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HLA genotype as a marker of Multiple Sclerosis prognosis

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Introduction

Multiple sclerosis (MS) is regarded as the most common neurological disease among young adults [1]. MS is an immune-mediated inflammatory, degenerative disease of the brain and spinal cord that causes demyelination, and central nervous system (CNS) atrophy. The disease is characterized by neurological symptoms such as sensory and visual deficits, limb weakness, balance impairment, problems of coordination, bladder and bowel disturbances, and cognitive deficits [2-4]. According to the World Health Organization (WHO) reports in 2008, approximately 2.3 million people with MS disease were recorded worldwide [1]. MS typically starts in young adults of 20 to 50 years of age, with a peak at 30 years of age, and is most common in Caucasians of Northern European origin [5, 6].

Traditionally, MS pathology has been characterized by focal demyelination plaques in the CNS white matter (WM); however, there is growing understanding on the broader pathology of MS involving cortical demyelination and diffuse damage of the normal-appearing white and gray matter (NAWM, NAGM) [7, 8]. As described by many scientists, main features of MS lesions include blood brain barrier disruption, multifocal inflammation, demyelination, oligodendrocyte loss, reactive gliosis, and axonopathy [9, 10].

In about 80% of MS patients, the disease course starts with the relapsing-remitting phase (RRMS), characterized by reversible episodes of neurological disability and recovery that lasts for many years, which then transforms into progressive neurological decline (secondary progressive phase [SPMS]). However, there is a high degree of variability in the patterns of disease evolution and the rates of disability accumulation. In 5-10% of cases, there are no relapses, but a progressive accumulation of disability since the beginning of the disease (primary progressive MS [PPMS]). The diagnosis of MS is based on guidelines termed the “McDonald criteria”, which were agreed on by an international panel of MS experts [11-13].

1. What causes MS?

While the cause of MS remains unknown, it is well recognized that the interactions of several genetic and environmental factors play a role in triggering this disease [4]. The genetic pre-disposition to MS has been demonstrated by several studies [14-16], with susceptibility genes directly or indirectly linked to the immune system. A large number of presumed environmental

agents have been investigated, and several leading environmental risk factor candidates have been identified, including infections, ultraviolet (UV) light exposure, vitamin D status, smoking, and obesity [17]. It is understood that complex gene–environment interactions acting at an individual level determine the influence of these risk factors on MS susceptibility [18].

Genetic Factors

Genetic, epidemiological and molecular studies have confirmed the high risk of MS in predisposed individuals. MS has a familial recurrence rate of about 20%, with a higher rate of aggregation in first degree relatives and absence of MS excess in adopted relatives despite shared environment [14, 19, 20]. The strong role of an inherited component in determining MS susceptibility is further ascertained by the excess concordance rate in monozygotic twins (25%) compared with dizygotic twins (5%), as shown by population-based studies in twins, and the higher risk of MS in children with both parents affected than in those with single-affected parents [16, 21].

Large-scale genetic studies of multiple sclerosis have identified over 230 risk effects across the human genome, making it a prototypical common disease with complex genetic architecture [349]. Genetic susceptibility to MS is consistently associated with genes of the major histocompatibility complex (MHC) genomic region. Growing evidence of a complex and predominant role of human leucocyte antigen (HLA) genes continues to be identified [22, 23]. Studies have shown that the main MS susceptibility loci are located within the MHC genomic region, at chromosomal position 6p21 that encodes the HLA cluster of genes [253-255], with the majority of studies demonstrating predisposition to sporadic MS associated with the HLA-A3, B7, DR2 extended haplotype [256]. Confirmation of a true genetic effect residing in the MHC comes from demonstration of linkage disequilibrium. Such studies of sporadic MS have not supported linkage to the MHC, however for familial MS, most studies support specific allelic association with HLA-DR2 in the MHC. However, the MHC locus probably represents less than half of the entire genetic etiology of MS, and possibly as little as one-sixth of the overall effect. Families lacking the HLA-DR2 allele appear to have no linkage to the MHC and thus must be influenced by other genes [257]. Families lacking the HLA-DR2 allele appear to have no linkage to the MHC and thus must be influenced by other genes [257].

In 2007, the first GWAS in MS looked at 1540 parent-affected offspring trios and identified two loci outside the MHC, encoding the interleukin-2 receptor (IL-2RA) and the interleukin-7 receptor (IL-7RA), respectively [259]. Additional genetic determinants identified are the ecotropic viral integration site 5 (EVI5) and kinesin family member 1B (KIF1B) genes [258-261].

A comparison of allele frequencies for 551 642 SNPs in 978 cases and 883 controls and an assessment of genotypic influences on susceptibility, age of onset, disease severity, as well as brain lesion load and normalized brain volume from magnetic resonance imaging exams was performed. 242 susceptibility SNPs exceeding established thresholds of significance were identified, including 65 within the MHC locus in chromosome 6p21.3. Gene ontology-based analysis demonstrated a functional dichotomy between genes involved in the susceptibility pathway and those related to the clinical phenotype [258-261].

By 2011, based on several successive studies GWAS and meta-analysis, common variants in 26 genomic loci had been associated with MS risk and independently replicated, but only explained a fraction of MS risk attributable to genetic factors [291, 350-358]. A collaborative GWAS of 9772 cases and 17 376 controls, again of European descent, in 2011 replicated 23 of 26 previously identified associations and identified 29 novel risk loci [255]. These loci are strongly enriched for genes acting in T-cell activation and proliferation pathways. A further study on a targeted array (the ImmunoChip44) in 29 300 MS cases and 50 794 unrelated healthy individuals, identified 48 new susceptibility variants, bringing the total number of MS risk variants to 110 at 103 discrete loci outside the MHC [324].

More recently, the International Multiple Sclerosis Genetics Consortium (IMSGC) has completed an even larger GWAS including over 115 000 cases and controls. This latest report brings the total number of MS risk associations to 233, including 200 autosomal variants outside the MHC, one on the X chromosome and 32 independent effects in the broader MHC locus, covering both classical and nonclassical gene regions [359]. Careful pathway, transcriptomic and epigenetic enrichment analyses suggest T-cell biology is a major feature of the disease, but also highlight the involvement of many other components of both adaptive and innate immunity in pathogenesis. All these effects combined explain 19.2% of the total heritability for MS. The 32 MHC effects accounted for 4% of the overall heritability, with the bulk of the remaining signal resident in the

other regions of the genome associated with MS risk. However, a small portion – approximately 2% of the overall heritability – resides in regions that either did not show suggestive association in the initial GWAS or that failed to replicate in independent samples, suggesting that there remain additional loci to be found [349].

Identifying the causal variants in GWAS loci through fine mapping remains difficult: linkage disequilibrium means that many variants will show evidence of association to disease, but only one is likely to be the causal one. Additionally, even if fine mapping is successful in a locus, there is every chance that the relevant gene cannot be readily identified. To overcome these limitations, efforts are made to integrate GWAS information with other functional genomics data to identify relevant genes [349]. The first is to identify genes with an expression quantitative trait loci (eQTL) driven by an MS risk variant in a locus and the second is to identify specific regulatory elements driving disease risk, and through these, the genes were affected, which must by definition be pathogenic.

Interestingly, the vast majority of GWAS loci encode genes obviously active in the immune system [324, 360], and particularly in the lymphocyte lineage [361], placing beyond a doubt the nature of the disease as autoimmune. In the most recent IMSGC GWAS, 104/200 non-MHC risk loci overlapped eQTLs active in prefrontal cortex or immune cells [359]. These sometimes involve more than one eQTL per locus, for a total of 212 eQTLs potentially being relevant to pathogenesis. Of these, 45 are present only in prefrontal cortex and do not appear to affect gene regulation in immune cell subsets, suggesting that some effects may be restricted to CNS-resident cells (including microglia, which are part of the hematopoietic, rather than the neural, lineage).

Environmental Factors

The potential role of a number of environmental agents in MS pathology has been investigated using epidemiological, clusters migration, and other studies [24]. The most frequently studied environmental factors are presented below:

- *Geographical region and migration*: MS frequency is known to be minimal at the equator, to increase with distance from the equator, to be rare in Asia, tropics, and subtropics, and to be common in Europe, United States, Canada, New Zealand, and Australia. Although the distribution of MS patients is changing as a result of large migrations, the highest risk

is observed for individuals of Northern European origin [25, 26]. Studies investigating the impact of geographical region and migration suggest that the risk of MS is correlated with the place of residence in childhood [27-29]. Migration from high-risk to low-risk region during childhood reduces the risk of MS as compared to their region of origin, whereas the opposite is observed for migration from low-risk to high-risk region in childhood. This pattern indicates the effect of environmental factors in childhood on the susceptibility to MS [4].

- *Viral and bacterial infections:* A number of infectious agents have been studied in relation to MS pathology, and Epstein-Barr virus (EBV) infection in young adults has been recognized as a strong risk factor [30-34]. The results of these studies corroborate the hygiene hypothesis, where infectious agents result in aberrant immune responses when they are encountered by young adults and not early in life due to a clean environment. Case-control and cohort studies have concluded that there is almost no risk for MS among individuals seronegative for EBV, an intermediate risk among individuals infected with EBV at an early age, and the highest risk among individuals with a first EBV infection later in life (adolescents and young adults) [35]. The fact that B-cells accumulating at MS lesions have been found to be infected with EBV supports the hypothesis of a molecular mimicry mechanism of pathogenesis, based on cross-reactions of the immune response to myelin, which bears similarity with EBV proteins [33, 36]. It has also been suggested that deficiency in $CD8^+$-T cells, a recognized feature of MS, could lead to a compromised response to EBV infection in B-cells, allowing their accumulation in the CNS, leading to MS [37]

  Other studies have shown that the interaction between the HLA-DRB1*1501 allele and EBV infection has an effect on the risk of MS [36]. A potential explanation for this observation could be that the HLA-DRB1*1501 allele prevents the presentation of EBV antigens to $CD4^+$ Th cells, which inhibits immune defense recognition and leads to EBV accumulation in B cells. This dysfunctional immune regulation may induce/stimulate autoimmune responses leading to the development of MS. Another explanation could be that particular HLA molecules induce the development of auto-reactive T-cells. When EBV infection makes B-cells presenting self-antigens, auto-reactive T-cells may recognize these self-antigens and further accelerate the progression of MS [36].

- *Smoking:* Cigarette smoke has a well-established role in susceptibility to autoimmune diseases, suggesting a probable biological association between smoking and the immune system. Smoking has been identified as a leading environmental risk factor for MS, with evidence showing association with both onset and clinical course of the disease [38]. Epidemiological data including meta-analyses suggest that ever-smoking increases the risk of MS with an odd ratio of around 1.50 compared with never-smoking [39, 40]. The mechanism by which smoking affects the susceptibility to and progression of MS, and how smoking integrates with other established risk factors is unclear. With regards to MS pathogenesis, smoking influences the clinical manifestation and accumulation of disability. Smoking increases the risk of conversion from RRMS to SPMS, rates of conversion from clinically isolated syndrome (CIS) to confirmed MS, and the risk of PPMS compared with RRMS onset [18].

- *Exposure to solar radiation:* The influence of UV exposure on the risk of MS is supported by diverse investigations [41-43]. A correlation between MS and the mean annual amount of UV in geographic areas shows that risk of MS is lower in the sunniest regions, including high-altitude regions compared to lowland regions [44-46]. However, it has been proposed that higher vitamin D levels may be the mediator of the beneficial effect of UV radiation.

- *Vitamin D:* Epidemiological as well as experimental studies in the MS animal model autoimmune encephalomyelitis (EAE) favor a protective role of Vitamin D in MS susceptibility and pathogenesis [47]. Vitamin D significantly influences regulatory T lymphocyte cells [48], and 1,25-dihydroxyvitamin D3 (the hormonal form of vitamin D3, which is an immune system regulator) has been shown to completely prevent EAE. Many specialists have associated the effect of vitamin D on MS risk with the amount of sunlight exposure (i.e. low-sunlight conditions lead to insufficient vitamin D3 production and increase the risk for MS) [49, 50].

  A previous study of Lysandropoulos et al. has shown that the cytokine profile of EBV-specific $CD8^+$ T cells was affected by *in vitro* adjunction of 1,25-dihydroxyvitamin D3. Whether 1,25-dihydroxyvitamin D3 may have an anti-inflammatory effect on this EBV-specific $CD8^+$ T cell response *in vivo* warrants further studies [49].

Previous studies have also shown that vitamin D has an effect on HLA gene expression [51, 52]. A consensus binding site for Vitamin D receptor, next to the HLADRB1 gene, was identified. Direct functional interactions between HLADRB1, which is the main susceptibility locus for MS, and Vitamin D were shown [50].

Together, the concept of protective role of Vitamin D and UV radiation may explain the striking geographic distribution of MS, which is nearly zero in equatorial regions and increases dramatically with latitude in both hemispheres. It can also explain the low MS rates seen at high altitudes where UV light intensity is higher.

- *Obesity*: Obesity in young adults, particularly between 18-25 years old, is associated with susceptibility to MS. A BMI of BMI ≥ 30 kg/m2 in this age group has been shown to confer an approximately 2-fold increased risk of developing MS [53].

2. Symptoms of MS

The clinical picture of MS is characterized by fatigue, and physical and cognitive disability. Symptoms include sensory disturbances like numbness, dysesthesia and paresthesia, visual impairment (visual loss or double vision), autonomic dysfunction (bowel and bladder urgency, incontinence or retention), weakness, ataxia, spasticity, and cognitive difficulties such as lack of attention, impaired memory, and loss of verbal fluency.

Clinical manifestations of disability may reflect the topical distribution of the lesions and damage in the brain and spinal cord. The predominance of each type of symptom may vary with individuals and with the clinical course. Even though the RRMS phase is characterized by symptoms' variability among individuals, the PPMS and SPMS phases tend to be quite similar with gait disturbance, spasticity, visual deficits, and cognitive decline.

3. Diagnosis of MS

The diagnosis of MS is based on clinical and para-clinical assessments, following the McDonald Criteria of the International Panel on Diagnosis of MS, which were established in 2001 [11] and were revised in 2005 [13] and 2010 [12]. The primary emphasis of the criteria is placed on the demonstration of the dissemination of lesions in space (DIS) and time (DIT), and the exclusion of alternative diagnoses. While MS diagnosis can be based on clinical presentations

alone, the McDonald Criteria assert the role of CNS magnetic resonance imaging (MRI) to support, supplement, or even replace some clinical criteria, to maintain a high degree of both specificity and sensitivity, and to allow a rapid and early diagnosis of the disease.

The 2010 revision of the McDonald Criteria further simplifies the requirements for the demonstration of both DIS and DIT, with fewer required MRI examinations, and increases the diagnostic sensitivity without compromising specificity [12]. The McDonald Criteria for the standardized integration of clinical presentation and other findings for establishing MS diagnosis are:

- No additional findings are needed for the diagnosis in case of clinical presentation of 2 attacks with objective clinical evidence of 2 lesions, or 1 lesion with reasonable historical evidence of a prior attack.
- Clinical presentation of ≥2 attacks with objective clinical evidence of 1 lesion must be further confirmed by DIS demonstrating ≥1 T2 lesion in ≥2 of the 4 CNS regions typical for MS lesions (periventricular, juxtacortical, infratentorial, or spinal cord) or by a further clinical attack implicating a different CNS site.
- One attack with objective clinical evidence of ≥2 lesions must be supported by DIT demonstrating the simultaneous presence of asymptomatic gadolinium (Gd)-enhancing and non-enhancing lesions at any time; or a new T2 and/or Gd-enhancing lesion(s) on follow-up MRI irrespective of its timing with reference to a baseline scan. In the absence of supporting MRI findings, diagnosis is confirmed after a second clinical attack.
- One attack with objective clinical evidence of 1 lesion (CIS: clinically isolated syndrome) must be supported by DIS and DIT, and demonstrated by MRI: a) ≥1 T2 lesion in at least 2 of the 4 CNS regions typical for MS (periventricular, juxtacortical, infratentorial, or spinal cord), or a second clinical attack implicating a different CNS site; and b) simultaneous presence of asymptomatic Gd-enhancing and non-enhancing lesions at any time, a new T2 and/or Gd-enhancing lesion(s) on follow-up MRI irrespective of its timing with reference to a baseline scan, or a second clinical attack.

The diagnosis of PPMS (insidious neurological progression suggestive of MS) is confirmed by 1 year of disease progression determined retrospectively or prospectively, and at least 2 of the following criteria:

- Evidence for DIS in the brain based on ≥1 T2 lesions in periventricular, juxtacortical, or infratentorial regions.
- Evidence for DIS in the spinal cord based on ≥2 T2 lesions in the cord.
- Positive cerebrospinal fluid (CSF), evidence of oligoclonal bands by isoelectric focusing, and/or elevated Immunoglobulin G (IgG) index.

To use the McDonald Criteria in clinical practice, it is required that a) the criteria be only applied in patients who present with a typical CIS suggestive of MS or symptoms consistent with a CNS inflammatory demyelinating disease (monofocal or multifocal presentation involving the optic nerve, brainstem/cerebellum, spinal cord, or cerebral hemispheres), and b) alternative diagnoses must be considered and excluded by applying other accepted supportive tests.

The most recent criteria, MAGNIMS, have modified the 2010 MRI criteria based on evidence and expert consensus. The new criteria have introduced a new DIS location in the CNS, the optic nerve (≥3 lesions in the periventricular regions are needed to confirm the involvement of this region in the DIS). Other important proposed MAGNIMS revisions include: the extension of the juxtacortical lesion concept with the combined term cortical/juxtacortical, and the absence of distinction between symptomatic and asymptomatic MRI lesions for the DIS or DIT. The committee also confirmed the use of identical criteria for the DIS for PPMS and RRMS, and the use of CSF results for clinically uncertain cases of PPMS. The MRI criteria for DIS and DIT can also be used for children above 11 years of age in the absence of acute disseminated encephalomyelitis presentation. The committee recommended cautious application of the 2010 criteria in children younger than 11 years old (when used solely at baseline). The new criteria recommend careful exclusion of alternative neurological disorders before application of MRI criteria to patients in Asia, Latin America, Europe, or Northern America. Further, the committee confirmed that the criteria should be used for MS diagnosis in radiologically isolated syndrome (RIS) patients [54].

4. Clinical course of MS

The clinical subtypes of MS were defined in 1996 by the US National Multiple Sclerosis Society (NMSS) Advisory Committee on Clinical Trials in Multiple Sclerosis [55] and were revised in 2013 [56]. In 1996, the NMSS Advisory Committee defined the 4 standardized MS clinical courses: RRMS, SPMS, PPMS, and progressive relapsing (PRMS), and incorporated them into clinical practice (Figure 1a). Later, it was noted that the clinical course descriptions were determined by subjective views of MS experts and lacked objective biological support. Hence, the classical MS phenotypes were re-examined, exploring the progress made in imaging and biomarker research, and understanding of MS pathogenesis. The consensus is to consider the disease course as a dynamic process, and to assess clinical phenotype based on current status and historical data (i.e., the subtype on initial assessment may change over time). Thus, the 2013 revisions proposed refined descriptors of the clinical course of MS, which include consideration of disease activity (based on clinical relapse rate and imaging findings) and disease progression (Figure 1).

Clinically Isolated Syndrome

The first clinical presentation of the disease is included in the MS phenotype spectrum as CIS, which shows characteristics of inflammatory demyelination without evidence for DIT. MS treatment with disease-modifying agents in clinical trials have shown to delay the development of a second exacerbation and conversion to clinically definite MS (CDMS) [57-59]. Natural history studies and clinical trials of MS disease-modifying therapies (DMTs) indicate a high risk for meeting the diagnostic criteria for MS when CIS is supported by brain MRI lesions [60-62] and oligoclonal bands in the CSF. Prospective follow-up of CIS patients should determine their subsequent disease phenotype.

Basic Phenotypes

MS phenotypes are classified into two core types: relapsing disease or progressive disease (PPMS or SPMS). Both types are subject to modification in disease activity, as defined by clinical assessment of relapse occurrence or lesion activity detected by CNS imaging (Gd-enhancing lesions, or new or unequivocally enlarging T2 lesions), and occurrence of progression of disability

over a given time period. According to the current understanding of MS pathogenesis, the two modifiers reflect principally ongoing inflammatory or neurodegenerative processes, respectively [2].

Relapses are signaled by the occurrence, recurrence, or worsening of neurologic symptoms. These symptoms usually develop sub-acutely over a few hours to days, last between a few days and weeks, and then spontaneously remit partially or completely [63]. The pattern of relapses (symptoms and severity) and their recovery have great inter-individual variability. Although there is no correlation between exacerbations and long-term disability, incomplete recovery may contribute to accumulation of disability. Relapse rates have prognostic value in MS. When progression is involved, a steady deterioration of the neurologic function is associated with new symptoms and signs that take place over a period of at least 6 or 12 months. Once progression has begun, it tends to continue, although temporary plateaus or minor improvements may also be present [64-66]

SPMS is diagnosed retrospectively by a history of gradual worsening after an initial relapsing disease course, with or without acute exacerbations during the progressive course. As written by Lassmann et al., PPMS represents a distinct, non-inflammatory pathologic form of MS [2]. However, clinical, imaging, and genetic data suggest that PPMS is a part of the spectrum of progressive MS phenotypes and that any differences are relative rather than absolute. Although PPMS is characterized by the absence of exacerbations prior to clinical progression, pathophysiologically it may not have distinct features compared with SPMS. The similarity in the accumulation of disability (when measured from the onset of the progressive course), the age of onset, and the time to reach disability milestones between the PPMS and SPMS unifies the two disease phenotypes [65, 67, 68]. This supports the concept that MS has as a complex rather than a heterogeneous pathology.

With the inclusion of activity as a modifier of the basic clinical course phenotype of PPMS, a patient who has an acute attack (thus fulfilling prior criteria for PRMS) would be considered to have active PPMS. Thus, MS is no more described as PRMS because this is equivalent to PPMS patients with disease activity. On the other hand, a patient with PPMS with no acute attacks and no MRI activity would be considered to have non-active PPMS. Similarly, a PPMS patient who has not progressed over a period of 1 year would be classified as having non-progressing PPMS,

and an SPMS patient who has gradually worsened and has Gd-enhancing lesions on MRI would be classified as having active and progressing SPMS. Activity and progression are determined by clinical presentations (new or increasing neurologic dysfunction) as well as by imaging findings (Figure 1b).

Benign MS

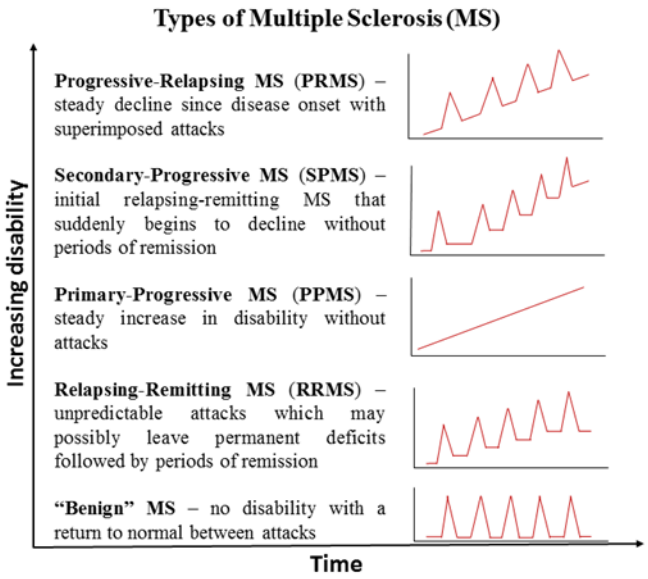
The mildest form of clinically apparent MS was labelled "benign MS (BMS)", not as a phenotype descriptor but rather as an indication of disease severity/disability over time that can apply to any MS phenotype. The term "benign" should always be determined retrospectively for patients who are "fully functional" >10-15 years after onset with score on the expanded disability status scale (EDSS) below 2-3. BMS is transitory and a significant number of BMS patients progress to other subtypes of MS 10 to 15 years after onset [69-72].

Routinely, BMS diagnosis relies on disease duration and patient disability, measured by EDSS scores, which gives weight to physical disability. With the absence of accepted standards for measuring BMS, frequencies reported across studies varied between 5% and 64% of all MS cases. Recent findings show that deterioration of cognitive function, fatigue, pain, and depression also occur in BMS patients, causing negative impact on work and social activities despite complete preservation of motor function. [73, 74]. However, without defined standards for measuring BMS, prognostic assessment is controversial.

Radiologically Isolated Syndrome

RIS is defined for patients with imaging findings suggestive of inflammatory demyelination in the absence of clinical signs or symptoms. The suspicion of MS in patients with RIS depends on the morphology and location of the MRI lesions, with changes on brain imaging suggestive of demyelinating pathology being proposed to carry the highest risk of future MS clinical symptoms [75]. However, RIS is not considered as a separate MS phenotype.

a)



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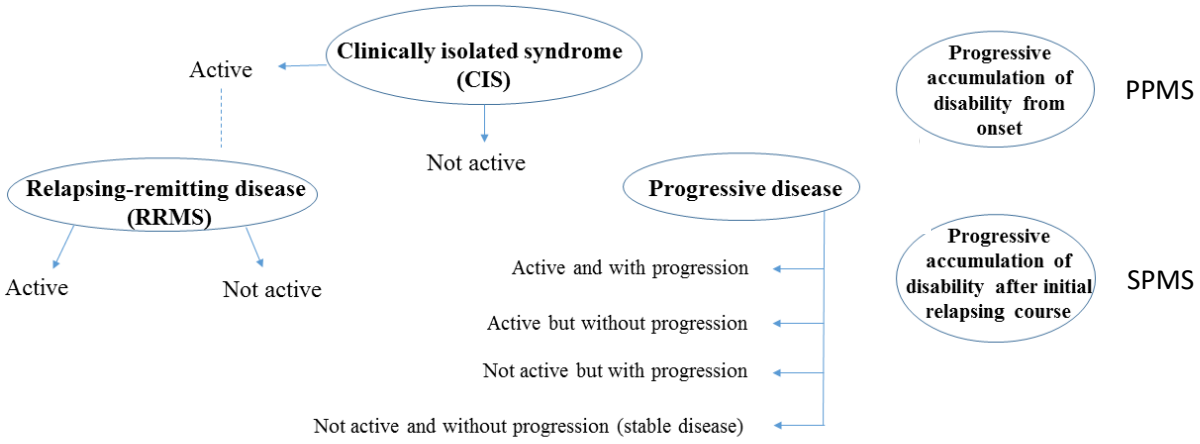

Figure 1 Types of MS as per Reingold classification 1996 and its revision in 2013

5. MS - Autoimmune inflammatory and degenerative disease of the CNS

Over the recent decades, substantial advances into the understanding of MS pathogenesis have been made, and it has increasingly been recognized as a complex disease with different pathways to tissue injury and clinical development. There are different clinical and pathological phenotypes of MS, involving engagement of the immune system, acute inflammatory injury of axons and glia, recovery of function and structural repair, progression to microglial activation, and neurodegeneration. The clinical correlate is characterized by early course of neurological dysfunction that recovers, but over time leads to accumulation of neurological disability [4].

The autoimmune pathogenesis of MS

Classically, MS is considered an inflammatory autoimmune disease, as supported by several findings in animal models and in active MS lesions [76, 77]. Pathologically, chronic inflammation in MS leads to focal demyelinated plaques in the CNS [78], and the diagnosis of MS is confirmed by the presence of multifocal inflammatory demyelinated plaques distributed over time and space within the CNS. Recurrent inflammation and appearance of such disseminated lesions are associated with clinical relapses. The resolution of the inflammation is considered to be the main factor leading to clinical improvement. The following evidences support the autoimmune concept of MS pathogenesis:

1. Active MS lesions display common inflammatory features across different MS sub-types. Lesions are dominated by inflammatory infiltration, mainly T-cells and their mediators (macrophages/monocytes, cytokines). Detailed immunopathological studies of early acute lesions revealed profound heterogeneity in the patterns of demyelination and the factors of the immune system involved [76, 79, 80].
2. MS susceptibility is controlled by genes affecting T-cell reactivity (HLA-DR).
3. Myelin autoreactive T-cells (specific for myelin basic protein [MBP]) are present in the blood of MS patients as well as in healthy donors. Animal studies have shown that a) myelin-specific T-cells can transfer EAE in monkeys, and b) transgenic mice with human T-cell receptor α- and β-chains ancillary genes, which are required for productive presentation and recognition of (auto) antigens, can develop spontaneous EAE [81-84].

4. B-cells play also a role in the pathogenesis of MS plaques, as evidenced by inflammatory infiltrates of B lymphocytes and plasma cells. B-cells also serve as antigen presenting cells for the processing of intact myelin antigen, and subsequent activation and pro-inflammatory differentiation of T-cells. There is also evidence of humoral auto-antibodies specifically binding to myelin and other components of the CNS [77, 85].
5. Immune therapies directed against T- and B-cells have an ameliorating effect.

In summary, as described by Wekerle, 2008, there are strong evidences suggesting that MS is caused by autoimmune lymphocytes (T- and B-cells), but there is a lack of formal proof [77]. Despite several arguments supporting the hypothesis that inflammation is the core process in MS pathogenesis, it is accepted that injury and clinical disability take different clinical and pathological phenotypes, and are the result of a complex sequence of events and not solely the result of inflammation [76].

MS, a degenerative disease

Although the primary pathology of MS involves immune-mediated mechanisms, the irreversible neurological dysfunction seen in MS patients is attributed to progressive axonal injury and neuronal loss, posing MS as a neurodegenerative disease [3, 10, 86]. Disease progression and accumulation of clinical disability correlate with early, diffuse, and chronic axonal loss [87], which is greatly supported by imaging (including functional MRI) [88-90] as well as by morphological studies [3, 91, 92]. Significant correlations exist between decreases in brain volume and other MS neuronal markers, indicating that atrophy reflects axonal loss and may serve as a marker of the degenerative phase of the disease [93-95]. Although the mechanism of axonal loss remains uncertain, it is proposed to involve degeneration subsequent to demyelination, or structural damage (cytoskeleton) mediated by inflammatory components. Moreover, studies show that inflammatory agents directly contribute to demyelination as well as neurodegeneration as evidenced by the presence of proteases, reactive oxygen species, and cytokines in lesions [96]. However, epidemiological data and treatment studies provided evidence that axonopathy is not caused by inflammation alone. Altered ion channel activity, endogenous neuroprotective pathways that counteract oxidative stress and mitochondrial dysfunction have all been investigated as possible non-immuno-related mechanisms which may play a role in the degeneration associated with

MS[97, 98]]. Current immunomodulatory therapies have limited effects on progressive atrophy, and reveal a dissociation between inflammation and disease progression once a certain level of clinical disability has been reached [93]. As mentioned by Wilkins et al. [99], the slow and insidious loss of neurological function that occurs during the progressive phase of the disease implies a degenerative process (Figure 2) [99], therefore, the non-immuno-related mechanisms offer alternative drug targets that may modulate disease progression beyond that offered by current immunomodulatory therapies [98].

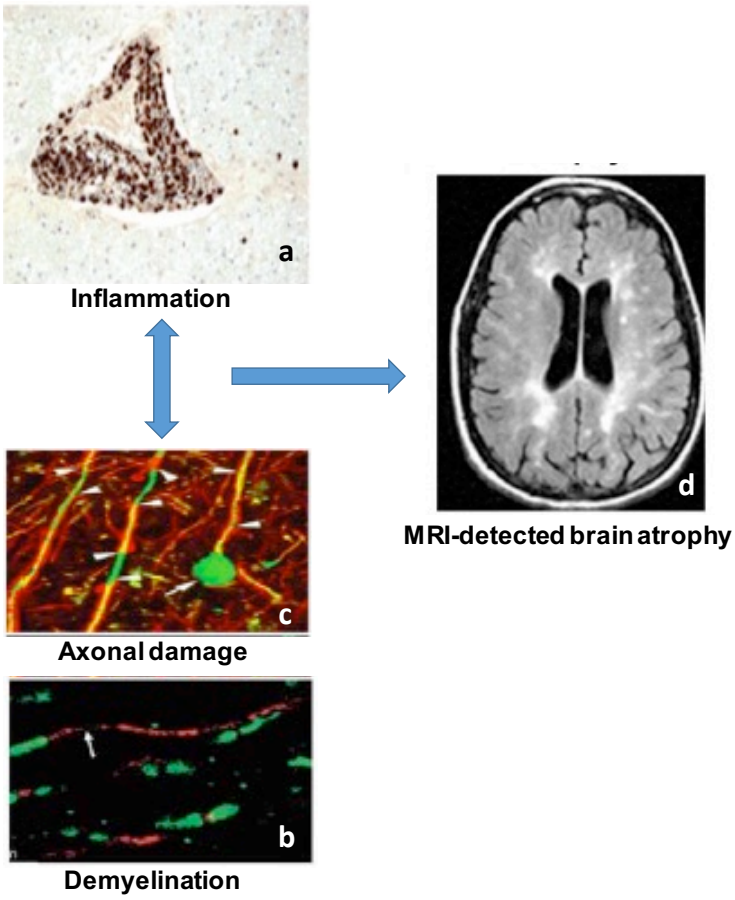

Figure 2 Brain volume loss in MS is a combination of inflammatory and degenerative process.

Prolonged demyelination may lead to axonal dysfunction even before degeneration. Inflammation (a), demyelination (b), and axonal damage (c) produce brain atrophy in MS (d). (a) T-cell infiltration detected by $CD3^{+}$ immunohistochemistry in MS plaques. (b) Actively demyelinating axons. (c) Three large axons staining for dephosphorylated neurofilaments (green) undergoing active demyelination (arrowheads). (d) Multiple focal hyper-intense lesions can be detected at the corpus callosum edge for example, and the enlarged CSF spaces in the region of the corpus callosum, as well as WM and GM atrophy are also

visualized. Images in Figures 2a, 1b, and 1c are reproduced with permission from Frischer et al. [100], Waxman et al. [101], and Craner et al. [102], respectively. The MR image in Figure 2d is presented from the courtesy of Dr. Robert Zivadinov and Dr. Stanley Krolczyk.

Given the evidences supporting the significance of inflammation and the neurodegenerative process in the pathogenesis of MS, it was believed that this disease has a two-step mechanism; an initial inflammatory phase with focal WM lesions followed by a neurodegenerative phase. The degenerative outcomes were considered as the consequence of recurrent inflammatory episodes and irreversible demyelination. However, recent studies have shown the presence of diffuse NAWM damage, significant GM involvement, and cortical functional reorganization in the early clinical stages of the disease, revealing the presence of neurodegenerative components of MS with limited association with MRI markers of inflammatory demyelination [103]. These observations suggest that MS is as much a neurodegenerative as an inflammatory disease, with a simultaneous interplay between the two components in its pathogenesis (Figure 2) [79, 87, 91, 96, 101-103].

6. MS prognosis

The principal endpoints for MS prognosis are physical disability milestones (accumulation over time) assessed using the 10-point EDSS. The general prognosis is a decline in mobility, with an EDSS score of 4 for limited ambulation, a score of 6 for unilateral aid for walking, and a score of 7 for wheel chair bound patients [104-107]. The average time to reach these disability milestones is 8, 20, and 30 years from onset, respectively. However, owing to the high inter-individual variation in the rate of accumulation of irreversible disability, prediction on an individual level is unreliable [65].

In CIS patients, the development of a second clinical episode (in a different CNS location) permits a diagnosis of CDMS to be made [11, 13]. The median time between the first and second relapse is approximately 2 years, and the highest probability of developing a second relapse is immediately after the initial one and diminishes progressively thereafter [64, 65]. This probability is not influenced by gender, age at onset, mono- or multi-focal initial symptoms, or degree of recovery from the initial relapse. MRI can be used to characterize the occurrence of the second relapse, and the presence and the number of MRI lesions at the clinical onset of MS, which are associated with the increased probability of a second neurologic relapse [61, 62, 108-113].

In patients with RRMS, which is the initial clinical phenotype of 85% of patients, successive episodes of neurologic disability may affect the optic nerves, brain stem, cerebellum or long tracts [65]. The average initial rate of relapse episodes is one in two years, but this rate tends to decrease over time. The majority of RRMS patients show an ultimate conversion to SPMS, with a median time of 12 years between diagnosis and SPMS conversion, and relapses that persist in about 40% of cases [64]. Clinical factors predictive of MS prognosis are interdependent, but the most important factor that influence the long-term outcome is the development of a progressive course. A few predictors of secondary progression are age at disease onset (>30 years), initial symptoms (motor symptoms), relapse frequency (5 or more attacks in the first 5 years), incomplete and shorter remission, cognitive symptoms within 5 years of disease onset, the presence of multifocal symptoms at progression onset, and family history [57, 65, 68, 114-116].

The course and prognosis of MS are essentially a function of the chronological age [64]. A previous study has suggested that the median age of patients with initial RRMS is 29.0 years at onset, while the median age of patients at the time of progressive phase onset is 39.1 years (either the onset of PPMS or SPMS) [104, 107, 117]. While the time to reach irreversible disability varies between patients with a RRMS and PPMS onset, the chronological age for onset of disability remains similar. The median estimated age of MS patients at the time of reaching DSS 4, 6 or 7 is 44.3, 54.7, and 63.1 years, respectively, regardless of the initial disease course [64, 104, 105]. However, although the age of reaching the different disability milestones and the initial course of MS are not dependent, earlier MS onsets predict presence of various disability landmarks at younger ages and the interaction between age at onset and chronologic age is complex [105]. Life expectancy is only minimally reduced in MS patients, and the estimated median survival time from onset to death has been estimated to be 31 years in a previous study conducted in the Danish population [64, 117].

The prognosis of MS is also a function of the gender; the male gender is associated with shorter time to reach SPMS conversion and disability milestones. Early MS onset, spinal cord-related symptoms, incomplete recovery, shorter interval between the first and second attack, a greater attack rate in the first 2 or 5 years, and a higher disability score in the first 5 years are more frequently observed in men than in women [114, 118, 119]. In contrast, BMS is more frequent in young women who initially have an initial RRMS disease course [106], and the median survival time from MS onset to death is significantly longer in women. In only 2% of cases, MS onset is in

childhood; these cases have a female preponderance, characterized by female-to-male ratios ranging from 2.2 to 3, i.e. higher than in adult-onset MS [120].

An important predictor of MS prognosis is brain atrophy, which impacts the cognitive dysfunction, physical disability, and the quality of life (QoL). It involves demyelination, gliosis, and axon loss/neurodegeneration. Although MS was regarded as a WM disease, GM loss during MS progress is correlated with disability outcomes [121]. The brain volume loss (BVL) during MS progresses at rates ranging between 0.5 to 1.35 % in a year: 5 to 13.5 times more rapidly than in healthy individuals [122]. The rate of WM loss in MS is approximately 3 times that of the healthy people, while that of GM loss increases over time with disease stage, from 3.4 times in early RRMS to 14 times in SPMS. WM and GM atrophy in MS may already occur in patients with CIS [123, 124].

MRI has a major role in the prediction of the disease prognosis [54]. Larger MRI T2 brain lesion volumes are associated with an increased risk of a relapse occurrence. Detection of at least one T2-weighted MS compatible brain lesion in patients with CIS increases the probability of conversion to CDMS from 3% to 65% of cases within 5 years [113], from 11% to 83% of cases within 10 years [61], and from 19% to 88% of cases within 14 years [125].

In patients who meet the Barkhof/Tintore criteria for MRI DIS at the first potential MS relapse time point, the risk of conversion to CDMS within 2 years increases from 10% to about 45% compared to asymptomatic patients [126]. Detection of juxtacortical, infratentorial, and periventricular lesions on T1-weighted brain MR images may also be used to predict occurrence of a second relapse in the short term. Although it has been shown that MRI detected brain abnormalities have a strong predictive value with respect to disability in MS, new biomarkers or technological advances would be useful to evaluate individual disease prognosis [64].

7. Outcome measures in MS

Outcome measures that can capture the fluctuations in health status over time are needed to evaluate the clinical progression of MS and the impact of treatment. Currently, there is a lack of well-defined endpoints to measure the long-term functional outcomes in MS patients in clinical studies [66]. Use of longevity as an outcome measure is limited due to the long follow-up needed and the insufficient documentation about causal relationship to MS. Likewise, conversion from

RRMS to SPMS has large inter-individual variability and necessitates a long follow-up, which is not feasible in a clinical study set up. Moreover, determining the onset of an SPMS course has been shown to be imprecise [66]. In clinical studies, relapse rates between groups are frequently compared to study the effect of a therapy. Such assessments presented at the group level are subject to biased interpretation due to individual drop outs and cannot be translated to individual patients.

The EDSS scale is the most widely used, regulatory approved outcome measure in clinical studies evaluating DMTs [127]. EDSS measures impairment in seven functional systems, such as visual, sensory brainstem, bowel/bladder, pyramidal, cerebellar, and cerebral impairments. Short-term EDSS changes may be seen as a surrogate marker for long-term disability. In RRMS patients, changes in EDSS scores may indicate exacerbations, but are not always correlated with disability accumulation. Yet, the EDSS scoring does not capture cognitive impairment, nor is it sensitive to disability in SPMS patients[128]. For that reason, two other dimensions are used[129], namely, leg (Timed 25-Foot walk (T25FW)) and arm (9-Hole Peg Test (9-HPT)) performance tests. Measure of cognitive impairment is poorly represented in the EDSS scale. More recent scales have been developed to try and provide a more multi-dimensional assessment of the disease including the Multiple Sclerosis Functional Composite (MSFC), which, as well as including the T25FW and 9-HPT to measure physical function, also measures cognitive function via the Paced Auditory Serial Addition Test (PASAT) that specifically assesses auditory information processing speed and flexibility, as well as calculation ability [130, 131]. Additionally, the symbol digit modalities test (SDMT) is suggested to be a useful, simple, fast, and economic screening tool to measure cognitive impairment in MS patients in everyday clinical practice [132].

The impact of the disease is also measured by QoL (Quality of Life) questionnaires. The MSQOL-54 questionnaire combines both generic and MS-specific items [133, 134]. The subscales include: physical function, role limitations (physical and emotional), pain, emotional well-being, energy, health perceptions, social function, cognitive function, health distress, overall QoL, and sexual function. Scoring is based on summary scales for mental and physical health, and an individual score for sexual function. Since fatigue is one of the most common and debilitating symptoms of MS, the disease outcome is also assessed based on fatigue reported by patients [135]. However, the absence of a clear definition of fatigue poses a limitation for its quantification. Among the different scales available, the Neurological Fatigue Index–MS is a validated scale

developed from reported experience of fatigue by patients in accordance with the latest guidelines of the Food and Drug Agency for scale development.

Advancement in MRI techniques have demonstrated the presence of active disease process within the CNS even in clinically stable patients, which cannot be detected by clinical measures [136]. Thus, MRI plays an important role in predicting outcomes of MS, and is used as a tool in many outcome scales. No evidence of disease activity (NEDA) is a composite measure of disease activity that is emerging as the target outcome of new DMTs. NEDA takes the following parameters into account; relapses, disability progression, and MRI activity (lesion load and/or brain volume). NEDA (NEDA-3) is defined as the absence of new or enlarging T2 lesions or T1 gadolinium-enhancing lesions on MRI and no sustained EDSS score progression or clinical relapse [137]. NEDA-3 assessment is regarded as a comprehensive measure of treatment response in RRMS, although weighted towards inflammatory activity. NEDA-4 is an expanded conception of NEDA [138] that was adopted to included change in BVL over time as the fourth factors. Accelerated BVL is an objective measure of disease worsening and progression in RRMS patients. NEDA-4 thus has the potential to capture the impact of therapies on both inflammation and neurodegeneration [139]. NEDA-4 status at one year can predict subsequent disability and structural damage for as long as 7 years.

The Rio Score and modified Rio Score use early relapses and MRI detection of new T2 lesions to predict long-term progression in RRMS patients [140]. This tool is being used for treatment management of RRMS patients under interferon Beta treatment and glatiramer acetate [141-143].

8. The role of MRI in MS

Brain MRI has evolved as an essential element in the diagnosis of MS, as re-emphasized in the latest revisions of the McDonald criteria [12]. The sensitivity of conventional MRI for the diagnosis of MS within the first year after a single attack is 94%, with a specificity of 83% [144]. MRI represents also the most important imaging tool used to monitor MS progression, make therapeutic decisions, and evaluate treatment efficacy [145-147]. Current recommendations warrant at least one MRI follow-up per year for the monitoring of the disease [54].

The most important biomarker is the lesion load, defined as the total volume of lesions in the brain WM and GM. MS lesions are detected using conventional MRI, based on the signal intensity

value corresponding to the lesion region where they appear: 1) a hypointense signal on T1-weighted images represents chronic stage lesions with axonal destruction and irreversible damage; 2) an enhanced signal on Gd-enhanced images represents "active" lesions, indicating inflammation and breakdown of the blood-brain-barrier, and corresponding to ongoing disease activity and, 3) a hyperintense signal on T2-weighted images [146]. It is also evident from many studies that MS is characterized by WM and GM atrophy [7, 148, 149]. Several MRI data on brain volumetry correlate whole brain and GM atrophy with the accumulation of physical and cognitive disability [150-152] and propose brain atrophy as a biomarker for disease progression [153]. Hence, besides lesion load, quantification of brain volumes and atrophy rates are especially crucial in disease management [150, 151, 154].

Quantitative biomarkers of brain atrophy and lesion load have been proposed as standardized parameters for the diagnosis and monitoring of the disease. These biomarkers can be used as surrogate measures to evaluate the disease processes in the brain of MS patients and can impact clinical decision; e.g., evidence of disease activity (new lesions, high atrophy rate) in patients on treatment, or evidence of too fast disease progression at early MS stage are prognostic markers of worse outcomes and disease evolution. However, clinical application of MRI in making decisions about disease management is limited by the absence of standardized MRI imaging protocols or guidelines for clinical interpretation.

9. MRI abnormalities in the CNS of MS patients

MRI evaluation of MS-specific WM lesions

Water diffusion is anisotropic in WM, because axon membranes limit molecular movement perpendicularly to the nerve fibers. This feature can be exploited to produce stunning maps of the orientation in space of the WM tracts and brain connections in just a few minutes [155].

The appearance of WM lesions on conventional MRI is related to the different stages of the disease (early versus chronic), and to the severity of tissue damage. Conventional MRI showed that the damages seen in lesions are higher than in NAWM [146]. Correlation between the regional distribution of WM lesions and long-term disability [146, 156] or cognitive deficits [146, 157-159]

in MS was achieved based on new refined analysis tools developed for non-conventional MRI using volumetry [160], multiscale spectral analysis [156], magnetic resonance chemical shift imaging [157], 1H-magnetic resonance spectroscopy (MRS) [158], diffusion, susceptibility, or MT [159] techniques.

Diffuse MRI abnormalities in NAWM

Several quantitative MRI techniques have been developed and evaluated to characterize the diffuse abnormalities in NAWM. Recently, new image post-processing techniques have been proposed to separate different fiber populations in regions of crossing WM pathways, with improved performance of whole-brain fiber tractography [161]. These MRI techniques have consistently shown that damage in NAWM is present in all MS phenotypes, starting from the earliest stage of the disease to more widespread involvement in the progressive phases of MS [146]. Correlation between histopathology and MRI findings have brought new insights into the more specific processes involved in MS, such as inflammation, demyelination, axonal loss, and gliosis histopathology [146].

Small focal lesions visible only on MR images acquired at ultrahigh magnetic field strengths are correlated with microstructural abnormalities in NAWM. Axonal injury drives NAWM damage, which may be assessed by diffusion and MT close to WM lesions, while microglia activation is prevalent in the distant NAWM close to the cortex [146]. In addition, tissue damage in NAWM of MS patients was detected using high b-value diffusion MRI [162]. New multicomponent relaxometry MRI techniques have been developed and used to estimate the signal related to myelin water and volume, demonstrating that diffuse WM damage is an important pathological feature in PPMS that can be monitored using MRI [146].

MRI evaluation of MS-specific GM atrophy or damage

Automated intensity-based brain segmentation algorithms are used to segment the brain into GM, WM and CSF. The volumes of the segmented brain regions can then be measured, and GM atrophy or its diffuse damage can be assessed. Diffuse damages of the NAGM involve both cortical and subcortical structures, start from early MS stages, and worsen over time [145-147].

Depending on the lesion extent, cortical GM lesions are classified as leucocortical type I (with possible involvement of WM lesions), purely intracortical type II, or subpial type III and IV [163]. Intense cortical inflammation and demyelination is potentially related to adjacent meningeal inflammatory activity and may characterize early MS. The cortical inflammation continues in latter stages, such as SPMS, and is mainly produced by the glial activation [164].

According to results in MRI studies using relaxometry and susceptibility-weighted techniques, the iron excess deposited in GM at the beginning of MS precedes GM atrophy and its concentration increases with progression of MS [145, 146, 165].

The direct quantitative relationship between the regional and whole-brain GM damage and/or atrophy and the associated physical disability and cognitive impairment has been confirmed and assessed using volumetry [160], relaxometry, and MT MRI techniques [146, 147]. In addition, recent evidence suggests that subcortical gray matter volume loss and in particular of thalamus, contribute to determine cognitive dysfunctions in MS, mainly influencing the executive functioning [348].

Benign MS and MRI

Recent investigations using quantitative MRI-based techniques, such as magnetization transfer (MT) MRI and diffusion MRI, have contributed to the understanding of pathological manifestations of BMS. Whereas, conventional MRI techniques show similar lesion loads in BMS, RRMS and SPMS, newer quantitative techniques have demonstrated a lower degree of tissue damage and/or higher reparatory and compensatory mechanisms in BMS, as compared with other disease subtypes. These measurements show milder brain damage, preservations of neuroaxonal integrity, and lower brain atrophy in BMS patients [166]. Although significant GM atrophy comparable to RRMS was observed in BMS patients, topographical distribution of lesion may account for the lack of disability. MRI studies also showed that, despite similar lesion load in the spinal cord between BMS and SPMS, atrophy is more dominant in SPMS.

With the advance in disease-modifying agents that can reduce both relapse rates and development of new MRI lesions, prediction of prognosis is important to make treatment decisions. The need for routine monitoring of non-motor symptoms and CNS imaging using quantitative MRI techniques in BMS patients is crucial for the management of the disease.

10. MRI as an outcome measure in MS

MRI can be used to assess both disease evolution over time and response to therapy.

T2-weighted MRI in MS

WM lesions in the brain and spinal cord can be detected using T2-weighted MRI. They correspond to focal T2-hyperintense regions on MR images, which are non-specific with regard to pathology but reflect a wide range of abnormalities. Physicochemically, T2-hyperintensity is produced by high-water content regions, while pathophysiologically, they correspond to edema, demyelination, axonal loss, matrix disruption or gliosis [167, 168].

Especially during the early MS stages, MRI sensitivity can be too low to separate each region of active remyelination, and several regions may be visualized in one single T2-lesion on the MR image [169, 170]. Despite their non-specificity, the T2-hyperintensity-based measurements are convenient and valuable for the measurement of prior and new MS-related activity on serial MR images [171].

Conventional T2-weighted MRI is very insensitive to focal cortical GM lesions at all MS stages [121, 172].

T2 burden of disease

The brain T2 lesion volume in MS has a dynamic decreasing evolution over time. During this evolution, the lesion reaches a maximum volume in approximately 2 to 8 weeks, then shrinks over a period of a few weeks to months, leaves a smaller residual region of MRI signal abnormality, representing the "T2 footprint" of a prior acute neurologic event, and becomes stabilized after many months. The vast majority of chronic lesions remain constant over many years. The severe pathology in some focal MS lesions may be associated with repeated cumulative inflammatory events within the lesions. These cumulative inflammatory events may possibly interfere with gliosis and other factors to produce loss of remyelination. Once demyelinated, the axon becomes more vulnerable to injury, but these latter events cannot be detected using conventional MRI [121].

The total brain or spinal cord T2 lesion MRI volume is called the T2 burden of disease (BOD). Many factors, including measurement errors, waxing, waning and development of new lesions,

contribute to the considerable monthly T2 BOD variation in patients with active MS. Despite this, in properly sized clinical trials, T2 BOD represents an informative measure of subclinical MS pathology. The total number of T2 lesions in the brain or the spinal cord and the T2 BOD increase typically over time. However, T2 BOD evolution may be unpredictable in treated or untreated patients, and its increase over time may slow down, become stable, or even decrease [121].

The total number of T2 lesions and BOD may already be substantial at the time of diagnosis or during the earliest MS stages, reflecting an initially subclinical MS pathology. At the time of CIS, 2 to 5 cc average T2 lesion volumes are measured on demyelination, suggestive of abnormal MR images. These vary considerably, and range from 2.6 to 21 cc in RRMS, and from 3 to 28 cc in SPMS. The rates of annual increase in T2 brain lesion volume, which range from 5% to 15% in RRMS and from 3.6% to 9% in SPMS, may level off and even decrease after long MS duration, possibly due to the tissue collapse or atrophy [121].

T1 black holes

Approximately 5–20% of the brain lesions detected using T2-weighted MRI techniques, are hypointense on corresponding T1-weighted images. The chronic or non-enhancing hypointense areas on the T1-weighted images are referred to as T1 black holes. They correspond to WM regions having relatively more severe focal pathology, and become larger in regions corresponding to irreversible injured tissues. Considerable T1 black hole-to-T2 lesion volume ratio variation can be present in different patients, but, despite this, the extent of more severe T1 relative to T2 pathology can be measured [121].

T1 black holes correspond to brain regions with reduced myelin and axonal density, and relatively severe matrix disruption. The reduced MT ratios and N-acetylaspartate (NAA) levels detected by MRI and MRS, respectively, confirm the increased severity of the injury in the black hole brain regions [121].

T1 black holes may be chronic or acute, and are mainly produced by edema or tissue damage. Considerable or complete MRI signal recovery over time is detected in the case of acute black holes. Age assessment of a particular T1 black hole is not always possible, and a T1 black hole is classified as chronic if the detected T1-hypointensity fails to enhance after contrast agent

administration [121]. T1 black hole brain lesion volume increases with disease duration and severity in all MS phenotypes [121].

Gadolinium-enhanced MRI

Different MS phenotypes are characterized by different Gd-MRI enhancing brain lesions. Compared to RRMS, fewer and lower enhancing regions are detected in patients with PPMS and SPMS. Despite the inter-individual variability, on the conventional 5-10 minutes delayed post-contrast MR images, most lesions are initially small (sub-centimeter), have a homogeneous enhancement, and may later progress to a ring-shape. Other lesions are larger and ring-shaped from the beginning [173]. When dynamic Gd-enhanced MRI is used, the lesions have a centrifugal enhancement pattern progressing from the region of a central vein outward [174]. This pattern of outer enhancement over days presumably corresponds to changes from perivascular egress to organizing rims of the underlying inflammatory pathology [121].

The pattern of lesion enhancement changes with the severity of the pathology. More severely damaged tissue corresponds to ring-shaped enhancement regions, which are associated with macrophage and protein-specific infiltration, larger lesion size and longer enhancement duration. These regions are more likely to develop into T1 black holes. Sometimes, Gd concentrations as high as triple the normal ones are needed (i.e.: 0.3 mmol/kg) to detect BMS pathology (i.e. low grade inflammation), corresponding to smaller enhancing lesions [121].

The Gd-enhanced MRI signals detected in MS correspond to a spectrum consisting of very different pathologies ranging from simple (and partially reversible) interstitial edema to severe demyelination and matrix disruption. Other MS pathologies include axonal injury in early macroscopic inflammation, generating acute focal enhancing lesion on MR images and decreased NAA detected using proton MRS [121].

Brain and Spinal cord atrophy

Atrophy of the brain or spinal cord indicate irreversible and destructive pathology of MS, and has become an important biomarker of this disease [175]. BVL seems to be more important in patients with the progressive forms of MS compared with RRMS, but previous studies have suggested that it already occurs at the early stages of disease [176]. Brain atrophy is a potential

marker of irreversible tissue damage because it has a stronger correlation with clinical disability than T2-hyperintense lesion load or other MRI measures [177].

The structures involved in brain atrophy differ according to the MS phenotypes. Ventricular enlargements is more often observed in patients with RRMS, while cortical atrophy predominates in SPMS and PPMS [178]. Moreover, the impact of treatment on BVL is correlated with its impact on disease progression [179].

Brain atrophy may impact differently the WM and GM, with often a greater GM rather than WM loss during the early stages of disease [180, 181]. Previous studies have shown correlations between GM loss and neuropathology [182] and suggested that there is a stronger clinical correlation for GM compared to WM atrophy [183].

Spinal cord measurements are secondary outcome measures in MS treatment trials and are thought to result from secondary axonal and myelin loss, from fiber tract degeneration, as well as from regional tissue contraction from focal pathology [184-187].

MRI assessment of response to and efficacy of therapy in MS

MRI in conjunction with other clinical measures is used to estimate response to MS treatment. Many of the available MS therapeutic agents impact the inflammatory disease. Presence of focal inflammatory activity in the brain can be detected using Gd-enhanced MRI and MRI volumetry. These MRI techniques can, therefore, be used to assess treatment efficacy in MS. More important, Gd-enhancing MRI lesions are now accepted as a red flag for aggravated immune responses, primary outcome measures, and secondary outcome measures in dose escalation and safety, preliminary efficacy, and definitive phase I, II, and III, MS trials, respectively [121].

Advances in MRI techniques

Non-conventional MRI, based on hardware and software developments consisting of novel image acquisition techniques and post-processing methods, has brought new insights into the mechanisms underlying MS [145-147]. New MRI scanners, using higher magnetic field strengths of up to 7 T, offer the possibility of acquiring human brain images with a higher spatiotemporal resolution, revealing the best anatomical human brain information achieved *in vivo* [147, 188,

189]. Other hardware developments in MRI consist of more performant protocols used to acquire faster images with improved quality.

Non-conventional MRI techniques, such as volumetry [158], diffusion [155], MT [159], perfusion-weighted [190], spectroscopy [158], susceptibility-weighted [165], and relaxometry [191] techniques, have been used to assess the clinical and pathological manifestations of MS based on multiparametric quantitative analyses [159, 165]. In brains affected by MS, these techniques allow the evaluation of whole or regional brain atrophy [160], integrity of WM tracts in specific neuronal circuits [155], demyelination and remyelination [192], concentrations of metabolites [191], and changes in water or iron concentration [165, 193].

11.Other MS biomarkers

Since MRI lacks specificity during the initial stages of MS, other markers would be helpful for the diagnosis of MS. Biomarkers are also needed to evaluate MS prognosis, subtypes and stage, to monitor the response to treatment and to predict adverse effects [194]. Besides MRI, other types of markers of interest are body fluid biomarkers (blood and CSF) [194], and genetic biomarkers [195].

Body fluid biomarkers

The first body fluid biomarkers of MS that were discovered were humoral immunity biomarkers, which can be used for the early diagnosis of MS and for the identification of patients with CIS who are most likely to convert to CDMS [194]. CSF-specific IgG oligoclonal bands (OCBs) [196] have a high sensitivity for MS, but their poor specificity compared to other inflammatory diseases of the CNS confirms that other biomarkers are needed. CSF-specific IgG OCBs [197], but also CSF-specific IgM OCBs, are also strong prognosis markers for CIS conversion to CDMS [198, 199]. Moreover, CSF-restricted IgM-OCB-positive patients with RRMS have been reported to convert earlier from RRMS to SPMS [200].

IgG directed against the neurotropic viruses measles, rubella and varicella zoster are also specific markers used to diagnose MS [201, 202]. They also have a prognostic value in CIS conversion to CDMS [203], but their identification is technically challenging. IgG directed against

EBV have also been reported in the serum and CSF samples from MS patients, and are indicative of high inflammatory activity and early disease onset. HLA-DRB1*1501 and the EBV interact on the additive scale and each can trigger MS alone or based on their interaction [36, 204].

Previous studies [204] have also shown that the levels of CSF immunoglobulin Kappa free light chains [205, 206] produced by plasma cells are increased in patients with CIS or RRMS and are predictive for CIS conversion. Other potential diagnostic biomarkers of MS include antibodies directed against the glial inwardly rectifying potassium channel KIR4.1 [207, 208], which could be a brain tissue-specific antigenic target in MS, and antibodies against myelin oligodendrocyte glycoprotein (MOG) [209], which were detected in children with either MS or other demyelinating diseases. Another essential biomarker that is currently used is the serum anti-aquaporin 4 IgG (AQP4-IgG), which is highly specific for neuromyelitis optica and can be used to differentiate this pathophysiologically distinct entity from MS [210].

Besides humoral immunity biomarkers, inflammatory and immunological markers can also be used for the diagnosis of MS. The B-cell-attracting C–X–C motif chemokine 13 (CXCL13) has a high potential as prognostic biomarker for CIS conversion [211, 212], but its use as diagnostic marker is limited by its lack of specificity [211]. Chitinase-3-like protein 1 (CHI3L1) is another biomarker that can be used for the prognostic of CIS conversion to CDMS and for earlier progression to high EDSS scores in patients with RRMS [213, 214].

Another category of body fluid biomarkers (e.g. miR-20a-5p [215], miR-22-5p [216]) that is currently evaluated to support the diagnosis of MS are small noncoding microRNAs, which regulate gene expression and have the advantage of being blood-based. Noncoding RNAs (miR-223 and miR-15b) can also be used to discriminate PPMS from RRMs [217] and to evaluate the level of disease severity [218]. Other family of molecules that can be used as biomarkers of disease activity are cytokines, which are liberated by the inflammatory activity in active demyelinating lesions, and adhesion molecules, which correlate with higher disease activity [219-221]. Osteopontin is also a biomarker of immunological activation, which is a macrophage-derived phosphoprotein that is upregulated during an MS relapse [222].

Two biomarkers of neuroprotection can also be measured: the vascular endothelial growth factor (VEGF), which is at lower levels in patients with SPMS compared with RRMS [223], and

vitamin D, which display an inhibitory role in MS, also at a genetic level, by interacting with a vitamin D response element, close to the HLA-DRB1*1501 coding area [50].

Axonal damage markers, including CSF-restricted antibodies against neurofilaments, represent promising biomarkers for MS diagnosis and CIS to CDMS conversion [224]. Neurofilaments consisting of light chains (NfL) can also be detected in the blood after axonal damage [225]. In patients with RRMS, high CSF NfL levels at diagnosis are correlated with more severe disease in the long-term and with higher rates of conversion to SPMS [226]. In patients with SPMS or PPMS, NfL was a predictor of EDSS increase and neurofilaments consisting of heavy chains (NfH) were identified as a predictor of ongoing disability [227]. NAA is another axonal damage biomarker that can be used to differentiate SPMS from RRMS and CIS. Decreased NAA levels are correlated with increased EDSS scores, increased MRI lesions, and decreased brain volume [227]. Glial fibrillary acidic protein (GFAP) is a third axonal damage marker that has a predictive value for neurological disability and is associated with earlier progression of the disease [213]. A fourth biomarker of axonal damage is a cytoskeleton protein, the Tau protein, which has a high predictive value of conversion of CIS to CDMS [228]. The Tau protein is associated with microtubules, and elevated CSF tubulin and actin values have been observed in patients with progressive MS [229].

Biomarkers of blood-brain barrier disruption have also been identified, such as matrix mettalloproteinase proteins (MMPs) that are at higher levels in patients with RRMS [230], Ninjurin-1 that was found up-regulated in active demyelinating lesions [231], CSF sICAM-1 [232] and endothelin-1, endothelin type B receptor, and endothelin-converting enzyme-1 levels [233].

Two biomarkers of demyelination have been identified: MBP, which are found in the CSF of MS patients during relapses [234], and αB-Crystalline, which is considered as primary target molecule for T-cells in MS [235]. Three biomarkers of remyelination repair have also been identified: the neuronal cell adhesion molecule (N-CAM), which is usually reported immediately after MS relapse and correlates with clinical improvement [236], the Brain-Derived Neurotrophic factor (BDNF), which is at lower levels in SPMS patients compared to RRMS patients and also correlates with clinical improvement [237], and the Soluble Molecule Nogo-A, which constitutes a bad prognostic marker of axonal repair [238].

Two biomarkers of oxidative stress may also be evaluated: nitric oxide and its metabolites, which are correlated with higher disease progression rates in MS [239], and reactive oxygen species, which have been reported to be elevated in the CSF of MS patients [240].

Biomarkers of specific cellular populations have also been identified, such as mature B-cells and plasma-blasts that were found to accumulate in the CSF of RRMS patients [241], autoreactive memory T-cells [242], natural killer (NK) cells, which are at higher levels in RRMS patients in a remission phase [243], and Lipocalin 2, which is found at increased levels in MS patients [244].

Although the number of available treatments against RRMS is growing, only a few biomarkers are available for treatment response monitoring and adverse events prediction. In patients treated with a protein drug, anti-interferon neutralizing antibodies assays or bioactivity measurements can be used to determine if the response to treatment is attenuated and a switch to an alternative drug should be considered [245]. The identification of patients who can continue natalizumab with only a minor risk of PML and those who should be switched on other medications can be done via the measure of other biomarkers of therapeutical response: anti-JCV antibodies [246], L-selectin-expressing CD4+ T cells in peripheral mononuclear blood cells [247] or lipid-specific IgM oligoclonal bands in the CSF [248]. Moreover, cholesterol has been identified as a potential biomarker of membrane homeostasis that could be used to monitor the response to statin therapy [249].

Optical coherence tomography (OCT)

Retina through its unmyelinated nerve fiber layer (RNFL) represents the ideal model for the assessment of the axonal degeneration extent in MS. Spectral domain OCT (SD-OCT) offers the most rapid and accurate overall retinal and retinal layer thickness quantitative information at present [250-252]. Total macular volume (TMV), peripapillary RNFL, and ganglion cell and inner plexiform layer (GC-IPL) thicknesses measured using OCT represent the most easily employed and reliable indicators of neural changes in MS patients. Thinner peripapillary RNFL and GC-IPL in MS patients correspond to reduced visual acuity assessed by low and high contrast visual acuity, respectively [251].

SD-OCT is used to assess the correlation between the impaired color vision of MS patients and RNFL, papillomacular bundle, and TMV thicknesses. Time domain OCT (TD-OCT) is used to

measure the mean deviation, which is correlated with RNFL thickness in MS eyes with a history of optic neuritis [251].

The EDSS scale correlates disability in MS negatively with RNFL, ganglion cell and inner plexiform layer, and GCL and, in RRMS and SPMS, positively with macular thicknesses, while ambulatory ability correlates with total macular volume.

Genetic biomarkers

As discussed section 1 of the introduction, previous studies have shown that the main MS susceptibility loci are located within the MHC genomic region, at chromosomal position 6p21 that encodes the HLA cluster of genes [253-255], with the majority of studies demonstrating predisposition to sporadic MS associated with the HLA-A3, B7, DR2 extended haplotype [256]. Confirmation of a true genetic effect residing in the MHC comes from demonstration of linkage disequilibrium. Such studies of sporadic MS have not supported linkage to the MHC, however for familial MS, most studies support specific allelic association with HLA-DR2 in the MHC [257]s. However, the MHC locus probably represents less than half of the entire genetic etiology of MS, and possibly as little as one-sixth of the overall effect.

12.Human Leucocyte Antigen (HLA) and MS

The HLA complex

The HLA complex encompasses approximately 3,500 kilobases of DNA, contains at least 150 genes, and encodes mainly proteins that function in the immune system [262]. This small segment of the human genome has been associated with more than 100 diseases, such as MS, diabetes, rheumatoid arthritis, psoriasis, and asthma [263]. Among these diseases, MS was one of the first autoimmune conditions proven to be mediated through HLA associations [264].

The HLA region has a very high genetic diversity, and hundreds of alleles have been described for some of the genes [254, 265]. Genes from the HLA complex are categorized in the HLA class I, class II, and class III groups. There are three main HLA class I genes, known as HLA-A, HLA-B, and HLA-C, and six main HLA class II genes, known as HLA-DPA1, HLA-DPB1, HLA-

DQA1, HLA-DQB1, HLA-DRA, and HLA-DRB1 [266]. While HLA class I molecules are specifically recognized by CD8+ T-cells that activate a cytotoxic response, HLA class II genes encode molecules involved in the recognition and presentation of antigens to T-cells [266] and are primarily expressed by antigen-presenting cells such as macrophages, B-cells, and dendritic cells [267]. Each HLA class II molecule is a combination of an alpha chain and a beta chain, which are encoded by separate genes.

HLA and risk of developing MS

In the 1970s and 1980s, linkage analysis revealed different variations in the HLA region that affected the risk of developing MS [268-275]. More recent studies have demonstrated that the genetic risk for MS is dominated by a series of class II risk alleles, while protective signals are mainly driven by class I alleles [255, 276, 277].

Among class II HLA alleles, previous studies have shown that the HLA-DRB1 and HLA-DQB1 classes of genes have the highest effect on the increased risk of MS [253, 255, 278-284]. In almost all populations studied, MS was found to be associated with the following haplotype: HLA-DRB1*15:01-DRB5*01:01-DQA1*01:02-DQB1*06:02 [24, 255, 285, 286]. High HLA genetic burden, primarily mediated by HLA-DRB1*15:01, is associated with a younger age of onset, and drives the shrinkage of subcortical gray matter fraction and cortical white matter fraction in women and the ratio of cervical cord gray matter area to upper cervical cord area in men. [284]. The association of MS with this DR2 haplotype may result from a propensity of T-cells to produce increased amounts of lymphotoxic tumor necrosis factor, controlled by a polymorphic gene in this region [257, 287].

Among the class II HLA alleles, although the partially dominant HLA-DRB1*15:01 has been shown to have the strongest association with MS, especially in Caucasian populations [253, 255, 276, 279, 284, 286, 288-290], in patients from Southern Europe, the largely recessive HLA-DRB1*03:01 allele and the HLA-DRB1*04:01 allele were also overrepresented in MS patients [276, 291-294]. In addition, the DRB1*13:01 and HLA-DRB1*08:01 alleles were another class II HLA genes from the HLA-DRB1 group that showed an additive risk effect on MS [276, 295]. Among the genes from the HLA-DQB1 group, the HLA-DQB1*06:02 allele has been identified

as a major susceptibility allele [280, 292, 296-298]. The dominant HLA-DQB1*03:02 allele was also identified as risk factor for MS [276].

Although most class II HLA alleles increase the risk of MS, some of them, such as the HLA-DRB1*01, HLA-DRB1*07, HLA-DRB1*12, and HLA-DRB1*14 alleles, have a protective effect against this disease, while others, such as the HLA-DRB1*08, HLA-DRB1*10 and HLA-DRB1*16 alleles, do not appear to have a significant effect on the MS risk [253, 277-280, 284, 298].

Besides the individual effect of each class II HLA allele, their interactions may also have an impact on the risk of MS. A recent meta-analysis has shown a strong protective effect of the HLA-DQA1*01:01 allele only in the presence of the HLA-DRB1*15:01 allele, and an abolishment of the risk associated with the HLA-DQB1*03:02 allele in the presence of the HLA-DQB1*03:01 allele [276].

Although considered as secondary to the HLA class II contribution, HLA class I alleles could also be associated with reduced (HLA-A*02:01, HLA-B*44:02) [293, 299-302] or increased (HLA-A*03, HLA-B*07) susceptibility to MS [293, 301, 303, 304]. Among HLA class I alleles, HLA-A*02:01 was shown to drive the protective signal. A recent meta-analysis suggested that the lack of interactions between class I and class II alleles may indicate that the respective risk and protective effects of alleles within these two systems act through very different mechanisms [276].

HLA and disease features

A study using brain MRI has shown that HLA-B*44 positive patients, especially those who were HLA-DRB1*1501 negative, were characterized by lower brain atrophy and T2-lesion volume [305]. A more recent study has shown that HLA-DRB1*15:01 was not strongly associated with MRI-visible GM pathology [306], but a connection was found between HLA-DRB1*15:01 and the main epidemiological (gender, exposure to vitamin D) features of the disease [50, 307].

A previous study suggested that the HLA-DRB1*01 allele is detected in BMS patients and missing in malignant MS patients, suggesting that the DRB1*01 allele acts as a modifier of disease progression in MS [308] or an elevated IgG index with novel SNPs in HLA identified [309].

HLA-DRB1*1501 was associated to a reduction N-acetyl-aspartate (NAA) concentration within normal appearing white matter (NAWM), an increase in white matter T2 lesion volume, a reduction in normalized brain parenchymal volume and an impairment in cognitive functions measured by Paced Auditory Serial Addition Test (PASAT-3). In this study, HLA-DRB1*1501 was also associated to women and a younger mean age et disease onset [276]. Other studies confirmed this latter element [276, 289, 290]. This allele has been also associated to the presence of IgG oligoclonal bands in the CSF [276].

An association was also found between the HLA risk score and age at onset, which was driven by the HLA-DQB1*06:02–HLA-DRB1*15:01 haplotype and the HLA-DQA1*01:01–HLA-DRB1*01:01 haplotype [255, 276, 310].

HLA-DR2 haplotype was found to be associated with a higher risk of clinically definite MS (CDMS) 5 years following an optic neuritis (ON) [276].

In another study, a higher T2 lesion load was associated with the presence of HLA-DRB1*04 and HLA-B*07. Higher T1 lesion load was associated with HLA-B*07 and DRB1*12. Moreover, brain parenchymal fraction (BPF) was predicted by the presence of DRB1*12 [276].

Nevertheless, other studies failed to confirm results about haplotype HLA-DR2, like a cohort of MS patients followed for 30 years or the Swedish and UK large datasets [276]. Likewise, unfavorable outcomes have been reported with regards to haplotype HLA-DR1, which were not confirmed by others [276].

HLA in MS treatment

Although the role of HLA in clinical response to immunotherapy is not completely known, Hoffmann et al. [311] identified genetic factors determining the development of antibodies to interferon-beta, which is a protein-based disease-modifying agent for the treatment of MS. The HLA-DRB1*04:01 and HLA-DRB1*04:08 (odds ratio: 5.15) alleles, but not other HLA alleles, were strongly associated with the development of antibodies to interferon-beta, which is a problem because these antibodies may neutralize the biological effects of the protein drug [311, 312]. The associated HLA-DRB1*04 alleles differ from non-associated HLA-DRB1*04 alleles by a glycine-to-valine substitution in position 86 of the epitope-binding alpha-helix of the HLA molecule. The

peptide-binding motif of HLA-DRB1*04:01 and HLA-DRB1*04:08 might promote binding and presentation of an immunogenic peptide, which may eventually break T-cell tolerance and facilitate antibody development to interferon-beta [311].

Can HLA genotypes be markers of Multiple Sclerosis prognosis?

Concept, Aims and Initial Research

There are a wide variety of available treatments in MS (first and second line) and the choice of first treatment and switch strategy is usually influenced by the severity of the disease. Prognostic biomarkers upon diagnosis that could inform on future progression and severity of MS would be a useful tool to guide treatment choice. Many doctors choose "light" drugs initially for all patients, or do not switch early enough to more potent drugs. If they had more information on how severe MS progression might be for some individuals, they might be more convinced to treat the early disease more aggressively thus leading to better patient outcomes.

Therefore, identification of a biomarker with prognostic value is an unmet need in MS. The aim of this thesis was to establish how different HLA genotypes correlate to MS severity and disease progression and whether they could be used as additional disease biomarkers.

In order to identify the most common HLA genotypes related to MS risk I undertook an extensive literature search detailed within the introduction to this thesis. Then based on results of this search and discussion with other MS experts and physiotherapists I identified the most relevant clinical tools to assess MS severity and prognosis, as well as the most likely targets for prognostic biomarkers.

The study was supported via the Erasme MS fund that I set up and managed and was based on various grants.

Summary of studies

Studies were undertaken using a study population derived from my patient cohort, based at Erasme University Hospital, Brussels (Belgium). Initial work was undertaken to establish that the HLA genotype profile of this group was comparable to MS cohorts described in the literature [313][Lysandropoulos 2017a]. This was then followed by a pilot study which attempted to identify HLA biomarkers for disease progression and severity within this population [314]

[Lysandropoulos 2017b], and a subsequent extension study in a larger cohort with a longer follow-up [315] [Lysandropoulos 2019]. In addition, since initially my patients were scanned in either 1.5 and 3.0 T 1 MRIs, I also undertook work to confirm that brain MRIs preformed at these different magnetic field strengths were comparable [193]. [Lysandropoulos 2016 – full paper is appended to this thesis].

Protocol for MRI analysis

In order to undertake the MRI analysis for this study I identified, and established links with, a spin off company associated with Antwerp University and worked together with them to set up the MRI brain volume analysis (MSmetrix)[316, 317]. I was a pioneer of this technique in my hospital and one of the first to undertake such analysis in Belgium.

Patients were either scanned on Philips Healthcare MR systems (Philips, Best, The Netherlands) Intera (1.5T) or and Achieva (3T). On each scanner, a clinical MRI protocol was acquired, including a transverse 3D FLAIR (Fluid Attenuated Inversion Recovery) sequence and a sagittal 3D T1-weighted turbo field echo sequence. Exact scan protocol parameters are detailed in my previous paper appended to this thesis [193]. Scan images were analyzed with MSmetrix, the newly developed method to measure brain volume changes for MS patients. MSmetrix is a CE approved automatic method for segmentation of GM, WM, cerebrospinal fluid (CSF) and white matter lesions based on unsupervised classification, as well as for a longitudinal atrophy measurement of whole brain or parenchymal volume (PV) and GM [316, 317]. Full details of the MSmatrix process steps are detailed in my previous paper appended to this thesis [193].

Once MRI scans were performed, I collected them from the patient's electronic files and uploaded them into the MSmatrix system for analysis, before assessing the results of this analysis for validity and performed group analysis.

Study 1 – Establishing HLA genotype profile in the study population

[Lysandropoulos 2017a]

The aim of this study was to compare the incidence of specific HLA alleles and haplotypes in a cohort of MS patients and a cohort of healthy controls in Belgium, and to establish if the HLA genotype profile was comparable with cohorts in the wider literature.

Having initially diagnosed MS in patients attending my clinic at the Erasme University Hospital, Brussels (Belgium), I followed 119 consecutive MS patients for the purposes of this study. MS diagnosis was established according to the criteria proposed by McDonald et al. [Polman 2010]. Patients with relapsing/remitting MS (RRMS), primary progressive MS (PPMS) or secondary progressive MS (SPMS) were included. As control population, I used data from 124 anonymized consecutive healthy organ donors at the Erasme University Hospital for whom the HLA typing of studied loci was available.

Having explained the study and confirmed consent, I took blood samples from all participants and HLA typing was performed on DNA extracted from peripheral blood mononuclear cells, by low- to intermediate-resolution polymerase chain reaction using sequence specific oligonucleotides (PCR-SSO). Reverse dot blotting was carried out on a nylon membrane containing immobilized sequence-specific oligonucleotide probes used for the typing of HLA class I (HLA-A*02, HLA-B*07, HLAB*44) and HLA class II (HLA-DRB1*15, HLA-DRB1*04, HLA-DRB1*07, HLA-DQB1*06) alleles (INNO-LiPA®, Fujirebio).

Statisical analysis is detailed in the full paper appended to this chapter

The frequencies of HLA class I and class II alleles in patients with MS and in the control population are detailed in the full paper appended to this thesis. My results confirmed that HLA class II alleles are associated with MS risk in this population. The HLA-DRB1*15 allele and the DRB1*15-DQB1*06 haplotype were clearly associated with the disease, which is in line with the results of broader studies conducted in Europe, and especially in Caucasian populations [refs in paper].

Other alleles were underrepresented in MS patients compared with healthy controls. The HLADRB1*07 allele had a protective role against MS development, confirming the results of previous studies [refs in paper] and, although not significant, the HLA-B*44 allele tended to be less frequent in MS patients, which is also in line with the results of previous studies [refs in paper]. Finally, the HLADRB1*04 allele also tended to be less frequent in MS patients when compared with controls.

These results confirmed that the HLA genotype profile of the study population was comparable with that of other MS cohorts described in the literature.

Human Leukocyte Antigen (HLA) Class I and II typing in Belgian Multiple Sclerosis Patients

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Abstract

This is one of the first study to compare frequencies of different Human Leucocyte Antigen (HLA) Class I and II alleles and haplotype HLA-DRB1*15-DQB1*06 in a cohort of 119 patients with multiple sclerosis (MS) and a cohort of 124 healthy controls in Belgium. An association with MS was found for the HLA-DRB1*15 (odds ratio [OR] = 2.60 [95% confidence interval (CI): 1.51–4.50]) and HLA-DQB1*06 (OR = 1.97 [95% CI: 1.18–3.29]) alleles, and for haplotype DRB1*15-DQB1*06 (OR = 2.63 [95% CI: 1.52–4.56]). The HLA-B*07 allele also tended to be more frequent in MS patients (OR = 1.46 [95% CI: 0.80–2.65]), and was more frequent among MS patients with than in those without the HLA-DRB1*15 allele (26/54 [48.1%] versus 6/65 [9.2%]; p-value <0.0001). Other alleles were underrepresented in MS patients, such as the HLA-DRB1*07 (OR = 0.39 [95% CI: 0.21–0.73]) and HLA-A*02 (OR = 0.56 [95% CI: 0.34–0.94]) alleles, showing a protective role against the disease. The HLA-B*44 (OR = 0.58 [95% CI: 0.31–1.09]) and HLA-DRB1*04 (OR = 0.75 [95% CI: 0.42–1.34]) alleles tended to be less frequent in MS patients. Altogether, the significant results observed in this population are in

line with those from other countries and confirm that propensity to MS can be due to a complex presence of various HLA class I and class II alleles.

Keywords: Human Leucocyte Antigen, multiple sclerosis, Belgium

Introduction

Multiple sclerosis (MS) is a common autoimmune neurological chronic disease, mostly affecting young adults [318]. The causative agents for MS are still unclear, but it is well established that genetic predispositions combined with environmental factors play a central role in the development of the disease [318-323]. Even though a recent study analysing sample from almost 30,000 MS patients has identified 110 MS risk variants at 103 discrete loci outside the major histocompatibility complex (MHC) [324], the genetic susceptibility to MS is mainly determined by Human Leucocyte Antigens (HLA), a cluster of genes located within the MHC on the short arm of chromosome 6 at p21.3 [253, 320]. In almost all populations studied, MS was found to be associated with HLA class II risk alleles [253, 277-283, 320]: mainly the DR15 specificity and its individual alleles (DRB1*15:01-DQA1*01:02-DQB1*06:02) [24, 285, 286, 320], but also other class II risk alleles (HLA-DRB1*13:03, HLA-DRB1*03:01, HLA-DRB1*08:01 and HLA-DQB1*03:02) [325]. Although considered as secondary to the DR15 contribution, HLA class I alleles could also be associated with reduced (HLA-A*02:01, HLA-B*44:02, HLA-B*38:01 and HLA-B*55:01) or increased (HLA-A*03, HLA-B*07) susceptibility to MS [301, 303, 325]. Among class II risk alleles and class I protective alleles, a recent study analysing data from almost 17,500 MS patients has identified two interactions that modulated the risk of MS: HLA-DQA1*01:01–HLA-DRB1*15:01 and HLA-DQB1*03:01–HLA-DQB1*03:02 [325]. Here, we present the results of a study that compared the incidence of specific HLA alleles and haplotypes in a cohort of MS patients and a cohort of healthy controls in Belgium, where the disease affects about 10,000 people.

Methods

Study population

This study included 119 consecutive patients with MS who were followed at the Hôpital Erasme, Faculty of Medicine, Université Libre de Bruxelles, Belgium. All patients were diagnosed with MS according to the criteria proposed by McDonald et al. [12]. Patients with either relapsing/remitting MS (RRMS), primary progressive MS (PPMS) or secondary progressive MS (SPMS) were included in the study. As control population, we used data from 124 anonymised consecutive healthy organ donors at the Hôpital Erasme for whom the HLA typing of studied loci was available. The study was approved by the local ethics committee (Approval N°: P2013/098/B406201316929).

HLA typing

HLA typing was performed on DNA extracted from peripheral blood mononuclear cells, by low- to intermediate-resolution polymerase chain reaction using sequence-specific oligonucleotides (PCR-SSO). Reverse dot-blotting was carried out on a nylon membrane containing immobilised sequence-specific oligonucleotide probes used for the typing of HLA class I (HLA-A*02, HLA-B*07, HLA-B*44) and HLA class II (HLA-DRB1*15, HLA-DRB1*04, HLA-DRB1*07, HLA-DQB1*06) alleles (INNO-LiPA®, Fujirebio).

Statistical methods

The allele carrier frequencies were determined by direct counting and were calculated as the number of participants carrying the specific allele (either at homozygous or heterozygous status) divided by the total number of participants. Comparisons of frequencies for the HLA-DRB1*15, HLA-DRB1*04, HLA-DRB1*07, HLA-A*02, HLA-B*44, HLA-B*07 and HLA-DQB1*06 alleles, and the DRB1*15-DQB1*06 haplotype between the MS patients and the controls were carried out using Chi-square test. Associations of particular alleles with MS were expressed as

odds ratios (ORs) with 95% confidence intervals (95% CIs). For every comparison, p-values <0.05 were considered to be statistically significant. All p-values were based on 2-tailed tests. Bonferroni's method was used for correction by multiplying the p-value obtained by the total number of alleles considered at each locus. Statistical analysis was performed by using STATA 12.

Results

Characteristics of participants

The demographic characteristics of the 119 MS patients and 124 healthy controls were comparable. This study included 79 women and 40 men with MS. Among these patients, 105 had RRMS, 8 had SPMS, and 6 had PPMS. The mean age of the MS patients was 44 years.

Frequency of HLA alleles

The frequencies of HLA class I and class II alleles in patients with MS and in the control population are shown in Table 1. The HLA-DRB1*15 allele (OR = 2.60 [95% CI: 1.51–4.50]), the HLA-DQB1*06 allele (OR = 1.97 [95% CI: 1.18–3.29]) and the haplotype DRB1*15-DQB1*06 (OR = 2.63 [95% CI: 1.52–4.56]) were more frequent in patients with MS. Although not statistically significant, the HLA-B*07 allele also tended to be more frequent in MS patients (OR = 1.46 [95% CI: 0.80–2.65]). Moreover, the HLA-B*07 allele was more frequent among MS patients with the HLA-DRB1*15 allele than in patients without the HLA-DRB1*15 allele (26/54 [48.1%] versus 6/65 [9.2%]; p-value <0.0001).

Other alleles were found to be underrepresented in MS patients, like the HLA-DRB1*07 (OR = 0.39 [95% CI: 0.21–0.73]) and HLA-A*02 (OR = 0.56 [95% CI: 0.34–0.94]) alleles. Although no statistically significant effect of the HLA-B*44 (OR = 0.58 [95% CI: 0.31–1.09]) and HLA-DRB1*04 (OR = 0.75 [95% CI: 0.42-1.34]) alleles was observed, both alleles tended to be less frequent in MS patients when compared to the control group. Within the cohort of MS patients, no

statistically significant difference was detected in terms of frequencies of HLA-A*02 (20/54 [37.0%] versus 22/65 [33.8%]; p-value = 0.72) and HLA-B*44 (6/54 [11.1%] versus 14/65 [21.5%]; p-value = 0.13) alleles between MS patients with and without the HLA-DRB1*15 allele.

Discussion

Different alleles and combination of alleles have been identified in MS patients across the world, stressing the importance of the genetic background in this disease. The present study was one of the first to investigate HLA class I and class II alleles and their effect on MS susceptibility in Belgium. Two previous studies conducted in the 90's showed that the DR2 haplotype (now preferentially covered by HLA-DR15 and HLA-DR16 serotype group, primarily recognizes gene products of the HLA-DRB1*15 and HLA-DRB1*16 allele groups) (RR = 3.71), the DRBl*1501 allele (RR=2.47; p=0.0035), and the DRBl*1501-DQA1*0102 haplotype (RR=2.65; p=0.0013) were positively associated with MS in Belgium [326, 327]. A more recent study conducted in 468 Belgian patients with MS and 482 controls confirmed the association of the DRB1*1501 allele with MS (OR= 2.67 [95% CI: 2.11–3.38]; $p=5*10^{-21}$) [328]. The significant results observed in the present study are in line with these previous findings and with other studies conducted in other countries, and confirm the complex role of HLA class I and II genes.

Our results confirmed that HLA class II alleles are associated with MS risk. The HLA-DRB1*15 allele and the DRB1*15-DQB1*06 haplotype were clearly associated with the disease, which is in line with the results of broader studies conducted in Europe, and especially in Caucasian populations [279, 280, 282, 310, 320, 326, 328-332]. A positive association was also found for the HLA-DQB1*06 allele, in accordance with previous studies conducted in 282 patients with MS in Slovakia (OR = 1.99 [95% CI: 1.38–2.87]) [280], in 94 MS patients compared with 98 healthy patients in Italy (DQB1*06:02; relative risk = 3.32) [297], and in 149 patients with MS in Spain (DQB1*06:02; OR = 3.1 [95% CI: 1.9–5.2]) [298]. Previous association studies performed in African Americans to better localize the HLA gene responsible for MS susceptibility showed that the DRB1*1501 disease associations were independent of DQB1*0602, indicating a primary role

for the DRB1 locus in MS and a potential modulating influence of the DQB1 locus on clinical outcome [286]. In our study, the frequency of HLA-B*07 allele tended to be higher in MS patients, although the association was not significant, and this allele was more frequent among MS patients with the HLA-DRB1*15 allele than in patients without the HLA-DRB1*15 allele. This observation is in line with a previous study that found an increased frequencies of HLA-B*0702 in MS patients (secondary to DRB1*15, DQB1*06) [293]. The association of MHC class I alleles with susceptibility to MS supports a possible role of certain immune cell types, such as $CD8^+$ T cells, in the onset of MS [301]. In a previous study, $CD8^+$ T cells recognizing Epstein Barr virus-derived peptides (in the context of HLA-A*02 or HLA-B*07) tended to be more frequently observed in MS patients than in controls [333].

In our study, other alleles were underrepresented in MS patients compared with the healthy controls. The HLA-DRB1*07 allele had a protective role against MS development, confirming the results of previous studies conducted in 282 patients with MS in Slovakia (OR = 0.53 [95% CI: 0.34–0.83]) [280], in 1,784 patients from Scandinavia [277], and a previous meta-analysis in Caucasians [279]. The HLA class I HLA-A*02 allele was also underrepresented in patients with MS, and these results remind of previous studies conducted in 532 patients with MS in the US (OR = 0.67 [95% CI: 0.55–0.81) [301], in 1,273 Italian MS patients (OR = 0.61 [95% CI: 0.51–0.72]) [299], in Portuguese MS patients [302], in 1,084 Swedish patients (OR = 0.63; $p = 7*10^{-12}$) [300], and in 200 Swedish patients (OR = 0.52; p = 0.0015) [293]. Although the effect was not significant, the HLA-B*44 allele tended to be less frequent in MS patients, which is in line with a previous study showing that this HLA class I allele reduced the susceptibility to MS (OR = 0.62 [95% CI: 0.48–0.80]) [301]. Finally, the HLA-DRB1*04 allele also tended to be less frequent in MS patients when compared to controls, which confirmed the results of a study previously conducted in Spain [298].

Altogether, these results confirm that propensity to MS can be due to a complex presence of various HLA class I and class II alleles. The frequency of different HLA alleles playing a role in MS observed in this study conducted in Belgian patients, shows that while some HLA class I and class II alleles are associated with MS risk, especially the HLA-DRB1*15 and HLA-DQB1*06 alleles and the haplotype DRB1*15-DQB1*06, other confer protection against MS, especially the HLA-A*02 and HLA-DRB1*07 alleles.

Allele	MS group		Control group		OR (95% CI)	p-value	p-value$_{corr}$
	N	n (%)	N	n (%)			
A*02	119	42 (35.3)	124	61 (49.2)	0.56 (0.34–	**0.03**	**0.03**
B*07	119	32 (26.9)	124	25 (20.2)	1.46 (0.80–	0.22	
B*044	119	20 (16.8)	124	32 (28.8)	0.58 (0.31–	0.09	
DRB1*15	119	54 (45.4)	124	30 (24.2)	2.60 (1.51–	**0.001**	**0.003**
DRB1*04	119	28 (23.5)	124	36 (29.0)	0.75 (0.42–	0.33	
DRB1*07	119	18 (15.1)	124	39 (31.5)	0.39 (0.21–	**0.003**	**0.009**
DQB1*06	119	73 (61.3)	121	54 (44.6)	1.97 (1.18–	**0.009**	**0.009**
DRB1*15-DQB1*06	119	53 (44.54)	124	29 (23.39)	2.63 (1.52–	**0.001**	

Table 1. *Phenotypic frequencies of HLA alleles in patients with MS and controls*

HLA, human leucocyte antigen; MS, multiple sclerosis; N, number of participants tested in each group; n (%), number (percentage) of participants expressing the allele; OR, odds ratio; CI, confidence interval. Bonferroni's method was used for correction for multiple comparisons (p-value$_{corr}$). ***Bold*** *indicates that there is a statistically significant difference at the p <0.05 level.*

Study 2 – Pilot study to establish if HLA genotype is a marker of MS prognosis [Lysandropoulos 2017b]

The aim of this study was to further investigate a possible association of HLA genotype with disease status and progression in MS, by using comprehensive and sensitive clinical and MRI parameters to measure disease effects. This was the first study applying various clinical (physical and cognitive) scales and using a validated scanner-independent software to extract whole brain atrophy, lesion volume changes, and the number of new lesions between two time points from MRI measurements.

I evaluated the HLA genotype of a total of 118 MS patients (79 females, 39 males). The demographic characteristics of the patients are presented in the full paper. No treatment change occurred during the observation period. I also assessed patients' MS status at two time points in a 2-year interval, based on clinical scores including the
EDSS, the MSSS (MS Severity Scale), the T25FW (Timed-25-Foot-Walk), the 9-HPT (9-Hole Peg Test), the SDMT (Symbol Digit Modalities Test), the BVMT (Brief Visual Memory Test) and the CVLT-II (CaliforniaVerbal Learning Test-II).) and MRI evaluations. The majority of patients were scanned on a Philips Healthcare MR system (Achieva or Intera), but field strengths differed between scans, i.e. 1.5 T or 3 T, however my previous work has confirmed that brain MRIs preformed at these different magnetic field strengths were comparable [193].
During the 2-year follow-up period, disease evolution was described by the extracted NEDA-3 (no evidence of disease activity based on absence of relapses, EDSS progression, and new T2 or gadolinium-enhancing lesions), the 24-week confirmed SDMT progression, and the EDSS plus.
Quantitative brain MRI values were also obtained for whole brain atrophy, FLAIR lesion volume change and number of new lesions using MSmetrix. Predefined HLA patient groups were compared for disease status and progression.

Statisical analysis is detailed in the full paper appended to this thesis.

Full details of the effects of HLA genotypes on disease status and progression are described in the

full paper appended to this thesis. The HLA-A*02 allele was associated with better outcomes in terms of MSSS, EDSS and new lesion count (Welch test p-value<0.05). The HLA-B*07 and HLA-B*44 alleles showed a global negative effect on disease status, although none of the measurements reached significance. Results for the HLA-DRB1*15, HLA-DQB1*06 and HLA-B*08 alleles were inconclusive, probably hampered by the limited number of patients. Therefore, larger MS cohorts with more extended follow-up and high-resolution HLA typing of multiple loci are needed.

HLA genotype as a marker of Multiple Sclerosis prognosis: a pilot study

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Abstract

Objective: The objective of this study is to examine the role of HLA on disease status and progression of Multiple Sclerosis (MS).

Method: A total of 118 MS patients (79 females, 39 males) underwent HLA typing. Patient status was assessed at two time points (2013 - 2015) based on clinical scores (including EDSS, SDMT, BVMT, 9 HPT, T25FW, CVLT) and evaluation of magnetic resonance imaging (MRI). Quantitative brain MRI values were obtained for whole brain atrophy, FLAIR lesion volume change and number of new lesions using MSmetrix. A statistical analysis was performed to compare the patient status of predefined HLA groups, focusing on HLA-A*02, B*08, B*07, B*44, DRB1*15 and DQB1*06. Global assessment was achieved by an overall t-statistic and assessment per measurement using a Welch test and/or Mann Whitney U-test. The effect of multiple covariates, incl. age, gender, disease duration as well as scan parameters, was also evaluated using a regression analysis.

Results: HLA-A*02 indicates a global protective effect with significant values for MSSS, EDSS and new lesion count (Welch test p-value <0.05). HLA-B*07 and B*44 show a global negative effect on disease status of Multiple Sclerosis, although none of the measurements reached

significance (p-value <0.05) without covariates. Results for HLA-DRB1*15, DQB1*06 and B*08 are inconclusive. The influence of the cofounding variables on the statistical analysis was limited.

Introduction

There is emerging evidence that HLA is linked to Multiple Sclerosis (MS) [282, 301, 333]. Studies have majorly focused on HLA analysis between normal controls and patients with MS, rather than on the direct influence on MS progression. In this study, we focus explicitly on the relationship of HLA and disease progression by evaluating MS patients over time (two-year interval) in terms of clinical scores (both cognitive and physically) as well as MRI measurements.

Materials and Methods

Data set

The study includes 118 MS patients of which 79 females (67%) and 39 males (33%). The cohort consists of 104 RR-MS, 8 SP-MS and 6 PP-MS, with an average age of 43.2 ± 11.2 years (min 19.6 years - max 66.9 years), average disease duration of 11.8 ± 7.1 years (min 0.7 years - max 38.8 years) and average EDSS score of 2.4 ± 1.4 years (min 1 - max 6.5). 93% of the MS patients is treated of which 44.5% using first line medication and 55.5% second line medication. An overview of the patient information is also provided in Table 1.

This study was approved by our institutional review board and written informed consent was obtained from all participants (reference P2013/098 / B406201316929).

118 MS patients	
Gender	39 males (33%) - 79 females (67%)
MS types	104 RR-MS, 8 SP-MS, 6 PP-MS
Age	43.2 ± 11.2 years (min 19.6 years - max 66.9 years)
EDSS scores	2.4 ± 1.4 years (min 1 - max 6.5)
Disease duration	11.8 ± 7.1 years (min 0.7 years - max 38.8 years)
Treatment	93% of the MS patients of which 44.5% first line medication and 55.5% second line medication

Table 1: Description of the patient population.

HLA analysis is performed by blood draws. Patients are evaluated at two time points with an interval of 2 years (resp. 2013 and 2015) based on both clinical scores and MRI scans. An overview is provided in Table 2. Clinical scores include SDMT, EDSS, MSSS, 9HPT (left and right), T25W, BVMT and CVLT. Furthermore, NEDA-3, SDMT progression and EDSS progression are extracted to describe the disease evolution. Brain MRI scans are available from clinical routine and include a FLAIR (Fluid Attenuated Inversion Recovery) sequence and a T1-weighted turbo field echo sequence (pre- or post-gadolinium injection). The majority of the patients is scanned on a Philips Healthcare MR system (Achieva or Intera). Also field strengths differ between scans, i.e. 1.5T or 3T. One scan showed insufficient image quality and is therefore excluded from the analysis.

General information	■ Age ■ Gender ■ MS type ■ Disease duration ■ Type of medication (first line vs second line)
Clinical scores (status)	● CVLT (2015) ● BVMT (2015) ● EDSS (2013, 2015) ● SDMT (2013, 2015) ● T25FW (2013, 2015) ● 9 HPT R (2013, 2015) and 9 HPT L (2013, 2015) ● MSSS
Clinical scores (progression)	● EDSSplus ● SDMT progression ● NEDA 3
MRI scores (progression)	● Whole brain atrophy ● Lesion volume change ● Number of new lesion count

Table 2: Information available for all patients in the cohort.

Clinical scores glossary [127, 334-340]

EDSS: Expanded Disability Status Scale

SDMT: Symbol Digit Modalities Test

MSSS: MS Severity Scale

9HPT: 9-Hole Peg Test

T25W: Timed-25-Foot-Walk

BVMT: Brief Visual Memory Test

CVLT-II: California Verbal Learning Test-II

NEDA-3: None Evidence of Disease Activity-3 (relapses, EDSS progression, T2/Gd+ MRI lesions)

EDSS plus: EDSS+SDMT+T25W

Quantitative MRI measurements

All MRI scans are analyzed using MSmetrix [316, 317], a scanner-independent software developed by icometrix, to extract whole brain atrophy, lesion volume changes and the number of new lesions between both time points.

In a first step, the algorithm iterates until convergence between (1) segmentation of healthy tissue (WM, GM, CSF) on T1 lesion filled images, based on (2) FLAIR lesion segmentation estimated using knowledge of the healthy tissue segmentations (from (1)). In a second step, the baseline and follow-up scan are analysed simultaneously by a Jacobian integration approach using the segmentations of step 1 as input. This longitudinal step is performed to guarantee consistency between the segmentations of the individual time points, resulting in measurements for brain atrophy and FLAIR lesion change.

MSmetrix received market approval in the EU (CE) and other countries including Canada, Brazil and Australia. The corresponding clinical report for the US market is named icobrain, for which icometrix has received 510(k) clearance from the FDA.

Statistics analysis

General

The relation between HLA and MS progression is evaluated by the statistical difference in clinical scores and quantitative MRI measurements of MS patients with different HLAs. As 74 different HLAs are identified within the cohort of 118 MS patients, a combined analysis of all HLAs would result in an underdetermined problem. Hence, predefined subgroups of HLAs are analyzed, extracted based on the available literature [282, 301, 333] and focuses on HLAs A*02, B*07, B*44, B*08, DRBA*15, DQB1*06, and combinations thereof. The different subgroups are provided in Table 3. The statistical analysis evaluates thus differences between the clinical scores and MRI measurements between each HLA group and its counterpart. The influence of different covariates is assessed such as gender, age, disease duration, MS type and treatment (first vs. second line). The statistical analysis of the quantitative MRI measurements also validates the effect of different scanner types, 1.5T vs a 3T scanner and/or pre- vs post-contrast scans. In other words,

the statistical analysis will consist of three major steps: (1) an overall assessment of the relation between Multiple Sclerosis and a specific HLA, (2) an individual assessment of the different clinical scores and MRI measurements describing the disease status and progression with respect to the predefined HLA groups, (3) the influence of diverse covariates (cofounding variables).

DRB1*15+ vs. DRB1*15-
B*07+ vs. B*07- DRB1*15+/B*07+ vs. DRB1*15+/B*07- DRB1*15-/B*07+ vs. DRB1*15-/B*07-
A*02+ vs. A*02- DRB1*15+/A*02+ vs. DRB1*15+/A*02- DRB1*15-/A*02+ vs. DRB1*15-/A*02+ A*02-/B*07+ vs. all others
DQB1*06+ vs DQB1*06- DQB1*06+ /DRB1*15 vs. all others
B*44+ vs. B*44-
B*08+ vs. B*08-

Table 3: Predefined HLA groups under study. + indicates "all patients with HLA value", - indicates "all patients without HLA value".

Global assessment of the relation between HLA and MS progression

The global effect of an HLA on disease status and progression will be evaluated by calculating an overall t-score over all clinical scores and MRI measurements of the group of subjects with HLA vs. without. This t-score indicates the overall effect size and direction of change of the disease progression/status. As such it can be interpreted as the protectiveness or negative effect of the HLA on Multiple Sclerosis.

The overall t-score is computed as the average of the t-statistics for each clinical and/or MRI measurement individually. In particular:

- For each clinical score/MRI measurement, the t-statistic is calculated as in the unpaired Welch test, i.e. two-sample t-test with assumption of unequal variance.
- If variables (i.e. clinical score / MRI measurement) are evaluated multiple times, the multiple t-scores for this variable will be averaged. As such, we avoid an overrepresentation of the variable in the global t-score overall variables. In other words, the variables EDSS, SDMT, T25FW are each evaluated at two time points, resp. 2013 and 2015. Hence, per variable, we will average both available t-scores. Furthermore, 4 t-statistics will be averaged for 9 HPT, respectively of two time points (2013, 2015) and for the left- and right-handed test.
- For some clinical scores an increasing effect is beneficial (e.g. SDMT), while for other clinical scores a decrease shows a positive effect (e.g. 9 HPT). Therefore, the corresponding t-scores are multiplied by 1 or -1 to make sure a positive t-score always indicates protectiveness of the first group.
- Finally, the corrected t-scores for all variables are averaged. A positive score can be interpreted as a protective effect on Multiple Sclerosis.

T-scores are an indication of the effect size of HLA for each variable. The t-score / effect size takes into account the variability of a variable. Further, they are dimensionless and even more, per HLA, the t-scores for all variables originate from the same samples (subjects). This justifies the comparison/averaging of t-scores to evaluate the global effect of a HLA.

Further interpretation of the t-score magnitudes (significance testing) requires knowledge on the underlying distributions. For sufficiently large sample sizes, normality of the arithmetic mean can, however, be assumed for all continuous and ordinal variables according to the central limit theorem.

For the extracted binary variables (NEDA-3, EDSS progression and SDMT progression), a similar approach is taken using the chi-squared score.

Assessment per clinical score/MRI measurements

A comparison between HLA groups is also performed at the level of the individual clinical scores and MRI measurements. Thereto, all binary variables (NEDA 3, EDSS plus, SDMT progression) will be evaluated using a Chi-squared t-test. The continuous and ordinal variables are analysed using a two-sample Welch test (two-sample t-test with unequal variance) under the normality assumption based on the central limit theorem. However, the underlying assumption of normality is explicitly validated using a Shapiro-Wilk test. A non-parametric Mann-Whitney U-test is performed additionally to the Welch test for group comparison in case the normality assumption is not fulfilled.

Cofounding variables

The heterogeneity of the cohort might influence the statistical tests. For each comparison, the groups are evaluated to be age-matched, gender-matched and matched in terms of disease duration. Additionally, their influence on the statistical tests is evaluated using these variables as covariates. The effect of MS type and treatment line (first line, second line or no treatment) is also evaluated.

The MRI scans are acquired on different scanners (Medical Philips Achieva and Medical Philips Intera), using different protocols (pre- vs post-contrast) and different field strengths (1.5T vs. 3T). Different studies have indicated the impact of inter- and intra-scanner variability on MRI measurements [193]. Thereto, we will also evaluate the covariates scanner type, contrast enhancement and field strength. In particular, for each of these covariates, three categories are introduced, (1) scan protocol 1 for both baseline and follow-up scans, (2) scan protocol 2 for both baseline and follow-up scan, (3) different scan protocol between baseline and follow-up scan.

All covariates are evaluated seperately using linear regression for continuous variables, ordered logistic regression for the ordinal variables and a factorial logistic regression for the binary (categorical) variables.

Results

Table 4 provides an overview of the assessment of HLA on disease status and progression. In particular, the corrected t-score (or chi-squared-score) is provided for every clinical/MRI measurement as well as globally for the different predefined HLA groups under study. A positive value means a positive effect on disease progression and the magnitude gives information about the effect size. Subsequently, statistical significant effects per measurement are indicated by * (p-value < 0.05) and ** (p-value<0.01). These are obtained from the individual scores, i.e. per year separately (2013, 2015), and indicated in case at least one of both evaluations is statistically significantly differently. Finally, (*) and (**) will be added in case a statistically significant effect is found for the variable for every covariate (age, disease progression, gender, MS type, treatment). Figure 1 and Figure 2 show the mean values and standard deviations of clinical variables wherefore a significant different effect was indicated between two HLA groups. Results for all statistical evaluations per measurement can be found in the appendix.

	DRB1*15+ vs. DRB1*15-	B*07+ vs. B*07-	DRB1*15+/B*07+ vs. DRB1*15+/B*07-	DRB1*15-/B*07+ vs. DRB1*15-/B*07-	A*02+ vs. A*02-	DRB1*15+/A*02+ vs. DRB1*15+/A*02-	DRB1*15-/A*02+ vs. DRB1*15-/A*02-	DQB1*06+ vs. DQB1*06-	DQB1*06+DRB1*15+ vs. rest	B*44+ vs. B*44-	B*08+ vs. B*08-	A*02-B*07+ vs.rest
# subjects	53 vs. 65	32 vs. 86	26 vs. 27	6 vs. 59	42 vs. 76	19 vs. 34	23 vs 42	72 vs. 46	52 vs. 66	20 vs. 98	18 vs. 100	19 vs 99
Clinical scores and MRI measurements (categorical / continuous variables)												
EDSS	-1.26	-1.43	-1.07	-0.25	2.18*	1.40	1.73	-1.14	-1.31	0.36	-1.20	-1.25
SDMT	-0.70	-0.23	0.53	-0.99	-0.79	-1.15	-0.03	-0.67	-0.71	-0.29	-1.48*	0.19
T25FW	-0.51	-0.54	-0.80	1.41	1.86	1.29	1.65	-1.20	-0.53	-0.18	0.73	-0.80
9 HPT	0.65	0.32	-0.74	1.32*	0.75	0.08	0.86	-0.80	0.74	-0.71(*)	0.34	0.35
MSSS	-0.89	-1.38	-0.91	-0.84	3.00**	2.38*	1.89	-1.33	-1.07	-0.53	-0.02	-1.25
CVLT-II	-0.31	-1.29	-0.86	-0.80	-0.07	-0.62	0.51	1.14	-0.48	-0.50	-0.22	-0.96
BVMT-R	0.04	0.31	0.16	0.32	1.14	-0.3	1.68	0.94	0.07	-0.43	-1.58	-0.34
lesions difference normalised	-1.51	-1.14	-1.32	0.73	-0.47	0.19	-0.75	-0.75	-1.50	-0.60	0.41	-0.78
new lesions count	2.20*	0.59	-1.26	-0.03	2.07*	-0.38	2.48*	-0.38	2.31*	-1.18(*)	1.52	0.40
annualised pbvc	-0.19	-0.63	-0.84	0.06	-1.4	-2.53*	0.11	-1.69	-0.16	-0.38	0.48	1.27
Average t-statistic	**-0.25**	**-0.54**	**-0.71**	**0.09**	**0.83**	**0.03**	**1.01**	**-0.59**	**-0.26**	**-0.44**	**-0.10**	**-0.32**
Protective effect (group 1)	**NO**	**NO**	**NO**	**YES**	**YES**	**YES**	**YES**	**NO**	**NO**	**NO**	**NO**	**NO**
Extracted values describing disease progression (binary variables)												
EDSS plus	0.11	-1.90	-1.25	-1.29	0.00	0.63	-0.54	-0.33	0.06	-0.02	0.04	-0.82
SDMT progression	1.16	0.00	0.00	-0.04	1.52	0.11	0.67	2.01	1.05	0.00	-4.21(*)	0.00
NEDA 3	-0.08	-0.06	-0.15	0.00	0.28	0.02	0.09	-0.99	-0.16	0.00	0.00	-0.01
Average signed Chi squared	**0.40**	**-0.65**	**-0.47**	**-0.44**	**0.60**	**0.25**	**0.07**	**0.23**	**0.32**	**-0.01**	**-1.39**	**-0.28**
Protective effect (group 1)	**YES**	**NO**	**NO**	**NO**	**YES**	**YES**	**YES**	**YES**	**YES**	**NO**	**NO**	**NO**

*Table 4: Overview of corrected t-score (or chi-squared-score) per clinical/MRI measurement as well as the averaged t-statistic (or chi-squared score) for the different predefined HLA groups under study. A corrected positive t-score means a positive effect. Statistically significant effects per measurement are indicated by * (p-value < 0.05) and ** (p-value<0.01), calculated on the*

original scores, i.e. per year separately (2013, 2015), and indicated in case at least one of both evaluations is statistically significantly different. (*) and/or (**) will be added in case a statistically significant effect is obtained for any of the covariates, but not without covariates.

Discussion

Multiple studies have evaluated HLA genotypes with respect to MS, by comparing HLA between healthy controls and MS patients [282, 301, 333]. The objective of this study is to examine the effect of HLA on the disease progression. Therefore, no control group is included here, but MS patients are evaluated in a two-year follow-up study based on multiple clinical scores (both cognitive and physically) and quantitative brain MRI measurements. Quantitative MRI measurements are extracted using MSmetrix [282, 301] providing whole brain atrophy, FLAIR lesion volume changes and number of new lesions between the baseline and follow-up scans. A statistical analysis is performed to compare the quantitative changes between baseline and 2-year follow-up between different predefined HLA groups. A total of 12 different predefined groups are evaluated focusing on the effect of 6 different HLA values on disease progression, resp. B*07, A*02, B*44, B*08, DQB1*06 and DRB1*15.

The statistical analysis is based on individual comparisons of 20 clinical scores and/or MRI measurements for these 12 different HLA groups. Moreover, the effect of 5 cofounding variables (age, gender, disease duration, treatment, MS type) is evaluated and an additional 3 variables in case of MRI measurements (scanner, field strength, contrast enhancement). This results in more than 1000 statistical tests for a cohort of 118 MS patients. Hence a correction for type I errors might be advised. Reduction in sensitivity is however not desirable as results are expected to be subtle. Instead, a global t-statistic is provided for each HLA group providing a general idea of direction of change and effect size, while individual results are seen as exploratory and no single result should be interpreted as decisive**.**

The statistical analysis indicates a protective effect of HLA-A*02, while patients with B*07 or B*44 seem to be more susceptibility for MS progression.

HLA-A*02

The evaluation of HLA-A*02 was based on a group of 42 MS patients compared to 76 MS patients without HLA-A*02. Both groups are balanced in gender, age and disease duration (no significant difference are found between both groups). HLA-A*02 seems to have a protective effect on MS status and progression according to the overall scoring based on the averaged t-statistic and chi-squared statistic. Moreover, the protective effect is significant (p-value<0.05) for EDSS, MSSS and new lesion count. The effect is close to significant for T25FW (Welch test p-value = 0.06 for 2013 and Welch test p-value =0.07 2015). For MSSS and EDSS, the differences remained significant (p-value <0.05) or close to significant in case one of the covariates was added. This indicates the limited impact of the divers cofounding variables. The lower new lesion count in the group with HLA-A*02 did however not remain after introduction for any of the covariates.

In case HLA-A*02 is evaluated for the subpopulation that contains also DRB1*15, we compare 19 vs. 34 MS patients. Both groups are still balanced in terms of gender, age and disease duration. The global protective effect is still present, although the protective vs. negative effect differs between variables. Only MSSS still shows a significant effect for protectiveness, while the annualized whole brain atrophy (annualized pbvc) shows even a negative significant impact of HLA-A*02 for the subpopulation that contains DRB1*15. Both effects remain significant in case any of the covariates is added.

Within the subgroup of MS patients without DRB1*15, 23 patients have HLA-A*02 vs. 42 patients without. Hence, there is quite an imbalance in sample size. Also both groups are not perfectly age-matched (significant difference in age: p-value Welch-test<0.05). Most scores as well as the overall score point towards a protective effect, although only new lesion count was significant. After covariates were added, the results were still close to significance.

HLA-A*02 seems to have a protective effect on MS. The influence of a combination of DRB1*15 is not conclusive and is probably hampered by the limited number of subjects. The protective effect of HLA-A*02 confirms the effects found in literature [301, 333].

HLA-B*07

The group with HLA-B*07 seems to have a negative effect on MS, although none of the scores reached statistically significance. The susceptibility of HLA-B*07 to MS is also confirmed in literature [282], in particular in terms of higher lesion load.

In this study, the evaluation relies on 32 patients with B*07, while 86 patients without B*07. The group of patients with B*07 is sufficiently large, however there is a relative imbalance in terms of sample size between both groups. Both groups are well matched in terms of age, gender and disease duration. No significant differences in clinical or MRI scores were detected between both groups. However, most scores (as well as the global t-statistic) point towards a negative impact of B*07 on MS.

In case we only evaluate the effect of HLA-B*07 on the subgroup with DRB1*15, we obtain similar findings. In particular, no significant effects are detected, but the majority of measurements point towards a negative effect of B*07.

For the evaluation of HLA-B*07 on the subgroup without DRB1*15, a very large discrepancy exists between sample sizes (i.e. 6 MS subjects vs 58 MS subjects). The HLA-B*07 group of patietns without DRB1*15 showed a trend towards a lower (better) score on 9 HPT L 2013, yet only as assessed by the Welch-test. In this case (non-normal distribution of one of both groups, and low sample size) the Welch-test should not be the only factor to rely on. The trend did not remain after evaluating for cofounding variables.

Combination HLA-B*07 without HLA-A*02

As both MS patients with HLA-B*07 and subjects with no HLA-A*02 seem to point towards being more prone to the MS progression, the combination of both might indicate an even stronger susceptibility. HLA-B*07 without HLA-A*02 group is compared to all other subjects. A large discrepancy in group sizes exists, but groups are matched in age, gender and disease duration. Most clinical scores and MRI measurements point in a direction of a negative effect. However,

none of the differences between both groups reached the threshold for significance or showed a clear trend towards significance. Therefore, it is not clear whether the effect of containing B*07 without A*02 further amplifies the negative overall effect.

HLA-B*44

Also patients with B*44 seem to be more susceptible for MS progression. However, a large discrepancy exists between the group sizes of patients with HLA-B*44 and without, respectively 20 vs 98 MS patients, but both groups are balanced in terms of age, gender and disease duration. Most clinical scores and MRI measurements point towards a negative effect of B*44 in terms of disease status and progression. However, none of these scores reaches significance (p-value <0.05). In case any of the covariates is included, we found a significant negative effect of the patients with HLA-B*44 for the 9 HPT 2015 test (p-value<0.05) and a higher number of new lesions (p-value <0.05). The negative effect of B*44 on disease progression does not confirm literature where B*44 has been indicated as protective [282].

HLA values DRB1*15, DQB1*06 and B*08 show inconclusive results.

HLA-DRB1*15

For patients with DRB1*15, the global t-statistic shows a negative effect and also most individual scores for disease status are negative. Different scores describing disease progression, i.e. new lesion count, EDSS plus and SDMT progression, show however a positive effect of DRB1*15. The number of new lesions reaches even statistically significance (p-value <0.05) according to a Welch test and stays significant after evaluating the effect of possible cofounding variables. The tests were evaluated on a group of 53 MS patients with DRB1*15 and 65 MS patients without DRB1*15. Both groups are comparable with respect to age, gender and disease duration. Patients with and without DRB1*15 were also often used for the analysis of subpopulations (e.g. see analysis of A*02 and B*07). Also for these analyses, no clear effect of DRB1*15 was found.

*HLA-DQB1*06*

Also for HLA-DQB1*06 no conclusive results are obtained as difference between the group with and without DQB1*06 are varying between the clinical scores and MRI measurements, while none of the tests reached significance (p-value <0.05). There is, however, a small imbalance between sample sizes of both groups within the cohort (72 vs. 46 MS patients), while also a small difference exists in gender distribution (p-value chi-squared test = 0.08), with slightly more female patients in the group with HLA-DQB1*06. This might have an influence on the results. The groups were however similar in terms of age and disease duration. The combination of DQB1*06 with DRB1*15 vs. all other subjects is also evaluated. However, for this cohort, only one subject differs from the analysis of DRB1*15 (with/without). Hence, conclusions are in line with the DRB1*15 analysis.

*HLA-B*08*

For HLA-B*08, there is a large discrepancy in group sizes of patients with and without the HLA, respectively 18 vs 100 MS patients. Although the groups are well matched in terms of age and gender, the disease duration is not completely similar. The group without HLA-B*08 has on average a slightly smaller time since onset (p-value disease duration = 0.06). Both protective or susceptible effects are observed for the different scores and measurements. The overall statistical score points towards a negative direction. A significant effect was observed for SDMT in 2013 (p-value of Welch-test = 0.05, normality is confirmed). Furthermore, also SDMT progression became significant after introducing of any of the cofounding variables (covariates). Both SDMT and SDMT progression point towards a negative effect of HLA-B*08 on MS.

In general, we observed only a limited influence of possible cofounding factors such as age, gender, disease duration, MS type, and treatment line as well as of the MRI variables such as scanner, contrast enhancement and field strength. Hence, it can be assumed that the population under study was sufficiently homogeneous for the statistical analysis. However, the variability in the MRI scan protocol, in particular between two subsequent scans of the same subject, should be taken into account when evaluating the quantitative MRI measurements. Scanner and protocol

changes have been indicated to result in larger measurement errors of the quantitative MRI analysis (e.g. [317]). Hence, subtle changes in brain atrophy and lesion load might not have been detected due to the inconsistency of the MRI protocols for patient follow-up.

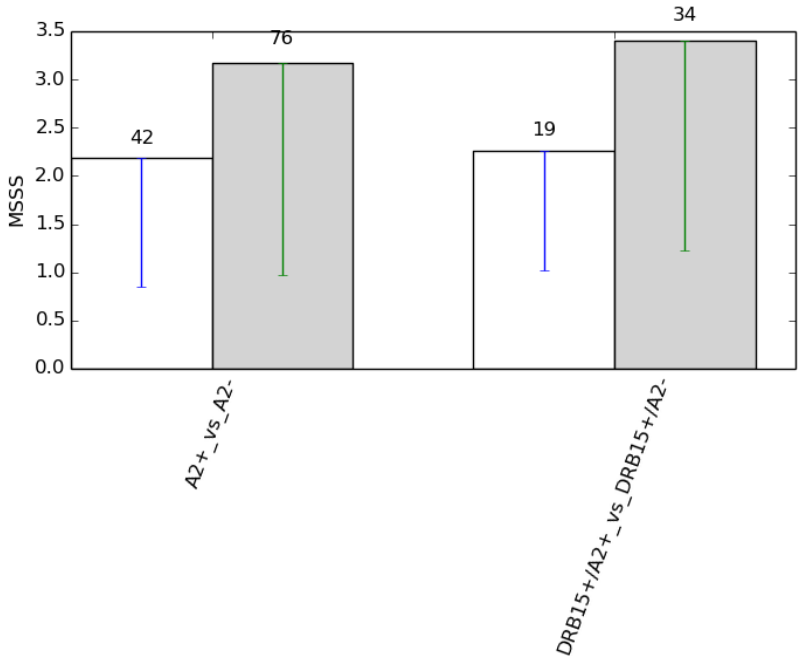

Figure 1: Mean value and standard deviation of significant differences in MSSS between two groups. Sample sizes of each subgroup are indicated.

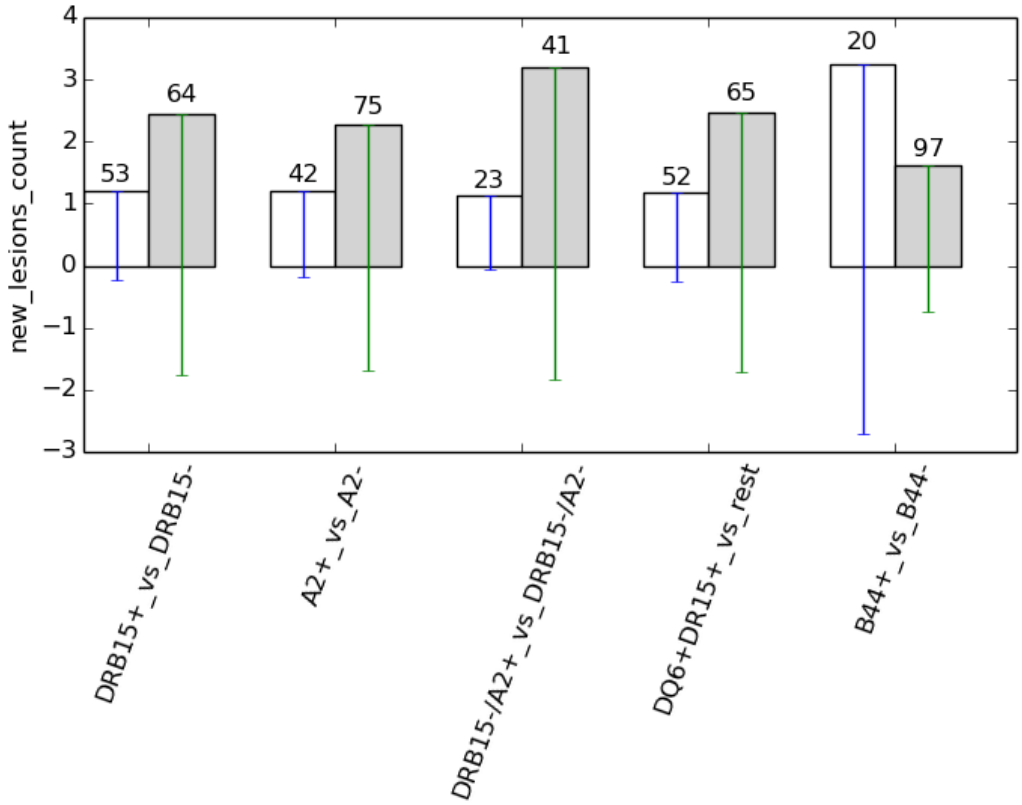

Figure 2: Mean value and standard deviation of significant differences in number of new lesions between two groups. Sample sizes of each subgroup are indicated.

Conclusion

This study evaluates HLA genotype as a marker of MS disease progression by comparing HLA groups within a cohort of 118 MS patients based on various clinical scores and quantitative MRI measurements. Our data indicate HLA-A*02 as a marker of a better prognosis, while B*07 or B*44 seem to predict a more severe disease evolution (Table 5).

	DRB15	B7	A2	DQ 6	B8	B44
Effect	inconclusive	towards negative	protective	inconclusive	inconclusive/towards negative effect	towards negative effect
Significant scores * = significant in case of covariate	x	x	MSSS EDSS new lesion count	x	SDMT SDMT progression*	9 HPT R 2015* new lesions*
Cofounding variables (covariates)	No	No	Limited (new lesion count)	No	Yes (large imbalance in population size)	Yes (large imbalance in population sizes)

Table 5: Summary of general trends of the statistical analysis for this cohort.

Study 3 – Larger extension study to establish if HLA genotype is a marker of MS prognosis

[Lysandropoulos 2020]

In this study, I aimed to further explore the possible association between HLA genotype and MS progression by incorporating already available data from an additional MS centre based in Lausanne University Hospital as well as by evaluating the patients over a longer period of time (3 time points over a mean follow-up of 4 years).

To expand the cohort for this study I established a collaboration with a team at the Lausanne University Hospital, Lausanne (Switzerland), then undertook work to make sure the datasets from their and my own cohorts were compatible. I was able to use their database to retrieve the relevant clinical data and MRI scans to send for analysis.

The study included patients with relapsing remitting (RR), secondary progressive (SP), or primary progressive (PP) MS based either my clinic at the Erasme University Hospital, Brussels (Belgium) or Lausanne University Hospital, Lausanne (Switzerland).

The two cohorts together consisted of 146 (102 females, 44 males) MS patients with available HLA genotype profile. The demographic characteristics of the patients are presented in the full paper. No treatment change occurred during the observation period. Patients' MS status was established during routine clinic appointments at three time points in a 4-year interval, based on clinical scores including the EDSS, the MSSS (MS Severity Scale), the T25FW (Timed-25-Foot-Walk), the 9-HPT (9-Hole Peg Test), and the SDMT (Symbol DigitModalities Test) and MRI evaluations. I uploaded all the MRI scans to be processed using 'icobrain ms' which analyzes two consecutive scans simultaneously, yielding robust and consistent measurements for whole brain volume and lesion load (volume and count).

Statisical analysis is detailed in the full paper appended to this thesis.

Full details of the effects of HLA genotypes on clinical and MRI parameters are described in the

full paper appended to this thesis. In accordance with my previous pilot study, HLAA*02 allele was associated with potentially better MS outcomes ($p < 0.05$ for MSSS; $p < 0.1$ for protective effects on EDSS and T25FW), whereas HLA-B*07, HLA-B*44, HLA-B*08, and HLA-DQB1*06 correlated to a worse disease status and/or disease progression as evaluated by multiple clinical and MRI outcomes. Results for HLA-DRB1*15 remained inconclusive.

This extension study on a bigger sample and with a longer follow-up mainly confirmed the results of my previous work.

Human Leukocyte Antigen genotype as a marker of Multiple Sclerosis prognosis

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Abstract

Objective In a previous pilot monocentric study, we investigated the relation between HLA genotype and Multiple Sclerosis (MS) disease progression over 2 years. HLA-A*02 allele was correlated with better outcomes, whereas HLA-B*07 and HLA-B*44 with worse outcomes. The objective of this extension study was to further investigate the possible association of HLA genotype with disease status and progression in MS as measured by sensitive and complex clinical and imaging parameters.

Methods: 146 MS patients underwent HLA typing. Over a 4-year period of follow-up, we performed 3 clinical and MRI assessments per patient, which respectively included EDSS, MSSS, T25FW, 9-HPT, SDMT, BVMT, CVLT-II and whole brain atrophy, FLAIR lesion volume change and number of new FLAIR lesions using icobrain. We then compared the clinical and MRI outcomes between predefined HLA patient groups.

Results: Results of this larger study with a longer follow-up are in line with what we have previously shown. HLA-A*02 allele is associated with potentially better MS outcomes, whereas HLA-B*07, HLA-B*44, HLA-B*08 and HLA-DQB1*06 with a potential negative effect. Results for HLA-DRB1*15 are inconclusive.

Conclusion : In the era of MS treatment abundance**,** HLA genotype might serve as an early biomarker for MS outcomes to inform individualised treatment decisions.

Introduction

The genetic risk for multiple sclerosis (MS) is related to a series of HLA class II and I alleles [276, 277, 320]. HLA-DRB1*15:01 allele has been shown to have the strongest association with MS, especially in Caucasian populations [253, 276, 279, 286, 288, 320]. HLA class I alleles have been associated with either reduced (HLA-A*02:01, HLA-B*44:02) [293, 299-302] or increased (HLA-A*03, HLA-B*07) susceptibility to MS [293, 301, 303, 304].

There is an unmet need for a biomarker with prognostic value in MS. Limited studies with contradictory results have investigated possible association of HLA genotype with MS severity by evaluating few and poorly sensitive clinical and MRI parameters [50, 305, 307, 308, 341-344].

In a previous study, we reported on the relationship between HLA genotype and MS disease progression over a two-year period, regarding both clinical including Expanded Disability Status Scale (EDSS) and Multiple Sclerosis Severity Scale (MSSS) and MRI outcomes (new lesion count and brain volume). We found that the HLA-A*02 allele was associated with better clinical and MRI outcomes, whereas the HLA-B*07 and HLA-B*44 alleles with a global negative effect on disease status. Results for the HLA-DRB1*15, HLA-DQB1*06 and HLA-B*08 alleles were inconclusive. The influence of confounding variables, such as age, gender, disease duration, MS type and treatment, scanner model, MRI field strength and Gadolinium (Gd) enhancement on the statistical analysis was limited [314].

In this study, we aim to further explore the possible association between HLA genotype and MS progression by incorporating data from an additional MS center as well as by evaluating the patients over a longer period of time (3 time points over a mean follow-up of 4 years).

Materials and Methods

Patients

We included patients with relapsing remitting (RR), secondary progressive (SP) or primary progressive (PP) MS followed in 2 MS centers: the Lausanne University Hospital, Lausanne (Switzerland) and the Erasme University Hospital, Brussels (Belgium). An overview of patients' characteristics is provided in Table 1.

# patients	146 (117 Erasme, 29 CHUV)
Median age, yr (range)	41.1 ± 11.2 (16.95 – 67.88)
Mean disease duration, yr (range)	11.5 ± 6.7(1.75 – 38.05)
Median EDSS (range)	2.4 ± 1.3 (1– 6.5)
Gender # (%) patients)	males 44 (30.1%), females 102 (69.9%)
MS types (n)	132 RR-MS, 8 SP-MS, 6 PP-MS
On treatment (RRMS and SPMS) (n, %)	93.8%
first line*	37.0%
second line*	56.8%

*Table 1. Description of the patient population at baseline. Abbreviations: EDSS= expanded disability status scale; RRMS =relapsing remitting MS; SPMS= secondary progressive MS; * as per EMA definition. All patients remained in the respective treatment line throughout the study period.*

HLA typing

HLA typing was performed on DNA extracted from peripheral blood mononuclear cells by low- to intermediate-resolution polymerase chain reaction using sequence-specific oligonucleotides. Reverse dot-blotting was carried out on a nylon membrane containing immobilized sequence-specific oligonucleotide probes used for the typing of HLA class I (HLA-A*02, HLA-B*07, HLA-B*44) (all patients) and HLA class II (HLA-DRB1*15, HLA-DRB1*04, HLA-DRB1*07, HLA-DQB1*06) alleles (Erasme patients) (INNO-LiPA®, Fujirebio).

Clinical and MRI evaluation

We assessed the patients at 3 time points over a 4-year period by evaluating various clinical and MRI parameters (table 2). Data were collected from patients' medical records. All clinical and MRI evaluations were performed as part of routine practice. Clinical tests were done at the same

time as the MRI. The mean interval in days between 1st and 2nd evaluation was 823.52 ± 382.52 and from 2nd to the 3rd 636.68 ± 241.90.

Clinical scores	EDSS at 3 time points and change over time (all patients)
	MSSS at 3 time points and change over time (all patients)
	9HPT at 3 time points and change over time (Erasme patients)
	T25FW at 3 time points and change over time (Erasme patients)
	SDMT at 3 time points and change over time (Erasme patients)
	BVMT-R at baseline (Erasme patients)
	CVLT at baseline (Erasme patients)
MRI scores	Whole brain volume at 3 time points and change over time (all patients)
	Lesion volume at 3 time points and change over time (all patients)
	New lesion count (all patients)

Table 2. Overview of MRI and clinical variables. Abbreviations: BVMT-R, Brief Visual Memory Test; CVLT-II, California Verbal Learning Test-II; EDSS, Expanded Disability Status Scale; MSSS, Multiple Sclerosis Severity Scale; PASAT-3, Paced Auditory Serial Addition Test; SDMT, Symbol Digit Modalities Test; T25FW, Timed-25-Foot-Walk; 9-HPT, 9-Hole Peg Test.

Clinical evaluation

Annualized percentage of change (apc) in EDSS, MSSS, T25FW, 9HPT and SDMT was computed, (apc-EDSS, apc-MSSS, apc-T25FW, apc-9HPT and apc-SDMT, respectively) by means of a linear regression on the available longitudinal results. Progression (Yes/No) in EDSS was defined as an increase by at least 1 point (for baseline EDSS below 6) or at least 0.5 points (for baseline EDSS of 6 or higher), confirmed ⩾24 weeks apart. For 9HPT and T25FW, progression was defined as an increase of the time by more than 20%, confirmed ⩾24 weeks apart. EDSS plus was defined as progression in either EDSS, 9HPT or T25FW confirmed ⩾24 weeks apart.

MRI measurements

Scans obtained from clinical routine included scans obtained from clinical routine included a Fluid Attenuated Inversion Recovery (FLAIR) sequence and a T1-weighted turbo field echo sequence (pre- or post-Gd injection). All MRI scans were processed using icobrain ms *(https://icometrix.com/products/icobrain-ms)* [316, 317, 345]. The method analyses two consecutive scans simultaneously, yielding robust and consistent measurements for whole brain volume and lesion load (volume and count). To obtain an overall atrophy score allowing multiple timepoints, a linear fit was applied on the whole brain volumes, and the annualized percentage brain volume change (aPBVC) was computed between first and last timepoint. In the same way, an annualized percentage in lesion volume change (aPLVC) was computed. The number of new lesions was summed to obtain the overall new lesions count since baseline.

Progression (Yes/No) in whole brain volume decrease was defined as aPBVC stronger than - 0.4%. To define progression in lesion count, a threshold was set to a minimal new lesion size of 5x3mm on 3D images.

Statistical analysis

The relationship between HLA genotype and clinical and MRI outcomes was evaluated by comparing group of patients with different HLA genotypes. Based on literature reports from previous studies [282, 302, 333], the analysis focused on specific and potentially relevant subgroups of HLA alleles: HLA-A*02, HLA-B*07, HLA-B*44, HLA-B*08, HLA-DRB1*15 and HLA-DQB1*06 alleles, and their combinations (table 3).

Thus, the statistical analyses evaluated differences in clinical scores and MRI measurements between patients from each HLA group and its counterpart. The statistical analysis followed three major steps: (1) an individual assessment of the different clinical scores and MRI measurements describing the disease status and progression with respect to the predefined HLA groups, (2) an

overall assessment of the relation between MRI/clinical outcomes and specific HLA genotypes, (3) an assessment of the influence of diverse covariates (confounding variables) : gender, age, disease duration, MS type, and treatment (first- vs. second-line), scanner type (1.5 T vs. 3 T scanner) and Gd injection .

Table 3. Overview of HLA groups assessed in this study.
B*07+ vs. B*07-
DRB1*15-/B*07+ vs. DRB1*15-/B*07-
DRB1*15+/B*07+ vs. DRB1*15+/B*07-
A*02-/B7+ vs. all others
A*02+ vs. A*02-
DRB1*15-/A*02+ vs. DRB1*15-/A*02-
DRB1*15+/A*02+ vs. DRB1*15+/A*02-
DRB1*15+ vs. DRB1*15-
B*08+ vs. B*08-
B*44+ vs. B*44-
DQB1*06+ vs. DQB1*06-
DQB1*06+DRB1*15+ vs. all others

Table 3 Overview of HLA groups assessed in this study

First, all data were evaluated to comply with the normality assumptions required for parametric statistical tests. The distribution of all variables was checked based on scatter plots and additionally normality was addressed based on the Shapiro-Wilk test. To gain normality, T2 lesion volume was cube root transformed, and 9-HPT and T-25FW scores were logarithmically (base 10) transformed.

Dependent variables for which multiple time points were available (whole brain volume, lesion volume, EDSS, MSSS, T25FW, 9HPT, SDMT), were analysed using linear mixed effects modeling (lmer function as implemented in the lme4 package in R). This allows for the joint analysis of patients with two or three scans. The standard model included the interaction between time (tp1, tp2 or tp3) and HLA group, and their main effects as fixed effects. A random effect was added to account for the repeated measures per patient. Dependent variables for which only one measurement per patient was assessed (aPBVC, aPLVC, total new lesions count, apc-EDSS, apc-

MSSS, apc-T25FW, apc-9HPT, apc-SDMT, CVLT-II, BVMT-R), were analyzed using a linear model with HLA group as fixed effect. From all models the main effect of group was assessed using a type 2 ANOVA and was considered significant for $p<0.05$. To obtain an overall impression of protectiveness of a certain HLA allele across clinical and MRI outcomes, we computed the weighted average of test statistics from the individual ANOVA. The T-statistics for main effect of group (in absence of a covariate) were multiplied by +1 or -1 to yield a positive value in case of a protective effect of the first of both groups. The weighted average across outcomes is then suggestive for an overall beneficial, adverse or neutral effect of a certain HLA allele's presence.

Each covariate's influence on a clinical or MRI measurement was tested by adding it to the model of interest (in absence of other covariates). Then the ANOVA type 2 analysis was repeated, and the significance of the main effect of group was reconsidered.

For progression markers, the odds ratio was computed for the different HLA groups, and progression was statistically compared between groups using the two-sided Fisher's exact test on the contingency table ($p<0.05$). Similar to the continuous outcomes, an overall impression of protectiveness can be given based on progression outcomes. To do so, the deviation from equal odds (i.e. 1-OR) was computed, after which a positive deviation is again indicative for a beneficial effect for the first mentioned group.

Results

Table 4 provides the overview of the effects of HLA genotypes on clinical and MRI parameters. An important note is that the T-statistics in this table should only be compared across (rows) and not between columns (group comparisons), because of different sample sizes. Furthermore, because not all clinical measures have been evaluated in all participants (as is the case for the MRI variables), the T-statistics of these clinical variables are also less comparable among each other. For this reason, Table 4 includes a separate average for the clinical and MRI measurements. Given the exploratory nature of this study, no correction for multiple comparisons was applied. Therefore, in the following paragraphs describing each of the HLA subtypes, the patterns of findings should be interpreted rather than the exact statistical results.

	B7+ vs B7-	DRB1 5-/B7+ vs DRB1 5-/B7-	DRB1 5+/B7+ vs DRB1 5+/B7-	A2-/B7+ vs rest	A2+ vs A2-	DRB1 5-/A2+ vs DRB1 5-/A2-	DRB1 5+/A2+ vs DRB1 5+/A2-	DRB1 5+ vs DRB1 5-	B8+ vs B8-	B44+ vs B44-	DQ6+ vs DQ6-	DQ6+ DR15+ vs rest
Two-sample t-statistic												
Norm. whole brain volume	0.15	1.00	-0.27	0.59	0.16	1.46	-0.80	-0.36	-2.07**	1.43	-0.37	-0.40
aPBVC	-1.82*	-0.71	-1.38	-0.01	-0.99	-0.07	-1.52	-0.37	0.38	-0.51	-1.04	-0.36
Lesion volume	2.11**	1.69*	0.58	0.54	0.84	0.72	-1.14	0.21	-0.71	2.11*	0.66	0.17
aPLVC	0.97	0.58	1.36	1.69*	-0.65	-0.78	0.10	0.55	-0.75	-1.78*	-0.03	0.57
Total new lesions count	1.09	0.04	-0.54	0.34	0.25	0.31	-0.52	1.83*	0.93	-3.08***	0.38	1.90*
BVMT-R	0.37	0.39	0.16	-0.33	1.20	1.78*	-0.29	0.11	-1.43	-0.45	0.90	0.14
CVLT-II	-1.21	-0.86	-0.86	-0.98	-0.05	0.55	-0.64	-0.30	-0.19	-0.55	1.12	-0.46
EDSS	-1.02	0.00	-1.02	-1.57	1.72*	1.11	1.46	-1.52	-0.47	0.68	-1.49	-1.58
Apc-EDSS	1.21	-0.17	0.22	0.31	0.58	0.11	-0.29	0.91	-2.54**	0.56	0.68	0.88
MSSS	-0.60	-0.30	-1.18	-1.38	2.03**	1.12	1.57	-1.01	0.61	-0.39	-1.74	-1.22
Apc-MSSS	1.09	-0.18	0.38	0.02	1.24	0.43	0.17	0.12	-2.48**	0.74	0.04	0.09
SDMT	0.10	-0.66	0.71	0.05	-0.25	0.18	-0.59	-0.23	-1.64	-0.31	-0.02	-0.36
Apc-SDMT	-0.33	1.18	-1.45	0.18	-0.68	-0.85	0.06	-0.20	1.05	-0.31	-0.72	0.18
T25FW	0.20	1.12	-0.16	-0.65	1.69*	1.29	1.08	-0.42	-0.44	0.17	-0.46	-0.41
Apc-T25FW	-1.12	-1.17	-0.35	-1.14	0.40	-0.11	0.80	-0.60	-0.25	0.24	-1.83*	-0.75
9HPT	0.10	0.70	-0.49	0.18	0.33	0.53	-0.19	-0.06	-0.49	-0.25	-0.49	0.10
Apc-9HPT	-0.20	-1.14	-0.04	0.32	-0.16	-0.57	0.74	1.09	1.77*	-2.06**	0.27	1.02
Average MRI	0.28	0.46	-0.18	0.57	-0.13	0.39	-0.84	0.25	-0.51	-0.23	-0.19	0.25
Average clinical	-0.16	-0.11	-0.34	-0.45	0.66	0.56	0.21	-0.16	-0.58	-0.21	-0.12	-0.19
Average all	-0.06	0.01	-0.30	-0.22	0.48	0.53	-0.02	-0.07	-0.57	-0.21	-0.14	-0.09
Deviation from equal progression odds ratio												
Atrophy	-0.23	0.05	-0.48	0.04	0.04	0.21	-0.51	0.18	-0.23	-0.44	0.04	0.16
New 5mm lesion	0.41	0.16	-0.02	0.72	-0.21	0.44	-0.82	0.81**	0.74	-1.41	-0.19	0.80**
EDSS prog	0.02	-0.35	0.35	-0.36	0.38	0.46	0.02	-0.38	-0.82	0.24	-0.38	-0.44
EDSS plus	-0.39	-0.78	-0.28	-0.26	0.22	0.24	0.19	-0.07	-0.09	0.19	-0.17	-0.10
T25FW prog	-0.83	-1.34	-0.62	-0.91	0.44	0.59	0.24	-0.25	0.03	-0.07	-0.61	-0.31
9HPT prog	-0.17	-0.49	-0.66	0.52	-0.28	-0.42	-0.07	0.34	1.00**	0.06	0.07	0.32

*Table 4. Weighted t-statistics of the main effect of group, in absence of covariates. A positive t-statistic indicates a protective effect of the first group. A negative t-statistic indicates increased susceptibility of the first group. A positive deviation from equal odds ratio indicates less probability of the first group to undergo progression. * p<0.1, ** p<0.05, ***p<0.01*

*HLA-B*07*

The evaluation relied on 45 patients harboring the HLA-B*07 allele and 101 patients without this allele. Both groups were balanced in terms of age, gender, and disease duration. Global t-statistic pointed towards a negative impact of the HLA-B*07 allele. A trend towards a negative effect was observed for aPBVC indicating more severe whole brain atrophy for HLA-B*07. After controlling for MS type, this effect became significantly different (t=-1.98, p=0.05). The FLAIR lesion volume was found to be significantly lower in the HLA-B*07 group (t=-2.11, p=0.029). This finding was

however not robust for controlling against scanner model, contrast, field strength, patient sex, age, treatment line, MS type or MS duration (all $p>0.05$).

With regards to disease progression, presence of HLA-B*07 allele appeared to have a negative effect on most clinical scores and whole brain atrophy.

Similar findings were obtained when the effect of the HLA-B*07 allele was evaluated in the subgroup of patients harboring the HLADRB1* 15 allele. The majority of measurements pointed towards a negative effect of HLA-B*07.

*HLA-A*02*

The evaluation of the HLA-A*02 allele was based on a group of 64 MS patients compared to 82 MS patients without HLA-A*02. Both groups were balanced in terms of gender, age, and disease duration. HLA-A*02 seemed to be associated with better prognosis according to the overall scoring based on the averaged t-statistic. This effect was significant (p-value <0.05) for MSSS, although it did not remain after controlling for treatment line. Furthermore, a trend ($p<0.1$) for protective effects of HLA-A*02 on EDSS and T25FW was found. Slightly higher brain volume and lower lesion volume were found for patients harboring HLA-A*02 allele, yet also more whole brain volume change and stronger increase in lesion volume were found.

Presence of HLA-A*02 was associated with a trend towards a protective effect for the majority of progression markers.

The effect of the HLA-A*02 allele was also evaluated in the subpopulation of patients harboring the HLA-DRB1*15 allele. In this analysis, 19 vs. 34 MS patients with and without the HLA-A*02 allele were compared. Both groups were still balanced in terms of gender, age, and disease duration. The global protective effect of HLA-A*02 was not present anymore, even though MSSS, EDSS and T25FW still leaned towards a protective effect of HLA-A*02. HLA-A*02 had a negative impact on annualized whole brain atrophy (annualized percentage of brain volume change) in the patients who were also HLADRB1*15 positive. In contrast to the overall population harboring HLA-A*02, in the subpopulation of HLADRB1*15 carriers, HLA-A*02 was associated to higher lesion volume, higher new lesions count and lower normalized whole brain volume, although not significant without covariates ($p>0.05$).

Within the subgroup of MS patients without HLA-DRB1*15, 23 patients had the HLA-A*02 allele and 41 patients did not. Most scores as well as the overall score pointed towards a protective effect. After controlling for age at baseline or MS duration, the increased BVMT-R in the group harboring HLA-A*02 became significant (with age: t=2.20, p=0.031, with MS duration: t=2.31, p=0.024). Presence of HLA-A*02 in the absence of HLA-DRB1*15 had a protective effect for the majority of clinical and MRI markers of progression, although not significant (p>0.05).

*Combination HLA-B*07 without HLA-A*02*

As both MS patients with HLA-B*07 and patients without HLA-A*02 seemed to be more prone to MS progression, the combination of both alleles might indicate an even stronger susceptibility. The group of patients with HLA-B*07 and without HLA-A*02 (22 patients) was compared to all other patients (124 patients). Groups were matched in terms of age and disease duration. Global t-statistics and various clinical scores pointed in the direction of a negative effect. However, none of the differences between both groups reached the threshold for significance. Overall, it was not clear whether the effect of HLA-B*07 without HLA-A*02 further amplified the negative overall effect. Presence of HLA-B*07 in the absence of HLA-A*02 tended to have a negative effect on clinical markers of disease progression.

*HLA-DRB1*15*

The effect of this allele was evaluated in a group of 53 MS patients with HLA-DRB1*15 and 64 MS patients without. Both groups were comparable with respect to age, gender, and disease duration. The effect of HLA-DRB1*15 allele was unclear. The global t-statistic was marginally negative and most individual scores for clinical and MRI outcomes were negative. Presence of HLA-DRB1*15 had a positive effect on some MRI markers of disease progression. In particular, from the odds ratio analysis it became clear that patients harboring the HLA-DRB1*15 allele were about 5 times less likely to get new lesions at follow-up (odds ratio 0.19, p=0.02). On the other hand, lower brain volume and worse whole brain atrophy were found for HLA-DRB1*15 carriers, yet not significant.

Patients with and without HLA-DRB1*15 were also used for the analyses of subpopulations (e.g. see analyses of HLA-A*02 and HLA-B*07), which showed no clear effect of HLA-DRB1*15.

*HLA-B*08*

For HLA-B*08 allele, there was a discrepancy in group size between patients with and without the allele (24 vs. 122 patients). The groups were well matched in terms of age and gender, but slightly imbalanced for disease duration. The overall statistical score pointed towards a negative direction. In absence of any covariates, the HLA-B*08 group had significant lower whole brain volume compared to the rest of the patients (not protective, t=-2.07, p=0.046), yet not significant after controlling for scanner model, field strength, contrast, patient age, MS duration, sex or treatment line. In terms of clinical scores, HLA-B*08 had lower apc-EDSS (protective, t=-2.54, p=0.012) and lower apc-MSSS (protective, t=-2.48, p=0.014). Both findings remained significant after controlling for single covariates. Furthermore, a trend towards lower apc-9HPT was found for HLA-B*08 carriers, which turned significant ($p<0.05$) when controlling for MS type (t=-2.48, p=0.015) or MS duration (t=-2.01, p=0.047).

Effect of HLA-B*08 on disease progression was ambiguous. None of the HLA-B*08 carriers showed progression in the 9HPT score, explaining the significant better odds ratio (OR=0, p=0.03). Also, HLA-B*08 carriers were 4 times less likely to develop a new 5mm diameter lesion. On the other hand, they more often experienced EDSS and whole brain atrophy progression (not significant).

HLA-B*44

Patients with HLA-B*44 seemed to be more susceptible to MS progression. Group sizes between patients with and without HLA-B*44 were different (24 vs. 122 patients), but both groups were balanced in terms of gender and disease duration. Patients without HLA-B*44 were slightly older (mean age at baseline of HLA-B*44 carriers 36.3 vs 42.1-year-old without this allele).

The overall statistical score pointed towards a negative direction. The overall count of new lesions was significantly higher in HLA-B*44 carriers compared to non-carriers (t=3.08, p=0.002), which was still the case after controlling for any covariate. Although there was a trend for smaller total

lesion volume in the carriers, the significant larger count of new lesions was paralleled by a trend towards larger increase in total lesion volume (not significant), driving the overall adverse effect of the HLA-B*44 allele. Furthermore, after controlling for age at baseline, an increased aPLVC in HLA-B*44 carriers (t=2.40, p=0.018) was found, indicative for vulnerability. We found no significant odds ratio for progression of clinical or MRI outcomes between carriers and non-carriers, although the odds for new lesion count were 2.4 times as high for HLA-B*44 carriers (not significant). Regarding clinical outcomes, there was a larger increase over time in 9HPT duration for HLA-B*44 (t=2.06, p=0.042). This remained after controlling for any covariate. Furthermore, the absolute value of 9HPT was found higher in the carrier group (not protective) after controlling for baseline age (t=1.39, p=0.017), MS duration (t=1.48, p=0.013), treatment line (t=1.02, p=0.029), patient sex (t=1.20, p=0.029), or MS type (t=1.23, p=0.023).

*HLA-DQB1*06*

The evaluation of HLA-DQB1*06 was based on a subset of patients, consisting of 71 patients harboring HLA-DQB1*06 and 46 without this allele. There was a trend towards a negative effect on MRI outcomes on average, mainly because of a trend for stronger atrophy (not significant). Regarding clinical parameters, a trend for increased apc-T25FW (not protective) was found in HLA-DQB1*06 carriers, yet this only became significant after controlling for MS duration (t=2.04, p=0.044)

Presence of HLA-DQB1*06 had a negative effect on the majority of disease progression MRI and clinical markers.

Results are summarised in (Table 5).

	DRB15	B7	A2	DQ6	B8	B44
Effect	inconclusive	Towards negative	Protective	Towards negative	Towards negative	Towards negative
Significant scores *= became or remained significant after controlling for covariates	New lesions odds ratio	aPBVC* Lesion volume	MSSS	Apc-T25FW*	Brain volume Apc-EDSS* Apc-MSSS* Apc-9HPT*	New lesions count aPLVC* 9HPT* apc-9HPT

Table 5. Summary of general trends of the statistical analysis of the relationship between HLA genotype and clinical/MRI outcomes

Discussion

This extension study on a bigger sample and with a longer follow-up mainly confirmed the results of our previous work [314]. The presence of HLA-B*07, HLA-B*08, HLA-B*44 and HLA-DQB1*06 was correlated to a worse disease status and/or disease progression as evaluated by multiple clinical and MRI outcomes, whereas HLA-A*02 to a better disease status. Presence of HLA-DRB1*15 had an ambiguous effect. Presence of this allele in combination with HLA-A*02 seemed to damp the protective effect of HLA-A*02.

This is the first study to investigate the relation between HLA genotype and overall MS progression by applying many clinical and MRI outcomes and using a validated software for MRI measurements. MS being a heterogeneous disease, multiple disease parameters should be evaluated in order to explore any correlation between a biomarker like HLA genotype and disease progression. The clinical relevance of the potential impact of HLA genotype on each individual clinical parameter is difficult to extrapolate from this study and remains to be explored in bigger cohorts and longer follow-up periods. Our results are in line with prior studies on this subject [282, 301, 333] and further strengthen the role of HLA genotype as a potential biomarker of disease trajectory that can be used early in the MS course to inform treatment decisions. There are currently many available drugs for the treatment of MS and yet it is challenging to predict treatment response at the individual level. Therefore, such biomarkers of disease progression could be of great interest to help neurologists in the treatment choice.

MHC class I and II alleles cannot yet be considered as causal variants; they probably represent markers of independent protective haplotypes within the MHC. It is also possible that other alleles in linkage disequilibrium with these HLA markers (HLA-A*02, HLA-B*07, HLA-B*08, HLA-B*44, HLA-DRB1*15) could be required to achieve the protective or non-protective effects observed. Several hypotheses have been made to explain the potential causal relationship between specific HLA alleles and MS risk and prognosis, including those related to effect of CD8+ T cells, Vitamin D and Epstein-Barr virus (EBV) [50, 282, 333, 346].

This study presents some limitations. The large number of statistical tests may require a type 1 error correction. However, given the exploratory nature of this study and, in order to enhance sensitivity, we did not correct for multiple comparisons. Furthermore, not all HLA subtypes were equally prevalent in our sample, leading to differing sample sizes for the various evaluations. Some group differences may therefore be underestimated. As a result, results of individual statistical tests should be interpreted with caution. This is why we opted to interpret the pattern of positive or negative weighted T-statistics providing a general idea of the direction of changes and effect size. Another limitation is the lack of MHC Class II data as well as some clinical data from one MS center (CHUV, Lausanne). Finally, as outlined in Table 1 our cohort was predominantly composed of MS patients of the relapsing remitting type. While we included the MS type as a covariate in statistics, our results may therefore be mostly representative for RR and less for progressive types.

Larger and longer studies with additional potential biomarkers, such as neurofilament in CSF, Optic Coherence Tomography (OCT) are needed to confirm these results. Moreover, the investigation of the potential effect of HLA genotype on treatment response especially in patients who switched from one compound to another would be of interest.

Statement of authorship

AL, GP, TB, AR, RDP, CPK, PM, MT participated in the concept, design, analysis, writing and revision of the manuscript.

Disclosures

AL has nothing to disclose

GP has nothing to disclose

TB reports personal fees from icometrix, during the conduct of the study; personal fees from icometrix, outside the submitted work.

AR reports personal fees from icometrix NV, during the conduct of the study; personal fees from icometrix NV, outside the submitted work; In addition, Dr. Ribbens has a patent EP2996085 issued.

RDP reports grants from Biogen, personal fees from Biogen, personal fees from Celgene, personal fees from Merck, personal fees from Novartis, personal fees from Roche, personal fees from Sanofi-Genzyme, outside the submitted work.

CPK has nothing to disclose

PM has nothing to disclose

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General conclusions

This project evaluates the relative frequencies of different HLA alleles in an MS population in comparison to healthy controls, before investigating the potential for HLA genotype to be a marker in MS prognosis based on various clinical and MRI measurements. In addition, as MRI scans in this study were conducted with either 1.5T or 3T magnetic resonance, it was important to establish if this variation had any impact on the data obtained, therefore, the comparability of 1.5 versus 3.0 Tesla brain MRI with regards to brain volume measurements is also investigated[193]. The MSmetrix median percentage error of the brain volume measurement between a 1.5T and a 3T scanner was found to be 0.52% for GM and 0.35% for PV. For Siena this error equaled 2.99%. When data from the same scanner were compared, the error was in the order of 0.06-0.08% for both MSmetrix and Siena. Therefore, MSmetrix appears robust on both the 1.5T and 3T systems and the measurement error becomes an order of magnitude higher between scanners with different field strength. Subsequently, we concluded that comparisons between groups of individuals scanned in 1.5 and 3.0T is feasible with no significant error. More important variability has to be considered when comparing MRI from the same individual.

Data from previous studies identified during my literature searches, suggest that the HLA genotype could be a valid candidate as a marker of MS prognosis. Among class II HLA alleles the HLA-DRB1 and HLA-DQB1 classes of genes have the highest effect on the increased risk of MS with the partially dominant HLA-DRB1*15:01 shown to have the strongest association with MS, especially in Caucasian populations [253, 255, 276, 278-284, 286, 288]. Among the genes from the HLA-DQB1 group, the HLA-DQB1*06:02 allele has been identified as a major susceptibility allele [280, 292, 296-298].

In my investigative studies, when comparing HLA alleles frequencies in MS patients and healthy controls, an association with MS was found for the HLA-DRB1*15 and HLA-DQB1*06 and for haplotype DRB1*15-DQB1*06. The HLA-B*07 allele also tended to be more frequent in MS patients, and was more frequent among MS patients with than in those without the HLA-DRB1*15 allele. Other alleles were underrepresented in MS patients, such as the HLA-DRB1*07 and HLA-A*02 alleles, showing a protective role against the disease. The HLA-B*44 and HLA-DRB1*04 alleles tended to be less frequent in MS patients.

Interestingly, based on both the pilot and extension studies, HLA-A*02 was a marker of a better prognosis (EDSS, MSSS, T25W and new lesion count) and, in contrast, HLA-B*07, B*08 and B*44 seem to be associated with a worse prognosis. However, a role for HLA-DBR1*15 as a marker in MS prognosis was unclear based on these studies, a phenomena which is shared by previous studies by others [276, 289, 290]. The HLA-DBR1*15 allele clearly correlates to younger age of onset, but the lack of association with disease outcome or duration suggests that the contribution of HLA in MS is probably linked with onset and initial triggering mechanisms rather than influencing disease progression, chronicity and severity[290].

MHC class I and II alleles exist on haplotypes that extend over long physical distances and encompass many different polymorphisms. Thus, based on this data and that of others, they cannot yet be considered as causal variants; they probably represent markers of independent protective haplotypes within the MHC. It is also possible that other alleles in linkage disequilibrium with these HLA markers (A*02, B*07, B*08, B*44, DRB1*15) could be required to achieve the protective or non-protective effects that we have validated.

Some hypothesis have been made with concern to the explanation of a potential causal relationship between some HLA alleles and MS risk and prognosis. CD8+ T cells and natural killer (NK) cells harbor receptors for MHC class I. The association between MHC class I and MS risk could fit well with the reported dysfunction of CD8 and NK cells in early stages of MS [346]. Others support the hypothesis that microglial activation, defined genetically by a particular HLA genotype, may act as a marker for oligodendrocyte/myelin and axonal pathology in MS [282]. A vitamin D response element (VDRE) in the HLA-DRB1 promoter region has been identified and functionally characterized [50]. The role of HLA Class I may occur in the context of environmental factors such as EBV and involve CD8 cells [347]. HLA-B*07 and A*02 have been associated with higher baseline anti-VCA IgG levels and follow-up anti-EA IgG levels lower anti-VCA IgG levels respectively [333].

This project uses for the first time in published literature, various clinical and MRI scores as part of an MS HLA study. Moreover, the software used for MRI measurements is validated and results seem to confirm previous studies. However, because of the large amount and variety of data, statistical analyses become more complicated, making it harder to establish significance.

Furthermore, the sample size and the duration of follow-up are limitations of the project. The results should therefore be treated with caution, since they reflect an estimation of trends.

Larger MS cohorts with more extended follow-up and higher resolution HLA typing of multiple loci to supplement the ongoing large SNP-based efforts are needed. Examining more homogeneous populations in terms of MS type that share more clinical, MRI and pathophysiological similarities, i.e. RRMS or CIS and Radiologically Isolated Syndrome (RIS), can ensure more robust data generation. The development and use of a new composite score encompassing many of the more meaningful clinical and MRI parameters could allow less complex statistical analysis and therefore more solid conclusions.

Other potential biomarkers, like GM volumetry, neurofilament, OCT, proteomics could be included in MS-HLA studies. In addition, further investigation of the immunological profiles of patients with the different HLA genotypes to identify potential correlations could also prove useful, however any such study would need to be performed before treatment initiation as therapy usually involves immunomodulators which would confuse the analysis.

One of the critical barriers to largescale genetic mapping is data availability: retrieving detailed disease data from medical charts if available and written in many languages and scattered across hundreds of medical centers on several continents might be a challenging task.

This thesis aimed to establish how different HLA genotypes correlate to MS severity and disease progression and whether they could be used as additional disease biomarkers and to a large extent the work has succeeded in this task. Association of MS with the alleles HLA-DRB1*15 and HLA-DQB1*06 and haplotype DRB1*15-DQB1*06 was identified, and under representation of other alleles, such as the HLA-DRB1*07 and HLA-A*02 alleles, showed a potentially protective role against the disease. HLA-A*02 was shown to be a marker of a better prognosis and, in contrast, HLA-B*07, B*08 and B*44 seem to be associated to with a worse prognosis. It is my hope that the work contained within this thesis, along with the ongoing research it may inspire, can be used to better the treatment outcomes of MS patients by enabling clinicians to better predict disease progression and severity.

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Appendix 1

Lysandropoulos 2016 [193]

Quantifying brain volumes for MS patients follow-up in clinical practice comparison of 1.5 and 3 Tesla MRI

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Abstract

There is emerging evidence that brain atrophy is a part of the pathophysiology of Multiple Sclerosis (MS) and correlates with several clinical outcomes of the disease, both physical and cognitive. Consequently, brain atrophy is becoming an important parameter in patients' follow-up.

Since in clinical practice both 1.5Tesla (T) and 3T magnetic resonance imaging (MRI) systems are used for MS patients follow-up, questions arise regarding compatibility and a possible need for standardization.

Therefore, in this study 18 MS patients were scanned on the same day on a 1.5T and a 3T scanner. For each scanner, a 3D T1 and a 3D FLAIR were acquired. As no atrophy is expected within one day, these datasets can be used to evaluate the median percentage error of the brain volume measurement for gray matter (GM) volume and parenchymal volume (PV) between 1.5T and 3T scanners.

The results are obtained with MSmetrix, which is developed especially for use in the MS clinical care path, and compared to Siena (FSL), a widely used software for research purposes.

The MSmetrix median percentage error of the brain volume measurement between a 1.5T and a 3T scanner is 0.52% for GM and 0.35% for PV. For Siena this error equals 2.99%. When data of the same scanner are compared, the error is in the order of 0.06-0.08% for both MSmetrix and Siena.

MSmetrix appeares robust on both the 1.5T and 3T systems and the measurement error becomes an order of magnitude higher between scanners with different field strength.

Introduction

Brain atrophy is a global marker of neuro-axonal loss resulting from demyelination and neuronal pathology (Filippi et al. 2010, Giorgo et al. 2008). It is now known that brain atrophy occurs in all clinical stages of Multiple Sclerosis (MS) at a rate of 0.5-1.0%/year *vs* 0.1-0.3%/year in healthy subjects (Filippi et al. 2010, Giorgo et al. 2008).

Different hypotheses have been addressed to explain atrophy in MS: dysfunction in neuronal connectivity, anterograde transynaptic degeneration, retrograde degeneration, wallerian degeneration or neuronal soma and dendritic shrinkage (Siffrin et al. 2010).

Brain atrophy is generally measured on 2D/3D T1-weighted images and it is analyzed using cross-sectional methods comparing patients to controls (e.g. brain parenchymal fraction (BPF), Structural Image Evaluation, using Normalisation, of Atrophy (SIENAX), voxel-based morphometry (VBM)) as well as longitudinal methods (e.g. SIENA)) (Giorgo et al. 2008).

Focal white matter (WM) lesions are the classic hallmark of MS. Profound alterations in normal-appearing WM (NAWM) and grey matter (GM) are associated with progressive loss of brain

volume (Smirniotopoulos et al. 2007, Marcovic-Plese et al. 2001, Kutzellnig and Lassmann, 2014). As a result, brain volume loss in MS occurs in both GM and WM (Filippi et al. 2012) in early and during all disease stages and subtypes (Giorgio et al. 2010). In addition, it has been demonstrated that brain volume loss is a predictor of long term disability progression (Popescu et al. 2013) and a marker of cognitive decline in MS (Morgen et al. 2006, Amato et al. 2007, Christodoulou et al. 2013, Houtchens et al. 2007). Therefore, brain volume evolution is emerging as one of the four parameters of MS to be considered when evaluating disease activity (NEDA-4 (no evidence of disease activity: relapses, EDSS, T2/Gd lesions, brain volume) (Giovannoni et al. 2015).

Since brain atrophy is related to clinical outcomes in MS, there is need for brain atrophy analysis on individual subjects in order to monitor treatment efficacy. However, in order to use brain atrophy measures in clinical practice, it is of paramount importance that the measurement error is very small. As the whole brain atrophy rate in MS patients is in the order of 0.5%-1%, reliable detection of subtle changes in brain volume is needed. MSmetrix brain volume measurements have been extensively tested for accuracy and precision in order to make it suitable for clinical practice. The method has obtained the CE mark and is approved for clinical use in Europe, Australia, India, Canada, Brazil and Iran. An additional challenge for using automated measurements in clinical practice is that the methods should be robust among different scanner types.

In this manuscript, we assess the intra and interscanner variability of two methods for automated brain for automated brain volume measurements at 1.5T and 3T MRI estimation at 1.5T and 3T MRI. To demonstrate the potential use in clinical practice, the measurement error within these scanners and between the scanners is evaluated. To this end, MS patients were scanned twice on both scanners during the same day.

Materials and methods

This prospective study was approved by our institutional review board and written informed consent was obtained from all participants (reference P2013/098 / B406201316929).

Patient population

18 MS patients (13 Relapsing-Remitting MS, 4 Secondary Progressive MS and 1 Primary Progressive MS) were enrolled. Inclusion criteria were MS diagnosis according to McDonald Criteria 2010 and no MRI contraindication. The mean age was 40 years old (range from 21 to 63 years old) and the female to male ratio 14:4. The mean EDSS was 3.1. The mean disease duration was 10 years. See Table 1 for the full overview of the population.

Table 1. Population overview with mean value, standard deviation and minimum and maximal values for age, disease duration since 1st symptoms, EDSS, brain volume, and lesion volume.

	Age (years)	1st Symptoms (years)	EDSS	Lesion Volume (ml)	Whole-Brain Volume (ml)	Gray Matter Volume (ml)
Mean	40	10	3.1	28.60	1021.40	637.91
SD	11	6	1.6	18.84	69.54	44.24
Min	21	3	1.5	2.64	891.75	560.63
Max	63	25	6.5	60.96	1134.88	704.42

MRI protocol

The patients were scanned on two Philips Healthcare MR systems (Philips, Best, The Netherlands): Intera (1.5T) and Achieva (3T). On each scanner, a clinical MRI protocol was acquired, including a transverse 3D FLAIR (Fluid Attenuated Inversion Recovery) sequence and a sagittal 3D T1-weighted turbo field echo sequence. The exact parameters are given in Table 2. This protocol was obtained twice on each scanner on the same day for all patients. Note that patients were not removed from the scanner in between the acquisition of the two MRI protocols.

Table 2. Scan protocol parameters for the 3D T1-weighted sequence (upper) and the FLAIR sequence (lower) on the Intera (left) and Achieva (right) systems.

	Intera	Achieva
3D T1 TFE		
Field strength	1.5 T	3.0 T
Acquisition voxel	0.87 × 1.25 × 1.2 mm^3	0.88 × 1.19 × 1 mm^3
FOV (field-of-view)	236 × 236 mm^2	200 × 239 mm^2
TR (repetition time)	8.8 msec	9.8 msec
TE (echo time)	4.2 msec	4.6 msec
Flip angle	8°	8°
Number of slices	130	160
Total scan duration	4 min 31 sec	5 min 35 sec
3D FLAIR		
Field strength	1.5 T	3.0 T
Acquisition voxel	1.36 × 1.77 × 1.5 mm^3	1.31 × 1.34 × 1.3 mm^3
FOV	240 × 192 mm^2	230 × 167 mm^2
TR	11000 msec	10000 msec
TE	140 msec	140 msec
TI (inversion delay)	2800 msec	2750 msec
Number of slices	96	105
Total scan duration	5 min 08 sec	7 min 30 sec

Image analysis

Scanning the patient twice on each scanner, allows three different test-retest datasets to be analysed. The first dataset includes for each patient 2 scan sessions on the Intera (1.5T), the second dataset is similar but all scans are acquired on the Achieva (3T) and the third dataset combines the first session from the Intera with the first session of the Achieva.

The different test-retest datasets, containing a 3D T1 and 3D FLAIR for two scan sessions on the same day, are analyzed with MSmetrix, a newly developed method to measure brain volume changes for MS patients.

MSmetrix is a CE approved automatic method for segmentation of GM, WM, cerebrospinal fluid (CSF) and white matter lesions based on unsupervised classification, as well as for a longitudinal atrophy measurement of whole brain or parenchymal volume (PV) and GM (Jain et al. 2015). It is an iterative method in order to optimize the segmentations of WM, GM and CSF based on the WM lesion segmentation and vice versa until convergence of the results. Figure 1 shows a schematic overview of the method.

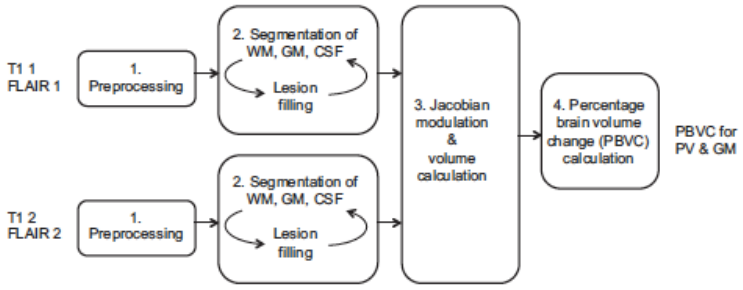

Figure 1. Schematic representation of MSmetrix, where the T1 and FLAIR of the two scan sessions that need to be compared are first preprocessed. Based on the preprocessed images, the segmentation of WM, GM and CSF is performed together with lesion filling in the second step. The third step is to calculate the volumes and perform the Jacobian modulation and only in the fourth step the actual PBVC is obtained.

The first step is a preprocessing step, during which for each session the FLAIR image and the T1-weighted image are rigidly co-registered to each other, followed by a skull-stripping of the T1-image. In addition, probabilistic anatomical priors for WM, GM and CSF are brought to the image space of the T1-image (Cardoso et al. 2012).

In the second step, the segmentation of the different brain structures is carried out for each session, using an expectation-maximization (EM) algorithm (Van Leemput et al. 2001) to model the intensities of each tissue class. In this step, also the white matter lesions are detected and filled so the lesion-filled image can be segmented again. This iterative process is repeated until the results for WM, GM, CSF and lesions do no longer change. Step 1 and 2 are still cross-sectional, i.e. the two scan sessions are processed separately.

In the third step, a jacobian modulation of the T1 images of each session to the T1 image of the other session provides us with a change in volume of one time point to the other. Now the information of both scan sessions is used together, which makes the method a longitudinal one.

In the last step, the volume changes of step three are averaged to obtain a robust measurement of the percentage brain volume change (PBVC) for PV and GM volume.

The results of MSmetrix are compared to the outcome obtained by SIENA (FSL, http://www.fmrib.ox.ac.uk/fsl), a commonly used software package for measuring whole brain atrophy (Smith et al. 2001, Smith et al. 2002) First, the Brain Extraction Tool (BET) is applied, by making a histogram of intensities and transforming the image into a binary mask (Jenkinson et al. 2001, Jenkinson et al. 2002). Subsequently, voxels within the obtained brain mask are classified in several classes, depending on the image intensities. As a result, CSF, WM, GM and background are segmented, and resulting cross-sectional volumes can be obtained, referred to as SIENAX (Gonzalez Ballester, 2000). Optimized brain extraction parameter settings were applied to ensure a correct masking of the brain (Popescu et al. 2012). A quality check was performed visually.

Based on the segmentation, brain parenchyma, or the combination of WM and GM, is classified and the edge between brain parenchyma and CSF is determined. When this is done for two MRI data sets of the same subject, they can be both transformed to an intermediate space using an affine transformation. Brain parenchyma/CSF edge displacement between the 2 time points is then estimated by aligning the peaks of the spatial derivatives of the intensity profiles of both images. Finally, the mean edge displacement is converted into a global estimate of percentage brain volume change between the 2 time points, referred to as SIENA.

Statistics

Based on the acquired MRI data sets, within scanner test-retest measurement errors for both 1.5T and 3T scanners, as well as the between scanner measurement errors are evaluated. For these experiments, the median over the patient population of the absolute values of the PBVC is calculated and denoted as the median percentage error. This is done for the PBVC of GM and PV obtained by MSmetrix and for the PBVC of PV obtained by SIENA. As these absolute values of the measurement errors are not normally distributed, the non-parametric paired Wilcoxon signed rank test was used to compare the errors between MSmetrix and SIENA for the within- and between-scanner comparisons. In order to visually compare the results of MSmetrix and SIENA

on the same data sets, Bland-Altman plots were generated for the measurement errors of both methods.

Results

In Figure 2, some visual results of the MSmetrix segmentations on a 1.5T and 3T scan of the same randomly selected subject are displayed. In Figure 2 (a) and (b), an axial slice of the 1.5T 3D T1 and 3D FLAIR are shown, respectively. For visualization purposes, the GM and lesion segmentation are visualized on the T1 (c) and the WM and lesion masks on the FLAIR (d). A similar slice was selected for the 3T scan, as shown in Figure 2 (e) and (f), for the 3D T1 and 3D FLAIR, respectively. Similar as in Figure 2 (c) and (d), the GM, lesions, and WM segmentations of the 3T MRI are displayed in Figure 2 (g) and (h). These lesion segmentations are then used to fill the 3D T1 with normal appearing white matter, as explained in Figure 1. The cross-sectional brain tissue segmentations that are shown in Figure 2 will be used as input for the longitudinal pipeline, to calculate the Jacobian of the deformation fields between both scans, resulting in a measure of brain and GM PBVC.

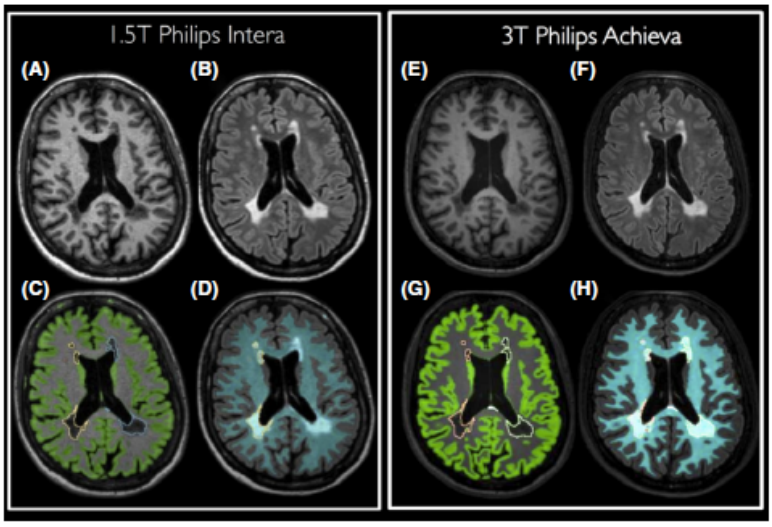

Figure 2. Visualization of MSmetrix segmentation results for the 1.5T (A–D) and 3T (E–H) scan of a randomly selected MS patient. T1 scans are shown in (A) and (E), FLAIR scans in (B) and (F). Lesion and GM segmentations are superimposed on the T1 scan, as displayed in (C) and (G). Finally, lesion and WM segmentations are visualized on the FLAIR scan in (D) and (H).

Boxplots of the measurement errors of the scan-rescan evaluations are presented in Figure 3. For the within scanner comparisons of the 1.5T and 3T scanner as well as the between-scanner

comparisons, boxplots of the absolute value of the measurement error (Figure 3 (a) and (b)) and of the measured scan-rescan PBVC (Figure 3 (c) and (d)) are displayed for both PV and GM. In Figure 3, MSmetrix results are shown in green, SIENA results in blue. The corresponding median and interquartile range of the absolute value of the measurement errors are displayed in Table 3.

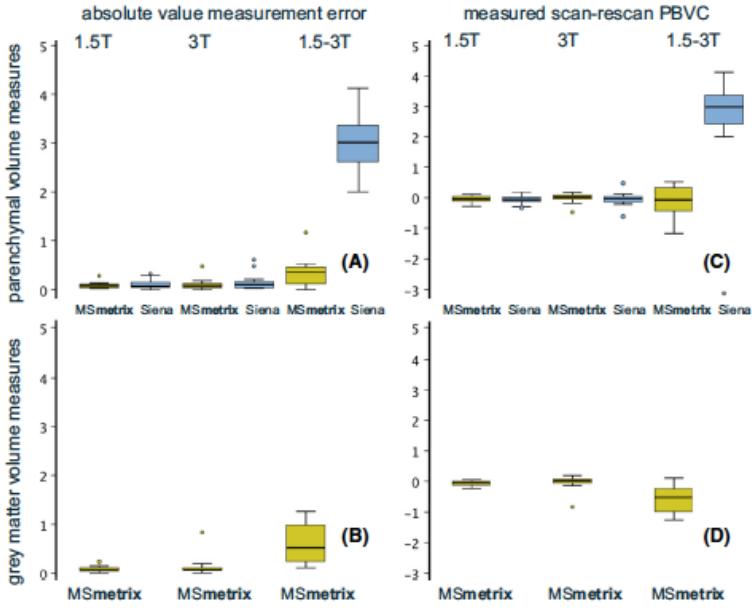

Figure 3. Boxplots of the measurement error. On the left, the boxplots of the absolute values of the measurement errors are shown for the parenchymal volume (A) and gray matter (B). On the right, boxplots of the measured scan–rescan PBVC (without taking the absolute value) are displayed for the parenchymal volume (C) and gray matter (D).

Table 3. Median and interquartile range (IQR) of the intra and inter-scanner test–retest measurement errors for PV and GM (in %).

	1.5T Intera		3T Achieva		1.5T versus 3T	
	Median	IQR	Median	IQR	Median	IQR
GM MSmetrix	0.061	0.07	0.067	0.04	0.52	0.70
PV MSmetrix	0.078	0.06	0.071	0.08	0.35	0.32
PV Siena	0.065	0.09	0.100	0.12	2.99	0.85

PV, parenchymal volume; GM, gray matter.

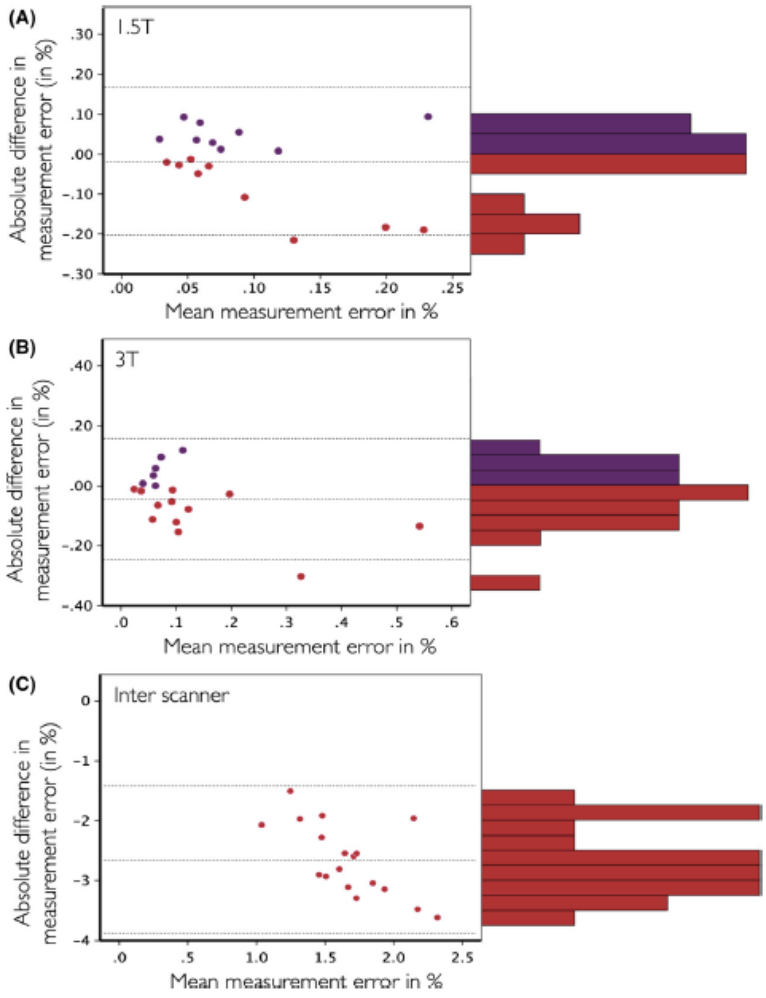

Figure 4. Bland–Altman plots of the comparison MSmetrix versus SIENA on the same datasets for the 1.5T within scanner (A), 3T within scanner (B), and 1.5T versus 3T between scanner (C) comparisons. On the *Y*-axis of all plots, the difference of the absolute value of the measurement errors is calculated as 'MSmetrix – SIENA', on the *X*-axis of all plots the mean of MSmetrix and SIENA is displayed. Purple dots were used when 'MSmetrix – SIENA' is positive, red dots when this difference is negative. In addition, the histogram of the 'MSmetrix – SIENA' difference is shown on the right of each Bland–Altman plot.

In Table 4, the median of the calculated PBVC measures (without taking the absolute value) are shown. These numbers represent the potential bias to measuring negative or positive atrophy within and between scanners.

Table 4. Median and interquartile range (IQR) of the intra and inter-scanner PBVC measures for PV and GM (in %).

	1.5T Intera		3T Achieva		1.5T versus 3T	
	Median	IQR	Median	IQR	Median	IQR
GM MSmetrix	−0.047	0.10	0.013	0.12	−0.52	0.70
PV MSmetrix	−0.036	0.14	0.023	0.12	−0.061	0.72
PV Siena	−0.056	0.12	−0.033	0.19	2.99	0.85
Wilcoxon PV	0.47		0.078		0.0002	

PV, parenchymal volume; GM, gray matter; PBVC, percentage brain volume change.

Discussion

Brain atrophy is a part of MS pathophysiology and is correlated to clinical outcomes, both physical and cognitive. Therefore, there is a need for measuring brain volume, and especially brain atrophy, in clinical practice for individual MS patients. In this manuscript, a longitudinal, Jacobian based method for measuring whole brain and grey matter atrophy is used. One of the main challenges of translating methods for brain atrophy from research analyses on groups of subjects to clinical practice in an individual patient is minimizing the measurement error of the assessment. To this end, in order to assess the use of this method in clinical practice on MRI data sets of individual MS patients, the measurement error of whole brain and grey matter volume measurements was evaluated in this manuscript. Results were compared to SIENA, a well-validated method for measuring brain atrophy. Note that only whole brain volume results are compared with SIENA, as no grey matter volume is measured with this software. To evaluate the measurement error of the brain volume measurement software packages, two sets of MRI data from a 1.5T and a 3T MRI scanner were acquired in 19 MS patients on the same day. It is then assumed that the brain volume would be the same between all MRI exams of each individual MS patient. The MRI protocol on each scanner consisted of a standard, non-optimized or harmonized 3D T1 and a 3D FLAIR. We notice that SIENA shows a large bias due to contrast differences. Volumes are consistently bigger when measured on a 3T image compared to a 1.5T image. MSmetrix is more robust to these

contrast differences due to regularization, where the whole brain is considered to determine the atrophy and not only the borders.

The MSmetrix software pipeline is specifically designed to measure atrophy in patients with MS, by including iterative lesion segmentation and lesion filling based on FLAIR and T1-weighted MRI scans. In this context, it is known that applying brain atrophy measures without performing lesion filling can introduce errors between 0.3% and 2.5%, depending on the lesion size and lesion intensity (Chard et al. 2010, Battaglini et al. 2012, Popescu et al. 2014). As all MRI scans were acquired on the same day, no changes in lesion volume or distribution are expected in the data that were analyzed. Performing lesion filling before the volume measures did not have an effect on the presented results and no additional errors have been added to the errors mentioned in this manuscript.

To the best of our knowledge, this is the first paper describing measurement errors of brain atrophy methods based on scan-rescan MRI data sets from different scanners on patients with MS. Other studies already evaluated scan-rescan errors in healthy subjects or patients with dementia (Smith et al. 2001, Nakamura et al. 2014, Cover et al. 2014). Another difference with these studies is that the MRI data sets used in our study were acquired using a clinical MRI protocol with 3D sequences. No optimized and typically longer research sequences were used, and the T1 and FLAIR sequences were not optimized within each scanner or harmonized between both scanners. In this context, in order to introduce brain atrophy measures in clinical practice, they should have an acceptable measurement error on MRI scans that can be obtained in a clinical setting with a limited acquisition time. As a result, the reproducibility results presented in this paper can be seen as representative for a clinical setting for patients with MS.

Our results demonstrate that a small brain volume measurement error can be achieved, especially when data of the same scanner are compared, in the order of 0.06-0.08% for both MSmetrix and SIENA. However, it should be noted that in this study, patients were not removed from the scanner in between both acquisitions on the same scanner. As a result, for the intra-scanner comparison, patients were positioned in the same way, which did not affect the measurement error results. This

can explain the lower measurement errors that were reported here for SIENA, compared to previously published studies, where errors in the order of 0.2% were found (Smith, De Stefano et al. 2001). Obviously, on the different scanners, patients were repositioned. Due to the repositioning, different sequences, different contrasts, the measurement errors were larger when scans from 1.5T and 3T were compared. Especially for SIENA, a significant larger measurement error was observed for the between-scanner analysis. In addition to an increased absolute error, it can be observed that a large bias was found. Although a trend was observed of a smaller measurement error for MSmetrix compared to SIENA for the within-scanner tests, only for the between-scanner comparison the Wilcoxon signed rank test indicated a significant difference. In contrast to SIENA, MSmetrix is able to also measure GM atrophy using a longitudinal approach.

Our study has other limitations. First, a small cohort of patients was included (18). Second, it is important to notice that all scans were acquired on Philips systems. Further research is needed to evaluate brain volume measurement errors on other MRI scanners. In conclusion, results of this study provide insights in the difference between 1.5T and 3T scanners and the clinical usability of automated measures on both scanner types. MSmetrix appeared robust on both the 1.5T and 3T systems, where it should be noted that the measurement error becomes an order of magnitude higher between scanners with different field strength.

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