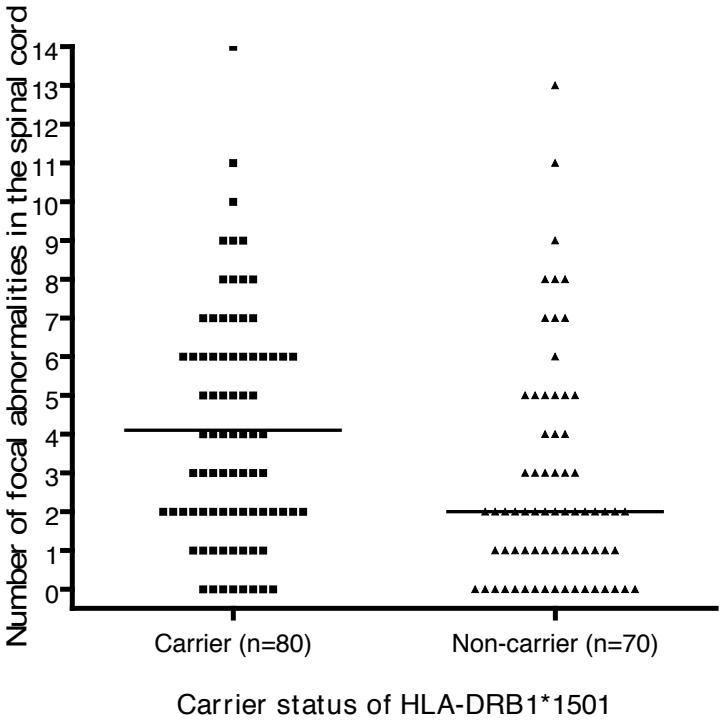

Figure 1: Carriership of HLA-DRB1*1501 and focal spinal cord lesions.
Scatterplot of focal spinal cord lesions for carriers and non-carriers of HLA-DRB1*1501 (measured as presence of A-allele of rs3135388). Carriers of HLA-DRB1*1501 had a median of 4 spinal cord lesions compared with 2 lesions for noncarriers (P < .001, Mann Whitney test). Line reflects the median number of focal abnormalities.

Comment

In the present candidate gene study, we observed an association between several SNPs within the MHC class II region and the number of focal abnormalities in the spinal cord. The most significant results were found for rs3135388. This SNP is a surrogate marker for the HLA-DRB1*1501 allele ($r^2 = 0.97$)[28].

After correction for multiple testing, no genes were significantly related to T2-weighted lesion load in the brain. The MHC class II SNPs that were associated with more lesions in the spinal cord and with a higher lesion volume in the spinal cord do not seem to affect the presence of T2-weighted lesions in the brain.

Studies have unambiguously shown that HLA-DRB1*1501 strongly influences MS susceptibility[6,29,30]. There is some evidence that this haplotype might also be associated with

a more severe disease course[1,4,6,31]. Recent findings show a correlation between carriership of HLA-DRB1*1501 and Expanded Disability Status Scale scores using an extremes of outcome strategy (comparing severe and mild disability only)[1]. In our study we could not demonstrate a correlation between carriership of HLA-DRB1*1501 and Expanded Disability Status Scale scores. This might be because of the impossibility to perform the extremes of outcome analysis owing to a small sample size. However, findings from this study suggest that spinal cord lesions might be an additional explanation for the described relationship between HLA-DRB1*1501 and MS disease severity.

Abnormalities in the spinal cord correlate with the degree of disability and with the date of diagnosis[32,33]. In our study we also noticed a relation between EDSS scores and the number of focal abnormalities in the spinal cord (p=0.02, ρ=0.185). Future studies should include additional MR imaging variables of the spinal cord such as atrophy (cross-sectional area), as this variable might correlate better with clinical disability [34].

In a recent article, Okuda et al. showed in their patients that HLA-DRB1*1501 affects disease severity as measured by clinical variables and by brain MR imaging variables[35]. In our patients, we could not demonstrate this effect on T2-weighted lesion load in the brain or on cognitive function (using Paced Auditory Serial Addition Task scores)[23]. Post-hoc power analysis detected a power of less than 25% to detect a T2-weighted lesion load difference of 1 ml, suggesting that a type II error cannot be ruled out. This also warrants careful interpretation of the suggestive positive finding of rs2107538 (CCL5) in association with brain T2-weighted lesion load, although previous findings have shown an influence of this gene on other MR imaging variables[36].

Previous data were sometimes conflicting about the effect of HLA haplotypes on brain MR imaging features. Some studies found a relationship between HLA genotypes and brain MR imaging quantitative markers[31,35], while other studies did not observe this effect of HLA-DRB1*1501[31,37]. In addition, no effect of HLA-DRB1*1501 was shown on lesion distribution within the brain using T1 and T2 lesion maps[38].

To date we are unaware of any study that specifically relates HLA-DRB1*1501 to spinal cord abnormalities on MR imaging in a Caucasian population.

The molecular basis of the association with a greater involvement of the spinal cord in patients carrying the HLA-DRB1*1501 allele remains unknown. Major Histocompatibility Complex class II genes are involved in self versus non-self immune recognition[39]. These genes encode for polymorphic surface glycoproteins. Variability in this region may determine individual differences in T-cell responses. It is postulated that MHC class II may present variable central nervous system antigens to T cells, possibly producing different lesion distribution in animals and in humans[40].

Moreover, Stromnes et al. reported different lesion distribution in the central nervous system (spinal cord vs brain parenchyma) in two mouse experimental autoimmune encephalitis models with different MHC strains [41]. This was found to be mediated by variable preferential Myelin Oligodendrocyte Glycoprotein (MOG) epitope presentation and ultimately by an alternative ratio of T helper 17 to T helper 1. The present study indicates a different mechanism of lesion formation in the brain vs the spinal cord, with a possible indirect role of the MHC class II genes. The role of the genes on this mechanism is unclear. The translation of these findings from animal models to the human situation warrants further studies.
Because of the complexity of the MHC class II region (epistatic effects and high linkage disequilibrium), future studies using high-density HLA mapping are warranted to unravel the genetic influence on lesion distribution and determine the responsible HLA allele.
In conclusion, this study demonstrates more focal lesions in the spinal cord among carriers of the HLA-DRB1*1501 allele. If confirmed in independent samples these observations may provide important insight in MS disease heterogeneity and its underlying mechanisms.

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Supplementary Table 1: SNPs included on MS-chip. HWE = Hardy-Weinberg Equilibrium in our sample; MAF = minor allele frequency in our sample.

Gene	rs-nr	Chromosome	Polymorphism	HWE *	MAF
ADAMTS14	rs4747075	10q22	A/G	P<0.01	0.30
ADAMTS14	rs7081273	10q22	C/G	n.s.	0.34
ADAMTS14	rs4746060	10q22	C/T	n.s.	0.08
Apo I/Fas	rs1800682	10q23	C/T	n.s.	0.47
Apo I/Fas	rs3781202	10q23	C/T	P<0.01	0.40
Apo I/Fas	rs2234978	10q23	C/T	n.s.	0.31
BTNL2	rs2076530	6p21.3	A/G	P<0.01	0.26
CIITA	rs3087456	16p13	A/G	n.s.	0.26
CACNG4	rs4790896	17q24	A/G	n.s.	0.41
CCR5	rs333	3p21	-/+	n.s.	0.11
CD24	rs8734	6q21	C	NA	0.00 §
CNTF	rs1800169	11q12	A/G	n.s.	0.12
CRYAB	rs14133	11q21-q23	C/G	n.s.	0.27
CRYAB	rs762550	11q21-q23	A/G	n.s.	0.42
CRYAB	rs2234702	11q21-q23	C	NA	0.00 §
CTLA4	rs231775	2q33	A/G	n.s.	0.37
CTLA4	rs5742909	2q33	C/T	n.s.	0.09
EBF	rs1368297	5q34	A/T	n.s.	0.38
GABBRA1	rs1805057	6p22	C	NA	0.00 §
HELZ	rs2363846	17q24	C/T	n.s.	0.48
HLA	rs2395166	6p21.3	C/T	n.s.	0.47
HLA	rs2213584	6p21.3	A/G	n.s.	0.40
HLA	rs2227139	6p21.3	C/T	n.s.	0.40
HLA	rs3135388	6p21.3	A/G	n.s.	0.33
HLA	rs9268458	6p21.3	A/C	n.s.	0.20
HLA	rs6457594	6p21.3	A/G	P<0.01	0.40
HLA-DRA	rs2395182	6p21.3	G/T	n.s.	0.38
HLA-DRA	rs2239802	6p21.3	C/G	n.s.	0.38
IFNAR1	rs2257167	21q22	C/G	n.s.	0.08
IFNGR2	rs9808753	21q22	A/G	n.s.	0.14
IKBL	rs3130062	6p21.3.	C/T	n.s.	0.18
IL10	rs1800896	1q32	A/G	n.s.	0.46
IL1B	rs1799916	2q14	A	NA	0.00 §
IL1B	rs1143627	2q14	A/G	n.s.	0.34
IL1B	rs1143634	2q14	C/T	n.s.	0.23
IL1RN	rs419598	2q12-q14	C/T	n.s.	0.31
IL1RN	2073 C/T	2q12-q14	C/T	n.s.	0.30
IL2	rs2069763	4q26	G/T	n.s.	0.36
IL2	rs2069762	4q26	G/T	n.s.	0.27
IL4R	rs1801275	16p12	A/G	n.s.	0.20
IL7R	rs11567685	5p13	C/T	n.s.	0.25
IL7R	rs7718919	5p13	G/T	n.s.	0.13
IL7R	rs11567686	5p13	A/G	n.s.	0.34

MC1R	rs1805009	16q24	C/G	NA	0.01 §
MC1R	rs1805006	16q24	A/C	NA	0.00 §
MEFV	rs28940577	16p13.3	A	NA	0.00 §
MGC33887	rs987931	17q24	G/T	n.s.	0.32
MOG	rs3130250	6p22	A/G	n.s.	0.19
MOG	rs3130253	6p22	A/G	n.s.	0.12
NDUFA7	rs2288414	19p13.2	C/G	NA	0.03 §
NDUFA7	rs561	19p13.2	A/G	n.s.	0.21
NDUFS5	rs2889683	1p34.2	C/T	n.s.	0.31
NDUFS5	rs6981	1p34.2	A/G	NA	0.04 §
NDUFS7	rs2074897	19p13.3	A/G	P<0.01	0.47
NOS2A	rs1137933	17q11.2	A/G	n.s.	0.25
NOS2A	rs2779248	17q11.2	C/T	n.s.	0.39
NOTCH4	rs367398	6p21.3	A/G	n.s.	0.16
PD-1	rs11568821	2q37	G/A	n.s.	0.11
PITPNC1	rs1318	17q24	A/G	n.s.	0.21
PITPNC1	rs2365403	17q24	C/G	n.s.	0.18
PNMT	rs876493	17q11-q23	A/G	n.s.	0.39
PRKCA	rs7220007	17q24	A/G	n.s.	0.49
PRKCA	rs887797	17q24	C/T	n.s.	0.30
PRKCA	rs2078153	17q24	C/G	n.s.	0.23
PRKCA	rs3890137	17q24	A/G	n.s.	0.37
PTPN22	rs2476601	1p13	A/G	n.s.	0.11
PTPRC	rs17612648	1q31	C/G	n.s.	0.03 §
PTPRC	rs4915154	1q31	A/G	n.s.	0.00 §
CCL5	rs2280788	17q11.2-q12	C/G	n.s.	0.02 §
CCL5	rs2107538	17q11.2-q12	C/T	n.s.	0.18
Spp1	rs1126616	4q21	C/T	n.s.	0.23
Spp1	rs1126772	4q21	A/G	n.s.	0.18
Spp1	rs2853744	4q21	G/T	n.s.	0.05
Spp1	rs9138	4q21	A/C	n.s.	0.24
Spp1	rs4754	4q21	C/T	n.s.	0.24
TNF	rs1800629	6p21.3	A/G	n.s.	0.17
TNFSF10	rs1131568†	3q26	C/T	n.s.	0.32
UCP2	rs659366	11q13	C/T	n.s.	0.37
VDR	rs1544410	12q13	A/G	n.s.	0.48
VDR	rs731236	12q13	A/G	n.s.	0.48

* P-value of Hardy Weinberg equilibrium (HWE). A p-value < 0.01 indicates deviation from HWE. NA: not applicable. N.s.: not significant.
§ Excluded due to minor allele frequency below 5%.
† Previous rs-number: rs9880164.

See list of abbreviations on page 181

Chapter 3.2

Genetic correlations of brain lesion distribution in Multiple Sclerosis - an exploratory study -

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Abstract

Background and Purpose: In MS, the total brain lesion volume and spatial distribution of lesions across the brain vary widely among individual patients. We hypothesized that spatial distribution may be partially driven by genetic predisposition and aimed to explore relations between candidate genes and the spatial distribution of white matter brain lesions in MS.

Material and methods: Genotypes of 69 single nucleotide polymorphisms (SNPs) in 208 MS patients were related to spatial distribution of T2 brain lesions. Lesions were manually outlined on magnetic resonance imaging scans and binary lesion masks were produced and registered to a common space. With Randomise software, the lesion masks were related to genotype using a voxelwise nonparametric General Linear Model approach, followed by clusterwise analysis. We used a DNA chip with SNPs selected from the literature on MS susceptibility, severity and phenotypes.

Results: For eleven of these SNPs, one of the genotypes expressed significant clusters of increased or decreased lesion probability in varying, predominantly periventricular, brain regions. When statistically controlling the voxelwise analyses for effects of total brain lesion volume, only one SNP remained significant: rs2227139, located within the Major Histocompatibilty Complex (MHC) class II region. This SNP retained its periventricular cluster of significantly increased lesion probability for the heterozygote genotype.

Conclusion: Heterozygosity of rs2227139 (MHC class II region) is associated with increased right frontal periventricular lesion probability ($p<0.01$). Ten other SNPs showed associations between genotype and spatial lesion distribution that are partly explained by total lesion volume.

Introduction

Multiple Sclerosis (MS) is a multifocal inflammatory demyelinating disease of the central nervous system (CNS). MS lesions can be found throughout the entire CNS parenchyma, histopathologically characterized by inflammation, demyelination and axonal loss resulting in a highly variable clinical presentation. Lucchinetti et al. showed the histopathological heterogeneity of MS lesions among patients with MS, with a distinct homogeneity *within* each patient, which might partially depend on the immunogenetic background of the individual patient with MS.[1,2]

The spatial distribution of MS lesions across the brain is not homogeneous, showing particularly high frequencies in the periventricular white matter. Moreover this spatial distribution is highly variable between patients.[3-5] Lesion probability mapping (LPM) studies in combination with a nonparametric General Linear Model (GLM) approach have been proven useful in studying associations between lesion distribution and clinical disability parameters. This voxel-wise method does not require a priori assumptions. Previous studies showed differences in lesion distribution across disease subtypes and disability status,[5-7] as well as between different lesion subtypes: T1 gadolinium-enhancing lesions showed a spatial distribution differing from non-enhancing T2 lesions and from T1 hypointense lesions ("black holes").[8,9]

We hypothesized that spatial lesion distribution might depend on individual immunogenetic background. Although genotype has been shown to influence MS susceptibility in several studies,[10-12] there are relatively few studies of genotype-phenotype associations in MS and positive results await confirmation in larger studies.[13-18]

We suspect a genetic influence on the anatomical distribution of MS lesions for three main reasons. First, studies confirm a tendency of patients to develop relapses in the same locations.[19,20] Moreover, in relatives who both suffer from MS, lesions have been observed more frequently in similar locations.[14] This tendency for localised exacerbations could be genetically predetermined. Second, in Experimental Autoimmune Encephalomyelitis (EAE) (the animal model used to study MS), involvement of a genetic factor was already demonstrated in lesion distribution. Butterfield et al. showed that in mice EAE the lesions in the spinal cord and brain were controlled by different quantitative trait loci.[21] Third, recently we found that carriership of HLA-DRB1*1501 is associated with more lesions within the spinal cord, but not with the total lesion volume in the brain.[22] This allele, also known to be involved in MS susceptibility, is hence suggested to specifically affect lesion development in the spinal cord, a particular region of the central nervous system. Similarly, lesion development in certain regions *inside* the brain may be partially dependent on genetic parameters. A recent study found that the HLA-DRB1*1501 allele did not exhibit an effect on lesion distribution.[23] We hypothesized that an effect on lesion distribution may be even more likely for genes associated with disease phenotype.

To explore this hypothesis, the present study compared spatial lesion distribution on MRI between different genotypes of a set of genes -selected based on their potential contribution to phenotypes of MS-, in a large cohort of MS patients, by using LPM.

Material and Methods

Patients

This cross-sectional study used clinical and genetic data and MRI scans of 208 Caucasian patients sampled from MS natural history studies at our center. Patients were included based on availability of brain MRI and DNA material. The selected patients are a subgroup of the patients in which the clinical correlates of brain lesion distribution were studied previously.[7] All had a diagnosis of MS according to Poser criteria[24], McDonald criteria[25] or revised McDonald criteria[26] depending on the date of data acquisition. Expanded Disability Status Scale (EDSS)[27] scores were obtained within a median interval of 0.0 (IQR 0.0 – 4.8) months from MRI scanning. Approval by the local ethics review board was obtained and informed consent was obtained from all participants.

Selection of SNPs

Pubmed (http://www.ncbi.nlm.nih.gov/sites/entrez) and genetic association (http://geneticassociationdb.nih.gov/cgi-bin/tableview.cgi?table=geneview) databases were searched for genes and polymorphisms suggested to be involved in pathogenesis, prognosis and treatment response in MS. Resulting polymorphisms were confirmed and associated to an identifier by using dbsnp database (www.ncbi.nlm.nih.gov/SNP). Their frequency in the Caucasian population was assessed using the HapMap project (http://www.hapmap.org/index.html.en). Nucleotide sequences for the design of allele-specific probes and PCR primers were retrieved in the SNPper database (http://snpper.chip.org/bio). Sequence specific probes and primers were designed using Primer3 software (freely available at http://frodo.wi.mit.edu/). If a polymorphism was not present in the database, position and sequences were established by performing a blast search (http://blast.ncbi.nlm.nih.gov/Blast.cgi,) using data available in the literature. SNPs within the MHC class II region were predominantly selected based on the described linkage disequilibrium with the HLA-DRB1*1501 allele.[28,29] All genetic databases were accessed in 2006. The SNP rs-numbers and gene symbols were actualized at February 9th 2009.

A total of 80 validated polymorphisms located in 44 different genes were finally chosen (**Supplementary Table 1**). Eleven of these 80 SNPs were excluded: five because they were monomorphic, and six because they had a minor allele frequency below five percent. Deviations from Hardy-Weinberg equilibrium were determined by Chi-square testing.

Genotyping

Genomic DNA was isolated from anti-coagulated blood with DNAzol reagent (Molecular Research Center, Inc., Cincinnati, OH). Genotyping was carried out using a previously described low-density DNA microarray based on allele-specific probes with minor modifications.[30]

Image acquisition and creation of lesion masks

Different image acquisition protocols were used for the different clinical studies from which our patients were sampled: we used 2D conventional or fast spin-echo PD- and T2- weighted images, acquired using either 1.0 Tesla (Siemens Magnetom Impact) (77.4% of patients) or 1.5 Tesla (Siemens Magnetom Vision) (22.6%) scanners using standard head coils, with slice thicknesses of 3-5 mm, an in-plane resolution of 1x1 mm^2, and a maximum inter-slice gap of 0.5 mm. On these images, lesions were identified by an expert reader, and then outlined using home-developed semi-automated seed-growing software (Show Images)[31] based on a local thresholding technique, thus creating binary lesion masks. T2 weighted images were created from each patient's T2 weighted image using Brain Extraction Tool (FSL).[32] We created a common space T2 template with 2x2x2 mm voxels by linearly registering all T2 brain images (allowing 12 degrees of freedom) to the ICBM brain (MNI-152) image,[33] using the registration tool FLIRT (part of the FMRIB software library (FSL)),[34] followed by averaging and smoothing using a 4mm FWHM Gaussian kernel. In order to bring all lesion masks to this common space template, all individual T2 brain images were then registered to the common space T2 brain template (again using FLIRT with 12 degrees of freedom), and the registration matrices thus obtained were then applied to the corresponding binary lesion masks. Nearest-neighbour interpolation was used to generate individual lesion maps that showed lesion presence or absence for each voxel. Registration quality was ensured by visual inspection. To be able to investigate the effect of the global whole-brain lesion load, we calculated total brain lesion volume. To account for head size differences, lesion volumes were calculated in common space after registration (therefore representing relative lesion volumes).

Statistical analysis

Voxel- and clusterwise statistical inference was accomplished using the non-parametric *Randomise* method as implemented in FSL 4.0 (part of the FMRIB software library: FMRIB Analysis Group, University of Oxford, http://www.fmrib.ox.ac.uk), using a general linear model design to model each voxel independently from the other voxels (5000 permutations; cluster-forming threshold pseudo-t = 2, corresponding to voxelwise p-threshold = 0.01).[34,35] For each SNP, three comparisons were made to test the association of genotypes with voxelwise lesion probability, always comparing one genotype to the combination of both other

genotypes (i.e. the comparisons made were: homozygote frequent allele vs heterozygote and other homozygote; homozygote rare allele vs heterozygote and other homozygote; and heterozygote vs both homozygotes). The lesion masks of the patients carrying a certain genotype were put together and compared to the lesion masks of patients that did not carry this genotype. For each comparison we separately tested for both *increased* and *decreased* lesion probability associated with that genotype.

Although SNP markers with low genotype frequencies may be of importance in complex trait disorders like MS, such markers can result in unreliable observations in voxelwise analyses. Therefore, homozygote genotypes with frequencies below 5% in our cohort were combined with the heterozygote genotype and compared to the other homozygote genotype.

Because total brain lesion volume has been found to influence lesion distribution,[7] the following two steps were taken. First, for genotypes showing significant clusters in the LPM analyses, these LPM analyses were repeated while statistically controlling for total brain lesion volume. Second, we investigated the effect of genotype on the total brain lesion volume at a patient level by directly comparing the total brain lesion volume between genotypes, while statistically controlling for disease duration (without taking anatomical distribution into account).

Finally, for the LPM and total brain lesion volume analyses, we tested whether correction for disease duration affected the results by using general linear model (after logtransformation of the total lesion volume to obtain a normal distribution).

Results

Patient characteristics

Our cohort of 208 MS patients was representative of the general MS population regarding patient characteristics (**Table 1**), with a slightly high proportion of primary progressive MS patients (19.2%). These patients had a quasi-symmetrical brain lesion distribution typical for MS (**Figure 1**), with the highest lesion frequencies in periventricular regions.

Table 1: Patient characteristics per MS subgroup.

	Total MS group: (n=208)	RR MS (n=126)	SP MS (n=42)	PP MS (n=40)
Gender; n (%) Male	77 (37%)	37 (29.4%)	25 (59.5%)	15 (37.5%)
Age in years; mean (SD)	41.1 (11.1)	35.6 (8.6)	47.8 (7.7)	51.6 (9.7)
Disease duration in years; median (IQR)	6.3 (10.1)	3.9 (7.8)	11.8 (12.2)	8.3 (9.5)
Lesion volume* (ml); median (IQR)	12.4 (35.5)	10.3 (22.9)	38.6 (102.4)	13.5 (40.0)
EDSS score; median (IQR)	3.5 (2.9)	2.0 (2.0)	6.0 (2.5)	4.5 (2.0)

* Lesion volumes were calculated in common space (therefore representing relative lesion volumes).

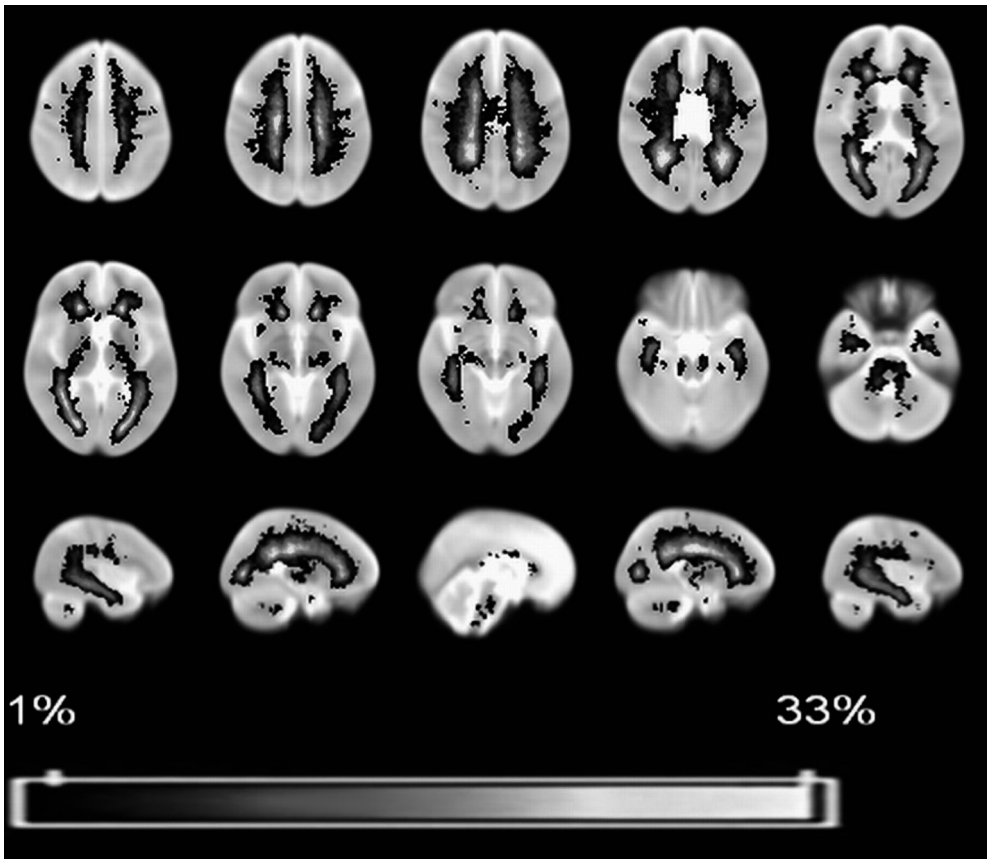

Figure 1: Lesion frequency map for our group of 208 MS patients, indicating for every voxel the lesion frequency throughout our patient sample, showing a range from 1% (n=2 patients having a lesion in that voxel) through the maximum of 33% (n=69). *For colour figure see page 187.*

Correlations between genotype and spatial lesion distribution

Of the 69 SNPs analysed, 11 genotypes of 10 SNPs showed significant clusters of either increased or decreased lesion probability, predominantly in periventricular clusters, for one of the genotypes (**Figures 2 and 3)**. All clusters were located periventricularly, abutting the frontal or occipital horn of the lateral ventricles, often in an asymmetrical configuration.

An increased lesion probability, in different periventricular regions, was observed for the heterozygous genotype of three SNPs: rs2227139 (within the MHC class II region), rs2076530 (within butyrophilin-like 2 gene) and rs876493 (within phenylethanolamine N-methyltransferase gene). Furthermore, there were two SNPs in which the homozygous major allele was associated with a higher lesion probability; i.e., rs2107538 within the chemokine (C-C motif) ligand 5 (CCL5) and rs9808753 within the interferon gamma receptor 2 (IFNGR2) gene (**Figure 2**).

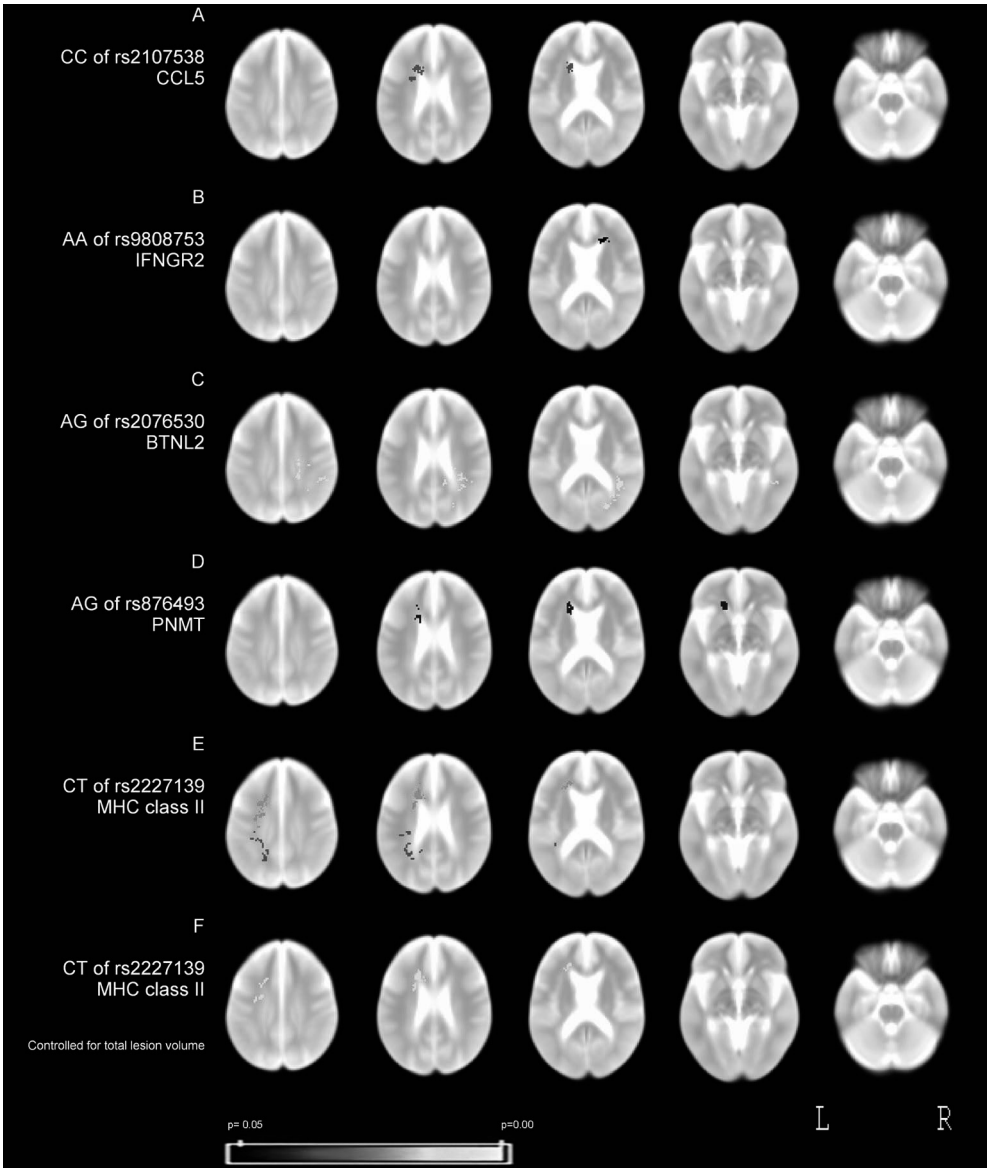

Figure 2: Association between *increased* lesion probability and genotype. Each row shows results for the comparison of one genotype compared to the other two. **A**: CC genotype of rs2107538 within the CCL5 gene (chemokine (C-C motif) ligand 5). **B**: AA genotype of rs9808753 within IFNGR2-gene (IFNGR2: interferon gamma receptor 2). **C**: AG genotype of rs2076530 within Butyrophilin-like 2 gene (BTNL2) (MHC class II associated). **D**: AG genotype of rs876493 (within phenylethanolamine N-methyltransferase-gene). **E**: CT genotype of rs2227139 (within MHC class II region). **F**: CT genotype of rs2227139 (within MHC class II region), when statistically controlled for total T2 brain lesion volume. Images show several axial slices of the T2-weighted template (the same slices in each case), with color overlay (indicating p-values) of the clusters in which local lesion probability was significantly increased. *For colour figure see page 188.*

For six SNPs, a genotype was associated with a decreased lesion probability (for details on cluster location and genotypes see **Figure 3**). These six SNPs were located within the following genes: Butyrophilin-like 2 gene (BTNL2, MHC class II associated), Alpha B crystalline (CRYAB), NADH dehydrogenase (ubiquinone) Fe-S protein 7 (NDUFS7), Uncoupling protein 2 (UCP2) and two SNPs within the TNF receptor superfamily, member 6 gene (also called FAS). When total brain lesion volume was included in the statistical model, only rs2227139 (within the MHC class II region) retained a significant cluster in which the CT genotype was associated with an increased local lesion probability compared to the other two genotypes (**Figure 2F)**.

This result of rs2227139 also remained after correction for disease duration.

To corroborate our findings, we calculated the voxelwise average lesion frequencies for each of the three genotypes of the SNP rs2227139, the only SNP that remained significant after controlling for total brain lesion volume. In the voxel with the maximum pseudo-t-value observed in the significant cluster (voxelwise pseudo-t = 4.1), a lesion was present in 29% (n=26 / 89) of the patients with the heterozygote genotype CT, compared to 16% (n= 12 / 77) of the patients with the CC genotype and 2% (n= 1 /42) of the patients with the TT genotype. There were no genotypes of any SNP associated simultaneously with both a decreased lesion probability in one location and an increased lesion probability in another, indicating consistency of the observed relations across the brain.

Influence of genotype on total brain lesion volume

We assessed whether genotype was directly related to total brain lesion volume for the SNPs with significant results in the clusterwise analysis. Of the 11 genotypes associated with an increased or decreased lesion probability at the described locations, six genotypes were significantly associated with total lesion volume in the brain (**Table 2)**. When we controlled these results for disease duration three SNPs were significantly associated with total lesion volume. The results were consistent with the LPM results- that is genotypes associated with a higher total brain lesion volume were also associated with an increased local lesion probability, and a decreased local lesion probability.

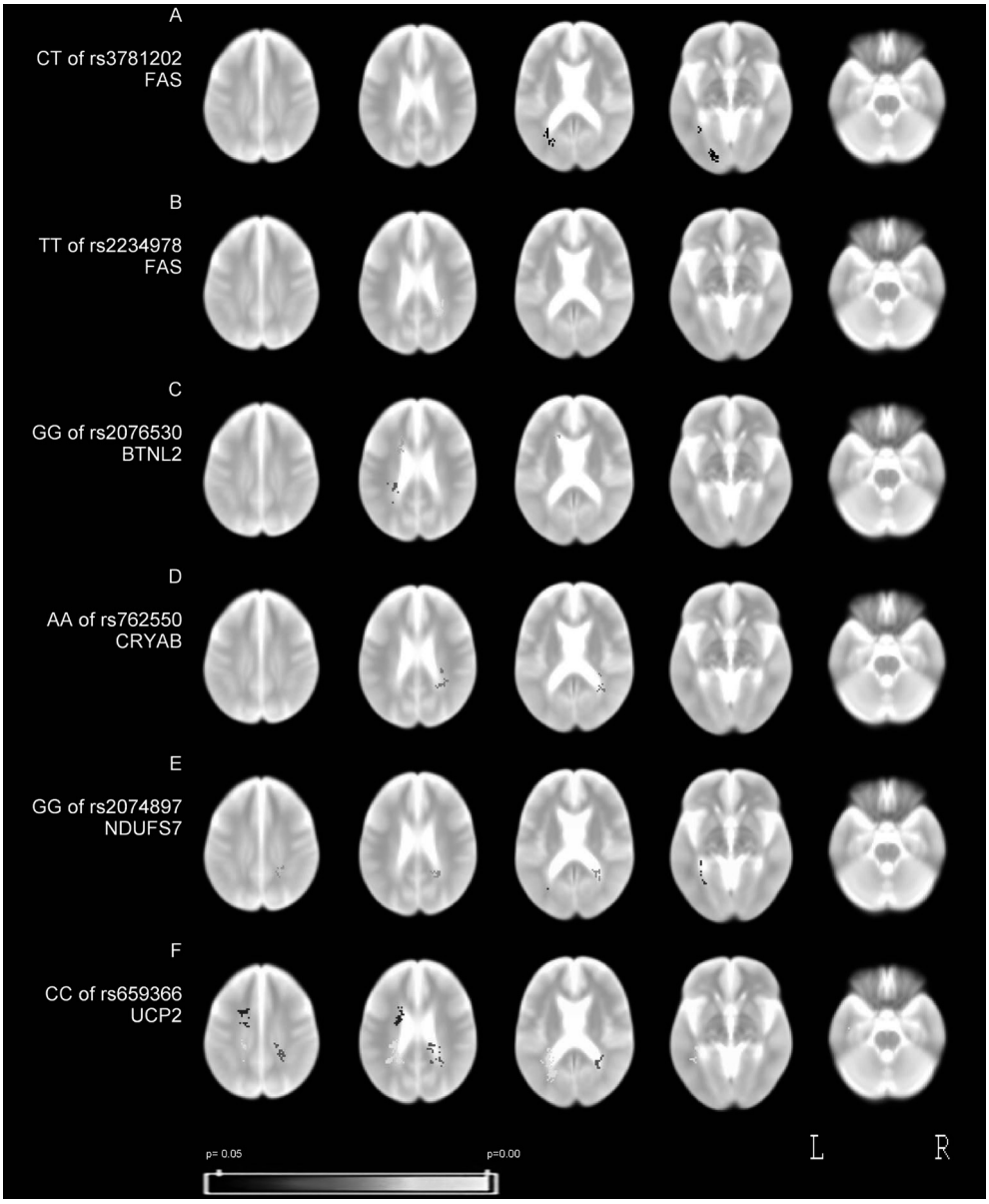

Figure 3: Association between *decreased* lesion probability and genotype. Each row shows results for the comparison of one genotype compared to the other two. **A**: CT genotype of rs3781202 (within FAS-gene: TNF receptor superfamily, member 6). **B**: TT genotype of rs2234978 (within FAS-gene: TNF receptor superfamily, member 6). **C**: GG genotype of rs2076530 within Butyrophilin-like 2 gene (BTNL2) (MHC class II associated). **D**: AA genotype of rs762550 within Alpha B crystallin gene. **E**: GG genotype of rs2074897 within NDUFS7-gene (NADH dehydrogenase (ubiquinone) Fe-S protein 7). **F**: CC genotype of rs659366 within UCP-2 gene (Uncoupling protein 2).
Images show several axial slices of the T2-weighted template (the same slices in each case), with color overlay of the clusters in which local lesion probability was significantly decreased. *For colour figure see page 189.*

Table 2: Association of genotypes of the SNPs significant in LPM analyses with total T2 lesion volume in the brain. P-values of significant associations to total T2 lesion volume are printed in bold.

Gene	Rs-number	Mean whole brain T2 lesion volumes in mL (SD)		P-value of differences in total lesion volume between genotypes (Mann Whitney U-test)	p-value of differences in total lesion volume, corrected for disease duration (using GLM)
		Genotype of interest†	Other genotypes		
Association with local *increased* local lesion probability in LPM analyses					
BTNL2	rs2076530§	AG 32.0 (32.9)	AA and GG 23.8 (27.4)	**0.04**	**0.05**
CCL5	rs2107538	CC 28.2 (29.0)	CT and TT* 20.9 (28.1)	**0.02**	0.07
IFNGR2	rs9808753	AA 28.5 (30.5)	AG and GG* 18.3 (22.6)	**0.04**	0.29
PNMT	rs876493	AG 28.1 (29.0)	AA and GG 23.2 (28.6)	0.15	0.13
HLA	rs2227139	CT 28.0 (29.9)	CC and TT 23.9 (28.0)	0.35	0.25
Association with local *decreased* local lesion probability in LPM analyses					
BTNL2	rs2076530§	GG 18.7 (22.7)	AA and AG 26.6 (29.5)	0.13	**0.04**
CRYAB	rs762550	AA 19.8 (21.0)	AG and GG 27.0 (30.2)	0.28	0.30
FAS	rs2234978	TT 12.5 (14.7)	CC and CT 27.1 (29.7)	**0.02**	0.06
FAS	rs3781202	CT 23.1 (28.5)	CC and TT 28.4 (29.1)	0.18	0.37
NDUFS7	rs2074897	GG 18.4 (23.7)	AA and AG 28.2 (30.1)	**<0.01**	0.08
UCP2	rs659366	CC 18.6 (23.1)	CT and TT 29.8 (31.1)	**<0.01**	**<0.01**

* Because of frequency < 5% of homozygote genotype, these genotypes (TT for CCL5 and GG for IFNGR2) were only analysed when combined with heterozygote genotype.
§ Different genotypes of this same SNP are involved in decreased and increased lesion probability.
† Genotype(s) that are significantly associated with altered local lesion probability in LPM analyses.

See list of abbreviations on page 181

Discussion

Based on the hypothesis that genetic background may determine spatial lesion patterns, this exploratory study combined lesion probability mapping with genetic data of 69 SNPs in 208 MS patients. To our knowledge, this is the first study that in a large cohort of MS patients tests the role of several genes on lesion distribution within the brain. Five SNPs showed a locally increased lesion probability in certain brain regions for one genotype, while six SNPs showed a decreased lesion probability for one of the genotypes. The most robust finding was the increased probability of having a lesion in the left periventricular region next to the frontal and to a lesser extent the occipital horn of the ventricles, for the heterozygous genotype of rs2227139 (**Figures 2E and 2F**). This was the only SNP for which a significant cluster remained after applying a correction for total brain lesion volume. Rs2227139 is located on chromosome 6 within the highly polymorphic major histocompatibility complex (MHC) class II region, which is involved in self versus non-self immune recognition.[36] These genes have consistently been shown to have a major effect on susceptibility to MS and other auto-immune diseases[36] and may be involved in disease severity as well.[15,37] No data is available on the effect of this SNP on region-specific differences in the brain. However, previously multiple HLA alleles showed effects on several MRI severity markers, such as T2 lesion volume, T1 hypo-intense lesion volume and atrophy measures,[38,39] suggesting a role for several HLA alleles in the MS phenotype. These findings highlight the importance of HLA genes on MRI severity markers. Future studies on the genetic influence on lesion location should preferably include high resolution HLA typing, enabling comparison of the effect of different HLA alleles on lesion distribution (taking possible interaction between genes into account) and detecting the true causative allele(s).

As can be seen from **figures 2 and 3**, the SNPs showed altered lesion probability in predominantly four regions: the bilateral periventricular white matter next to the frontal and occipital tip of the horns of the lateral ventricles. The periventricular clusters are distributed in an asymmetrical manner. We have no explanation for these asymmetries, which persisted after post-hoc repeated analyses with a lower significance threshold. A priori, a genetic influence on this asymmetrical distribution is theoretically unlikely. Another study also noted an asymmetrical distribution of MS brain lesions at the individual patient level, possibly reflecting a different lineage pattern.[9] Although our number of patients is relatively high, bias due to, for example, the high number of progressive patients is possible and cannot be excluded.

Rs2076530 within BTNL2 (Butyrophilin-like 2-gene) was the only SNP showing significant effects for more than one genotype. The AG genotype was associated with an increased lesion probability in the periventricular white matter adjacent to the right occipital horn, while the GG genotype was associated with a decreased lesion probability in the periventricular white

matter adjacent to the left occipital and frontal horn. Both effects were non-conflicting and disappeared after correction for total brain lesion volume.

The relation between HLA-DRB1*1501 (the MHC class II allele strongly associated with susceptibility to MS)[12,40,41] and spatial lesion distribution within the brain was studied in one previous report,[23] demonstrating no significant influence of the allele. Our results (by using rs3135388, a SNP in the MHC region predicting the HLA-DRB1*1501 haplotype with very high sensitivity)[28] confirmed in a larger sample the negative result of this previous study of Sepulcre et al. In a subset of the current patient group, we previously found the HLA-DRB1*1501 allele to be related to increased lesion loads in the spinal cord but not the brain. In the present study on a larger patient group encompassing the former, we confirmed that the HLA-DRB1*1501 allele was not related to the total volume of brain lesions,[22] and importantly, added the new finding that it is also unrelated to their anatomical distribution across the brain.

Limitations of this study include the relatively low field strength of the MRI scanners we used, which in general leads to lower sensitivity to lesions than current state of the art MRI scanners, operating at 3T or above.[42]

Furthermore, the linear registration method used to warp the lesion masks of individual patients to the template, by definition, cannot correct for the variability in ventricular and sulcal sizes that is present in MS patients as a result of differences in brain atrophy. Therefore, the matching between individual patient's brains will be imperfect; this discrepancy leads to less accurate overlay of corresponding anatomical areas and may explain, to some extent, why we only found clusters in the periventricular region and not in areas with a lower lesion frequency. In spite of these limitations, we deliberately chose a linear registration method (FLIRT), because of the difficulties associated with non-linear registration methods. In this study we were limited in the sequences available (dual-echo images only), which pose problems for non-linear registration regarding the distinction between periventricular lesions and ventricular CSF, because of their similar signal intensities. Second, non linear registration could result in displacement of lesions inside the brain, thereby affecting the very aspect of the disease that we intended to study (namely lesion distribution).

The third limitation is the absence of grey matter lesions in our study, since these lesions go mostly undetected when using standard MRI techniques.[43] From post-mortem studies we know that grey matter lesions are extensively present in MS patients[43] and it can be expected that a different genetic background may influence the lesion distribution between the white and grey matter compartment, as well as the anatomical distribution within the white and grey matter.

Total brain lesion volume seems to be an important covariate in LPM analyses.[7] Apart from the left frontal horn cluster in rs2227139, no clusters retained significance when the model was controlled for lesion volume. Thus, the influence of genetic background on

spatial lesion distribution, as assessed in the current study, may partly act through total brain lesion volume and come to expression in voxelwise analyses especially in regions with relatively high frequencies of MS lesions, where differences between groups can become statistically significant (i.e., the periventricular white matter). Therefore, in future studies, when comparing groups, a correction for total brain lesion volume should be considered.

The genes investigated in this study are putatively associated with susceptibility or disease severity in MS, and we hypothesized that the variation in anatomical distribution of brain lesions between MS patients may be partly genetic in nature. Our results suggest that such a genetic influence may indeed be present. Independent studies should now be conducted to confirm these findings, including studies using alternative methodology in addition to LPM, e.g., using pre-defined regions of interest defined on the basis of LPM results, to increase sensitivity to detect differences. For the genes with significant results, there are no obvious relationships between their functions and spatial MS lesion distribution. However, several of the SNPs that were significantly associated with lesion distribution, were also significantly associated with the total lesion volume in the brain. The previously reported effect of these genes on the overall lesion burden (T2 lesion volume, T1 hypo-intense lesion volume or atrophy,[38,44,45]), acting through their functions in different processes such as mitochondrial energy metabolism and inflammation, is likely to generate differences in lesion distribution between the genotypes. Our finding that statistically controlling for total brain lesion volume removes the observed effects on lesion distribution, suggests that these genes possibly exert an effect mainly on the overall lesion burden. However, to fully address this issue, a more focused approach should be applied, using both an LPM approach and predefined areas of interest (hereby increasing the statistical power) and select more homogenous patients to limit the effect of confounding factors. In this context a longitudinal study on the spatiotemporal development of new lesions (including T1 hypo-intense lesions and T1 gadolinium enhancing lesions) would be valuable. Eventually, the insights gained from this approach may yield ways of predicting from patients genotype whether they are likely to develop lesions in clinically eloquent areas.

Recently, pathway analysis in relation to susceptibility to MS revealed, not surprisingly, immune-related pathways.[46] However, the same study also detected significant neural pathways, implicating a primary neural dysfunction in susceptibility to MS. This pathway analysis approach may provide a basis for patient stratification (for instance patients carrying a predominantly inflammatory profile versus patients carrying a more neurodegenerative profile) and it would be valuable to characterize these patient groups in terms of their lesion distribution. Previously, some of the SNPs selected for this study were found related in relatively small studies, to a more neurodegenerative profile (CCR 5 and CRYAB),[18,45] while others have found several HLA alleles associated with a more inflammatory profile.[37,38] Such profile differences may be able to explain the previously described differences in lesion

distribution between T1 gadolinium enhancing lesions (acute inflammatory activity) and T1 persistent hypo-intense lesions (neurodegenerative activity).[9]

Conclusion:

This exploratory study revealed an association between heterozygosity on rs2227139 in the MHC class II region and preferential periventricular lesion formation in MS. Other SNPs also showed associations between genotype and spatial lesion distribution, which are worth studying in future studies using a more focused approach and considering genetic pathways in addition to single genes. In these future studies, the potential role of lesion volume influencing the relation between genotype and lesion distribution should be explored further, as well as the distribution of grey matter lesions, contrast-enhancing lesions, and persistent T1-hypointense lesions.

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Supplementary Table 1: SNPs included in our analyses. MAF=minor allele frequency in our sample.

Gene	rs-nr	Chromosome	Polymorphism (major allele / minor allele)	MAF
ADAMTS14 *	rs4747075	10q22	G/A	0.30
ADAMTS14	rs7081273	10q22	G/C	0.35
ADAMTS14	rs4746060	10q22	C/T	0.09
BTNL2 *	rs2076530	6p21.3	A/G	0.24
CACNG4	rs4790896	17q24	A/G	0.38
CCDC46	rs987931	17q24	T/G	0.31
CCL5	rs2280788	17q11.2-q12	C/G	0.02[§]
CCL5	rs2107538	17q11.2-q12	C/T	0.19
CCR5	rs333	3p21	+/-	0.10
CD24	rs8734	6q21	C	0.00[§]
CIITA	rs3087456	16p13	A/G	0.26
CNTF	rs1800169	11q12	G/A	0.12
CRYAB	rs14133	11q21-q23	C/G	0.25
CRYAB	rs762550	11q21-q23	G/A	0.42
CTLA4	rs231775	2q33	A/G	0.38
CTLA4	rs5742909	2q33	C/T	0.09
EBF1	rs1368297	5q34	T/A	0.38
FAS	rs1800682	10q23	T/C	0.46
FAS	rs3781202	10q23	C/T	0.41
FAS	rs2234978	10q23	C/T	0.31
GABBR1	rs1805057	6p22	C	0.00[§]
HELZ	rs2363846	17q24	C/T	0.50
HLA *	rs2395166	6p21.3	T/C	0.48
HLA-DRA	rs2213584	6p21.3	A/G	0.42
HLA	rs2227139	6p21.3	C/T	0.42
HLA-DRA	rs3135388	6p21.3	G/A	0.33
HLA	rs9268458	6p21.3	C/A	0.19
HLA *	rs6457594	6p21.3	A/G	0.38
HLA-DRA	rs2395182	6p21.3	T/G	0.39
HLA-DRA	rs2239802	6p21.3	C/G	0.39
HSPB2	rs2234702	11q21-q23	C	0.00[§]
IFNAR1	rs2257167	21q22	G/C	0.07
IFNGR2	rs9808753	21q22	A/G	0.15
IL1B	rs1799916	2q14	A	0.00[§]
IL1B	rs1143627	2q14	A/G	0.35
IL1B	rs1143634	2q14	C/T	0.23
IL1RN	rs419598	2q12-q14	T/C	0.31
IL1RN	2073 C/T	2q12-q14	C/T	0.31
IL2	rs2069763	4q26-27	G/T	0.34
IL2	rs2069762	4q26-27	T/G	0.26
IL4R	rs1801275	16p12	A/G	0.20
IL7R	rs11567685	5p13	T/C	0.25
IL7R	rs7718919	5p13	G/T	0.13

Supplementary Table 1: Continued

Gene	rs-nr	Chromosome	Polymorphism (major allele / minor allele)	MAF
IL7R	rs11567686	5p13	A/G	0.34
IL10	rs1800896	1q32	A/G	0.45
MC1R	rs1805009	16q24	G/C	0.01§
MC1R	rs1805006	16q24	C/A	0.01§
MEFV	rs28940577	16p13.3	A	0.00§
MOG	rs3130250	6p22	G/A	0.20
MOG	rs3130253	6p22	G/A	0.11
NDUFA7	rs2288414	19p13.2	G/C	0.03§
NDUFA7	rs561	19p13.2	G/A	0.19
NDUFS5	rs2889683	1p34.2	T/C	0.31
NDUFS5	rs6981	1p34.2	G/A	0.05
NDUFS7	rs2074897	19p13.3	G/A	0.46
NFKBIL1	rs3130062	6p21.3.	T/C	0.17
NOS2	rs1137933	17q11.2	G/A	0.24
NOS2	rs2779248	17q11.2	T/C	0.38
NOTCH4	rs367398	6p21.3	G/A	0.18
PDCD1	rs11568821	2q37	G/A	0.11
PITPNC1	rs1318	17q24	A/G	0.22
PITPNC1	rs2365403	17q24	C/G	0.17
PNMT	rs876493	17q11-q23	A/G	0.42
PRKCA	rs7220007	17q24	A/G	0.48
PRKCA	rs887797	17q24	C/T	0.30
PRKCA	rs2078153	17q24	G/C	0.23
PRKCA	rs3890137	17q24	A/G	0.37
PTPN22	rs2476601	1p13	G/A	0.10
PTPRC	rs17612648	1q31	C/G	0.02§
PTPRC	rs4915154	1q31	A/G	0.01§
SPP1	rs1126616	4q21	C/T	0.23
SPP1	rs1126772	4q21	A/G	0.19
SPP1	rs2853744	4q21	G/T	0.06
SPP1	rs9138	4q21	A/C	0.24
SPP1	rs4754	4q21	T/C	0.24
TNF	rs1800629	6p21.3	G/A	0.21
TNFSF10	rs1131568†	3q26	C/T	0.35
UCP2	rs659366	11q13	C/T	0.40
VDR	rs1544410	12q13	G/A	0.44
VDR	rs731236	12q13	A/G	0.44

* This SNP deviated from Hardy-Weinberg Equilibrium (p<0.01)
§ Excluded due to minor allele frequency below 5%.
† Previous rs-number: rs9880164.

See list of abbreviations on page 181

Chapter 4

Spinal cord MRI in predicting diagnosis and prognosis of Multiple Sclerosis

Chapter 4.1

Spinal cord lesions in CIS patients: A powerful tool in diagnosis and prognosis

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Abstract

Objective: Spinal cord (SC) lesions are frequently found in Multiple Sclerosis (MS), but are rare in healthy ageing and cerebrovascular patients. Our aim was analyzing the contribution of SC involvement in Clinically Isolated Syndrome (CIS) in diagnosing MS according the McDonald 2010 criteria and in predicting conversion to Clinically Definite MS (CDMS).

Methods: We prospectively followed monofocal, relapsing onset CIS patients with either SC or brain symptom onset (including optic neuritis). MRI of the brain and SC were performed shortly after onset and patients were followed for 24-119 months (median 64). SC MRI findings were assessed for their contribution to the McDonald 2010 diagnostic criteria and their effect on conversion to CDMS.

Results: 121 patients were included (63 spinal-CIS). Based on the brain scan only, 36 patients fulfilled the McDonald criteria; by including SC findings 6 additional patients fulfilled these criteria. To diagnose one additional non-spinal CIS patient, the number needed to scan is 7. In non-spinal CIS patients that did not fulfil McDonald brain MRI criteria (n=42), presence of a SC lesion was associated with a higher risk of conversion to CDMS (OR: 14.4 (95% CI: 2.6 – 80.0) and shorter time to conversion to CDMS (Hazard ratio: 51.4 (95%- CI: 5.5 – 476.3).

Conclusions: Presence of SC lesions facilitates diagnosing MS and is predictive for conversion to CDMS, especially in non-spinal CIS patients that do not fulfill brain MRI criteria. We therefore recommend performing a SC scan in non-spinal CIS patients that do not fulfil McDonald brain MRI criteria.

Introduction

Spinal cord (SC) abnormalities on Magnetic Resonance Imaging (MRI) are frequently found in clinically definite multiple Sclerosis (CDMS) patients[1]. In patients presenting with clinically isolated syndromes (CIS) the prevalence of SC lesions is lower[2,3]. In most other neurological diseases, especially in cerebrovascular disorders, auto-immune inflammatory disorders and also in healthy ageing, SC lesions are rather rare, whereas cerebral white matter changes are common[4-7].

By introducing MRI criteria, early diagnosis was facilitated and treatment could be started early, in an attempt to reduce disability accumulation[8-12]. SC lesions have been incorporated in the MS diagnostic criteria since 2001[10,11]. In the 2010 revisions, SC lesions have become increasingly important and now have the same weight as brain lesions[12]. Despite the recognized contribution of SC imaging to the early diagnosis of MS[13-17], scanning of the SC is not routinely performed in all patients. As a rule, mostly patients presenting with a CIS with SC symptomatology, will be referred for a spinal MRI scan to identify the cause of the symptoms (demyelination, compression of the SC etc). However, in CIS patients presenting with brain symptoms, SC imaging is often not performed, although asymptomatic SC involvement is frequent[1,18] and may add independent prognostic information by contributing to the diagnostic criteria[12].

The aim of this study was to prospectively assess the occurrence of brain and SC lesions in a large monofocal CIS cohort, to test the contribution of SC imaging in diagnosing MS applying the McDonald 2010 criteria. The second aim was to investigate the impact of presence of SC lesions on future conversion to CDMS in patients with SC symptoms (spinal CIS) and brain symptoms (non-spinal CIS).

Methods

Patients

Patients with a monofocal CIS were selected from an ongoing natural history at the VUmc Medical Center[19]. Recruitment for this natural history study started in December 2000 and lasted until September 2007. Patients with CIS or early MS were only included when a demyelinating cause of the complaints was most likely, as assessed by MS-neurologists. These patients were invited to participate in this natural history study. Spinal cord and brain MRI were obtained at baseline per study protocol from all participants entering the natural history study, irrespective of the clinical course of the patient. Of our total cohort of 319 CIS / early MS patients, patients diagnosed with CDMS at entry of the study (n= 102) or with a progressive onset (n= 28) were excluded. Of all CIS patients (n= 189) only the CIS patients fulfilling the criteria mentioned below were included:

1) Patients presenting with a monofocal CIS, suggestive of central nervous system demyelination, not attributable to other diseases, without previous neurological episodes suggestive of demyelination; symptoms started within 12 months preceding the recruitment (27 CIS patients were excluded). Patients with a multifocal onset (signs and symptoms) were excluded from further analysis, because they already fulfilled the dissemination in space criteria (22 multifocal CIS patients were excluded).
2) Age between 16 and 60 years at onset of first symptoms.
3) Availability of an MRI scan of the brain and SC at baseline preferably made on the same day, but no more than 3 months apart (13 CIS patients were excluded).
4) Clinical follow-up of at least 24 months (6 were excluded).
All patients fulfilling these criteria were enrolled (n= 121)

Standard Protocol Approvals, and Patient Consents
Written informed consent was obtained from all patients. The study was approved by the Institutional Review board of the VU University Medical Center.

Clinical data
Symptoms at onset were classified according to the presenting symptoms as “spinal-CIS” (onset of symptoms originating from the SC) and “non-spinal CIS” (onset of symptoms originating from the brain, infratentorial brain region (brainstem and cerebellum) or optic nerve). Patients were seen yearly and interviewed for new symptoms and use of disease modifying therapy. If new symptoms occurred and patients were seen by a neurologist in a different hospital, this neurologist was contacted for information on the signs and symptoms of the relapse. Conversion to CDMS was defined as a second episode of new symptoms occurring after an interval of at least 1 months and not attributable to other disease[21]. The time from disease onset to conversion to CDMS was calculated in months.
In our analyses the new McDonald 2010 diagnostic criteria for dissemination in space and time were applied[12].

MRI
All patients, irrespective of the onset symptoms, had an MRI scan of the brain and the SC. MRI scans of the brain and SC were acquired at baseline either on 1.0 Tesla or 1.5 Tesla MRI whole body MRI systems (Siemens Magnetom Impact Expert, Siemens Vision and Siemens Sonata) (Siemens AG, Erlangen, Germany). For the brain, we used standard 2D dual-echo spin-echo Proton Density (PD) - and T2-weighted images (TR: 2200-3000 ms, TE: 20-30 & 80-100 ms) and T1-weighted images after administration of intravenous gadolinium (TR 500-600 & TE 20 ms) with slice thicknesses of 3-5 mm, a maximum gap between slices of 0.5 mm, and an in-plane resolution of 1x1 mm2. Lesions on T2 (hyperintense) and T1

weighted images (gadolinium-enhancing lesions) were identified by a blinded experienced reader (Image Analysis Center) and then outlined using local thresholding software. Lesion location was scored using the following regions: periventricular, juxtacortical, infratentorial (brainstem and cerebellum). SC scanning included a cardiac-triggered sagittal PD and T2-weighted dual-echo spin-echo sequence with a slice-thickness of 3mm covering the whole SC (TR: 2500 – 3000 ms and TE: 20-30 & 80-100 ms), with a gap between slices of 0.3 mm, and an in plane resolution of 1x1 mm, using spinal phased-array coils. The number of focal lesions was scored by one experienced reader, being unaware of the clinical condition of the patient.

Statistical analyses

The effect of SC scanning on diagnosing MS was evaluated by applying the McDonald 2010 criteria first without the findings of the SC scan and secondly when including the findings of the SC. This allows assessment of whether the SC findings contributed to the DIS and/or DIT criteria. The number of patients that needs to be scanned to diagnose one additional MS patient was calculated.

To assess our second aim (determining the contribution of SC lesions on conversion to CDMS) odds ratios (including 95% confidence-intervals (CI)) were calculated in four subgroups: 1. Spinal-CIS fulfilling McDonald MRI criteria at baseline brain scan 2. Spinal-CIS not fulfilling McDonald brain MRI criteria at baseline 3. Non-spinal CIS fulfilling McDonald brain MRI criteria at baseline and 4. Non-spinal CIS not fulfilling McDonald brain MRI criteria at baseline. Furthermore, survival analysis was performed on time to conversion to CDMS in the abovementioned four groups. Kaplan-Meier curves were constructed. Cox-regression analysis was used to calculate hazard ratios (including 95% CI) for the effect of presence of SC lesions on time to conversion to CDMS and corrected for use of DMT before conversion to CDMS. Analyses were performed using SPSS for Windows 15.0 (SPSS Inc, Chicago, Illinois).

Results

Patient characteristics

In this study 121 CIS patients were included with a mean age-at-onset of 35 years. The mean disease duration at follow up was 5 years and 4 months (range: 2 years - 10 years), during which 55 patients (45.5%) converted to CDMS according to the Poser criteria. For more details on the patient clinical and MRI characteristics see **Table 1**. In 63 patients, the presenting symptoms were attributable to the SC (referred to as “spinal-CIS”) while 58 patients experienced the first symptoms originating from the brain (“non-spinal CIS”: optic nerve, infratentorial region or hemispherical region). The brain MRI showed T2

abnormalities in 95% of patients, while 44 patients had gadolinium enhancing lesions in the brain (36.4%). In total 82 patients had focal lesions in the SC (67.8%). No patients showed SC lesions extending longitudinally over 3 or more segments. The patients with spinal CIS did not differ from the non-spinal CIS group in total number of lesions in terms of juxtacortical, periventricular or enhancing lesions. However, spinal CIS patients more frequently had SC lesions (p=0.001) and a trend towards more frequent infratentorial lesions (p=0.06) (**Table 1**).

Effect of adding SC findings on diagnosing MS using the McDonald 2010 criteria

When we applied the McDonald 2010 criteria, 36 patients fulfilled the criteria for DIS and DIT, based on the baseline brain MRI scan only. When the findings of the SC scan were added, 2 extra patients fulfilled DIS criteria (however still lacked DIT) and 6 additional patients fulfilled both DIS and DIT criteria: 2 patients went from not fulfilling DIS and DIT to fulfilling both DIS and DIT, 2 patients fulfilled DIS criteria based on the brain scan and after taking into account SC findings also fulfilled the DIT criteria and 2 patients fulfilled DIT criteria based on the brain scan and now additionally fulfilled DIS criteria when SC findings were added. These 6 additional patients were all non-spinal CIS patients, because in the McDonald 2010 criteria all lesions in the SC in spinal CIS patients are considered symptomatic and therefore excluded from contributing to the diagnostic criteria. From our study, the number needed to scan in non-spinal CIS patients to be able to early diagnose one more patient with MS is 7.

Table 1: Patient characteristics.
Patient characteristics of all CIS patients in our study, subdivided by spinal and non-spinal CIS.

Patient characteristics		Total CIS (n=121)	Spinal-CIS (n=63)	Non-spinal CIS (n=58)
Gender (n; % Male)		42 (35)	18 (28.6)	24 (41.4)
Mean age at onset (SD)		34.9 (9.0)	35.3 (8.5)	34.5 (9.6)
Presenting symptoms:				
	Spinal cord symptoms (%)	63 (52)	63	
	Optic neuritis (%)	20 (17)		20
	Brainstem / cerebellar syndrome (%)	34 (28)		34
	Hemispherical (%)	4 (3)		4
Mean time between date of onset and baseline-scan in months (SD)		4.7 (2.9)	5.1 (2.9)	4.2 (2.7)
Mean disease duration at most recent visit in months (SD)		64.4 (20.3)	67.2 (18.5)	61.3 (21.9)
Mean EDSS at baseline (SD)		2.0 (1.2)	2.1 (1.2)	2.0)1.2)
Number of patients using DMT before conversion to CDMS (%)		35 (28.9)	18 (28.6)	17 (29.3)
Brain MRI findings at baseline				
Number of patients with ≥1 periventricular lesion (%)		96 (79.3)	50 (79.4)	46 (79.3)
Number of patients with ≥1 juxtacortical lesion (%)		84 (69.4)	42 (66.7)	42 (72.4)
Number of patients with ≥1 asymptomatic infratentorial lesion (%)		18 (14.9)	13 (20.6)	5 (8.6)
Number of patients with:	**0 T2 lesions (%)**	6 (5.0)	4 (6.3)	2 (3.4)
	1-3 T2 lesions (%)	16 (13.2)	7 (11.1)	9 (15.5)
	4-8 T2 lesions (%)	24 (19.8)	12 (19.0)	12 (20.7)
	≥9 T2 lesions (%)	75 (62.0)	40 (63.4)	35 (60.3)
Number of patients with gadolinium enhancing lesion (%)*		44 (36.4)	22 (34.9)	22 (37.9)
Spinal cord MRI findings at baseline				
Number of patients with focal abnormalities in the spinal cord (%)		82 (67.8)	51 (81.0)	31 (53.4)
Number of patients with gadolinium enhancing lesions (%)**		19 (16.1)	12 (19.7)	7 (12.3)

* N= 120; in one patient no gadolinium was administered. ** n=118; in three patients no gadolinium was administered.
SD= standard deviation; EDSS= Extended Disability Status Scale.

Predictive power of the presence of focal SC lesions for conversion to CDMS

In the total CIS group (n=121), during a mean follow-up of 5 years and 4 months, the odds-ratio of presence of focal SC lesions for conversion to CDMS was 3.53 (95% CI: 1.52 – 8.17) compared to patients who do not have SC lesions. The odds-ratio for presence of SC lesions for conversion to CDMS in the subgroup spinal-CIS was 1.24 (95% CI: 0.35 – 4.44), while in the non-spinal CIS subgroup the odds was 6.48 (95% CI: 2.34 – 17.95). Moreover, the Cox-regression (with correction for use of DMT before conversion to CDMS) showed a significantly shorter time to conversion to CDMS in patients with SC lesions, compared to patients without SC lesions for the total CIS group (n=121) (Hazard ratio: 2.76 (95%-CI: 1.37 – 5.56; p=0.005) (for survival-curve see **Figure 1A**).

Odds-ratios and hazard ratios for the separate subgroups of spinal CIS and non-spinal CIS patients with or without an abnormal brain MRI scan, for predicting (time to) conversion to CDMS in patients with SC lesions compared to those without SC lesions, are presented in **Table 2**. The odds-ratio for presence of SC lesions on predicting conversion to CDMS in the non-spinal CIS subgroup that did not fulfil the diagnostic brain MRI criteria was 14.4 (95%-CI: (2.60 – 80.03). Only patients with spinal cord lesions in the nonspinal CIS group that did not fulfill the McDonald 2010 brain MRI criteria had a significantly shorter time to conversion to CDMS, compared with those patients without spinal cord lesions (see **figure 1B** for the survival curve for this group of patients). Please see **Figure 2** for an example of an MRI of a non-spinal CIS patient with typical MS brain lesions, however lacking spinal cord lesions on MRI. This patient did not convert to CDMS during follow-up.

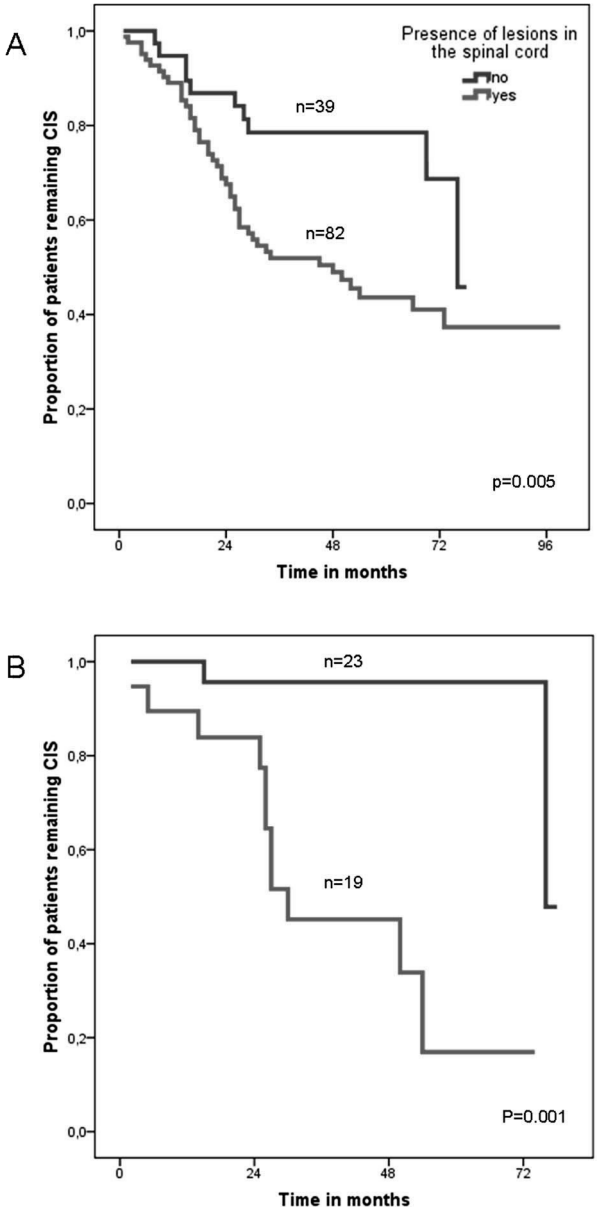

Figure 1: **Survival-curves on effect of presence of spinal cord lesions on time to conversion to CDMS. Figure 1A:** Survival-curve (Kaplan Meier) in total group of CIS patients (n=121), significant shorter time to CDMS for patients with compared to patients without SC lesions (p=0.005 Cox regression). **Figure 1B**: Kaplan Meier curve in non-spinal CIS patients *not* fulfilling McDonald brain MRI criteria (n=42). *For colour figure see page 190.*

Table 2: Effect of presence of spinal cord lesions on conversion to CDMS.
For every subgroup of CIS patients (spinal CIS and non-spinal CIS patients that fulfilled the McDonald 2010 brain criteria and spinal-CIS and non-spinal CIS patients that did not fulfill the diagnostic criteria), odds ratio on conversion to CDMS (including 95%CI) and hazard ratio for time to conversion to CDMS (including 95%CI) are shown.

	Number of patients	Number of patients with SC lesions	Number of patients without SC lesions	Odds ratio for patients with SC lesions to develop CDMS vs patients without SC lesions (95%CI)	Hazard ratio for time to develop CDMS, using Cox-regression* (95%CI)
1. Spinal-CIS fulfilling McDonald brain MRI criteria	20	18	2	NA**	0.69 (0.14 – 3.36) p-value: 0.65
2. Spinal-CIS ***not*** fulfilling McDonald brain MRI criteria	43	33	10	1.33 (0.29 – 6.14)	1.08 (0.29 – 4.06) p-value: 0.92
3. Non-spinal CIS fulfilling McDonald brain MRI criteria	16	12	4	1.67 (0.11 – 25.4)	0.89 (0.21 - 3.71) p-value: 0.87
4. Non-spinal CIS ***not*** fulfilling McDonald brain MRI criteria	42	18	23	14.4 (2.60 – 80.03)	51.38 (5.54 – 476.33) p-value: 0.001

* regression corrected for DMT use before conversion to CDMS ** Can not be calculated (one cell = 0). SC: spinal cord. CI: confidence interval.

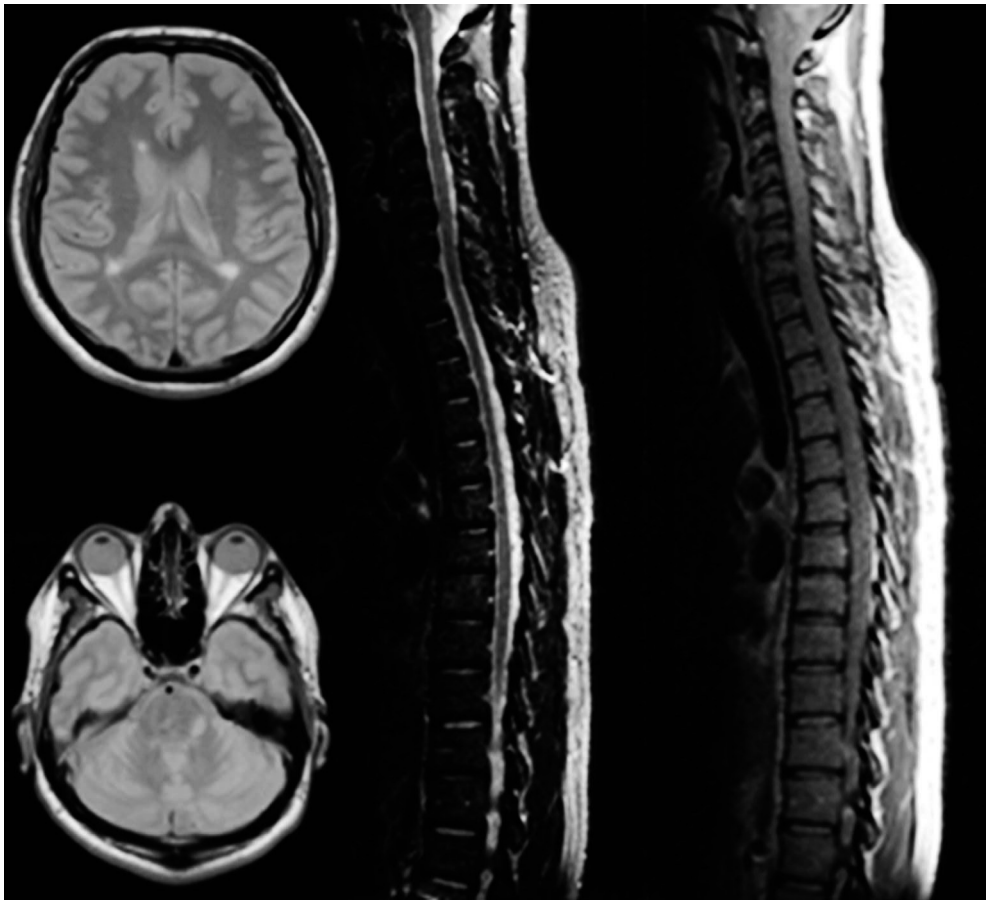

Figure 2: Brain and spinal cord MRIs in patients with and without spinal cord lesions.
MRI of a 36-year-old male that experienced a relapse with brainstem symptoms (an episode of sensory disturbances on the left side of his face). The baseline MRI scan of the brain showed multiple periventricular lesions (see left upper panel T2 weighted brain images), two infratentorial lesions (lower left panel) and a juxtacortical lesion (not shown). No abnormalities were found in the SC (see right panels: sagittal T2 weighted and proton-density images). No conversion to CDMS occurred during follow-up (4 years).

Discussion

Although SC abnormalities have gained importance in the most recent diagnostic criteria when compared to the previous diagnostic criteria for MS, SC scanning is not performed in all patients presenting with a CIS. Nowadays, state of the art MRI acquisition techniques allow us to image the whole SC with high resolution within a reasonable acquisition time. However, in many centers, patients will only be referred for SC MRI when presenting

symptoms indicate SC involvement (to rule out other causes). In this study we show however that especially in patients not presenting with SC symptomatology, presence of SC lesions can both aid in diagnosing MS by contributing to the diagnostic McDonald 2010 criteria, and have prognostic value in predicting conversion to CDMS.

In CIS patients presenting with SC symptomatology (spinal CIS), an MRI scan of the SC will be performed almost instantly to assess the cause of SC symptoms. When lesions are found consistent with demyelination, a brain scan will be performed to assess dissemination in space and time. In our study in this subgroup, presence of focal SC lesions did not aid in predicting future conversion to CDMS. To a certain extent this is an artifact of the McDonald 2010 criteria that ignore all SC lesions in cord-onset patients, while it might be argued that some (if 2 or more) are asymptomatic. However, spinal CIS patients in our cohort, with two or more SC lesions did not convert more often to CDMS than patients with zero or one SC lesion.

By contrast, in patients presenting with brain symptoms (non-spinal CIS), the first procedure will be a brain MRI scan. If there is good reason to assume that demyelination is the cause of the symptoms, the McDonald 2010 criteria will be applied. In our total CIS cohort, 36 out of 121 patients (in non-spinal CIS patients 16/58) could be diagnosed with MS after a single brain MRI scan. In this group of patients (either spinal-CIS or non-spinal CIS) SC lesions did not further contribute to predicting conversion to CDMS. When we added the findings of the SC scan in the brain CIS patients that could not be diagnosed with MS (n=42), 6 additional patients were diagnosed with MS. This means that in this subset for every 7 MRI scans of the SC 1 extra patient will be diagnosed with MS (number needed to scan = 7).

In addition to a moderate contribution to immediately diagnosing McDonald 2010 MS, we found that in the same group of non-spinal CIS patients, presence of SC lesions is a strong prognostic factor in predicting conversion to CDMS (see **Table 2** and **Figure 1B)**. Patients with SC lesions in this group have a 14.4 (95%-CI: 2.60 – 80.03) higher risk of converting to CDMS, compared to patients that do not have SC lesions. Moreover, patients that have SC lesions will experience a second relapse much faster (Hazard ratio: 51.38 (95%-CI: 5.54 – 476.33). Taken together, in patients with non-spinal CIS presentation, who do not meet McDonald criteria on their brain MRI, SC MRI allows for early diagnosis and much more adequate future risk estimation.

Performing additional SC scans in the non-spinal CIS patients somewhat increases the burden for the patients and increases the costs. However, it is to be expected that an early diagnosis and better prognostificaton of CIS patients might lead to less uncertainty and allows an early start of disease modifying therapy. Future studies should analyze these combined effects. Moreover, it would be interesting for future studies to assess whether SC imaging immediately after onset of non-spinal CIS increases the yield of DIS and DIT diagnosis, compared to repeating the brain MRI.

In a 2008 consensus paper[22], absence of SC lesions was considered a minor red flag based on the opinion of MS-experts, however this was not based on literature. Our results indeed indicate that absence of SC lesions is related to a much lower and delayed conversion to CDMS. Even though some of our patients will probably still convert to CDMS in the future, we feel that (with a median follow-up of 5 years and 4 months) this is a safe conclusion, since literature data show that most conversions to CDMS occur in the first five years[23,24]. Therefore, absence of SC lesions should be considered a warning sign and lead to reconsideration of the possibility that this patient might not experience a second relapse or will develop MS.

As a tertiary referral-center for MS patients, the characteristics of the patients in our study-cohort might also reflect this. When compared to other studies[25,26] our cohort has a lower frequency of optic neuritis and a higher frequency of SC onset symptoms. In addition, before being entered in this study, patients were carefully assessed by MS expert neurologists, which probably resulted in a low rate of patients with cerebrovascular disease being admitted into this study. We feel that the yield of one extra diagnosis in our, often complex, population, justifies the number of scans needed (=7). However, we do acknowledge that additional factors in other hospitals, such as availability of extra scan-time and extra costs need to be considered. Before we can generalize our conclusions to other centers, our findings should be tested in other cohorts especially in more general neurology practices.

Interestingly, in the spinal-CIS group “only” 81% showed SC abnormalities, although one would expect a percentage near 100%. Twelve patients with a clinically defined spinal cord syndrome did not show focal lesions in the spinal cord. Of these twelve patients five converted to CDMS during the follow-up. Ten out of twelve showed abnormalities on the initial brain MRI scan. This discrepancy between clinical symptoms and imaging parameters might be caused because of lack of sensitivity of the SC scan to detect the lesions (considering the time between scanning and symptoms) or small lesions escaping detection at the current resolution, or diffuse changes not leading to qualitative signal change (normal-appearing tissue). Another explanation could be that attribution of symptoms to either brain or SC involvement on clinical grounds alone can be difficult.

While it is already common practice to perform SC MRI in patients with CIS who present with SC symptoms, we advocate, based on the findings of this study, to more routinely perform SC MRI in patients with non-spinal clinical presentation who do not fulfill McDonald criteria on their baseline brain MRI. In these patients SC MRI helps to fulfill diagnostic criteria (1 extra MS diagnosis from 7 MRIs) and in addition it has pronounced prognostic value, mainly because it helps to identify a subgroup that has a very low risk to develop MS.

Acknowledgements

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Chapter 4.2

Relevance of spinal cord pathology for clinical disability: MRI findings in a large cohort of MS patients

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Submitted

Advances in Knowledge

- UCCA (upper cervical cord cross-sectional area) differs between MS subtypes, being smaller in the progressive disease courses.
- Atrophy of the spinal cord is more strongly related to clinical disability than spinal cord lesion number.
- UCCA can be determined from routine 3D brain MRI scans.

Implication for Patient Care

- Spinal cord atrophy and diffuse signal increase are helpful in assessing disease progression in MS.
- Number of affected spinal cord segments is more strongly related to clinical disability than the number of SC lesions.

Abstract

Purpose: To study whether spinal cord (SC) atrophy differs between disease types in multiple sclerosis (MS), and to determine its contribution to clinical disability over and above other MR imaging markers.

Material and Methods: The institutional review board approved the study; all subjects gave written informed consent. Mean upper cervical cord cross-sectional area (UCCA), brain and SC lesion loads and brain atrophy were measured in 440 MS patients (311 relapsing-remitting [RRMS]; 92 secondary-progressive [SPMS]; 37 primary-progressive [PPMS]) studied in two different centers. Disability was scored using the Expanded Disability Status Scale (EDSS), 25 feet timed-walk test (TWT) and Nine-Hole-Peg (9-HPT) test. UCCA was compared between groups using Mann-Whitney U-test. Correlations were assessed using Spearman's rho. Multivariate associations between UCCA and clinical and other MRI parameters were assessed using multiple linear regression, adjusted for center.

Results: Mean UCCA in SPMS (79 mm^2) and PPMS (77.3 mm^2) patients were significantly (both $p<0.001$) lower than in RRMS patients (84 mm^2). UCCA was inversely correlated with EDSS, the TWT and the 9-HPT (all: $rho \leq -0.29$; $p<0.001$). The UCCA, number of hypointense T1 brain lesions (T1LL), presence of diffuse abnormalities and number of involved segments in the SC were found to be significant explanatory factors for clinical disability ($R^2=0.564$). UCCA and brain T1LL were the strongest MRI predictors of EDSS.

Conclusion: SC pathology has a strong impact on clinical disability in MS. MRI derived UCCA was found to be the most significant SC predictor on EDSS.

Introduction

Multiple sclerosis (MS) is a heterogeneous neurological disease with a diverse spectrum of deficits and highly variable outcome. Focal abnormalities in the spinal cord (SC) are present in the majority of MS patients[1], affecting the cervical region more frequently than the thoracic and lumbar regions[2,3].

Structural abnormalities in the SC can be expected to dramatically affect the functional outcome of patients, but reports are ambiguous regarding the correlation between the number of focal SC lesions and the degree of disability[3-6]. Patients with primary progressive MS have a higher disease burden in the SC and a more pronounced walking disability than relapsing onset patients[7].

While newer imaging modalities have improved the detection of SC lesions in MS[8-11], conventional MR-imaging still lacks histopathological specificity[3], leading to increased attention for quantitative techniques, such as SC atrophy measurement. SC atrophy correlates with clinical disability[12-14] and is related to disability more strongly than focal lesion load[15]. It is assumed that axonal loss in MS is an important factor for the occurrence of atrophy, but whether or not MS-related neurodegeneration may occur in the absence of inflammation is unclear[16-18]. Because SC atrophy tends to occur in the first few years of the disease[2], quantification of SC volume or cross-sectional area by MRI seems to be a potential marker for monitoring the disease course or treatment efficacy in MS. SC atrophy has been described in patients with a clinically isolated syndrome (CIS) exhibiting brain abnormalities[19] as well as in small groups of RRMS patients[12,14]. In addition SC atrophy has been shown to be more pronounced in the progressive forms of the disease than in RRMS[4,12,20-22], although except by[20] most studies have not included a representative large number of major disease subtypes. Although atrophy is not limited to the spinal cord in MS, but can also be detected in the whole brain and it's compartment such as grey and white matter[23.24], large studies focusing on the clinical importance of spinal cord atrophy upon other MRI markers, especially brain parameters are lacking[25].

In the current study we used a large cohort of MS patients to investigate, first, whether SC atrophy differs between the major disease types; second, whether SC atrophy is associated with other conventional SC or brain measures of disease; and third, how SC atrophy is related to clinical disability.

Methods

Subjects

The study was approved by the institutional medical ethics committee. Informed consent was obtained from every patient. In total 440 patients were recruited in two centers. Patients were diagnosed with MS according to International Guidelines[26,27] and classified either as relapsing remitting (RR), secondary progressive (SP), or primary progressive (PP) MS[28]. Disability was assessed using the Expanded Disability Status Scale (EDSS) [29] and two subtests of the Multiple Sclerosis Functional Composite Measure (MSFC)[30]; the 25 feet timed-walk test (TWT) assessing ambulatory function and the Nine-Hole-Peg test (9-HPT) assessing upper limb function. For all patients, use of disease modifying therapy (DMT) before start of the study was recorded.

Neuroimaging

MRI scans were performed on 1.5T MRI Siemens scanners at both study sites. The head scan protocol included: a) sagittal MPRAGE (TR/TE/TI [msec]: 9.7-20.8/2-4/300-400; 1.0mm3 isotropic resolution); b) dual-echo PD/T2w (2000-4000/14-20/80-108; interleaved axial 3.0mm thick slices; in-plane resolution 1.0×1.0mm2); c) post-contrast T1w spin-echo (467-650/8-17; axial 3.0mm thick slices; in-plane resolution 1.0×1.0mm2). SC scanning included a cardiac-triggered sagittal dual-echo PD/T2-weighted sequence, covering the whole SC (2500-3000/20-30/80-100; slice-thickness 3mm; 10% gap between slices; in-plane resolution 1.0×1.0mm2).

Image analysis

Brain volume normalized for head size (NBV), as well as normalized gray matter volume (NGMV) and normalized white matter volume (NWMV) were analyzed in center A using SIENAX which allows analysis of brain atrophy state from a single time point after registering each individual scan to MNI-152 standard space, using the skull as a scaling constraint[31].

Marking and measurement of T2 hyperintense brain lesions and T1 hypointense brain lesions was performed in center B by experienced readers with more than 12 years experience, using commercial semi-automatic software (AMIRA 3.1.1; Mercury Computer Systems Inc.). Subsequently, volumes were calculated for these lesion categories. Number of enhancing lesions were scored on post-contrast T1-weighted images.

For SC images, number of focal lesions as well as number of involved segments and presence of diffuse abnormalities were scored by an experienced reader in center A with more than 3 years of experience. Diffuse abnormalities were defined as poorly delineated areas with increased signal intensity compared to signal intensity of spinal CSF on PD-weighted images (**Figure 1**) [32].

Figure 1: Sagittal PD (B,D) and T2-weighted (A,C) images of the spinal cord. (A-B) 36 year old male, 5yrs disease course, relapsing-remitting disease type, EDSS: 2, PD and T2-weighted images reveal a focal lesion in the upper cervical cord (arrow) without evidence of diffuse spinal cord abnormalities. (C,D) 45 year old female, 13yrs disease course, primary progressive disease type, EDSS: 6; diffuse signal hyperintensity indicating diffuse abnormalities of the entire spinal cord, best seen on the corresponding PD-weighted image (D, asterix).

Upper SC atrophy was measured by two authors (both with more than 8 years of experience) on each available sagittal 3D-T1 brain dataset on which the upper cervical cord was visible with sufficient image quality. For the current analysis 18 patients (RRMS, n=12; SPMS, n=4; PPMS, n=2) had to be excluded due to insufficient coverage of the upper cervical cord within the field-of-view leading to a major signal drop in this area, or image artifacts in the cord region due to swallowing or movement. Images were post-processed using a semi-automated volumetry method (NeuroQLab, Fraunhofer MEVIS, Germany) [33,34]. Briefly, volumes of interest with a fixed section length of 30mm were placed on the sagittal 3D-T1 images starting at the upper border of C2 (**Figure 2 and 3**). Thereafter, the SC was interactively defined by the operator and then separated from surrounding non-SC tissue based on an interactive watershed transform. Mislabeled objects not belonging to

the SC were removed after visual inspection of the segmentation results. After this initial exploratory step, volume computation was performed through an automated histogram based partial volume correction and volume results presented in milliliters were calculated. The mean upper cervical cord cross-sectional area (UCCA) was then calculated by dividing the volume by the section length.

Intra-rater reproducibility and inter-rater reproducibility were evaluated on 15 MS patients randomly selected from the study population. To evaluate the intra-rater reproducibility, one investigator repeated the volumetry on the same post-processed dataset twice, blind to his first. Similarly, inter-rater reproducibility was assessed by two authors (A,B), who did post-processing and volumetry independently on the same datasets.

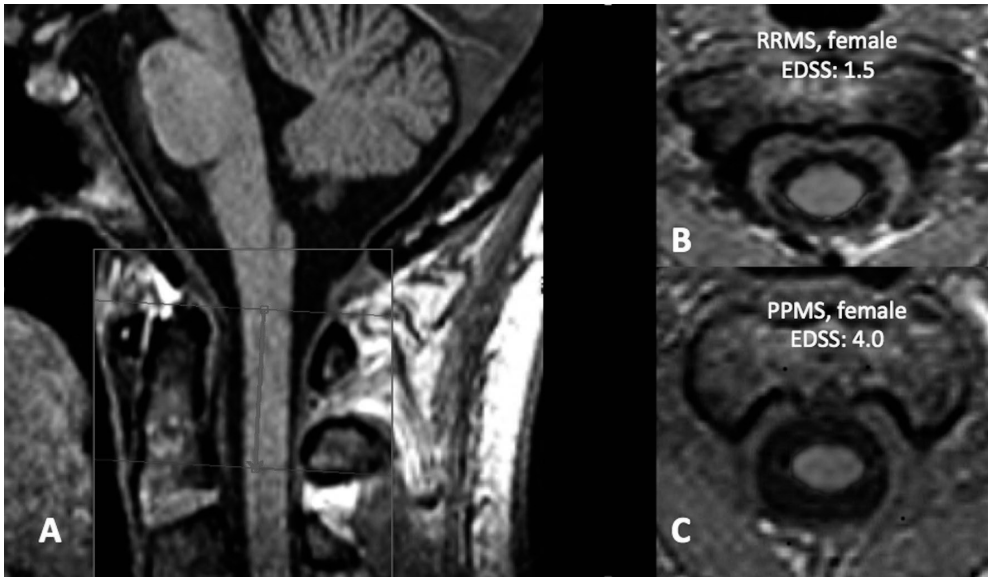

Figure 2: Sagittal T1-weighted image of the brain illustrating volume-of-interest selection (30mm section length) in the cervical cord (A) starting at the upper borders of C2. Representative axial T1w with overlaid (red) corresponding segmentation image of (B) a relapsing remitting MS patient (UCCA: 85mm^2) and (C) a primary progressive MS patient (UCCA: 66mm^2). *For colour figure see page 191.*

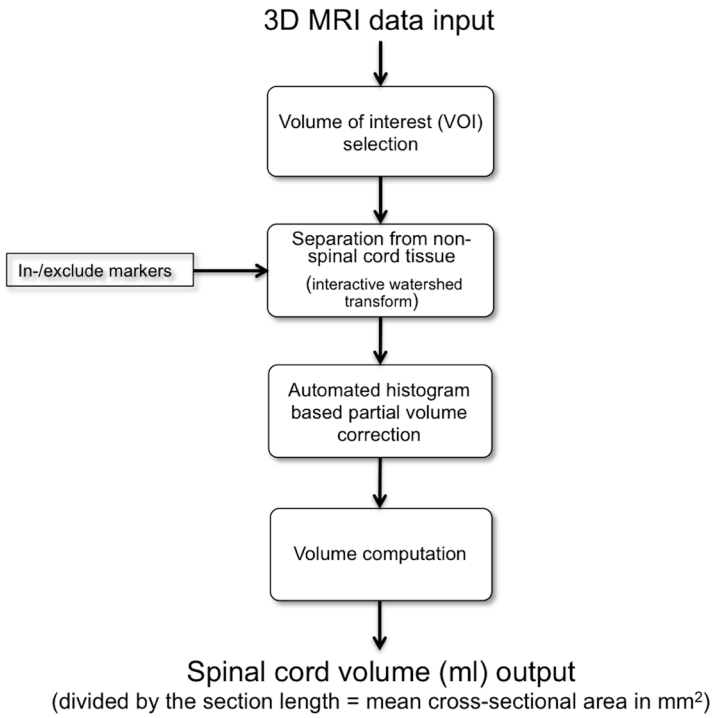

Figure 3: Flow chart illustrating the image processing to obtain UCCA from 3D-T1 weighted brain images.

Statistical analysis

Analyses were performed using SPSS18 (SPSS, Chicago, USA). Comparisons of the demographical data between disease subtypes and among centers were made using Mann-Whitney U test or Pearson's x2 test.

For UCCA reproducibility measures, intra-rater and inter-rater reproducibility were expressed by the coefficient of variation (COV), defined as the ratio of the standard deviation and the overall mean[35]. Correlations between UCCA and clinical and MRI parameters were performed using Spearman's rank correlation rho.

Associations between UCCA and clinical and other MRI parameters were first assessed in univariate models, statistically adjusted for center. In a multiple linear regression model with UCCA as dependent variable, all originally considered independent variables were entered together in a combined model and removed one by one using backward stepwise selection in order of descending p-values, until the remaining variables were all significant at $p<0.10$. To improve normal distribution a natural log transformation (ln) was used for all lesion volumes. To the number of SC segments and SC lesions, a value of 1 was added before

applying the natural log transformation. To avoid collinearity in the models, log transformed T2 and T1 lesion volumes (lnT2LV, and lnT1LV) were separately used by analyzing two different models, one including only lnT2LV, and one including only lnT1LV. Similarly, two separate models were conducted for NGMV and NWMV. In the final combined model, for each of these pairs only the variable with the most significant b coefficient was entered.
The degree to which clinical and MRI parameters explain EDSS was assessed using multiple linear regression. Again, this was done univariately for each variable adjusting for center, and subsequently by entering the independent variables together and removing them one by one using backward stepwise selection until the remaining variables were significant at $p<0.10$. The same approach was then applied using TWT as the dependent variable.

Results

Patient characteristics

Patient characteristics

Four hundred and forty patients (287 women and 153 men) were included in the analysis. Slightly more than half of the patients (n=225) originated from center B. Descriptive data for each center are provided in **Table 1**. The majority of these patients had an RR disease type (311 patients; 70.7%); there were 92 (20.9%) SP patients and 37 (8.4%) PP patients. Patients enrolled in center A had a higher median EDSS ($p<0.001$) and a shorter median disease duration ($p=0.02$). Median age of all patients was not different between centers ($p=0.11$). DMT (Interferon beta-1a, interferon beta-1b, glatiramer acetate) was received by 111 patients in center A, respectively 125 in center B; the proportion did not differ between centers.

Table 1: Clinical characteristics of patients separated by center

	Center A (n=215)	Center B (n=225)
Gender (f:m)	129:86	158:67
Disease type	RRMS : 142 SPMS : 49 PPMS : 24	RRMS : 169 SPMS : 43 PPMS : 13
EDSS (median, IQR)	4 (2.5-5)	3 (2-4)
Disease duration in yrs (median, IQR)	9 (4-16)	11 (6-19)
Age in yrs (median, IQR)	46 (39-54)	43.8 (36.1-53.4)

IQR: inter quartile range; yrs: years; RRMS: relapsing remitting MS; SPMS: secondary progressive MS; PPMS: primary progressive MS

Brain and SC lesions

For 420 patients an MRI scan of the entire SC was available. The results of the assessment of focal and diffuse SC lesions are listed in **Table 2**. SC lesions were present in almost 75% of these patients, while nearly all patients (n=440; 99.5%) had T2-abnormalities on the available brain MRI scans. Diffuse SC abnormalities were present in 61 (13.9%) patients. In the total group of patients a median of 2 SC lesions (IQR: 0-4) was found, with a median number of involved segments of 1.75 (IQR: 0-3.5). Number of SC lesions (p=0.03) and number of involved segments (p<0.001) in the SC were significantly higher in SPMS compared to RRMS patients, while no differences could be established between the other disease types (all: p>0.11). Diffuse abnormalities were found more frequently in the chronic disease types with the highest frequency in PPMS patients (**Table 2**).

Table 2: Clinical and MRI characteristics of patients separated for disease type.

	RRMS (n=311)	SPMS (n=92)	PPMS (n=37)
Gender (f:m)	222:89	50:42	15:22
Age, yrs (median, IQR)	42 (35-48.7)	54.1 (47.4-60.3)	50 (44-56.5)
Disease duration, yrs (median, IQR)	8 (4-14)	18 (13-25.8)	11 (6-19)
EDSS (median, IQR)	2.5 (2-3.5)	5.75 (4-6.5)	4.5 (4-6)
9-HTP_D, sec (median, IQR)	18.7 (16.8-21.4)	25 (21.1-33.6)	22.1 (18.9-28.6)
9-HTP_ND, sec (median, IQR)	19.8 (17.8-22.6)	27.2 (21.4-36.6	25.9 (22.7-37.1)
TWT, sec (median, IQR)	4.4 (3.8-5.3)	8.6 (6.2-17)	7.7 (5.2-13.7)
NBV, ml (median, IQR)	1567 (1506.4-1626.2)	1508 (1460-1555.5)	1564.7 (1473.3-1607.7)
NGMV, ml (median, IQR)	783 (741.7-825.8)	730.4 (684.9-758)	768.7 (731.5-797.5)
NWMV, ml (median, IQR)	775.9 (775.9-812.9)	768.8 (737.8-827.5)	786.7 (773-797.5)
UCCA, mm^2 (median, IQR)	84 (78.7-89.3)	79 (72.4-84.9)	77.3 (69-82.5)
No. of SC lesions (median, IQR)	2 (0-4)	3 (1-5)	3 (0-5)
No. of segments SC (median, IQR)	1.5 (0-3)	2.5 (1-4.75)	2.5 (0-4.25)
Presence of diffuse abnormalities SC (%)	11.9	17.4	21.6
Use of DMT (%)	56.6	57.6	16.2

IQR: inter quartile range; yrs: years; RRMS: relapsing remitting MS; SPMS: secondary progressive MS; PPMS: primary progressive MS; TWT: 25 feet timed-walk test; 9-HPT: Nine-Hole-Peg test (D: dominant hand, ND: non-dominant hand) ; NBV: brain volume normalized for head size; NGMV: normalized gray matter volume ; NWMV: normalized white matter volume; UCCA: mean upper cervical cord cross-sectional area; DMT: disease modifying therapy; SC: spinal cord.

Upper cervical cord cross-sectional area and MS subtypes

The intra-rater coefficient of variation (COV) of the method was 0.58%. Inter-rater COV was 0.99%. Mean UCCA did not differ between centers (p=0.75). For the whole cohort, both SPMS and PPMS patients had significantly lower mean UCCA than RRMS patients (both p<0.001), while mean UCCA did not differ between SP and PP patients (p=0.30) (**Table 2**). Separated by center, similar results were found (**Figure 4**) except for differences between

RRMS and PPMS that reached significance only in center A with sufficient numbers of such patients (p<0.001).

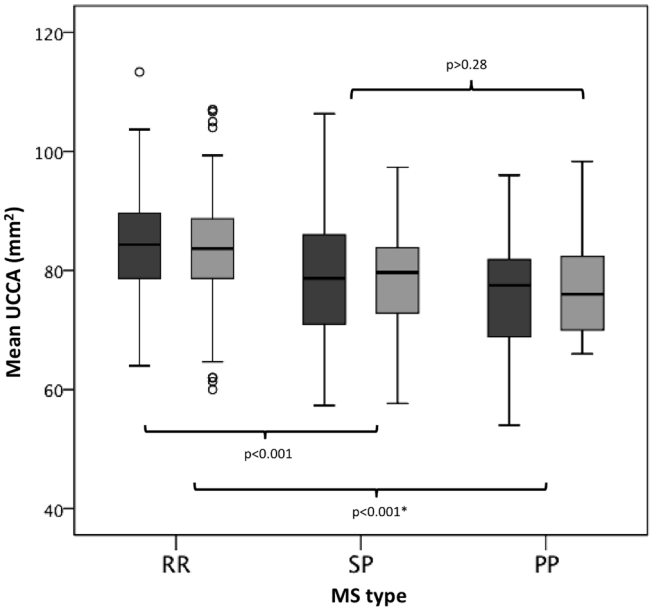

Figure 4: Box-plots for UCCA shown for each center (dark: Center A; light: Center B) and disease subtype. Highest UCCA were found in RRMS patients while patients with a progressive phenotype had lower UCCA.
* Significant difference between RRMS and PPMS patients was noted for patients included in Center A only.

Correlation between UCCA and clinical disability

In the whole group, UCCA was correlated with all investigated clinical parameters: including disease duration, age, EDSS, TWT and HPT. Details are provided in the supplementary material (**Supplementary Table 1**). For EDSS subscores, the pyramidal and sensory subscores as well as the bowel and bladder subscore were found to be moderate correlated with UCCA. Separated by center, findings were similar (**Figure 5 and Supplementary Table 1**).

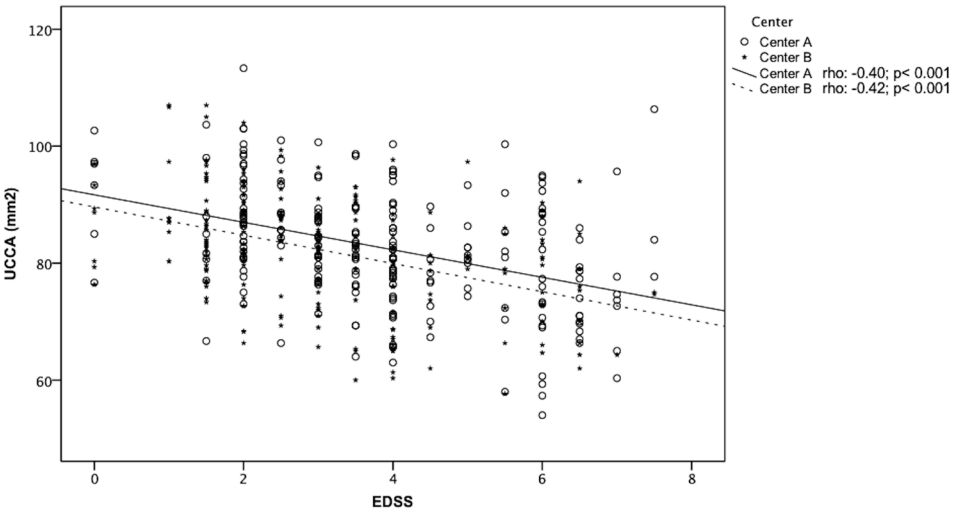

Figure 5: Scatterplots of EDSS and UCCA (mm^2) separated by center.

Relationship between SC atrophy and other MRI measures

UCCA was correlated will all brain MRI parameters in the overall group as well as separately in each center, being more significantly correlated to brain atrophy measures (Supplementary material: Table E1). In the univariate analysis, adjusted for center NBV, NGMV, NWMV, lnT1LV, lnT2LV, presence of enhancing brain lesions and presence of diffuse SC abnormalities were each significantly associated with UCCA (**Table 3**). The backward stepwise multiple regression analysis, controlling for center, yielded a combined model consisting of the clinical variables: disease type, disease duration and gender, and of MRI variables: NBV, NWMV, presence of enhancing brain lesions, and presence of diffuse SC abnormalities (**Table 3**). Among these, presence of enhancing brain lesions and presence of diffuse SC abnormalities were found to be most significantly associated with UCCA. Neither the number of SC lesions, nor the number of involved segments of the SC was included the final model. R2 –value for the final model was 0.276.

Table 3: Linear regression for UCCA, univariately for each center and multivariate statistically adjusted for center.

	Center A			Center B			UNIVARIATE			MULTIVARIATE		
	regression coefficient b	SE	p-value	regression coefficient b	SE	p-value	regression coefficient b	SE	p-value	regression coefficient b	SE	p-value
CLINICAL PARAMETERS												
Gender (female) [a]	2.14	1.43	0.14	4.3	1.39	0.002	3.19	0.99	0.001	3.81	0.97	<0.001
MS subtype Relapsing (RR) =1 PP and SP (0) [a]	6.6	1.42	<0.001	5.06	1.46	<0.001	5.88	1.02	<0.001	4.44	1.09	<0.001
Age [a]	-0.29	0.07	<0.001	-0.17	0.06	0.004	-0.22	0.04	<0.001	-	-	-
Disease duration [a]	-0.45	0.09	<0.001	-0.34	0.07	<0.001	-0.39	0.05	<0.001	-0.19	0.06	0.002
Ever use of DMT [a]	-0.48	1.41	0.73	-0.78	1.30	0.55	-0.63	0.96	0.51	-	-	-
MRI PARAMETERS												
NGMV	0.02	0.012	0.044	0.04	0.01	<0.001	0.03	0.007	<0.001	-	-	-
NWMV [a]	0.05	0.012	<0.001	0.04	0.01	0.005	0.04	0.009	<0.001	0.03	0.01	0.024
NBV [a]	0.04	0.008	<0.001	0.04	0.01	<0.001	0.04	0.005	<0.001	0.02	0.008	0.026
lnT2 LV	-0.35	0.47	0.46	-0.1	0.45	0.03	-0.67	0.32	0.04	-	-	-
lnT1 LV [a]	-0.48	0.36	0.19	-0.97	0.34	0.004	-0.73	0.25	0.003	-	-	-
Presence of gadolinium enhancing brain lesions [a]	3.38	1.69	0.046	3.03	1.82	0.01	3.23	1.23	0.009	3.22	1.12	0.004
Number of segments SC (ln-transformed) [a]	-1.25	0.92	0.176	0.42	1.01	0.68	-0.55	0.68	0.42	-	-	-
Number of SC lesions (ln-transformed)	-0.19	0.94	0.838	0.24	0.88	0.78	0.02	0.64	0.97	-	-	-
Diffuse abnormalities SC (0 absent; 1 present) [a]	-6.98	1.78	<0.001	-3.47	2.17	0.11	-5.63	1.37	<0.001	-5.76	1.27	<0.001

superscript letter ([a]) indicate independent variables that were initially entered in the multivariate model. RR: relapsing remitting MS; SP: secondary progressive MS; PP: primary progressive MS; DMT: disease modifying therapy; NGMV: normalized gray matter volume; NWMV: normalized white matter volume; NBV: brain volume normalized for head size; T2LV: volume of T2 brain lesion; T1LV: volume of T1 hypointense brain lesion; SC: Spinal cord; UCCA: mean upper cervical cord cross-sectional area; ln: natural log transformation; SE: Standard error.

Associations of MRI measures with disability

Results of the linear regression for EDSS and TWT separately for each center as well as univaritely, statistically adjusted for center are provided in the supplementary material (**Supplementary tables 2 and 3**).

Multiple linear regression, controlling for center, with EDSS as the dependent variable revealed significant independent predictive value for UCCA, lnT1LV, the presence of diffuse abnormalities, and the number of involved segments in the SC, with a R2 –value for the final model of 0.564. Among these MRI variables UCCA and lnT1LV were found to be the strongest predictors of EDSS based on p-values (**Table 4**). Using TWT as the dependent variable, multiple linear regression revealed significant associations with NGMV, presence of enhancing brain lesions as well with number of involved SC segments and UCCA (Table 4). In this model, NGMV and UCCA were found to be the strongest MRI predictors based on p-values. R2 –value for the final model was 0.389.

Table 4: Multivariate regression for EDSS and TWT, statistically adjusted for center.

	EDSS			TWT		
	regression coefficient b	SE	p-value	regression coefficient b	SE	p-value
CLINICAL PARAMETERS						
Gender [a,b]	-	-	-	-0.13	0.07	0.05
MS subtype Relapsing (RR)=1 vs PP and SP=0 [a,b]	-1.67	0.15	<0.001	-0.72	0.08	<0.001
Age [a,b]	0.02	0.01	0.001	-	-	-
Disease duration [a,b]	0.02	0.01	0.02	0.10	0.004	0.03
Ever use of DMT [a,b]	-	-	-	-	-	-
MRI PARAMETERS						
UCCA [a,b]	-0.03	0.01	<0.001	-0.01	0.003	0.007
lnT1LV [a,b]	0.11	0.03	0.001	-	-	-
Diffuse abnormalities SC (0 absent; 1 present) [a,b]	0.54	0.17	0.002	-	-	-
Number of segments SC (ln-transformed) [a,b]	0.25	0.08	0.004	0.1	0.04	0.02
Presence of gadolinium enhancing brain lesions [a,b]	-	-	-	0.18	0.08	0.02
lnT2LV	-	-	-	-	-	-
NWMV [a]	-	-	-	-	-	-
NGMV [a,b]	-	-	-	-0.001	0.001	0.006
NBV	-	-	-	-	-	-
Number of SC lesions (ln-transformed)	-	-	-	-	-	-

Superscript letters independent variables that were initially entered in the multivariate models ([a]: EDSS; [b]: TWT)

RR: relapsing remitting MS; SP: secondary progressive MS; PP: primary progressive MS; DMT: disease modifying therapy; UCCA: mean upper cervical cord cross-sectional area; T1LV: volume of T1 hypointense brain lesion; SC: SC; NGMV: normalized gray matter volume; NWMV: normalized white matter volume; NBV: brain volume normalized for head size; ln: natural log transformation; SE: Standard error.

Discussion

This study demonstrates, in a large cohort of patients, the importance of SC atrophy in explaining clinical disability in MS, and shows its relation to focal and diffuse pathology both in cord and brain. Specifically, UCCA, the presence of diffuse abnormalities in the SC, and the number of segments affected by MR-visible pathology, were found to be the most significant MRI predictors explaining physical disability as measured by the EDSS. Furthermore, the number of involved SC segments and especially UCCA, were associated with walking abilities as expressed by the TWT, confirming the importance of SC pathology regarding ambulatory dysfunctions in MS.

This confirms, in a large cohort, previous smaller studies reporting that cord atrophy was correlated with clinical disability[4,15,22,36,37]. Furthermore, SC atrophy was more pronounced in PPMS and SPMS, than in RRMS patients confirming findings by Rocca and colleagues[20] who also studied a large patient group. We additionally investigated how the clinical effect of SC atrophy compares to that of established MRI measures such as NBV, NGMV and SC lesion load. Although UCCA was the most significant MRI predictor of disability, other SC parameters were additionally important. The previously observed relation between EDSS scores and diffuse SC abnormalities[4] was largely confirmed. Moreover, our large group allowed us to detect associations between the number of affected SC segments and both EDSS and TWT, which has not been observed previously in smaller patient groups[3,4,38,39]. This relation may be understood because lesions in the SC more often cause sensory, limb motoric or urinary symptoms than do brain lesions[38-40] and they correlate better than do brain lesions to the degree of physical disability[41].

The absence of an association between SC atrophy and SC lesion metrics is in line with Rocca and colleagues[20] and with histopathological studies suggesting that SC atrophy is largely independent of lesional tissue loss[2]. Conversely, the presence of diffuse SC abnormalities was associated with more pronounced SC atrophy both in our work and in a previous study[4], consistent with more severe pathology in diffuse than in focal SC abnormalities[42].

Several MRI studies have investigated possible relations between brain and SC pathology[4,12,15,39,43-45]. When controlling for other variables, brain T2 lesion volumes were not associated with UCCA, suggesting that distant pathology e.g. by antegrade degeneration due to axonal damage in brain lesions does not seem to be a major contributor of SC atrophy. This is in line with previous findings suggesting that SC pathology may develop independently from brain pathology[4,44,46,47]. The brain atrophy measures NBV and NWMV however, did exhibit an association with UCCA, suggesting that brain and SC atrophy to some degree proceed hand in hand. Interestingly, presence of enhancing brain lesions was also associated with UCCA, suggesting that during acute inflammation the SC cross-sectional area is larger. This association may actually reflect the effect of local edematous swelling

of the SC around acute inflammatory SC lesions, because acute inflammatory activity in brain and SC are thought to be strongly correlated[39]. Unfortunately, we could not test this hypothesis in our dataset, because no contrast-enhanced imaging of the SC was available.
Mean UCCA was associated with clinical dysfunction similarly in both centers, suggesting that these findings reflect true disease-related associations that would generalize also outside the present study. Most notably, the clinical measures most likely to reflect SC damage, i.e. the EDSS subscores for pyramidal, sensory, and bowel and bladder dysfunction, as well as the TWT and 9-HPT scores were moderately associated with UCCA. In a smaller cohort of MS patients with 20-year disease, Bonati and colleagues similarly found that UCCA was independently associated with EDSS as well as with TWT and 9-HPT (48), suggesting that these associations are robust in the long-term.
Limitations of our study include the lack of a healthy control group, leaving us unable to assess the extent of atrophy especially in RRMS. Future studies should also include CIS patients to study whether or not spinal cord atrophy already occurs early in the disease course[12,14,19,20].
A second limitation is the absence of lesion-filling[49-51] in the determination of brain atrophy measures, which has probably yielded a (limited) degree of underestimation of WM volumes due to lesion misclassification[52,53], but with the images available no satisfactory automated lesion segmentation was feasible, and the cost of manual outlining on 3DT1 for 440 patients was considered prohibitive. Third, we used unnormalized UCCA, as recommended by[54]; although several normalization approaches have been suggested[54-57], normalization factors such as intracranial volume, body mass index and body surface area have shown limited, if any, impact on reliability and sensitivity to differences between subjects[57]. Finally, UCCA was derived from brain datasets because appropriate 3D SC sequences had not been acquired in the dataset analyzed in the current study. Nevertheless, segmentation of the upper SC was successful in 440/458 (96%) of cases, reliability of our measurements was good, and SC areas in our cohort were in concordance with literature[4,20,22,58]. Overall, our study shows that measuring upper cervical spinal cord area is feasible using brain datasets, which has important implications for clinical studies including treatment trials. Without the additional SC scan, total acquisition times can be shorter, reducing both cost and patient burden.
In conclusion, SC pathology, especially upper cervical SC atrophy, is an important determinant of clinical disability in MS, even surpassing brain atrophy in this large-scale cross-sectional study.

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Supplementary Table 1: Correlation between UCCA and clinical as well brain MRI findings separated by center.

		CLINICAL PARAMETERS								BRAIN MRI PARAMETERS					
		Disease duration (yrs)	Age (yrs)	FS PYRAMIDAL	FS SENSORY	FS Bowel & Bladder	9-HTP_D (sec)	9-HTP_ND (sec)	TWT (sec)	NBV	NWM	NGM	T1 LL	T2 LL	No. Gad lesions
Center A	rho	-0.35	-0.28	-0.44	-0.33	-0.29	-0.27	-0.27	-0.31	0.32	0.31	0.19	-0.12	-0.07	0.13
	p-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.008	0.08	0.32	0.07
Center B	rho	-0.31	-0.20	-0.40	-0.36	-0.32	-0.31	-0.31	-0.30	0.32	0.21	0.25	-0.16	-0.13	0.1
	p-value	< 0.001	0.003	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.003	< 0.001	0.02	0.057	0.13
Both centers	rho	-0.33	-0.23	-0.42	-0.33	-0.29	-0.27	-0.27	-0.31	0.32	0.26	0.22	-0.15	-0.1	0.115
	p-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.002	0.038	0.016

yrs: years; FS: Functional score; 9-HTP: Nine-Hole-Peg test (D: dominant hand, ND: non-dominant hand); TWT: 25 feet timed-walk test; NBV: brain volume normalized for head size; NWMV: normalized white matter volume; NGMV: normalized gray matter volume; T1LV: volume of T1 hypointense brain lesion; T2LV: volume of T2 brain lesion; No. Gad lesions; Number of gadolinium enhancing lesions.

Supplementary Table 2: Linear regression for EDSS separately for each center as well as univaritely, statistically adjusted for center.

	CENTER A			CENTER B			UNIVARIATE		
	regression coefficient b	SE	p-value	regression coefficient b	SE	p-value	regression coefficient b	SE	p-value
CLINICAL PARAMETERS									
Gender	-0.01	0.24	0.97	0.56	0.24	0.02	0.26	0.17	0.13
MS subtype Relapsing (RR)=1 vs PP and SP=0	-2.34	0.19	<0.001	-2.31	0.21	<0.001	-2.33	0.139	<0.001
Age	0.08	0.01	<0.001	0.06	0.01	<0.001	0.07	0.007	<0.001
Disease duration	0.11	0.01	<0.001	0.07	0.01	<0.001	0.09	0.009	<0.001
Ever use of DMT	0.31	0.23	0.19	-0.34	0.22	0.13	-0.02	0.162	0.90
MRI PARAMETERS									
UCCA	-0.07	0.01	<0.001	-0.07	0.01	<0.001	-0.07	0.007	<0.001
lnT1 LV	0.23	0.06	<0.001	0.30	0.06	<0.001	0.27	0.04	<0.001
Diffuse abnormalities SC (0 absent; 1 present)	0.57	0.31	0.06	1.51	0.35	<0.001	0.93	0.231	<0.001
Number of segments SC (ln-transformed)	0.35	0.15	0.02	0.81	0.16	<0.001	0.54	0.11	<0.001
Presence of gadolinium enhancing brain lesions	-0.61	0.28	0.03	0.26	0.31	0.42	-0.24	0.21	0.26
lnT2LV	0.19	0.08	0.02	0.31	0.08	<0.001	0.25	0.054	<0.001
NWMV	-0.003	0.002	0.11	<0.001	0.002	0.89	-0.002	0.002	0.2
NGMV	-0.01	0.002	<0.001	-0.01	0.001	<0.001	-0.01	0.001	<0.001
NBV	-0.007	0.001	<0.001	-0.007	0.001	<0.001	-0.007	0.001	<0.001
Number of SC lesions (ln-transformed)	0.09	0.16	0.56	0.58	0.14	<0.001	0.34	0.107	0.002

RR: relapsing remitting MS; SP: secondary progressive MS; PP: primary progressive MS; DMT: disease modifying therapy; UCCA: mean upper cervical cord cross-sectional area; T1LV: volume of T1 hypointense brain lesion; SC: SC; NGMV: normalized gray matter volume; NWMV: normalized white matter volume; NBV: brain volume normalized for head size; ln: natural log transformation; SE: Standard error.

Supplementary Table 3: Linear regression for TWT (lnTWT) separately for each center as well as univaritely, statistically adjusted for center.

	CENTER A			CENTER B			UNIVARIATE		
	regression coefficient b	SE	p-value	regression coefficient b	SE	p-value	regression coefficient b	SE	p-value
CLINICAL PARAMETERS									
Gender	-0.12	0.12	0.33	0.07	0.08	0.44	-0.03	0.075	0.67
MS subtype Relapsing (RR)=1 vs PP and SP=0	-0.99	0.11	<0.001	-0.67	0.08	<0.001	-0.84	0.067	<0.001
Age	0.03	0.006	<0.001	0.02	0.003	<0.001	0.02	0.003	<0.001
Disease duration	0.04	0.007	<0.001	0.02	0.004	<0.001	0.03	0.004	<0.001
Ever use of DMT	0.13	0.12	0.27	-0.09	0.08	0.25	0.02	0.071	0.77
MRI PARAMETERS									
UCCA	-0.02	0.006	<0.001	-0.02	0.004	<0.001	-0.02	0.003	<0.001
lnT1LV	0.09	0.03	0.004	0.08	0.02	<0.001	0.08	0.018	<0.001
Diffuse abnormalities SC (0 absent; 1 present)	-0.007	0.16	0.97	0.42	0.13	0.001	0.15	0.104	0.14
Number of segments SC (ln-transformed)	0.23	0.08	0.003	0.15	0.06	0.01	0.2	0.049	<0.001
Presence of gadolinium enhancing brain lesions	-0.001	0.15	0.1	0.05	0.11	0.65	0.02	0.092	0.82
lnT2LV	0.01	0.04	0.02	0.08	0.03	0.003	0.09	0.024	<0.001
NWMV	-0.001	-0.001	0.56	0.001	0.001	0.48	-0.000086	-0.001	0.9
NGMV	-0.004	0.001	<0.001	-0.003	0.001	<0.001	-0.004	0.001	<0.001
NBV	-0.003	0.001	<0.001	-0.002	<0.001	<0.001	-0.002	<0.001	<0.001
Number of SC lesions (ln-transformed)	0.09	0.08	0.25	0.09	0.05	0.07	0.09	0.048	0.054

RR: relapsing remitting MS; SP: secondary progressive MS; PP: primary progressive MS; DMT: disease modifying therapy; UCCA: mean upper cervical cord cross-sectional area; T1LV: volume of T1 hypointense brain lesion; SC: SC; NGMV: normalized gray matter volume; NWMV: normalized white matter volume; NBV: brain volume normalized for head size; ln: natural log transformation; SE: Standard error.

Chapter 5

Summary, Discussion and Future Perspectives

Summary and General discussion

This thesis aims to provide more insight in factors explaining parts of the heterogeneity in MS, in an attempt to increase the accuracy of the prognosis and diagnosis of individual MS patients. We therefore assessed the following two main issues: 1) genetic role in phenotypic aspects of MS (especially disability (**chapter 2**) and lesion distribution (**chapter 3**)); and 2) the role of spinal cord findings in diagnosis and prognosis of MS (**chapter 4**).
In this final chapter, the main findings of this thesis will be discussed and placed in a broader perspective, and finally directions for future research will be discussed.

Genetic susceptibility studies in MS

Since 2007, when the first Genome Wide Association Study (GWAS) aiming to find genes related to MS-susceptibility was performed[1], the understanding of the genetic background in MS susceptibility has increased enormously. Since then, multiple confirmation studies based on extensive international collaboration have been performed[2-5]. Recently, in a large cohort of patients (involving almost ten thousand MS patients and more than 17,000 controls), 24 loci have been replicated[6] and 29 new susceptibility loci have been identified.
But what are the clinical implications of these findings from genetic studies? Can they contribute to unraveling the complexity of MS? Indeed, these studies do increase our knowledge on the etiologic background of MS. Information from susceptibility studies can provide us with information on what processes are involved in starting the disease, or who is at risk for developing MS. This may confirm certain hypotheses on the pathological basis of MS (i.e. whether it is a primary or secondary neurodegenerative disease). However, the current strategy (Genome Wide Association Studies) is now facing the limits of its capacities in detecting genes related to MS susceptibility, because the expected effect sizes are too small to detect in medium or even large groups of patients. The most recent study included ten thousand patients from several countries. In my opinion it is not to be expected that further increasing the number of patients will result in many immediately relevant genes. Effect sizes are expected to be so small (OR below 1.1), such that no determining influence on disease susceptibility is to be expected. The likelihood of discovering a new focus for treatment with these low ORs is also expected to be low. In addition, the costs of performing a study of this magnitude require huge funding. Moreover, this amount of data will also lead to a higher complexity in analyzing it (i.e. increased heterogeneity due to genetic population differences). Challenges for the future are to incorporate the current findings from these genetic susceptibility studies into more functional or clinical studies leading to further understanding of MS pathology and hopefully ultimately leading to new treatment options.

Genotype-Phenotype associations in MS

In the past decade the emphasis in MS literature has been placed on these susceptibility studies. In this thesis, however, we focused on finding genotype-phenotype associations. It is not likely that gene identification in MS will lead to genetic counseling or prenatal diagnosis, as even full knowledge of the genetic background on MS susceptibility will allow "only" 30% accuracy in predicting if a person will develop MS during his lifetime. It is expected that the rest of a person's susceptibility is determined by (mostly unknown) environmental factors. Moreover, in the last ten years, several Disease Modifying Therapies (DMTs) have been proven helpful in reducing the number of relapses and the number of new MRI lesions in the brain, but no cure for MS is to be expected within the near future. Currently, knowledge of a possibly increased risk of developing MS during the lifetime of a healthy person (derived from already available commercial DNA-arrays) will not lead to the start of disease modifying treatment. I expect therefore, that it is only in the way that risk genes reveal details of disease triggering events, or that severity genes influence mechanisms driving the tissue damage, that genetic information (derived from genotype-phenotype-association studies) has the potential to more readily lead to clinical implications (or even therapeutical options). We aimed to predict more accurately MS diagnosis and prognosis using genetic variables and spinal cord MR findings. Initially, we focused on genotype-phenotype associations to predict disease course. We started by taking clinical disability measures as the primary outcome parameter (**Chapters 2.1, 2.2 and 2.3)**. Assessing clinical disability remains the most important factor in treating MS patients, although clear correlations with the pathological processes involved are often lacking. When trying to accurately assess the genetic role in disability development (by assessing genotype-phenotype associations), these clinical parameters seem to be unsatisfactory. These clinical outcome measures are considered less sensitive in reflecting the ongoing MS pathology as can be demonstrated on MRI scans. It has been proven that lesion location within the brain and spinal cord lesions have an impact on disability. Due to these characteristics, we also took these imaging variables as outcome-parameters (**Chapters 3.1 and 3.2)**.

To improve our understanding of genotype-phenotype associations in MS we used several strategies. In **chapter 2.1** we found that a combination of genes could explain part of the disease progression and disability accumulation, while individual effects of the genes were not detected (when controlled for multiple testing). We included several outcome parameters for disability to increase our confidence in the results (preferably relevant genes were associated to more than 1 outcome parameter). Our hypothesis was based on the assumption that disability accumulation in MS is determined by several genes with small effect sizes. Although the statistical method on combining several genes in predicting disease severity seems a promising method, our results need to be confirmed first, ideally

using information from the most recent GWAS and recent pathway studies or other candidate gene studies on MS phenotype. Our study demonstrates the possible advantage of a predictive model over assessing individual genetic effects.

A second strategy, which we applied in **chapter 2.2** is to combine genetic studies with studies on the mRNA level to gain more functional information, thereby increasing our understanding of how genetic polymorphisms exert their effect on the disease. It is generally accepted that disease progression and tissue damage are continuously or intermittently supported by several mechanisms (which are assumed to be predominantly pro-inflammatory). By gaining insight into the functional consequences of a genetic polymorphism supporting these tissue damaging mechanisms, we hope to find new possibilities in halting these mechanisms. In **chapter 2.2** we applied this strategy for the IL7RA risk allele (rs6897932-T), that was found to be related to susceptibility to MS. Of this SNP within the IL7R gene, it was known from the literature that it had direct consequences on the mRNA level[5,7] and also on protein functioning. In light of the function of this protein in inflammatory processes and the consequences of the polymorphism on protein functioning, we expected that this would be a promising gene to affect disease progression. Unfortunately, we could not demonstrate this effect neither at the gene-level, nor at the mRNA–level. Our observation that this susceptibility SNP within the IL7R gene was not associated with disability parameters, was confirmed by several (GWAS) studies[2,6,8]. These studies on disease phenotype showed that disability was associated with genes relatively new to MS literature[2]. Our study and others illustrate that in MS, disease-modifying genes are not necessarily identical to disease susceptibility genes[8,9]. In other diseases this has been shown (e.g. the effect of the ApoEє4-genotype on Alzheimer's susceptibility and progression[10,11]. Probably, genes that are related to susceptibility to MS provide information on mechanisms involved in disease initiation and are not directly related to mechanisms that are involved in ongoing tissue damage. Another possibility is that the genetic effects on disease phenotype are too small to detect. However, IL7RA remains an intriguing gene (within a relevant pathway) of which more and more functional data is becoming available. The relevance of IL7R in MS susceptibility was confirmed by another study in MS patients in 2010, using an IL7/IL7RA pathway approach[12]. Recently, in HIV, polymorphisms in the IL7RA gene were found associated with rapid progression to AIDS by using an extreme of outcome strategy[13]. This study analyzed several non-synonymous SNPs and haplotype analysis, demonstrating that different SNPs (on the same haplotype) may cause opposing effects on inflammation via mRNA. This elegant approach has not yet been applied in assessing the relationship with MS phenotype. However, it is worth assessing this to definitively exclude an effect of the IL7R gene on MS phenotype.

In **chapter 2.3** we started from the function of the gene and correlated this to a specific clinical phenotype. We assessed whether the risk allele of KIF1B (rs10492972) corresponded

with the expected functioning of the gene. KIF1B is responsible for axonal transport of mitochondria and synaptic vesicle precursors and is related to neurodegeneration. Rs10492972 was reported as the first MS susceptibility gene with a known function in neurodegeneration (instead of inflammation) in a large study[14]. We hypothesized that carriers of the risk allele might exhibit a disease course with a more neurodegenerative phenotype (clinically, and using MRI characteristics). We found no evidence for a determining influence of the risk allele on clinical and MRI measures related to neurodegeneration. Our findings further strengthen the hypothesis that susceptibility genes need not necessarily be related to phenotypic appearances of MS. Our findings are furthermore supported by a recent large genotype-phenotype study on MS, that showed that most genes identified as related to MS are related to immune system function (overrepresentation of genes that influence T-cell maturation) and a relative absence was noted of genes related to neurodegeneration independent from inflammation[6]. Based on these results it was strongly suggested that the pathogenesis of MS is mostly related to dysregulation of the immune system. This conclusion favours the "primary inflammatory hypothesis" (i.e. auto-immunity). However, this hypothesis is still heavily debated based on pathological findings in early MS comprising not only early inflammatory, but also meningeal and cortical gray matter pathology[15].
Although replication of the association of KIF1B-gene to MS susceptibility is still awaited, the KIF-family already is an interesting set of genes and proteins. Both in MS and in other auto-immune diseases, other members of the KIF-family have been found to be associated with susceptibility[16-20]. These new studies strengthen the idea that the KIF-family is involved in MS, possibly by influencing axonal transport and thereby increasing susceptibility to axonal damage, however, the exact mechanism is not clear and should be studied. Possibly a pathway approach would be able to provide insight into the relevant mechanisms.

In 2011 a huge GWAS (7000 MS patients) on MS phenotype did not find a genome-wide-significant association of any gene with disease phenotype (disability, age at onset, MS subtype)[6]. Despite the extremely high number of patients in this study, no strong correlation with a particular gene could be found, indicating that it is not to be expected that common variations within the genome will be identified with a large effect on disability accumulation. However, this negative study does not imply that disease progression is not genetically influenced. One must consider the many difficulties researchers have been faced with in their search for genetic predictive markers for disease phenotype in the last decade. Not only is it expected (based on familial phenotype studies) that effect-sizes on disability accumulation of genes are small, but also, several interaction within genes (gene-gene), and between genes and environment (gene-environment and gene-gene-environment) have been described, that should ideally be controlled for[21,22]. Moreover, epistasis and epigenetic effects (micro-RNA's, methylation, etc) and rare variants have not been taken into account

in GWAS studies using SNPs. These genetic variants may play an important role in mediating genetic effects in MS[23,24]. GWAS are not able to assess these effects and other genetic methods should be used to assess these effects (i.e. sequencing).

Furthermore, an important complicating factor in genotype-phenotype associations is the enormous heterogeneity in MS (variety in subtypes, neurological complaints, rate of disability accumulation and lesion distribution throughout the brain and spinal cord). Often the contrasts of the outcome parameters (phenotype) are insufficient, thereby decreasing the power of the results. The conservative statistical approaches that have been used often, are not capable of detecting these subtle differences. This problem highlights the urgent call for new statistical approaches that aim to keep adequate power but do not increase the number of false positives findings.

Clinical outcome parameters are often considered less sensitive than certain MRI parameters in reflecting the ongoing MS pathology. Clinical measures may underestimate the extent of damage to the brain due to functional reorganization, while this can be seen in an earlier phase on MRI. It is not well understood whether this variability in MRI parameters reflects genetic subgroups of MS patients. In this thesis we aimed to study the genetic role in lesion distribution (a uniformly assessed outcome parameter) that may be a clinically relevant[25]. In **chapter 3.1** we assessed whether the lesion distribution between the brain and spinal cord was influenced by genetic factors, while in **chapter 3.2** we assessed whether lesion distribution within the brain was associated with a set of candidate genes in MS. We expected a genetic role on lesion distribution based on certain demographic hallmarks. The prevalence of spinal cord lesions differs between distinct populations (Asian MS patients have more frequently spinal cord involvement than "Western-type" MS) [26,27] and between patients (for instance some patients exhibit repetitive spinal cord involvement with less brain involvement) [28,29]. In **chapter 3.1** we showed a genetic role on lesion distribution. In our cohort, spinal cord lesion number and volume were associated with the HLA-DRB1*1501 genotype (the most important susceptibility gene in MS that is possibly related to MS disability features). Our findings were recently more thoroughly investigated by an Australian group of researchers using high resolution HLA typing (4-digit). They showed a correlation between HLA-DRB1 alleles (not HLA-DRB1*1501, but DRB1*1104 and DRB1*0701) and lesion numbers in different parts of the spinal cord (cervical vs thoracic regions). They also hypothesize that regional differences in the levels of expression of MHC Class II antigens and the presentation of myelin antigens to the immune system may underlie these effects[30]. Previously, differences in Myelin Basic Protein (MBP) and PLP levels between brain and spinal cord were found in healthy controls[31]. However, this has not been demonstrated in MS patients. Two mice EAE models with different MHC strains reported different lesion distribution patterns in the central nervous system (spinal cord vs brain parenchyma)[32]. This was found to be mediated via different preferential MOG epitope presentation and ultimately via alternative Th17/Th1

ratio[32]. This study indicates a different mechanism of lesion formation in the brain versus spinal cord with a possible indirect role of the MHC class II genes. However, the exact role of the MHC class II genes on this mechanism is still unclear and future pathological studies of spinal cord lesions and brain lesions, including assessment of local expression profiles of MHC Class II molecules, could possibly help us to understand the lesion developing mechanisms in the brain and spinal cord and any differences between them.

In **chapter 3.2** we assessed whether lesion location within the brain is genetically influenced. We suspected a genetic role because of clinical observations that show a tendency of individual patients and relatives to develop relapses, related to pathology in the same location[28,29,33]. Our results suggest that such a genetic influence may indeed be present. The most significant association was found for rs2227139. This SNP is located in MHC class II region, which is involved in self versus non-self immune recognition. Another recent study (in MS patients) showed that carriers of HLA-DR4, DR7 and DR13 have a higher incidence of brainstem and cerebellar lesions (and a higher reactivity tot the PLP 184-209 region (a component of myelin))[34]. Some studies (in healthy controls) show that there are natural spatial variations in myelin content, which may in turn reflect areas with variable predilection for damage in demyelinating disease[35]. Moreover, in other white matter diseases (Krabbe's disease, late infantile metachromatic leukodystrophy) several predilection sites have been identified[36-38]. Unfortunately, MS lesions can reflect different pathological processes (edema, de- and remyelination, gliosis, etc), that can not be distinguished in detail on regular T2-weighted MRI sequences. Therefore, after this hypothesis-free study, a more focused approach using different MRI techniques that are able to detect specific pathologically determined lesion subtypes (for instance: T1 black holes (more related to axonal loss) and cortical lesions), may increase our understanding of the pathophysiological mechanisms involved in lesion distribution and its relationship to (HLA)genes. Careful interpretation of our results is furthermore warranted because our results were found in a clinical heterogeneous population and should therefore first be confirmed in different MS populations.

Spinal cord MRI in predicting diagnosis and prognosis of MS

In **chapter 4.1 and 4.2** we focus on the predictive capacities of spinal cord lesions and cervical spinal cord atrophy on diagnosis and prognosis of MS. In the past, the emphasis has been placed on brain MRI scanning in MS. However, we show in our studies that lesions on spinal cord MRI can both aid in the MS diagnosis and in the prognosis of a second relapse and are therefore of significant clinical relevance. Moreover, cervical spinal cord atrophy and diffuse abnormalities are associated with a higher disability (in our study even more associated to disability than brain parameters (atrophy or brain lesion volumes). Based on the results of our studies, we advise to more frequently perform an MRI scan of the spinal cord in order to contribute to a better diagnosis and prognosis of MS patients.

We show in our study in **chapter 4.1** that presence of spinal cord lesions in CIS patients is an important factor in prognosing (time to) a relapse and therefore we advocate to perform an MRI scan of the spinal cord early after onset of symptoms especially in brain onset CIS patients that do not fulfill the diagnostic criteria. Not only does information of this scan aid in diagnosing MS early, but presence of spinal cord lesions is also associated with a short interval to the second relapse. Scanning of the spinal cord can be performed easily in most hospitals and the burden for patients is minimal (especially when combined with the brain MRI scan). Moreover we show in our study that by adding the results from the spinal cord MRI scan, an earlier diagnosis could be made. We had to perform a spinal cord MRI scan in seven patients to be able to diagnose one extra patient early. Although we think that the benefits of an early diagnosis outweigh the costs and burden to the patients, others may argue that a yield of 1 in 7 does not justify this extra scan (depending on the local health care system).

The diagnostic criteria[39-41], were created to be able to early identify CIS patients that will convert to MS. An early diagnosis can reduce uncertainty for the patient and enables an early start of disease modifying therapy, that has shown to moderately reduce the number of relapses on the short term and may decrease disability accumulation in the long term. However, there is no standard test to confirm MS diagnosis. The diagnosis is made based on fulfilling the clinical and/or MRI criteria for dissemination in time and space. However, to my opinion, diagnosing MS should not only focus on fulfilling these important criteria but should also comprise a more accurate prognosis of future relapses and (preferably also) future disability. In light of these considerations, maybe the diagnostic criteria should be considered as a prerequisite to diagnose MS, however, this process should ideally be followed by an effort to provide the most accurate prognosis on disability accumulation and prediction of (time to) subsequent relapses. The uncertainty of the extent and timing of disability accumulation and relapses are highly relevant for the individual MS-patient. The range of disability accumulation and time to first relapse are extremely variable. We know that there is a subgroup of officially diagnosed MS patients, that have a “benign” course (none or little disability even after 15-20 years). Moreover, we also know that there is a subgroup of patients that will not experience a second relapse within 15 years, although the MRI has shown dissemination in time. We now can officially diagnose these patients with MS using the new diagnostic criteria[41]. However some of these patients will not benefit from the current disease modifying therapies (because no relapse would have occurred *with* or *without* DMT) and one could argue whether a diagnosis of MS has any consequences for the patients and whether the diagnosis increases their quality of life. Therefore, clinical, radiological or biomarker studies that focus on increasing the accuracy of prognosing the disease course of MS are equally or maybe even more important.

In **chapter 4.2** we evaluated the clinical relevance of cervical spinal cord atrophy and other spinal cord and brain parameters on disability. We confirmed in a large multicenter study that atrophy of the spinal cord (assessed by mean upper cervical cord area (UCCA)) is associated with a higher clinical disability. This variable was found to be the most significant variable correlated with disability in addition to clinical parameters. Also other spinal cord parameters (extent of focal lesions in the spinal cord and presence of diffuse abnormalities) were found related to disability. This study highlights the clinical importance of spinal cord findings (especially atrophy and to a lesser extent focal lesions). It was already known that brain atrophy is more correlated to disability at the long term when compared to the extent of lesions in the brain. Moreover, we applied a technique enabling assessment of the volume of the cervical spinal cord using the brain MRI scan, without the necessity of performing an extra spinal cord scan. This technique therefore, does not put extra burden on MS patients and is therefore easy to apply in clinical practice.

Future perspectives

Genotype-phenotype associations

Although GWA-studies have yielded enormous amounts of information about MS susceptibility, it has also been shown that it can answer only a limited number of questions on the mechanisms involved in MS phenotype, and therefore different strategies are needed. In this thesis we used several strategies to overcome some of the difficulties in genotype-phenotype correlation research to gain insight into the pathological mechanisms involved in MS.

Recommendations for future research would be to use a pathway-based approach to test specific hypotheses[42]. This strategy reduces the number of genes assessed considerably and is expected to assess specific hypothesis on the pathological basis of MS. In 2010 the glutamate pathway was assessed by Baranzini et al to assess the hypothesis of glutamate toxicity[43]. The authors composed a model of 70 genes with high relevance to glutamate biology (based on protein interaction networks) and correlated it with in vivo glutamate levels of MS patients. This study demonstrated that patients carrying a higher number of associated alleles from genes in this module showed the highest levels of glutamate (assessed via magnetic resonance spectroscopy imaging). Moreover the SNP that was found most strongly related to the brain glutamate concentration was also associated with the extent of neurodegeneration (as indicated before as a clinically relevant outcome). This elegant study demonstrates the genetic role in neurodegenerative processes and highlights the opportunities of pathway analysis in assessing MS genotype-phenotype associations.

Over the years more and more MRI parameters have been used as indirect outcome parameters. These MRI parameters (new lesion development or lesion location) might be more sensitive and objective than clinical outcome measures and may provide more direct linkage to pathophysiological mechanisms (gadolinium enhancing lesions are considered to reflect recent inflammatory MS lesions, while black holes represent chronic axonal loss). In other neurological diseases recently new imaging techniques display a higher pathological specificity: PIB-PET scans performed in Alzheimer's patients are for instance capable of revealing amyloid-beta depositions in the brain[44]. In MS, proton magnetic resonance spectroscopy ((1)H-MRS) measuring whole brain N-acetyl-L-aspartate (a neural marker) has recently proven to be a sensitive and stable marker even in small MS patient groups and might be incorporated in future studies[45]. Other high-end MRI measures, such as subtle abnormalities in normal appearing white matter in connectivity measures: Diffusion Tensor Imaging (DTI) or Magnetization transfer ratio (MTR) have recently shown to be, more specific than standard (T2-weighted) MRI measures, related to, pathological processes (microglial activiation) [46].

Finally, recent studies have shown that grey matter (cortical and subcortical) involvement (lesions and atrophy) is a frequent finding and can be demonstrated in vivo using specific MRI settings (e.g. Double Inversion Recovery (DIR)). Pathologically, these grey matter lesions show clear differences when compared to white matter lesions. It would be interesting to assess the genetic influence on the development of (heterogeneity in) these grey matter lesions (and atrophy), as cortical pathology is considered to be an important pathologic correlate of disability (cognitive impairment) [47].

These recent developments increase our knowledge on the pathological basis of MRI findings. When these results are confirmed in different data sets, this may ultimately enable us to assess, in vivo, the genetic role on distinct MS pathological processes. Future endo-phenotyping based on these specific MRI findings are an important strategy to dissect the pathological (genetic) basis of MS, without the necessity of including more than 10,000 patients in a study. For all future endo-phenotyping it remains important to use variables with a strong contrast (for instance by selecting the extremes of outcome).

Moreover, MS cohorts should be large and clinically and paraclinically (for instance by MRI) well defined and preferably longitudinally followed during their MS course. Confirmation in different populations remains essential, due to the high chance on false positive findings in this field.

Based on our findings (**chapter 3.1 and 3.2**) and on recent findings of others[48-50], the HLA –region remains an intriguing gene-region. HLA molecules play an important role in antigen binding and presentation and T cell repertoire determination[51] (associated with auto-immune inflammation) and would therefore be a logical candidate not only in MS susceptibility but also in determining MS phenotype[52,53]. Recently, alleles at the HLA-DRB1 locus have quite convincingly been shown to affect clinical disease severity[54,55]. Some HLA-DRB1 alleles additionally have been found to influence disease severity as assessed by MRI outcomes[56,57]. Thorough investigations using high-resolution HLA genotyping (and correction for interactions within the HLA-region), might yield important information on several phenotypical characteristics of MS.

In addition to the genetic and imaging strategies mentioned in this thesis, future studies incorporating epigenetics[23,24,58] and studies taking (gene-gene and gene-environment) interactions into account, are promising. Previous studies have shown that when building an algorithm to predict MS susceptibility, predictive accuracy will increase when environmental factors are also taken into account[59,60].

Spinal cord imaging:

Based on the findings in this thesis, we advise to more frequently perform an MRI scan of the spinal cord in patients with a clinical brain onset, that do not fulfil the diagnostic criteria. Presence of spinal cord lesions in this group of patients can aid in diagnosing MS,

but moreover highly predictive of a short time to relapse. Confirmation studies are awaited. New imaging techniques of the spinal cord probably will lead to a higher detection rate of spinal cord lesions and the presence of diffuse abnormalities in the spinal cord[61]. The effect of this higher sensitivity of lesion detection on diagnosis and prognosis in CIS patients should be assessed.
In the future, new diagnostic criteria, maybe with incorporation of cortical lesions or lesions in the corpus callosum are expected as they may play a role in prognosing MS[62,63]. However, we expect that spinal cord findings will remain an important part of these criteria. It is extremely important that our findings should be assessed in a more general neurology practice, since the diagnostic criteria are developed in MS specialized centers. A study in 2008 in Ireland showed a considerable gap between the clinical diagnosis of MS and compliance with the previous diagnostic criteria[64]. The new criteria have been simplified and it would be valuable to test the accuracy of the 2010 criteria in the general neurology practice.

We furthermore showed in our studies that atrophy of the spinal cord is clinically highly associated with disability. Possibly, atrophy of the spinal cord can be used as an outcome-parameter in trials assessing the effect of DMT on disability accumulation (and may reflect the neurodegenerative process). To assess this, future studies should involve longitudinal cohort-studies to assess the association of decreasing UCCA overtime in relation to disability accumulation. Maybe, this parameter can be used as a relevant outcome parameter when assessing the influence of DMT on prevention of disability.

Prediction of disease course and disability is extremely important and research should focus on this topic since the therapies available for MS today are not able to cure MS, but only seem to slow down the disease. An accurate selection of patients that could benefit from these therapies is essential. In this thesis we aimed to explain the role of genetics and spinal cord pathology (as two examples of (endo)phenotyping) in MS diagnosis and prognosis.
In the last five years the knowledge of the genetic role in MS has grown enormously due to new techniques and it is expected that genetic information can provide highly valuable information on disease mechanisms in MS. However, the challenge lies in translating these genetic findings to the individual patient. Currently, this translation to the individual patient is not possible yet, but to my opinion unraveling the genetic basis of disease phenotype is essential in understanding and predicting MS disease course. Spinal cord MRI scans are already regularly performed by neurologists for diagnostic purposes, but our findings indicate that it is also of clinical relevance for prognosing MS and we advocate a broader field of application.

Most likely a *combination* of clinical, radiological and biomarker studies (including genetics) at the start of the disease could increase our understanding of MS and will lead to a more accurate prediction of disease course for the individual patient in the future. The ultimate future goal is to build a clinical algorithm, integrating the different risk factors (clinical, genetic and using early MRI variables) to be able, early in the disease, to predict long-term disability for individual MS patients.

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Chapter 6

Nederlandse Samenvatting

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Nederlandse samenvatting

GENETISCHE EN RADIOLOGISCHE MARKERS VOOR DE PROGNOSE EN DIAGNOSE VAN MULTIPLE SCLEROSE

Multiple Sclerose (MS) is een aandoening van het centrale zenuwstelsel (hersenen en ruggenmerg) waarbij er schade optreedt aan de myelineschede (de isolerende laag om de zenuwen) en de axonen (de zenuwuitlopers) . Meestal begint de ziekte met aanvalsgewijze uitvalsverschijnselen. Deze verschijnselen zijn erg gevarieerd: bv krachtsverlies of gevoelsstoornissen van armen of benen, problemen met het zien, blaasproblemen, geheugen- of concentratieproblemen en vermoeidheid. De ziekte begint vaak (ongeveer 90%) met een episode van neurologische klachten (klinisch geïsoleerd syndroom, Clinically Isolated Syndrome (CIS)), die meestal daarna weer verdwijnen of in lichte mate aanwezig blijven. Bij het merendeel van de patiënten wordt dit later gevolgd door opnieuw episodes van neurologische klachten ("relapsing remitting" beloopsvorm). De diagnose kan worden gesteld als er op basis van de klinische verschijnselen aangevuld met MRI parameters wordt voldaan aan disseminatie in tijd (meerdere episodes van opvlammingen klinisch (door nieuwe terugval met neurologische klachten) of radiologisch (nieuwe ontstekingen zichtbaar op MRI hersenen) en plaats (ontstekingen op tenminste 2 voor MS karakteristieke lokaties in het centrale zenuwstelsel (klinisch danwel radiologisch aangetoond). Echter na enige tijd van terugvallen, met deels of volledig herstel, ontwikkelt de ziekte zich vaak tot een langzaam progressieve ziekte, waarbij de klachten geleidelijk aan steeds erger worden zonder duidelijke opvlammingen van de ziekte. De snelheid waarin dit proces zich voltooid (en MS patiënten geïnvalideerd raken) verschilt enorm: zo zijn er patiënten die binnen een jaar na de diagnose reeds rolstoel-gebonden zijn, terwijl andere MS patiënten soms tot 30 jaar na de diagnose vrij weinig tot geen blijvende klachten hebben. Het voorspellen van het ziektebeloop in de individuele patiënt is tot op heden niet mogelijk. Er bestaat tevens momenteel geen genezende behandeling voor MS. Er zijn wel ziekte-modulerende therapieën voorhanden, welke met name de hoeveelheid terugvallen verminderen, echter het langetermijneffect op het voorkomen van progressieve klachten is nog niet uitgekristalliseerd. Patiënten met een snel progressief ziektebeloop hebben naar alle waarschijnlijkheid het meeste baat bij deze therapieën, terwijl patiënten met een gunstiger beloop (benigne MS) misschien geen effect zullen merken (en mogelijk wel "onnodige" bijwerkingen van deze medicijnen ondervinden). Het is belangrijk om met name vroeg in de ziekte een betere voorspelling te geven van het verwachte ziektebeloop. Niet alleen vanwege bovengenoemde reden met betrekking tot de behandeling, maar ook vanuit het perspectief van de, vaak jonge, patiënt, om zo de mate van onzekerheid met betrekking tot de prognose te verkleinen. Uiteindelijk hopen we, door de kennis over factoren die van invloed zijn op ziekteprogressie te vergroten, dat

er nieuwe behandelstrategieën worden gevonden om de ziekteprogressie te vertragen of te voorkomen en de juiste patiënten te selecteren voor behandeling met medicatie met mogelijk ernstige bijwerkingen.
Tot nu toe zijn er slechts een beperkt aantal klinische variabelen gevonden die gecorreleerd zijn aan een sneller en ernstiger ziektebeloop van MS: de leeftijd waarop de eerste klachten ontstaan, progressieve vs. het relapsing remitting subtype, en de frequentie van klinische "relapses" vroeg in de ziekte. Echter uitbreiding van deze markers is essentieel voor een betere voorspelling van het ziektebeloop. Onze hypothese is dat genetische factoren en radiologische factoren vroeg in de ziekte, hierin zouden kunnen bijdragen om een deel van de variatie in MS te kunnen verklaren (hierbij hebben we ons met name gericht op ziekteprogressie en afgeleiden hiervan). Kunnen we op basis van genetische en radiologische factoren (in combinatie met bekende klinische factoren) subtypes onderscheiden met een bepaalde prognose? Er zijn in eerdere studies overeenkomsten gevonden in het ziektebeloop van meerdere MS patiënten binnen 1 familie. Deze bevindingen suggereren een genetische rol. Tevens zijn er radiologische studies beschreven waarin er een verband werd gevonden van (verandering in) afwijkingen op de MRI van de hersenen in het begin van de ziekte en het ziektebeloop tot 20 jaar na de eerste MRI scan. Tenslotte is bekend dat er een associatie bestaat tussen de hoeveelheid afwijkingen in het ruggenmerg met meer neurologische klachten (zoals loopproblemen en blaasproblemen).
Zowel genetische als radiologische factoren (zoals zichtbaar op MRI scans van hoofd en ruggenmerg) zouden dus vroeg in de ziekte waardevol kunnen zijn bij het verhogen van de accuratesse van de ziekte-voorspelling. Beide biomarkers kunnen bijdragen aan een betere voorspelling van het ziektebeloop op basis van hun eigen karakteristieken. Zo zullen genetische variabelen een bepaalde predispositie aan kunnen tonen voor een bepaald ziektebeloop, waarbij genetische variabelen een voordeel hebben dat deze niet veranderen gedurende de ziekte en daarmee dus al vanaf het begin van de ziekte betrouwbaar te bepalen zijn. Radiologische variabelen daarentegen (zoals veranderingen in het ruggenmerg afgebeeld middels MRI) veranderen gedurende de ziekte en zijn daarmee een goede afspiegeling van de activiteit tijdens (het begin van) de ziekte. Tevens visualiseren ze de aangedane delen van het centrale zenuwstelsel en kunnen daarmee bepaalde symptomen verklaren.

Het doel van dit proefschrift is om meer inzicht te geven in enkele factoren die mogelijk van invloed zijn op de bijna oneindige klinische en radiologische variatie binnen Multiple Sclerose teneinde de nauwkeurigheid van de prognose en diagnose van MS patiënten te verhogen. Hierbij hebben we ons gebaseerd op de volgende twee hoofdvragen:
1. Bestuderen van de rol van enkele genen op bepaalde klinische en radiologische variatie binnen MS (waaronder met name de mate van handicap van MS patiënten en de verdeling

van de laesies (onstekingshaarden) zoals zichtbaar op MRI van de hersenen en ruggenmerg). 2. Bestuderen van de rol van ruggenmerg-afwijkingen (zichtbaar op de MRI van het ruggenmerg) op de diagnose en prognose van MS. Hierbij hebben we zowel naar focale afwijkingen gekeken als atrofie (afname van het volume) van het ruggenmerg.

Na de algemene introductie in **hoofdstuk 1** proberen we in **hoofdstuk 2** het ziektebeloop te voorspellen aan de hand van een aantal genetische factoren. In **hoofdstuk 2.1** hebben we onderzocht of we de mate van handicap van individuele MS patiënten konden voorspellen op basis van de combinatie van meerdere genetische variaties in aanvulling op het effect van bekende klinische parameters. Hiertoe hebben we een DNA chip gebruikt waarop 80 genetische variaties werden onderzocht, die eerder beschreven waren als mogelijk gerelateerd aan MS. We vonden dat de genetische variaties afzonderlijk geen significant effect hadden op de mate van handicap van patiënten later tijdens de ziekte, tevens was er geen effect op de ziekteduur op het moment dat ze een hulpmiddel nodig hadden bij het lopen. Echter, een combinatie van genetische variaties laat wel een verhoging van de nauwkeurigheid van de prognose zien ten opzichte van alleen klinische variabelen.
In **hoofdstuk 2.2** hebben we specifiek naar 1 bepaald gen gekeken: het Interleukine 7 receptor-alfa gen. Een variatie (single nucleotide polymorfisme (SNP)) in dit gen is de laatste jaren duidelijk geassocieerd met het risico op het ontwikkelen van MS. Deze specifieke variatie binnen dit gen leidt tevens tot functionele veranderingen op mRNA niveau, wat zou kunnen leiden tot een veranderde functie van het eiwit zelf. Gezien deze veranderingen op het mRNA niveau was onze hypothese dat dit gen (betrokken bij ontstekingsprocessen) ook een rol zou kunnen spelen bij processen betrokken bij ziekteprogressie. Hier vonden we echter geen aanwijzingen voor in onze studie.
Tenslotte hebben we in **hoofdstuk 2.3** onderzocht of het KIF1B gen een rol speelt bij het neurodegeneratieve fenotype van MS. De variatie (rs10492972) in het KIF1B gen is de enige SNP in een gen dat geassocieerd is met een hoger risico op het ontwikkelen van MS, waarbij het eiwit een directe rol speelt in neurodegeneratie (de overige genen die betrokken zijn bij een hoger risico op MS zijn vooral geassocieerd met inflammatoire (ontstekingsgerelateerde) processen). Onze hypothese was dat MS patiënten die het met MS geassocieerde allel dragen meer neurodegeneratieve kenmerken hebben. Echter, in onze patiëntenpopulatie vonden we geen effect van dit gen op klinische en radiologische afgeleiden van neurodegeneratie (respectievelijk ziekte-ernst na langere tijd en de snelheid van hersenvolume afname). De hoofdstukken hiervoor genoemd geven aan (gesterkt door andere studies) dat de genen die geassocieerd zijn met het ontwikkelen van MS niet dezelfde zijn als de genen die betrokken zijn bij processen tijdens de ziekte die de ziekte-ernst bepalen.

In **hoofdstuk 3** hebben we gekeken naar de genetische invloed op de verdeling van laesies binnen het centrale zenuwstelsel. De lokatie van de laesies is een van de parameters die direct en indirect de ziekte-ernst kunnen bepalen. Sommige laesies in klinisch belangrijke gebieden zullen veel klachten geven, terwijl er ook nieuwe laesies op bepaalde plaatsen in het centrale zenuwstelsel op de MRI zichtbaar kunnen zijn, zonder dat deze klachten heeft gegeven bij de patiënt. We verwachten een genetische invloed gebaseerd op eerdere demografische studies. In **hoofdstuk 3.1** hebben we onderzocht of de laesie-verdeling tussen de hersenen en het ruggenmerg beïnvloed wordt door genetische invloeden. Onze studie laat zien dat het HLA-DRB1*1501 genotype (het genotype waarvan reeds bekend is dat het een vier keer verhoogde kans geeft op het ontwikkelen van MS) geassocieerd is met een hoger aantal en groter totaal volume van de ruggenmerg laesies.
In **hoofdstuk 3.2** hebben we tevens onderzocht of de laesieverdeling in de hersenen zelf onder invloed staat van genen. We vonden dat een bepaalde variatie binnen het MHC klasse II gen (rs2227139) geassocieerd is met een hogere kans op het hebben van een laesie op de MRI ter plaatse van de rechter voorhoorn. Nog tien andere genetische variaties waren borderline-significant geassocieerd met het ontwikkelen van laesies op specifieke plaatsen in de hersenen. Hoewel uiterst interessant dienen deze bevindingen verder worden onderzocht door andere onderzoeksgroepen.
In **hoofdstuk 4** onderzochten we de klinische relevantie van ruggenmerg-afwijkingen (laesies in het gehele ruggenmerg en het volume van het cervicale ruggenmerg) op het diagnosticeren van MS en op het voorspellen van de ernst van de ziekte. In het verleden heeft de nadruk gelegen op de MRI scan van de hersenen. Onze studie in **hoofdstuk 4.1** laat zien dat de aanwezigheid van laesies in het ruggenmerg zowel kunnen helpen bij het vroeg stellen van de diagnose MS in CIS patiënten, als ook op het voorspellen van de tijd tot een volgende klinische episode van klachten. Naar aanleiding van onze studie adviseren we om vaker een ruggenmerg MRI scan te maken om zo sneller de diagnose te stellen en om accurater een prognose te geven. Dit zou kunnen leiden tot minder onzekerheid bij patiënten in de vroege fase.
In **hoofdstuk 4.2** laten we tevens zien dat niet alleen laesies relevant zijn in de prognose van MS patiënten, maar dat ook het volume van het cervicale ruggenmerg (ruggenmerg ter plaatse van de nek) zeer gerelateerd is aan de mate van invaliditeit. Een lager volume van het cervicale ruggenmerg is geassocieerd met een hogere mate van invaliditeit.

Concluderend, is het nauwkeurig voorspellen van het ziektebeloop van MS patiënten erg belangrijk, mede ook gezien de recente ontwikkelingen op het gebied van nieuwe ziekte-modulerende medicatie die binnenkort zeer waarschijnlijk op de markt zullen komen. Een nauwkeurige selectie van patiënten die mogelijk baat zou kunnen hebben bij deze therapieën is uiterst belangrijk. Meest waarschijnlijk zal de combinatie van klinische

gegevens, radiologische bevindingen en resultaten van biomarker studies (incl. genetische informatie) in de toekomst kunnen leiden tot een betere voorspelling van de prognose van individuele MS patiënten.

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Biografie

Madeleine Henrieke Sombekke werd op 2 december 1980 geboren en groeide op in Denekamp. Ze ging naar de Alexander basisschool in Denekamp gevolgd door het Carmellyceum in Oldenzaal. In 1999 startte ze de opleiding geneeskunde aan de medische faculteit van de Vrije Universiteit. Ze volgde het profiel neurowetenschappen en deed haar wetenschappelijke stage op de Universitas Kristen Indonesia (UKI) in Jakarta, Indonesië. Naast haar studie volleybalde ze in het eerste van VVAmsterdam. In 2006 begon ze het onderzoek onder leiding van Prof. Dr. C.H. Polman en Prof. Dr. F. Barkhof dat leidde tot dit proefschrift. In 2010 startte ze met opleiding tot neuroloog onder Prof. Dr. J.J. Heimans.
In 2009 trouwde ze met Lars Klieverik. Samen hebben ze twee zoons: Stan (2009) en Guus (2012).

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List of abbreviations

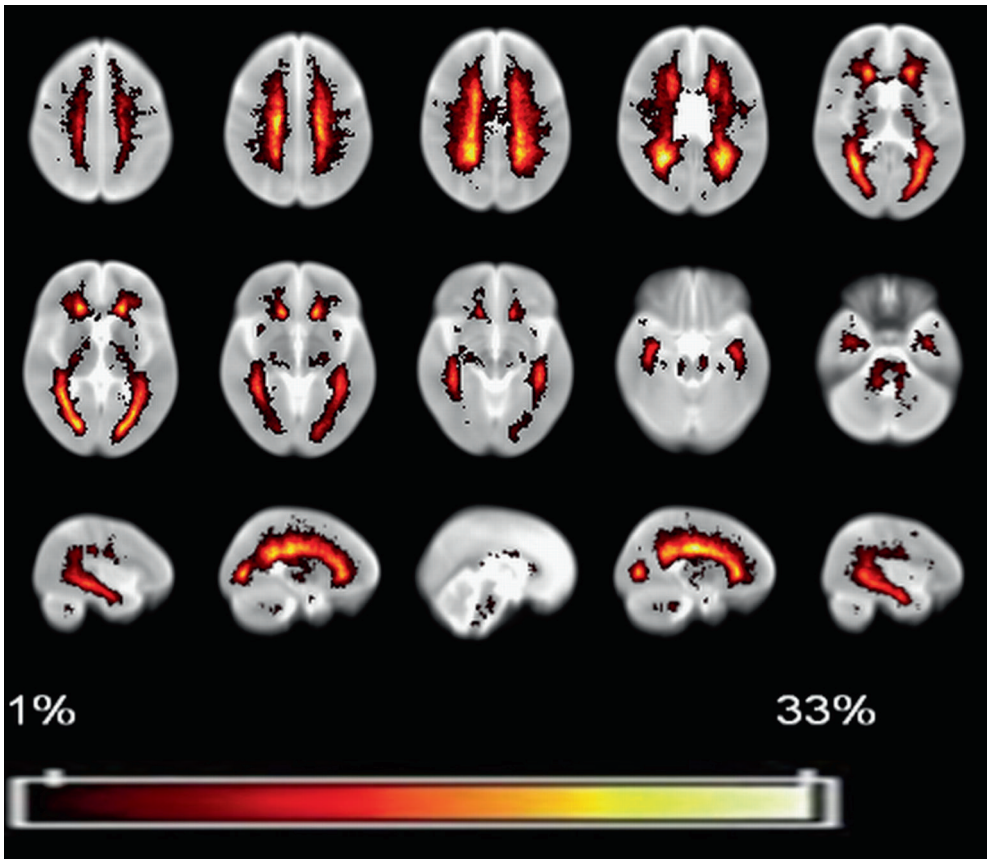
25-TWT:	25 feet timed walk test
9-HTP:	Nine-Hole-Peg test (D: dominant hand, ND: non-dominant hand)
ADAMTS14:	a disintegrin and metalloproteinase with thrombospondin motif, type 1 motif 14
BTNL2:	butyrophilin-like 2
CACNG4:	calcium channel, voltage-dependent, gamma subunit 4
CIS:	clinically isolated syndrome
CCDC46:	coiled coil domain containing 46
CCL5:	chemokine (C-C motif) ligand 5
CCR5:	chemokine (C-C motif) receptor 5
CIITA:	class II, major histocompatibility complex, transactivator
CNS:	central nervous system
CNTF:	ciliary neurotrophic factor
CRYAB:	Alpha B crystallin
CTLA4:	cytotoxic T-lymphocyte-associated protein 4
DMT:	disease modifying therapy
EBF1:	early B-cell factor 1
EDSS:	Expanded Disability Status Scale
FAS:	TNF receptor superfamily, member 6
GABBR1:	gamma-aminobutyric acid (GABA) B receptor, 1
GLM:	General Lineal Model
GWAS:	genome-wide association study
HELZ:	helicase with zinc finger
HLA:	human leucocyte antigen
HLA-DRA:	human leucocyte antigen DR alpha
HSPB2:	heat shock protein Beta 2
HWE:	Hardy-Weinberg equilibrium
IFNAR1:	interferon (alpha, beta and omega) receptor 1
IFNGR2:	interferon gamma receptor 2 (interferon gamma transducer 1)
IL1B:	interleukin 1, beta
IL1RN:	interleukin 1 receptor antagonist
IL2:	interleukin 2
IL4R:	interleukin 4 receptor
IL7R:	interleukin 7 receptor
IL10:	interleukin 10
IQR :	interquartile range

KIF1B:	kinesin family member 1B
Ln:	natural log transformation
LPM:	lesion probability mapping
MAF :	minor allele frequency
MC1R:	melanocortin 1 receptor
MEFV:	mediterranean fever
MOG:	myelin oligodendrocyte glycoprotein
MRI:	magnetic resonance imaging
mRNA:	messenger ribo nucleic acid
MS:	Multiple Sclerosis
ms:	millisecond
MSFC:	Multiple Sclerosis functional composite
MSSS:	Multiple Sclerosis severity score
NBV:	brain volume normalized for head size
NDUFA7:	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7
NDUFS5:	NADH = (Nicotinamide adenine dinucleotide) dehydrogenase (ubiquinone) Fe-S protein 5
NDUFS7:	NADH dehydrogenase (ubiquinone) Fe-S protein 7
NFKIBL1:	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1
NGMV:	normalized gray matter volume
NWMV:	normalized white matter volume
NOS2:	nitric oxide synthase 2
NOTCH4:	Notch homolog 4
PASAT:	paced auditory serial addition test
PD:	proton density
PDCD1:	programmed cell death 1
PITPNC1:	phosphatidylinositol transfer protein, cytoplasmic 1
PNMT:	phenylethanolamine N-methyltransferase
PP:	primary progressive
PRKCA:	protein kinase C, alpha
PTPN22:	protein tyrosine phosphatase, non-receptor type 22
PTPRC:	protein tyrosine phosphatase, receptor type, C
RR:	relapsing remitting
SC:	spinal cord
SD:	standard deviation
SE:	standard error
SP:	secondary progressive

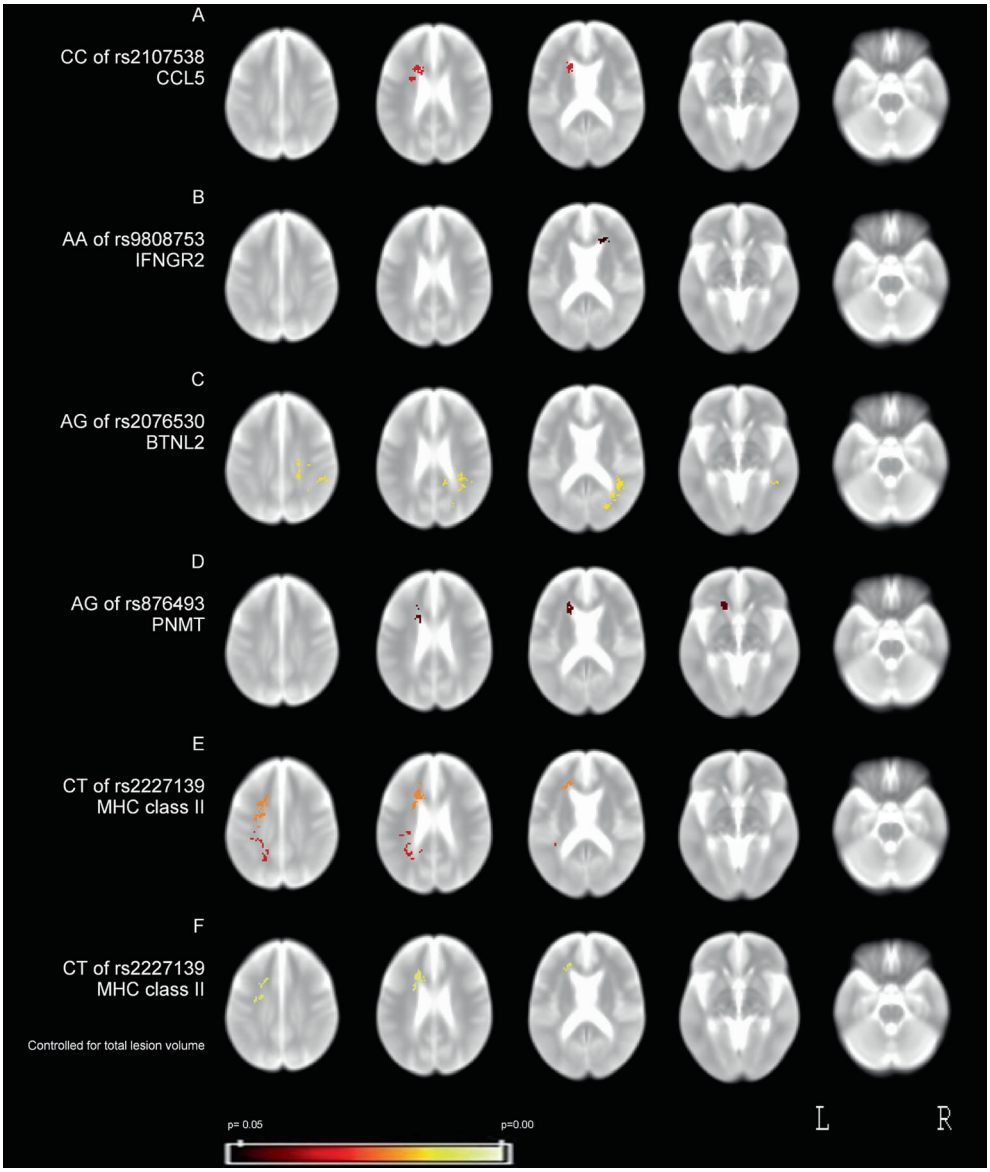
SPP1:	secreted phosphoprotein 1 / osteopontin
T1LV:	volume of T1 hypointense brain lesions
TE:	echo time
TR:	repetition time
TNF:	tumor necrosis factor
TNFSF10:	tumor necrosis factor (ligand) superfamily, member 10
UCCA:	mean upper cervical cord cross-sectional area
UCP2:	uncoupling protein 2
VDR:	vitamin D (1,25- dihydroxyvitamin D3) receptor

Appendix

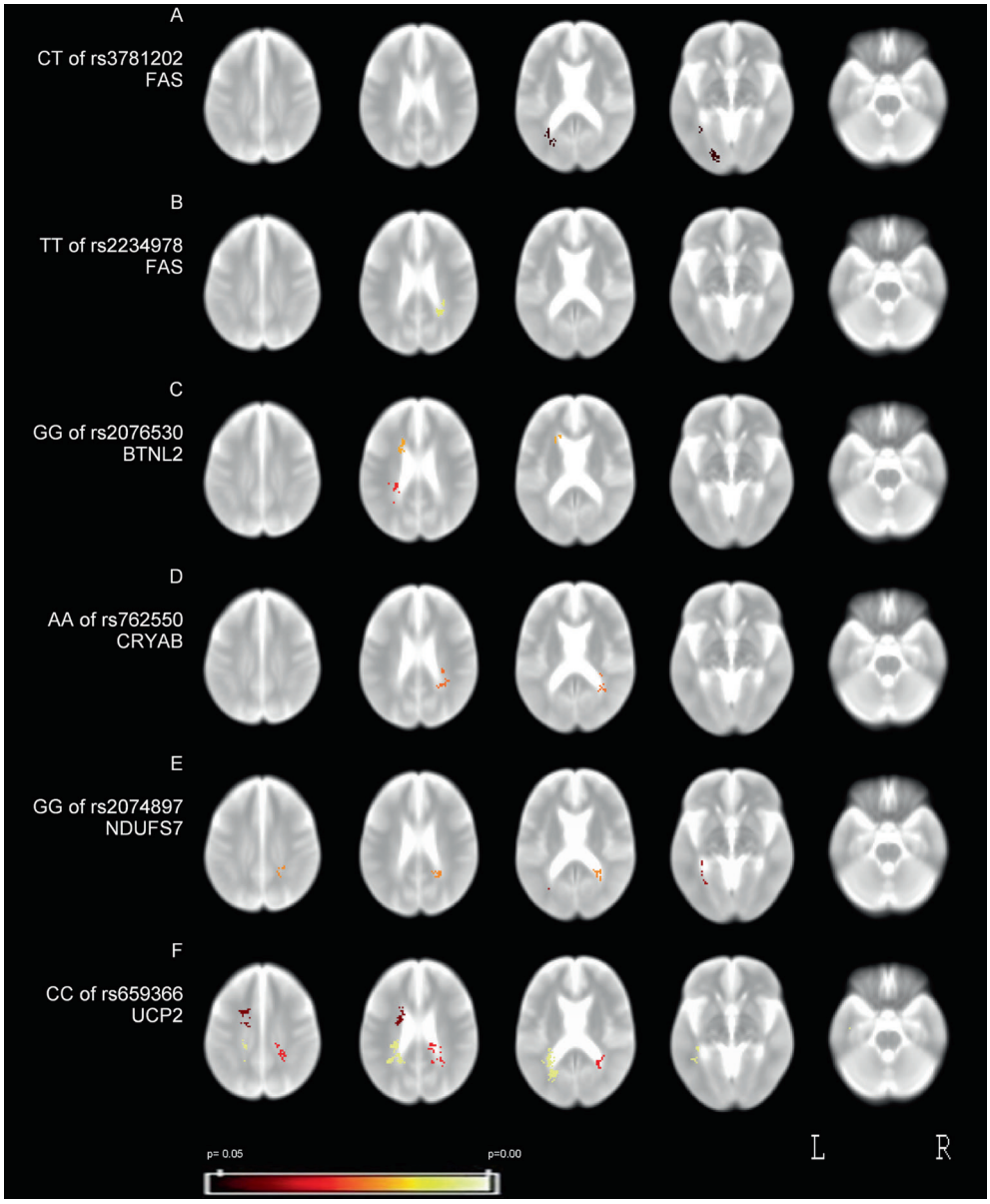
Colour figures



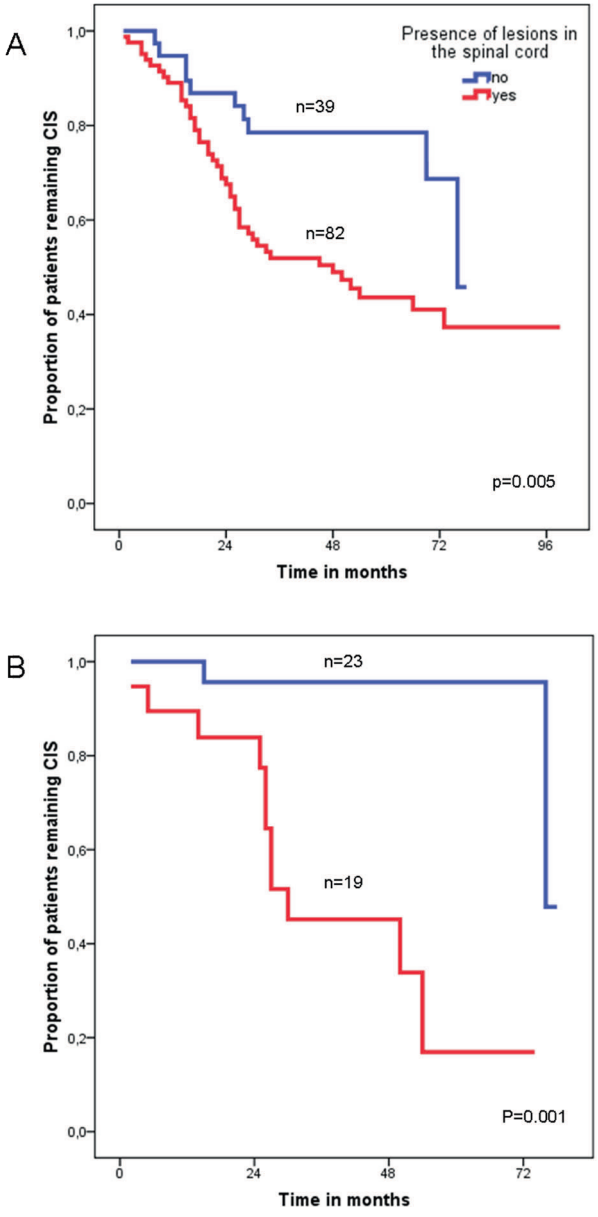
Chapter 3.2. Figure 1: Lesion frequency map for our group of 208 MS patients, indicating for every voxel the lesion frequency throughout our patient sample, showing a range from 1% (n=2 patients having a lesion in that voxel) through the maximum of 33% (n=69).



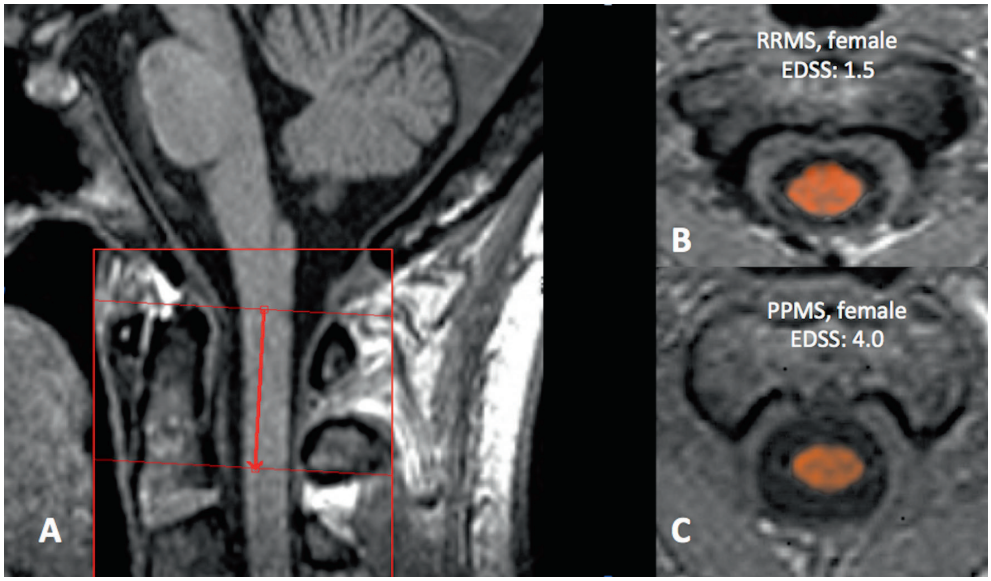
Chapter 3.2. Figure 2: Association between *increased* lesion probability and genotype. Each row shows results for the comparison of one genotype compared to the other two. **A**: CC genotype of rs2107538 within the CCL5 gene (chemokine (C-C motif) ligand 5). **B**: AA genotype of rs9808753 within IFNGR2-gene (IFNGR2: interferon gamma receptor 2). **C**: AG genotype of rs2076530 within Butyrophilin-like 2 gene (BTNL2) (MHC class II associated). **D**: AG genotype of rs876493 (within phenylethanolamine N-methyltransferase-gene). **E**: CT genotype of rs2227139 (within MHC class II region). **F**: CT genotype of rs2227139 (within MHC class II region), when statistically controlled for total T2 brain lesion volume. Images show several axial slices of the T2-weighted template (the same slices in each case), with color overlay (indicating p-values) of the clusters in which local lesion probability was significantly increased.



Chapter 3.2. Figure 3: Association between *decreased* lesion probability and genotype. Each row shows results for the comparison of one genotype compared to the other two. **A**: CT genotype of rs3781202 (within FAS-gene: TNF receptor superfamily, member 6). **B**: TT genotype of rs2234978 (within FAS-gene: TNF receptor superfamily, member 6). **C**: GG genotype of rs2076530 within Butyrophilin-like 2 gene (BTNL2) (MHC class II associated). **D**: AA genotype of rs762550 within Alpha B crystallin gene. **E**: GG genotype of rs2074897 within NDUFS7-gene (NADH dehydrogenase (ubiquinone) Fe-S protein 7). **F**: CC genotype of rs659366 within UCP-2 gene (Uncoupling protein 2).
Images show several axial slices of the T2-weighted template (the same slices in each case), with color overlay of the clusters in which local lesion probability was significantly decreased.



Chapter 4.1. Figure 1: **Survival-curves on effect of presence of spinal cord lesions on time to conversion to CDMS.**
Figure 1A: Survival-curve (Kaplan Meier) in total group of CIS patients (n=121), significant shorter time to CDMS for patients with compared to patients without SC lesions (p=0.005 Cox regression). **Figure 1B**: Kaplan Meier curve in non-spinal CIS patients *not* fulfilling McDonald brain MRI criteria (n=42).



Chapter 4.2. Figure 2: Sagittal T1-weighted image of the brain illustrating volume-of-interest selection (30mm section length) in the cervical cord (A) starting at the upper borders of C2. Representative axial T1w with overlaid (red) corresponding segmentation image of (B) a relapsing remitting MS patient (UCCA: 85mm^2) and (C) a primary progressive MS patient (UCCA: 66mm^2).