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GENETIC, MAGNETIC RESONANCE IMAGING AND BODY FLUID BIOMARKER ASSOCIATIONS WITH SEVERITY OF MULTIPLE SCLEROSIS

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Genetic, magnetic resonance imaging and body fluid biomarker associations with severity of multiple sclerosis

Thesis for Doctoral Degree (Ph.D.)

By

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To my friends and family, who were always so supportive through this journey.

Popular science summary of the thesis

Multiple sclerosis (MS) is a disease that leads to damage of the nerve fibers and their insulating myelin sheets in the brain and spinal cord through inflammatory activity. Previous research studies have revealed a complex interaction of many environmental, lifestyle and genetic factors that increase the risk of developing MS. However, factors influencing the severity of the disease and the disease trajectories have been less studied, and therefore largely remain unknown. In part this may be explained by the wide spectrum of disease symptoms, limitations in severity assessment tools, and the complex background impacting disease severity. In this doctoral thesis, I investigated how a wide range of genetic variants influence MS-related pathology in the brain using magnetic resonance imaging (MRI) and plasma levels of a biomarker for nerve damage called neurofilament light (NfL) in persons with MS. I also studied how NfL levels early in the disease course affected the rate of MS-related brain pathology using MRI and how these MRI measures affected the progression of the disease as measured by different clinical assessment scales.

Abstract

Multiple sclerosis is a chronic and progressive neuroinflammatory disease that leads to demyelination and neurodegeneration in the central nervous system (CNS). Previous research has identified a wide range of environmental, lifestyle and genetic factors which increase MS susceptibility. However, the pathomechanisms that influence the severity of MS are largely unknown, and adequate biomarkers of disease severity are consequently lacking. Therefore, the aim of my thesis was to; 1) assess associations between the nerve injury biomarker neurofilament light (NfL) and brain atrophy and lesion volumes; 2) assess which brain/lesion volume measures show the strongest longitudinal association with clinical MS disability measures and to what degree these associations were affected by age; and to 3) identify genetic variants associated with brain atrophy, lesion volumes and plasma NfL (pNfL) levels in persons with MS.

In **Study I**, we assessed how cerebrospinal fluid (CSF) and pNfL levels were associated with T1- and T2-lesion volumes as well as whole-brain, cortical and subcortical grey matter, white matter and thalamic volume fractions of total intracranial volume based on magnetic resonance imaging (MRI). High baseline CSF and pNfL levels were associated with lower whole-brain, subcortical grey matter, thalamic, white matter and corpus callosal volume fractions over time. A further analysis showed that there was an association between baseline pNfL and baseline cortical grey matter fractions also in absence of radiological signs of inflammatory disease activity. A topographic analysis of cortical thickness showed that loss of cortical volume preferentially involved frontotemporal cortical regions. These findings indicate that NfL levels contribute information about MS severity not provided by traditional MRI lesion metrics.

In **Study II**, we showed that associations between baseline MRI variables, and baseline physical disability and self-reported impact of MS rapidly increased in strength in individuals beyond approximately 40-50 years of age. In separate longitudinal analyses using linear mixed-effects models, we showed that among the recorded brain volume measures, cortical and subcortical grey matter and thalamic volume fractions at baseline were the strongest predictors of future worsening in clinical disability over a median of approximately ten years' follow-up time. They were also stronger predictors than T1- and T2-lesion volumes.

In **Study III,** we assessed if a weighted risk score comprising 12 known MS risk human leukocyte antigen (HLA) alleles was associated with baseline and longitudinal MRI measures as described in Studies I and II. While this risk score was not significantly associated with baseline MRI measures, we found that a high score was associated with lower cortical grey matter fractions longitudinally. A further analysis showed that this effect was primarily driven by the *HLA-DRB1*15:01* allele. These results suggest that MS HLA risk variants not only affect inflammatory, but also neurodegenerative aspects of the disease.

In **Studies IV and V**, we performed genome-wide association studies of pNfL levels and whole-brain volume fractions, respectively, in persons with MS (and controls in Study IV). While no genome-wide significant associations were found in Study IV, gene set analyses highlighted a neural crest and odontogenesis development pathway in the regulation of pNfL levels, and a weighted MS susceptibility polygenic risk score was associated with higher pNfL levels in MS with statistical significance. These findings suggest that there is some degree of genetic regulation of pNfL levels, which partially overlap with MS risk. In Study V, we identified a genome-wide significant locus upstream of the glycerol kinase

2 (GK2) gene, previously implicated in the propensity for tobacco smoking, which is a known MS risk and severity factor. Gene set analyses in Study V also implicated Hypoxia Inducible Factor-1 (HIF1) in the regulation of whole-brain volume fractions, indicating that iron metabolism and response to hypoxia play a role in the neurodegenerative processes in MS.

List of scientific papers

I. Benjamin V. Ineichen, **Thomas Moridi**, Ewoud Ewing, Russell Ouellette, Ali Manouchehrinia, Leszek Stawiarz, Daniel Ferreira, Sebastian J. Muehlboeck, Jens Kuhle, Eric Westman, David Leppert, Jan Hillert, Tomas Olsson, Ingrid Kockum, Fredrik Piehl, Tobias Granberg.
Neurofilament light chain as a marker for cortical atrophy in multiple sclerosis without radiological signs of disease activity.
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II. **Thomas Moridi**, Leszek Stawiarz, Kyla A McKay, Benjamin V Ineichen, Russell Ouellette, Daniel Ferreira, J-Sebastian Muehlboeck, Eric Westman, Ingrid Kockum, Tomas Olsson, Fredrik Piehl, Jan Hillert, Ali Manouchehrinia, Tobias Granberg.
Association between brain volume and disability over time in multiple sclerosis.
Mult Scler J Exp Transl Clin. 2022 Dec 18;8(4):20552173221144230. doi: 10.1177/20552173221144230. PMID: 36570871; PMCID: PMC9768834.

III. **Thomas Moridi**, Pernilla Stridh, Ali Manouchehrinia, Leszek Stawiarz, Daniel Ferreira, Russell Ouellette, J-Sebastian Muehlboeck, Eric Westman, Tomas Olsson, Fredrik Piehl, Jan Hillert, Ingrid Kockum, Tobias Granberg.
HLA genetic burden is associated with longitudinal cortical atrophy in multiple sclerosis (submitted)

IV. **Thomas Moridi**, Pernilla Stridh, Adil Harroud, Klementy Shchetynsky, Ali Manouchehrinia, Jens Kuhle, Jan Hillert, Fredrik Piehl, Tomas Olsson, Ingrid Kockum.
A genome-wide association study of plasma neurofilament light chain levels in multiple sclerosis (in manuscript)

V. **Thomas Moridi**, Pernilla Strid, Adil Harroud, Klementy Shchetynsky, Leszek Stawiarz, Daniel Ferreira, J-Sebastian Muehlboeck, Eric Westman, Jan Hillert, Fredrik Piehl, Tomas Olsson, Tobias Granberg, Ingrid Kockum.
A genome-wide association study of whole-brain volume fractions in multiple sclerosis (in manuscript)

Contents

List of abbreviations

ARMSS	Age-Related Multiple Sclerosis Severity
BMI	Body Mass Index
BMP	Bone Morphogenic Protein
CIS	Clinically Isolated Syndrome
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
DMT	Disease-Modifying Treatment
EDSS	Expanded Disability Status Scale
EBNA-1	Epstein-Barr Nuclear Antigen 1
EIMS	Epidemiological Investigation of Multiple Sclerosis
ELISA	Enzyme Linked Immunosorbent Assay
eQTL	expression Quantitative Trait Loci
FGFR2	Fibroblast Growth Factor Receptor 2
FLAIR	Fluid-Attenuated Inversion Recovery
FUMA	Functional Mapping and Annotation
GEMS	Genes and Environment in Multiple Sclerosis
GFAP	Glial Fibrillary Acidic Protein
GK2	Glycerol Kinase 2
GSA	Infinium Global Screening Array-24
GWAS	Genome-Wide Association Study
HIF1A/B	Hypoxia Inducible Factor 1-Alpha/Beta
HLA	Human Leukocyte Antigen
IMSE	Immunomodulation and Multiple Sclerosis Epidemiology
IMSGC	International Multiple Sclerosis Genetics Consortium
IL-22BP	Interleukin-22 Binding Protein
IQR	InterQuartile Range
LD	Linkage Disequilibrium
LPA	Lesion Prediction Algorithm
LST	Lesion Segmentation Tool
MAF	Minor Allele Frequency
MS	Multiple Sclerosis
MSIS-29	Multiple Sclerosis Impact Scale 29

MRI	Magnetic Resonance Imaging
(p/s)NfL	(plasma/serum) Neurofilament Light
OE	Infinium Human Omni Express Bead Chip
OR	Odds Ratio
PCA	Principal Component Analysis
PIRA	Progression Independent of Relapse Activity
PPMS	Primary Progessive Multiple Sclerosis
RAW	Relapse-Associated Worsening
RRMS	Relapsing-Remitting Multiple Sclerosis
SDMT	Symbol Digit Modalities Test
Simoa	Single Molecule Array
SIENA(X)	Structural Image Evaluation with Normalisation of Atrophy (Cross-sectional)
SPM	Statistical Parametric Mapping
SPMS	Secondary Progressive Multiple Sclerosis
STOP-MS	Stockholm Prospective Assessment of Multiple Sclerosis
VEGF	Vascular Endothelial Growth Factor

1 Introduction

1.1 Multiple sclerosis

Multiple sclerosis (MS) is a heterogeneous chronic, inflammatory and demyelinating disease affecting the central nervous system (CNS)[1]. The disease onset is usually between 20 and 40 years of age, and females have a two-fold increased risk compared to males to develop MS[2]. Currently, there are 2.9 million people in the world estimated to be living with MS, and among young adults in Europe and the United States, it is estimated to be the most common non-traumatic cause of neurological disability[3-5]. More than 22,000 people live with MS in Sweden where the incidence of the disease is approximately 10/100,000 person-years and the prevalence is 215/100,000 inhabitants[5, 6]. MS can lead to impairment in motor, sensory, visual, bladder, sphincter and cognitive functions, largely depending on the location of the focal inflammatory lesions in the CNS[7]. These lesions can be located anywhere in the CNS, but typically in the periventricular, cortical/juxtacortical, infratentorial, or spinal regions of the brain[8]. In addition, MS leads to a substantially lower self-reported quality of life that deteriorates as the disease progresses[9]. Albeit the exact etiology of MS unknown, it is considered to be a multifactorial disease with environmental, lifestyle and genetic factors that interact with each other to confer risk of MS[2].

According to the diagnostic criteria for MS, the diagnosis is based on dissemination of typical symptoms and/or magnetic resonance imaging (MRI) lesions in space and time[8]. Cases without evidence of dissemination in both space and time are denoted as clinically isolated syndrome (CIS). As of the latest revision in 2017, the time criteria can be substituted with the presence of immunoglobulin gamma oligoclonal bands in the cerebrospinal fluid (CSF), as it is strongly linked with emergence of future CNS lesions[8]. In approximately 85 % of the cases, the disease follows an initial relapsing-remitting phase (relapsing-remitting MS, RRMS) characterized by a high degree of localized inflammation caused by peripheral autoreactive immune cells entering the CNS through the blood-brain barrier and the CSF (Fig. 1) [10, 11]. The relapses can cause transient or permanent accrual of disability, commonly denoted as relapse-associated worsening (RAW). After 10-20 years, the initial relapsing-remitting phase is followed by a progressive phase (secondary progressive MS, SPMS) that is characterized by overall less inflammation and a higher degree of neurodegenerative processes[1]. This leads to a continuous and irreversible decline in neurological function, commonly referred to as progression independent of relapse activity (PIRA). The remaining 15 % of MS cases follow a progressive trajectory already from the onset of the disease, referred to as primary progressive MS (PPMS). However, the distinction between relapsing-remitting and progressive MS has been questioned in recent years as PIRA has been shown to be common also in relapsing-remitting MS, accounting for up 50% of sustained disability accrual[12]. This suggest that MS should rather be viewed as a continuum of disease processes and symptoms than clearly distinct phases.

Increasingly potent disease-modifying treatments (DMTs) that mitigate the inflammatory activity in the CNS have been developed in recent decades[13]. These include drugs that induce immune tolerance (e.g. glatiramer acetate), modulators of inflammatory mediators (e.g. interferons), modulators of the immune response through intracellular mechanisms (e.g. teriflunomide and dimethyl fumarate) inhibitors of lymphocyte migration (e.g. natalizumab and fingolimod) and lymphocyte depleting therapies (e.g. cladribine, anti-CD20 and anti-CD52 antibodies). Autologous hematopoietic stem cell transplantation is an option for individuals with highly active relapsing MS who have not adequately responded to (or are not eligible for) highly potent DMTs. However, there is currently no definitive cure for the disease, and no effective treatment options for persons with a progressive disease phenotype[1, 13]. Due to the

heterogeneity between individuals regarding symptom presentation and disease progression, there is a need for sensitive and specific biomarkers to accurately diagnose the disease, and monitor and predict long-term clinical outcomes.

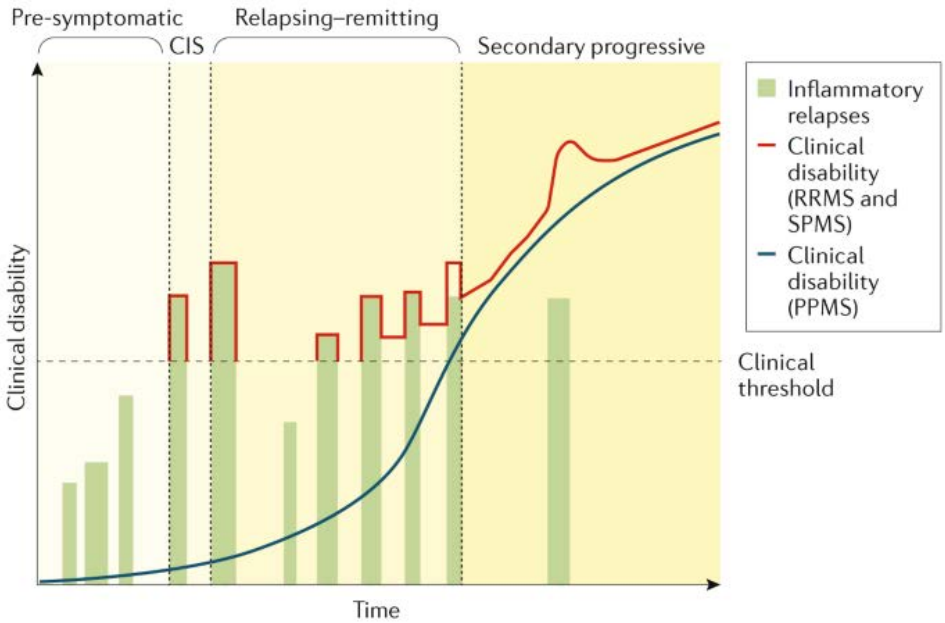

Fig 1. *The clinical course of multiple sclerosis. Abbreviations: CIS, clinically isolated syndrome; PPMS, primary progressive multiple sclerosis; RRMS, relapsing-remitting MS; SPMS, secondary progressive MS. Filippi, M., Bar-Or, A., Piehl, F. et al. Multiple sclerosis. Nat Rev Dis Primers* ***4****, 43 (2018). https://doi.org/10.1038/s41572-018-0041-4*

1.2 Pathophysiology of MS

During the 20th century, two main pathophysiological hypotheses of MS based on neuropathological findings emerged. The tendency of lesions to accumulate in the periventricular region of the brain led researchers to hypothesize that the pathogenic factor of MS entered the CNS from the CSF[14, 15]. However, findings of perivenular lymphocyte infiltration indicated an origin from the blood circulation through the blood-brain barrier[16]. In recent years, it has been evident that these are two co-existing immunological disease mechanisms that have different relative importance during the course of the disease[17, 18]. Activated lymphocytes have been suggested to enter the CNS through the blood-CSF barrier of the choroid plexus, as well as through the endothelial blood-brain barrier by upregulating adhesion molecules that facilitate lymphocyte migration and barrier disruption[13, 19, 20]. The myelin-reactive lymphocytes then cause focal demyelination identified as MS lesions on MRI and neuropathological examination, typically centred around veins in the white and grey matter[21]. The mechanisms by which lymphocytes become autoreactive in MS has remained elusive, although molecular mimicry between sequences of viral proteins – primarily from the Epstein-Barr Virus – and homologous sequences of CNS proteins in susceptible individuals have in recent years been proposed as one of the main mechanisms[22].

MS has traditionally been regarded as a T-cell-related disease, involving both activated cytotoxic $CD8^+$ and helper $CD4^+$ T-cells[1]. More recently, CNS resident microglia, peripheral myeloid cells and particularly memory B-cells in peripheral lymphoid organs have also been implicated in the pathogenesis of the disease[10, 23, 24]. Memory B-cells have been reported to have a crucial role in the activation of brain-specific autoreactive $CD4^+$ T-cells[10]. The importance of B-cells is also highlighted by the effectiveness of B-cells depleting therapies in substantially reducing clinical and radiological disease activity in the relapsing-remitting phase of the disease[13].

In the progressive disease phenotypes, the localized inflammatory activity of the adaptive immune system seen in relapsing-remitting MS has largely subsided due to age-related immunosenescence while the innate immune system is sustaining a more chronic and wide-spread inflammatory activity[23]. Activated microglia in normal-appearing white matter in the vicinity of lesions and slowly-expanding smouldering (chronic active) lesions with a rim of activated microglia have been suggested to be important drivers of progressive MS[25, 26]. Oxidative stress caused by myeloid cells, microglia and excessive accumulation of iron in the CNS has been shown to negatively impact the function of neuronal mitochondria, contributing to axonal degeneration[23]. These factors are compounded by ageing processes, including increased susceptibility to neuronal damage and decreased compensatory mechanisms, in particular remyelination and neuroplasticity[25].

1.3 What is a useful biomarker?

A biomarker is commonly defined as a measurable objective indication of a biological state and is usually measured using body fluids, soft tissues, clinical assessment scales or imaging techniques. The usefulness of a biomarker is determined by four different parameters: reliability, accuracy, assessability and accessibility.[27] Its measurement should be consistent under similar conditions (reliability) and give predictions with high sensitivity and specificity (accuracy) while also being affordable and logistically feasible (assessability). It should also be as non-invasive as possible (accessibility) to reduce the health risks for the individual being assessed. It is common to categorize biomarkers depending on the purpose of their use, and these include susceptibility, diagnostic, monitoring, predictive and prognostic biomarkers.[27] Some examples of both clinically established and more experimental biomarkers in each category are outlined in Table 1. It is important to note that no currently known susceptibility biomarkers have sufficient accuracy to be used for screening for MS in the general population. The current state of research regarding biomarkers for MS will be discussed in the coming sections of this work, mainly focusing on genetic, CSF and plasma protein as well as various MRI volumetric biomarkers of MS susceptibility and prognosis/severity.

Table 1. *Types of biomarkers for MS*

Type of biomarker	Example	Clinical use in Sweden
Susceptibility	*HLA-DRB1*15:01* allele (increases MS risk with OR ~3.9)[28]	No
	High body-mass index[29]	No
	Low serum levels of vitamin D[30]	No
Diagnostic	CSF Oligoclonal bands and kappa free light chain index (confirm dissemination in time)[8]	Yes
	Number of T2-lesions and contrast-enhancing T1-lesions on MRI (confirm dissemination in space and time)[8]	Yes
Monitoring	Neurofilament light in CSF and plasma (effect of disease-modifying treatments and confirmation of clinical relapses)[31]	Yes, in CSF
	Number of new T2-lesions and presence of contrast-enhancing T1-lesions on MRI (effect of disease-modifying treatments and confirmation of clinical relapses)[32, 33]	Yes
Predictive	JC virus antibody levels (risk of progressive multifocal leukoencephalopathy upon treatment with natalizumab)[34]	Yes
Prognostic	Age at onset, male sex, EDSS score, relapse rate and T2 lesion load on MRI at diagnosis (time to secondary progressive MS)[35]	Yes
	SNP rs10191329[A] (reducing the time to require a walking aid)[36]	No

Abbreviations: CSF, cerebrospinal fluid; EDSS, expanded disability status scale; HLA, human leucocyte antigen; JC, John Cunningham; MRI, magnetic resonance imaging; OR, odds ratio; SNP, single nucleotide polymorphism.

1.4 Clinical outcome measures

In clinical practice, the severity of MS is monitored over time with clinical scores based on objective neurological assessments or self-reported information, the number of inflammatory lesions on MRI of the brain and spinal cord, and more recently, CSF biomarkers of inflammation and neurodegeneration[37]. These assessments have become vital to select the appropriate type and timing of DMTs, given that a delay in treatment might cause further neurological disability[13].

Validated and widely used clinical measures of disease severity include the Expanded Disability Status Scale (EDSS), the Symbol Digit Modalities Test (SDMT) and the MS Impact Scale 29 (MSIS-29)[38-40]. The EDSS is the standard measure of physical disability. It is based on physical neurological examination of eight different functional systems of the CNS (e.g. motor, sensory, and cerebellar function) resulting in a score that ranges from 0 (no disability) to 10 (death). The SDMT is a test of information processing speed by measuring the number of symbols the person can pair with the right number using a key within 90 seconds. Each correct answer renders one point (range 0-110), and a high score thus indicates a high information processing speed. The MSIS-29 which is based on a self-report survey, includes 29 questions covering the psychological and physical impact of MS, and the results are converted into a score (range 0 – 100). A higher score indicates a more negative self-perceived impact of the disease.

1.5 Imaging and body fluid biomarkers for MS

MRI is currently the most pivotal method to objectively measure inflammatory activity and tissue damage in the CNS in persons with MS. It is a non-invasive imaging method that uses strong magnetic fields to align the rotational/spin axis of protons in the water of the tissues followed by pulses of radio waves to excite the protons changing their rotational axis. Antennas are then used to detect the radiofrequencies released from the protons as they return to equilibrium. The chemical composition of the tissues determines the amount of energy that is released and the time it takes for the protons to return to their equilibrium state, and this information is used to construct 2D or 3D images of the tissues. A major advantage over other imaging techniques such as computer tomography is that no ionizing radiation is used. While being relatively expensive and time-consuming, MRI does not confer any health hazards to the examined individual if safety procedures are followed correctly, and it can visualize MS-related pathology with high resolution and sensitivity.

The number and location of gadolinium-enhancing white matter lesions in the brain visualized on T1-weighted sequences and hyperintense white matter lesions visualized on T2-weighted sequences are standard markers for establishing diagnosis and measuring disease progression and treatment response in trials as well as the clinical setting[32, 33, 41]. T2- and contrast-enhancing T1-lesions reflect focal tissue oedema and axonal demyelination. The latter representing more recently developed lesions – usually within two months – hence capturing ongoing MS-related inflammation[33]. On the other hand, T1-hypointense lesions captured on native (without contrast) MRI sequences reflect the lipid and protein content of the tissue, and hence a permanent focal loss of white matter[42]. As a result, T1-lesions are more stable over time regarding their size than T2- and contrast-enhanced T1-lesions. Although not typically reported in clinical routine, T1-lesions have in clinical studies shown stronger correlations than T2-lesions with permanent physical disability in MS[42]. In older literature, T1-lesions were often referred to as “black holes” but this term is seldomly used today since this refers to the fact that only some lesions were detectable on 2D spin echo T1-weighted imaging. With modern MRI protocols, where T1-weighted imaging is often performed with 3D gradient-recall echo sequences, all lesions detectable on

T2-weighted imaging are also typically delineated on T1-weighted imaging, reducing the usefulness of the term.

While both the T1- and T2-lesion numbers are robust measures of white matter tissue damage in MS, they do not adequately measure the total lesion burden, since they do not account for the volumes of the individual lesions or their clinical impact based on location[43]. On the other hand, the total lesion volume is a metric that more exactly measures the lesion burden[44]. The lesion volume has, therefore, gained widespread use as an alternative or complementing outcome measure to total lesion number in MS studies. It should be noted that harmonization of MRI acquisition and processing protocols is of importance to reduce variability and increase comparability between study cohorts, albeit powerful statistical methods exist to account for such potential discrepancies[45, 46].

Traditionally, oligoclonal bands in the CSF have been the main routinely used MS body fluid biomarker in a clinical setting. However, it has only been shown to be informative in regards to establishing an MS diagnosis, while its correlation with clinical severity and progression has been notably weaker[8, 47]. A more recent alternative to oligoclonal bands is the CSF kappa free light chain index, which has a similar diagnostic accuracy while being less time- and labor-consuming and not relying on subjective interpretation.[48] On the other hand, the Neurofilament Light (NfL) chain protein has gained interest as the potentially most clinically useful body fluid biomarker of MS severity and treatment response[31]. NfL is a subunit of the neurofilament, a pivotal structural protein of the neuronal axon. It is released into the CSF upon neuronal death, and has received recognition as a clinically useful marker of neurodegeneration in MS and other neurodegenerative conditions such as Alzheimer´s disease[31]. NfL concentrations can be measured in the CSF with traditional Enzyme-Linked Immunosorbent Assay (ELISA). Recently, single molecule array (Simoa) digital ELISA has been a further development allowing for high-throughput analysis of proteins with concentrations at the sub-femtomolar level ($<10^{-15}$ M), including NfL in serum (sNfL) and plasma (pNfL), while conventional ELISA has a lower detection level at the picomolar level (10^{-12})[49]. It has been shown that CSF levels of NfL strongly correlate with s- and pNfL, providing a possibility for a simplified and safer measurement by a blood sample instead of spinal tap, although large body mass index (BMI)/blood volume have diluting effects on serum and plasma levels which needs to be adjusted for[50-52]. Studies have also shown that NfL levels in both CSF and plasma are reduced upon use of DMTs, with the most pronounced reduction for highly potent treatments including fingolimod, natalizumab, rituximab and alemtuzumab[50, 53-57]. Furthermore, s- and pNfL levels have been reported to associate with present and future brain and spinal cord atrophy and lesion accumulation, as well as relapses and permanent physical disability independently of other clinical or imaging variables[31, 58-60]. Additional studies in large cohorts are warranted to elucidate whether NfL can predict more long-term disability in MS, measured not only with clinical scales but also potentially more sensitive MRI outcomes, including brain atrophy metrics. The relationship between NfL levels and atrophy of different regions of the brain – such as white matter, cortical and subcortical grey matter – especially in the absence of lesion activity, also requires further investigation.

1.6 Brain atrophy – a more sensitive severity marker

It is evident that the clinical, imaging and body fluid markers outlined in this work do not account for the full extent of neurodegeneration and inflammation in MS. T2- and particularly T1-lesion count and volumes have in previous studies been reported to associate with progression of physical disability[42, 61, 62]. However, clinical progression can occur in the absence of new lesions or elevation of body fluid biomarkers[63]. Therefore, additional objective and robust measures of disease severity are warranted. As

a supplement to these existing disease measures, brain atrophy has been proposed to be a more sensitive measure of both physical disability and cognitive decline in persons with MS, potentially capturing MS-related neurodegeneration not evident by lesion volume/number or levels of body fluid biomarkers[64-69]. Its potential to predict future worsening of clinical symptoms, including the EDSS, has also been highlighted[70].

1.6.1 Brain and lesion segmentation methods

There are several available software packages for MRI-based automatic segmentation of regional and whole-brain and lesion volumes. Automated methods are user-independent, do not require raters with in-depth knowledge of neuroanatomy and are less time-consuming than manual approaches. These automated segmentation tools can incorporate different methods to classify the brain voxels to the correct tissue, mainly using signal intensity information from the images and/or a priori information from brain tissue probability maps that are based on a large collection of brain images that have been registered to a common space[71]. There are also other common features such as correction for signal intensity nonuniformity in the images, which is an important source of bias in the intensity-based tissue classification.

Structural Image Evaluation with Normalisation of Atrophy Cross-sectional (SIENAX), FreeSurfer and Statistical Parametric Mapping (SPM) are examples of widely used publicly available automated segmentation-based softwares for estimation of brain volumes using 3D T1-weighted images. SIENAX performs skull stripping after registration to a brain template and subsequently segmentation based on signal intensity.[72] FreeSurfer is a tool that uses a more complex pipeline that performs a combination of brain surface- and volumetric segmentations using within-subject templates that are unbiased regarding the time point of the scan, thereby increasing the robustness of the segmentations and reducing inter-individual variability.[73] SPM performs the image processing steps in a unified model that includes non-linear registration of the image onto a template and estimates voxel-wise tissue probabilities[74]. FreeSurfer has options for volumetric analysis of longitudinal MRI data, while SIENA is the corresponding longitudinal analysis software for SIENAX.

The advantages of SIENAX and SPM are their relatively short computational times and that these tools are widely used and validated, while a major limitation is that the segmentations are affected by the presence of MS lesions in the brain, although this can be overcome by using lesion filling techniques[75]. FreeSurfer is also widely used and validated, and it has been shown to give more robust estimates than SIENA(X) and SPM, even without lesion filling techniques since it already accounts for white matter hypointensities[75]. A major downside of FreeSurfer is that it uses a more computationally expensive algorithm than the other tools. Furthermore, none of these four tools are yet certified for clinical use. It is recommended to normalize the segmented brain volumes by dividing it with the total intracranial volume when using FreeSurfer and SPM, but this is not necessary for SIENA(X) since it already includes normalization for head size from the template registration process[72]. Albeit this normalization produces more robust estimates, especially if different scanners models and field strengths are used, it is still recommended to adjust any statistical analysis for the scanner model[75]. A graphical representation of the volumetric output of the FreeSurfer, SIENAX and SPM is shown in Fig. 2.

For segmentation of T2-hyperintense lesions, the Lesion Prediction Algorithm of the SPM Lesion Segmentation Tool (LST) and *nicMSlesions* are examples of robust commonly used publicly available softwares.[76-79] The LPA uses the model fit parameters from a high-dimensional logistic regression model from a training dataset from persons with MS to segment lesions in new images. The advantages of the

LPA are that it only requires T2-weighted fluid-attenuated inversion recovery (FLAIR) images as input and no parameters are required to be specified by the user, while a limitation is that the image acquisition parameters need to be somewhat standardized to obtain reliable results. *nicMSlesions* require FLAIR and T1-weighted images and uses a supervised deep learning method with two 3D convoluted neural networks. While this method was shown to be highly accurate, it has to be trained for each tested dataset, which may require more time and expertise than other methods such as LST.[77]

Major limitations of most of the available brain and lesion segmentation tools are the lack of clinical validation studies as well as the need for standardization of image acquisition (i.e., using the same pulse sequence, MRI system and acquisition parameters), although work is ongoing to develop tools that are less reliant on technical standardization.[69, 78]

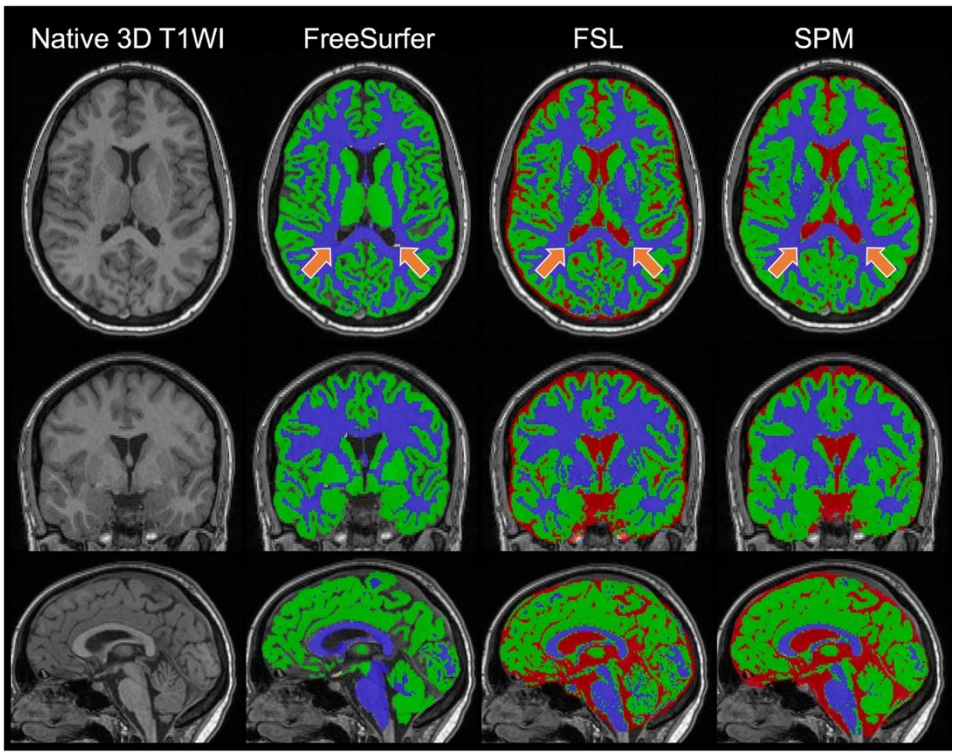

Fig 2. *Volumetric brain volume segmentation of a person with MS using different segmentation softwares. Only FreeSurfer performs segmentation of white matter hypointensities (at the orange arrows) and incorporates these in the total brain volume, while FSL-SIENAX and SPM segment these hypointensities as cerebrospinal fluid and/or grey matter. Green colour: grey matter. Blue colour: white matter. Black/red colour; cerebrospinal fluid; Yellow colour: white matter hypointesities. Segmentations by the SPM-CAT software were cropped out from the original figure. Guo C et al. Repeatability and reproducibility of FreeSurfer, FSL-SIENAX and SPM brain volumetric measurements and the effect of lesion filling in multiple sclerosis. Eur Radiol 29, 1355–1364 (2019). https://doi.org/10.1007/s00330-018-5710-x. Creative Commons CC BY (http://creativecommons.org/licenses/by/4.0/)*

1.6.2 Associations with clinical outcomes

Neurodegeneration and subsequent loss of brain volume is part of normal aging, but it is well established that there is a faster decline in brain volume in persons with MS than in healthy controls[80]. Furthermore, grey matter – in particular subcortical – atrophy measured with MRI has been shown to correlate with physical disability measured with the EDSS to a higher degree than lesion volume or other regions of the brain[64, 70, 81, 82]. Certain cortical regions, such as the insula and sensorimotor cortex, appear to be particularly associated with the EDSS as shown in one cross-sectional study[81]. The SDMT and other measures of cognitive function have likewise been shown to correlate with brain atrophy[83, 84]. The literature indicates that particularly strong associations exist between cognitive function and the cortical and subcortical grey matter regions – including the thalamic volume – compared with other segments or lesion volumes, albeit studies specifically focusing on the SDMT or other measures of information processing speed are relatively few[67, 85-87]. The importance of assessing the affected individual's subjective experience of the impact of the disease has also been highlighted in recent years. Indeed, the MSIS-29 has in a few studies been reported to correlate with objective disability metrics such as the EDSS, but its relationship with atrophy and lesion measures remains to be studied[40, 88, 89].

Large-scale, and in-depth longitudinal studies of the relationship of lesion and particularly regional atrophy metrics with physical and especially cognitive and self-reported disease severity are still lacking. Such studies are warranted in order for brain atrophy metrics to be adopted as informative and robust routine outcome measures in clinical practice in the future, although the complexity in processing and interpretation of the MRI images currently makes clinical implementation challenging[33]. Atrophy measures have the potential to add important information regarding, for instance, treatment response and sub-classification of the clinical trajectory of the disease.

1.7 Environmental and lifestyle factors for MS risk and severity

Several environmental and lifestyle factors have been reported to affect the risk of being diagnosed with MS. These include low serum levels of vitamin D, active and passive smoking, combustion-related air pollution, exposure to organic solvents, high BMI, shift work, sleep deprivation, head trauma, and Epstein Barr Virus, Human Herpes Virus 6, measles and influenza virus infections[2, 90-93]. The increased risk of MS conferred by high BMI, sleep deprivation and shift work has been reported to be age-dependent, reaching its peak during adolescence[2, 90, 91]. A correlation between high latitude and increased MS risk has also been observed, and it has been suggested that this association is mediated via vitamin D levels or directly via exposure to ultraviolet radiation[94]. Interestingly, low sun exposure statistically interacts with high levels of antibodies against Epstein-Barr nuclear antigen 1 (EBNA-1), suggesting that low sun exposure to some extent modulates – potentially via low vitamin D levels – the effect of EBV infection on MS risk through common pathogenic mechanisms.[95] Furthermore, most of these environmental and lifestyle factors have been shown to statistically interact with the *HLA-DRB1*15:01* and *HLA-A*02:01* alleles, stressing the importance of a comprehensive view of environmental, lifestyle and genetic factors in regards to MS risk (Fig. 3)[2]. While the associations between various environmental/lifestyle factors and MS risk have been extensively studied, the relationship between these factors and severity of MS is less established. However, some studies have shown that tobacco smoking is linked to brain atrophy and disability progression as measured with the hazard of reaching EDSS milestones[96, 97]. High BMI has likewise been reported to associate with disability progression in MS[98].

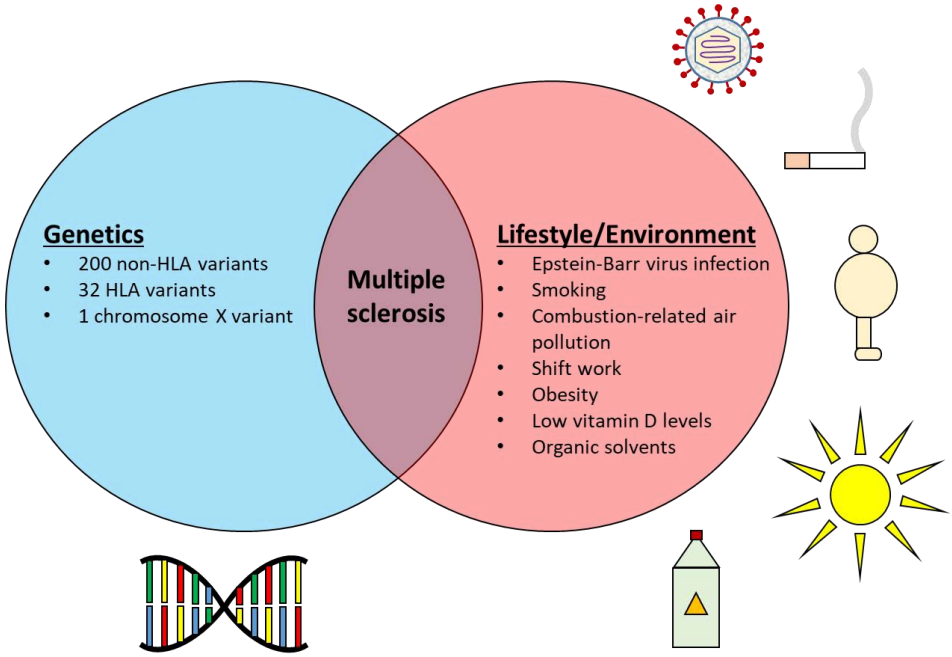

Fig. 3. *Genetic, environmental and lifestyle factors affecting multiple sclerosis risk*

1.8 MS genetics

1.8.1 Genome-Wide Association Studies

Over the past two decades, genome-wide association studies (GWAS) have shown to be a useful method to elucidate the influence of genetic variants across the whole genome on complex, multifactorial diseases such as MS[99]. Importantly, these studies may facilitate the understanding of the causal pathways of the disease, and provide new potential treatment targets and biomarkers. Knowledge of associated genetic variants may also facilitate personalized medicine by improving therapeutic choices and risk prediction on the individual level[99]. The GWAS methodology include the extraction of DNA from blood samples from the individuals, followed by genotyping of hundreds of thousands of single nucleotide polymorphisms (SNPs) using high-throughput genotyping arrays. Mostly common genetic variants – with minor allele frequencies (MAF) typically > 1 % – are selected for genotyping, as more rare variants require very large sample sizes to gain enough statistical power for genome-wide analyses. Thereafter, quality control is performed which typically includes removing SNPs with low minor allele frequency, low genotyping success rate and deviation from Hardy-Weinberg equilibrium; as well as filtering out study subjects with low genotyping success rate, mismatch between genetic and reported sex, high inbreeding coefficients, high degree of relatedness with other study subjects, or who are of a different ethnic origin than for the majority in the study population (i.e. outliers in the genetic population cluster calculated by, for instance, principal component analysis)[100]. After the quality control, imputation of additional genotypes can be performed as an optional step. SNPs that have not been genotyped in the study population, are statistically inferred (imputed) using the linkage disequilibrium (LD) structure in a reference population with a similar ethnic origin, which has been sequenced[101]. The result is a higher SNP density in the study population, which allows for association analyses with a higher resolution, hence increasing the probability of pinpointing causal variants.

Table 2. *Common terminology in genetic epidemiology*

Co-heritability	A measure of the overlap in genetic regulation of certain traits, i.e. the proportion of covariance between traits that is explained by genetics.
Hardy-Weinberg equilibrium/law	This law states that the allele and genotype frequencies will remain constant over generations assuming an infinitely large population and in the absence of mutation, migration, natural selection or genetic drift. Under this law, the genotype frequencies are a function of the allele frequencies at each locus. If the observed genotype frequencies in a population deviate greatly from the predicted frequencies, there is a high probability that the deviation is due to genotyping errors.
Heritability	The proportion of the total phenotypic variance that is explained by genetics.
Linkage disequilibrium (LD)	The estimated non-random correlation between genetic variants at different loci due to close positional proximity and co-inheritance within a chromosome. A higher the LD indicates a stronger correlation between variants than expected during random assortment.
LD pruning	A method to filter for SNPs that are uncorrelated within a sliding window of SNPs across the genome.
Minor allele frequency (MAF)	The frequency of the least common allele at a specific genomic location in a given population.
Population stratification/ substructure	Persons with different ethnic origins forming multiple subpopulations within a cohort. Allele frequencies can be different between subpopulations, thus potentially confounding genetic association analyses.
Principal component analysis	A statistical technique to reduce the dimensionality/complexity of data while preserving as much information as possible. It is a commonly used method to visualize and correct for population substructure in genetic association studies.
Single nucleotide polymorphism (SNP)	A variation of a single nucleotide, including A, C, G or T, at a certain genomic location. There are usually two different alleles for each SNP, e.g. an individual could have either C or T at a certain location. A pair of alleles, one from each chromosome, make up a genotype, e.g. C/C, C/T or TT.

Imputation also increases the overlap of SNPs in cohorts genotyped on different arrays, enabling meta-analysis of different cohorts. A schematic overview of the genotyping, quality control and imputation process is shown in Fig. 4. Finally, an association analysis is performed between the imputed genotypes and the phenotype of interest, while correcting for potential confounders. It is a requirement to correct for population substructure, by including, for instance, principal component vectors as covariates in the analysis[100]. Due to the high number of statistical tests in GWAS, it is necessary to perform conservative correction for multiple testing. It is now common practice to use Bonferroni correction for one million comparisons, corresponding to the number of independently inherited genetic "blocks" of the human genome[102]. Hence, the threshold for genome-wide significance is usually set to $p < 5 \times 10^{-8}$.

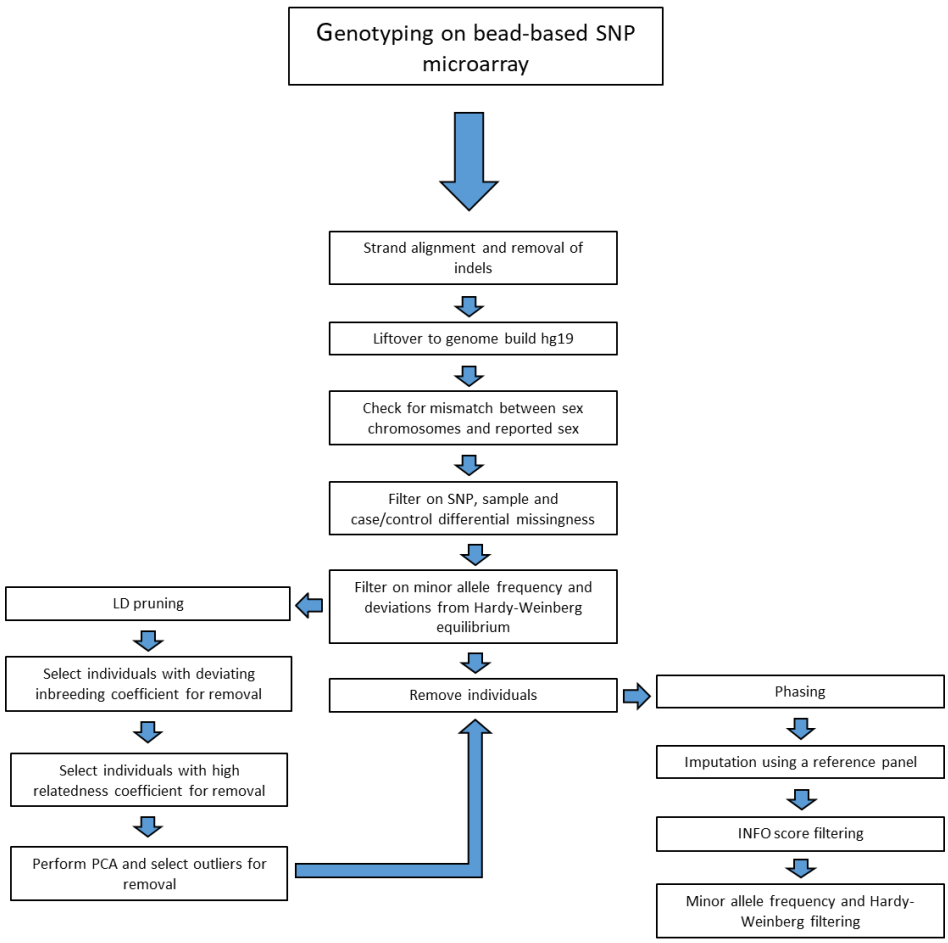

Fig. 4. *A flow chart with an example of the genotyping, quality control and imputation procedure for GWAS. Abbreviations: GWAS, genome-wide association study, LD, linkage disequilibrium, PCA, principal component analysis, SNP, single nucleotide polymorphism.*

1.8.2 The genetics of MS risk

The total heritability for MS has in a Swedish twin study been estimated to be 64 %, and an individual with a sibling or parent with MS is 6 times and 7 times, respectively, more likely to develop the disease compared with the general population[103]. A large number of genetic variants across the genome have in recent years been reported to affect MS risk. A recent GWAS by the International Multiple Sclerosis Genetics Consortium (IMSGC) showed that 232 genetic variants are independently associated with MS susceptibility with genome-wide significance[24]. These variants include ~30 human leukocyte antigen (HLA) alleles and ~200 non-HLA SNPs, including one variant on the X chromosome. The HLA alleles account for approximately 20 % of the estimated heritability of MS, while the non-HLA SNPs account for another 18 %. The primary MS risk variant is the *HLA-DRB1*15:01* allele with an odds ratio (OR) of ~3.9, while the main protective variant, *HLA-A*02:01* has an OR of ~0.7 (Fig. 5)[28]. In contrast, most non-HLA SNPs have a more modest OR of approximately 1.1-1.2. Albeit the exact mechanisms by which these genetic variants affect MS susceptibility are largely unknown, bioinformatical functional analyses of these GWAS hits have pointed to the involvement of peripheral T-, B- and myeloid cells as well as resident microglia in the CNS[104]. These results are corroborated by previous animal and *in vitro* studies suggesting that MS primarily is an immune-mediated disease, implicating both the innate and adaptive immune system[10, 23].

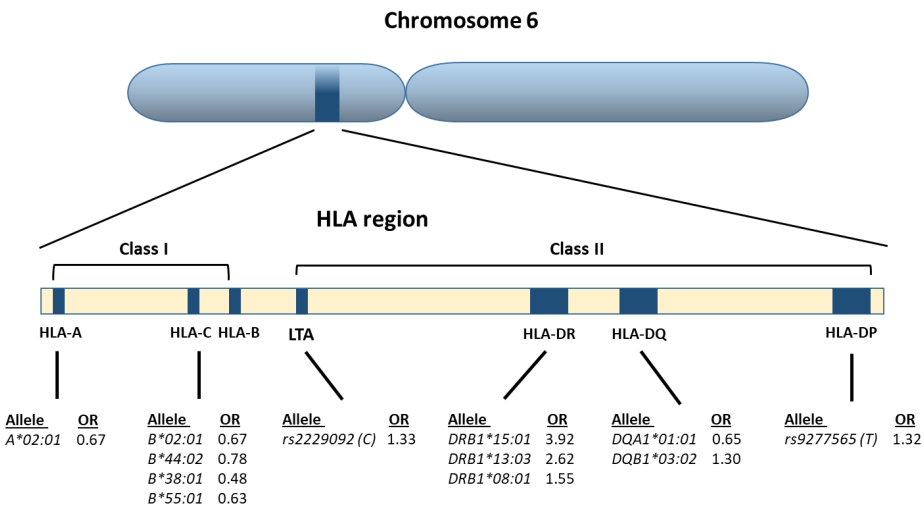

Fig. 5. *The HLA region on the short arm of chromosome 6. A total of 12 class I and II HLA variants are shown with their respective odds ratios for MS risk as estimated in Moutsianas L et al. Class II HLA interactions modulate genetic risk for multiple sclerosis. Nat Genet. 2015; 47: 1107-13. Abbreviations: HLA, human leucocyte antigen; LTA, lymphotoxin alpha; MS, multiple sclerosis; OR, odds ratio.*

1.8.3 The genetics of MS severity

In contrast to MS susceptibility, the potential genetic factors contributing to the severity and progression of the disease are still largely unknown. One recent large-scale severity GWAS included a discovery cohort of 12,584 persons with MS with a replication cohort of 9,805 individuals[36]. In this study, the SNP rs10191329 in the DYSF-ZNF638 gene was significantly associated with increased age-related

MS severity (ARMSS) scores, which is a ranked EDSS score within age-strata of persons with MS, and a median of 3.7 years of shorter time to require a walking aid.[105] A heritability enrichment analysis of the GWAS summary statistics showed enrichment in CNS tissues. Further assessment of the significant hit using Mendelian randomization and a real-world MS cohort showed associations with educational attainment. Taken together, these results suggest that genetically determined neurocognitive reserve plays a role for the clinical severity of MS. Another GWAS with discovery and replication cohorts of 506 and 485 persons with relapsing-onset MS, respectively, showed that the SNP rs11871306 within the WNT9B gene was significantly associated with a relapse hazard of 2.15[106]. A further gene set analysis showed an association between relapse hazard and a biological pathway related to response to vitamin D. However, to the best of our knowledge, no GWAS of CSF or pNfL levels, lesion number/volume or brain atrophy with a sufficient sample size to attain genome-wide significant results has yet been reported.

One study including 127 individuals having a first demyelinating event showed that 7 previously reported non-HLA MS risk SNPs were associated with longitudinal EDSS progression[107]. In one GWAS of cortical thickness including 675 MS cases, a gene set enrichment analysis with a protein interaction network was subsequently performed using sub-significant SNPs from the GWAS[108]. It showed enrichment for neural development, glutamate signaling and intracellular calcium regulation. These results could provide insights into the pathophysiological mechanisms of cortical thinning in MS, although no controls were used to differentiate the findings from other conditions or the normal aging process. In another GWAS of T1-lesion topology including 284 persons with MS, a similar protein interaction network analysis was performed, showing associations with proteins related to neural development and immune cell function[109]. The known MS risk variants/genes rs669607, *CYP27B1*, *IL12B*, *NFKB1*, *BATF*, *EVI5*, *PLEK*, *TAGAP* and *IL7* were in a cohort of 141 persons with MS reported to be associated with cervical atrophy[110]. Furthermore, the MS risk variant rs17066096 which encodes IL-22 binding protein (IL-22BP) was in a cohort of 84 persons with MS linked to higher CSF levels of IL-22[111]. High IL-22 levels were in turn associated with high MRI lesion numbers, but not with EDSS scores. Presence of the *HLA-DRB1*15:01* allele has in some studies been linked to a higher incidence of female than male MS cases, an earlier age at onset of MS, and a more beneficial effect of glatiramer acetate, a platform DMT for MS[112-116]. Small cross-sectional studies have also shown that the *HLA-DRB1*15:01* allele is associated with low whole-brain volumes measured with MRI in progressive and relapsing-remitting MS[117-119]. However, other studies of similar size have not shown any significant associations between the *HLA-DRB1*15:01* allele and whole-brain or T2-lesion volumes[96, 120]. One study assessed a genetic risk score comprising known MS risk HLA variants, and found it to be associated with low cross-sectional subcortical grey matter volumes in the relapsing-onset female sub-group comprising 439 individuals.[121] Another study showed that a combined HLA and non-HLA risk score was associated with thalamic atrophy in 467 persons with MS and that this association was replicated in a cohort of 132 MS cases.[122] Genetic risk scores leverage the combined effect of many genetic variants with individually modest effects, thus rendering more statistical power to the analysis[113]. Presence of the *HLA-B*44:01* allele, which previously has been linked to a reduced risk of MS, was shown to be associated with higher cross-sectional brain volumes, indicating a potentially protective effect regarding MS-related neurodegeneration[123]. However, these reports on HLA associations have not been consistent across studies, possibly due to differences in study design, the small sample sizes and the modest effect sizes that have been observed. Furthermore, large-scale longitudinal studies with adequate follow-up time addressing the genetic impact, including genetic risk scores, on brain atrophy in MS are currently lacking.

2 Research aims

In this doctoral thesis, I investigated large MS cohorts with a deep level of genetic, clinical, imaging and body fluid biomarker data. The overall aim was to identify factors that associate with the severity of MS in order to improve the understanding of the pathomechanisms of the disease. This knowledge may guide future research regarding more individualized disease prognostication, treatment selection and identification of new therapy targets.

Specifically, the aims of this research project were:

In **Study I**, to investigate whether baseline CSF and pNfL levels are associated with cross-sectional and longitudinal MRI-based brain and lesion volumes in persons with MS; to determine whether NfL levels are differentially associated between these MRI measures; and whether there were any associations between NfL and brain volumes in the absence of radiological signs of disease activity.

In **Study II**, to assess which MRI-based brain volume and brain lesion volume metric are most strongly associated with longitudinal physical disability, cognitive processing speed and self-reported impact of MS, respectively. I also aimed to investigate whether the strength of the cross-sectional associations between clinical and MRI measures differ with age.

In **Study III**, to identify HLA gene variants that are associated with cross-sectional and longitudinal MRI-based brain and lesion volumes in persons with MS.

In **Study IV**, to identify genetic variants and associated biological pathways affecting cross-sectional pNfL levels in persons with MS and whether these associations differ from healthy controls.

In **Study V**, to identify genetic variants and associated biological pathways affecting baseline whole-brain volume fractions in persons with MS.

3 Materials and methods

3.1 Study cohorts

I used the following real-world cohorts for the studies presented in this thesis: the Genes and Environment in MS (GEMS), Epidemiological Investigation of MS (EIMS), Immunomodulation and MS Epidemiology (IMSE) and Stockholm Prospective Assessment of MS (STOP-MS).[124-127] STOP-MS is a prospective cohort with ongoing recruitment since 2001 for which the aim is to assess how the timing of DMTs affects long-term disability in persons with MS. EIMS is an incidence-based cohort that includes persons with MS with ongoing recruitment since 2005 from neurological clinics across Sweden. GEMS is a cohort that included prevalent persons with MS recruited 2009-2010 identified through the Swedish national MS registry. IMSE 1 and 2 are post-marketing studies of DMTs for persons with MS. Study I-III and V comprised individuals from all four cohorts, predominantly the STOP-MS cohort, while Study IV comprised individuals from the EIMS and IMSE cohorts with partial overlap with the STOPMS and GEMS cohorts. Population-based controls from the EIMS study for Study IV were retrieved from the national population register and matched by age with five-year intervals, residential area and sex. MRI and clinical data including EDSS, MSIS-29 and SDMT scores were obtained on an approximately annual basis for all cohorts. Measurement of the clinical scores is ongoing while MRI data collection used for the current studies was collected up until 2015 (after which technical changes were made to the MRI scanners and protocols).

3.2 Genotyping and imputation

3.2.1 Study III

In Study III, the study participants were genotyped on the MS replication Chip, which is a customized Illumina SNP genotyping chip that has a dense coverage in the HLA region. The variant quality control included the exclusion of SNPs with: missingness rate >= 0.1; deviation from Hardy-Weinberg equilibrium at $p < 0.0001$; and minor allele frequency < 0.02. The sample quality control included exclusion of individuals with: SNP missingness rate >= 0.02; mismatch between genetic and reported sex; inbreeding coefficient deviating >= 3 standard from the mean. After quality control, the SNP data was used to perform imputation of HLA alleles with four-digit resolution using the HLA*IMP:02 software.[128, 129] An extended reference panel for HLA class II alleles was used that included 400 Swedish control subjects from the EIMS study.[130] After imputation, individuals related at the second degree or closer were removed. Principal component analysis was applied to account for population substructure. Specifically, samples that were > 6 standard deviations from the mean in any of the first 11 principal components were excluded. The first 11 principal components were also included as covariates in the statistical analyses to account for any remaining population substructure. None of the 12 established MS risk HLA alleles that were included in the study deviated significantly ($p < 0.0001$) from Hardy-Weinberg equilibrium[28].

3.2.2 Studies IV and V

In studies IV and V, the study participants were genotyped on the Infinium Human Omni Express Bead Chip (OE) and the Infinium Global Screening Array-24 (GSA) by deCode Genetics Inc (Iceland) following DNA extraction from blood samples. Genotype quality control, imputation and post-imputation quality control were performed as described in the multi-center MS severity GWAS by

Baranzini et al.[131] This is summarized in the following sub-sections about cohort-level quality control and stratum-level (all cohorts on the same genotyping arrays combined) quality control, respectively.

3.2.2.1 Cohort-level quality control

Indels as well as mitochondrial variants were excluded. Individuals with genotype missingness > 0.05 and/or a mismatch between genetic and reported sex were removed. To avoid the effects of population stratification, variant quality control was performed in individuals from the largest ancestral group derived from each cohort. In order to identify this ancestral group, high-confidence autosomal variants were selected using the following filtering criteria: genotype missingness < 0.01; MAF > 0.05; Hardy Weinberg equilibrium $p > 10^{-10}$; excluding palindromic variants (AT/CG); LD-pruning (*PLINK2 --indep-pairwise 1000 kb 1 0.01*); removing genomic regions with large principal component loadings; only keeping markers present in 1000 Genomes phase 3 reference panel. These high-confidence variants were used to perform PCA on 2,534 individuals from the 1000 Genomes phase 3 reference panel that were unrelated to each other (*PLINK2 --king-cutoff 0.1*). Samples from our cohorts were projected onto that space and clustering was then performed on the principal components. For samples that fell into the largest ancestral group, which overlapped with the 1000 Genomes European population, variants were filtered according to the following criteria: genotype missingness < 0.02 (< 0.05 in the replication sub-cohort in Baranzini et al); Hardy Weinberg equilibrium $p > 10^{-10}$ ($> 10^{-6}$ for controls in the replication sub-cohort); MAF > 0.01; absolute difference in allele frequency < 0.1 and log2 fold-change < 5 between cohorts in the discovery sub-cohort; difference in missingness between cases and controls with $p > 10^{-4}$ in the replication sub-cohort. For the individual quality control, persons with an inbreeding coefficient > 0.05 were removed. *PLINK2 --king-cutoff 0.0442* was used to remove individuals who were related at the third degree or closer. Finally, cohorts that were genotyped on the same genotyping array were merged together.

3.2.2.2 Stratum-level quality control

Sample quality control was performed on each stratum comprising all cohorts that have been genotyped on the same genotyping array. Duplicates across cohorts and related individuals were removed using the same approach as in the cohort-level quality control. To account for population stratification, PCA was used to remove samples that were > 6 standard deviations from the mean of each stratum on any of the first 10 principal components using 5 iterations. Then, principal components were calculated for the entire 1000 Genomes phase 3 reference cohort, onto which our participants were projected. Samples that were > 6 standard deviations from the mean of the 1000 Genomes European population on any of the first 10 principal components were excluded. Further variant filter criteria were then applied: genotype missingness < 0.05; deviation from Hardy Weinberg equilibrium $p > 10^{-10}$; < 0.4 or > 0.6 alternate allele frequency for palindromic variants; absolute difference in alternate allele frequency < 0.2 relative to individuals of European ancestry in the Haplotype Reference Consortium panel (version 1.1).

3.2.2.3 Phasing and imputation

Eagle2 (version 2.4.1) was used to perform phasing in 20 Mb segments with 5 Mb overlapping flanking regions[132]. To increase the imputation accuracy, each strata was merged with the Haplotype Reference Consortium. Minimac4 was then used to perform imputation on the phased genotypes.[133] Several different analyses were performed to examine imputation quality, chromosome continuity, differences

in allele frequency relative to the reference panel. Finally, imputed variants with MAF < 0.01, deviation from Hardy Weinberg equilibrium at $p > 10^{-6}$ or $R^2 < 0.8$ were excluded.

3.3 Body fluid biomarker measurement

For Study I, CSF samples were collected during routine neurological diagnostic work-up, centrifuged immediately thereafter and then stored at −80°C before analysis. Measurement of NfL levels was performed with a commercially available ELISA kit according to the manufacturer's instructions (Uman Diagnostics, Umeå, Sweden).

For Studies I and IV, ethylenediaminetetraacetic acid (EDTA)-treated blood samples were posted by mail at room temperature before plasma separation by centrifugation and then stored at −80°C before analysis. pNfL concentrations were measured at the University Hospital Basel using the Simoa immunoassay with the commercially available NF-Light Advantage kit with antibodies from Uman Diagnostics (Quanterix, Lexington, MA) according to the manufacturer's instructions. Both storage and measurement were done on pseudonymized samples and in the same way for cases and controls. The samples were analyzed in two batches, each of which comprised both EIMS and IMSE participants as well as cases and controls.

3.4 Image processing

For Studies I-III and V, 3D T1-weighted MRI scans were performed on 1.5 and 3 Tesla MRI scanners (Vision Plus, Aera, Avanto, and Trio; Siemens Healthcare, Erlangen Germany). The protocol was consistent with a magnetization-prepared rapid gradient echo (MPRAGE) sequence and a spatial resolution of 1.5 x 1.0 x 1.0 mm. The T1-lesion volume ("WM hypointensities") and volumes of the whole-brain ("BrainSegVol"), cortical ("CortexVol") and subcortical grey matter ("SubCortGrayVol"), thalamus ("Right Thalamus Proper" and "Left Thalamus Proper"), white matter ("CerebralWhiteMatterVol") and corpus callosum ("CC_Central", "CC_Posterior", "CC_Mid_Posterior", "CC_Anterior" and "CC_Mid_Anterior") were segmented using the longitudinal stream of the FreeSurfer 6.0.0 software (http://surfer.nmr.mgh.harvard.edu/), without any lesion filling techniques since white matter hypointensities are taken into account by FreeSurfer.[73, 75] This image processing was performed through the HiveDB database system.[134] We used the LPA of the SPM LST (https://www.statistical-modelling.de/lst.html) for segmentation of the T2-lesion volume using 2D or 3D FLAIR sequences, whichever was available.[79] Normalization of the brain volume measures was done by dividing the brain volumes by the estimated total intracranial volume, while no such normalization was performed for the T1- or T2-lesion volumes. All native and processed MRI images were quality controlled by a radiologist.

3.5 Statistical analysis

To assess the rate of change of the longitudinal outcome variables in Studies I-III, linear mixed effect models were used with adjustment for clinical and demographic fixed effect covariates and random effects.[46] Linear mixed-effects models can leverage the information from multiple data points within each group (e.g. repeated measurement of clinical scores from a study participant) while accounting for nested correlation structure of subgroups within groups (e.g. study participants within different cohorts). Multivariable linear regression models were used to assess the associations between cross-sectional NfL levels and MRI outcome measures in Study I, while multivariable rolling regression models were applied to determine whether the strength of the cross-sectional MRI-clinical associations in Study II

differed for different ages among the study participants. In Study I, we also used general linear models in FreeSurfer to topographically compare the cortical thickness of different brain regions between subgroups of study participants in regards to pNfL levels and lesion load. In Studies IV and V, we performed GWAS and polygenic score analyses of pNfL levels and whole-brain volume fractions applying multivariable linear regression on the imputed genotype dosages, adjusting for various clinical/demographic factors and genetic principal components to account for population sub-structure. Polygenic scores were calculated by multiplying the allele dosages with the reported effect size of the same allele in the base data (i.e. the MS risk HLA analysis by Moutsianas et al[28] as base data for Study III, the most recent IMSGC GWAS on MS susceptibility[24] as base data for Study IV, and the pNfL GWAS in Study IV as base data for Study V), and then adding the scores of all variants into a single score. Significantly associated SNPs were mapped to protein-coding genes using the SNP2GENE algorithm in the Functional Mapping and Annotation (FUMA) software and/or the Open Targets Genetics algorithm.[135, 136] In the same studies, gene set analyses were done using the GSA-SNP2 and MAGMA softwares in order to assess whether pNfL levels and whole-brain volume fractions were associated with specific biological pathways using the summary statistics from the GWA studies.[137, 138] Data curation and statistical analyses were performed in R, except for the GWAS which was performed in PLINK 1.9 and 2.0.[139-141] In all-sub-studies, transformation procedures were performed in case the continuous variables were substantially deviating from normal distribution.

3.6 Ethical considerations

There are four fundamental principles in medical research ethics that all researchers are morally and legally responsible to follow: non-maleficence, beneficence, justice and respect for the autonomy of the study participants[142]. Non-maleficience means that harm to the study participant must be avoided. My doctoral thesis includes large datasets with a wide range of sensitive personal data, including genetics, clinical/demographic characteristics as well as other types clinical and and paraclinical biomarker data. Thus, there is a risk that non-secure handling of this data can do great harm to the study participants. It has been imperative for me to minimize this risk by ensuring that all data was pseudonymized, stored on safe servers and in all aspects handled in compliance with Swedish data protection law and the European General Data Protection Regulation (GDPR). Another aspect of non-maleficience relates to the study-specific procedures, where harm also must be avoided and the benefits of the research must outweigh any potential harm. Most of the study procedures in this thesis were non-invasive, part of clinical routine and the risk of harm was considered to be outweighed by the benefits of these procedures to the patient. Beneficence is closely related to non-maleficience and means that the research should be aimed at doing good. Although the research results in this doctoral thesis likely will have no immediate and direct beneficial implication for the study participants or other persons with MS – which is the case for most research that is conducted – it may have that in future by facilitating future research discoveries applicable to the treatment and management of MS. Justice means that the research should be fair and comply with legal requirements and the rights of the study participant. Participation in any of the study cohorts in this thesis did not entail any direct advantage or disadvantage compared to non-participants in terms of access or quality of the health care, largely due to the fact that most procedures were part of clinical routine. Furthermore, all studies underwent ethical review to ensure that all ethical and legal standards were met. Respect for autonomy means that the participation in the study and any procedures and treatment are voluntary and that the participant has received sufficient information to make a . For all studies in this thesis, participation was voluntary and written informed consent was obtained from all study participants.

Ethical approval for all study-specific procedures, including clinical data collection, blood/CSF sampling and MRI acquisition and processing was obtained from the Stockholm Regional Ethical Review Board for the EIMS (04-252/1-4, 2017/1349-32 and 2018/2714-32), GEMS (2008/1617-31/2, 2017/1350-32 and 2018/2689-32), IMSE (2006/845-31/1, 2011/641-31/4, 2017/1426-32 and 2018/2639-32) and STOPMS (02-548, 2009/2107-31/2, 2017/1347-32 and 2018/2711-32) cohorts.

4 Results

4.1 Clinical characteristics

The clinical characteristics of the cohorts in Studies I-V are shown in Table 3.

Table 3. *Clinical characteristics of the study cohorts at baseline*

Variables at baseline	Study I	Study II	Study III	Study IV		Study V
				MS	Controls	
N	534	989	731	3706	822	644
Age, median (IQR) [a]	39 (11)	37 (17)	38 (18)	39 (16)	40 (17)	36 (16)
Disease duration at MRI scan, median (IQR)	2 (5)	1 (5)	3 (8)	4 (8)	NA	1 (1)
Sex, % females	70	71	75	71	72	75
MS phenotype (% relapsing-onset/% progressive-onset/ % NA)	97/2/1	93/7/0	93/7/0	97/3/0	NA	99.8/0.2/0
Charlson comorbidity score with weights according to Quan et al., median (range)	NA	NA	NA	0 (0 – 6)	NA	0 (0 – 6)
Whole-brain volume fractions (%), median (IQR)	74.3 (3.3)	74.2 (3.6)	73.9 (3.7)	NA	NA	74.3 (3.5)
T2-lesion volumes, median (IQR) [b]	2.0 (1.9)	4.0 (12.8)	4.1 (14.3)	NA	NA	NA
pNfL levels (pg/mL), median (IQR)	12.1 (10.1)	NA	NA	11.9 (10.2)	7.7 (4.1)	NA
CSF NfL levels (pg/mL), median, (IQR)	665 (972)	NA	NA	NA	NA	NA

[a] *mean (SD) is reported in study I.*

[b] *T2-lesion volumes were only available from 3D FLAIR images for Study I, while both 2D and 3D images were used for segmentation in the other studies.*

Abbreviations: CSF, cerebrospinal fluid; FLAIR, fluid-attenuated inverse recovery; IQR, interquartile range; MRI, magnetic resonance imaging; MS, multiple sclerosis; (p)NfL, (plasma) neurofilament light.

4.2 Study I

In Study I, a total of 534 persons with relapsing- (RR and SP) or progressive-onset (PP)MS were included in the final study cohort at baseline, and the median MRI scan follow-up time was 6.2 (IQR 6.0 - 9.0) years. We assessed how baseline levels of NfL in CSF and plasma associated with baseline and longitudinal MRI T1- and T2-lesion volumes as well as whole-brain, cortical and subcortical grey matter, white matter, thalamic and corpus callosal fractions of total intracranial volume. The models were adjusted for age at the baseline MRI scan, sex, disease duration, time difference between the first and the last MRI (in the longitudinal analyses), MRI scanner and time difference between blood/CSF sample and MRI (maximum 6 months difference). The longitudinal mixed-effects models included both random intercepts and time slopes. We observed a strong correlation between baseline CSF and pNfL levels but this correlation decreased with increased time intervals between CSF and blood sampling (e.g. $r \approx 0.85$ with 0.1 years' and $r \approx 0.75$ with 1 years' interval). Both high baseline CSF and pNfL levels were linked with high T1- and T2-lesion volumes and low baseline thalamic volume fractions using multivariable linear models. Applying linear mixed-effects models, high baseline CSF and pNfL levels were also associated with lower whole-brain, subcortical grey matter, thalamic, white matter and corpus callosal volume fractions over time. A further analysis showed that there was an association between baseline pNfL and baseline cortical grey matter fractions in the absence of radiological disease activity in the form of increasing T1-lesion volumes (Table 4). A topographic cross-sectional analysis of the cortical thickness using general linear models showed that high pNfL levels were associated with low cortical thickness in the temporal and frontal lobes in individuals with stable T1-lesion volumes.

Table 4. *Study I multiple regression models of baseline brain fraction measures and pNfL stratified according to T1-lesion volume accrual.*

T1+	**Low vs. high pNfL**	
	beta	**p-value (FDR)**
Whole Brain	0.209	0.61
Cortical gray matter	0.009	0.97
Subcortical gray matter	-0.066	0.1
Thalamus	**-0.029**	**0.039**
White matter	0.183	0.60

T1-	**Low vs. high pNfL**	
	beta	**p-value (FDR)**
Whole Brain	1.441	0.05
Cortical gray matter	**-0.247**	**0.001**
Subcortical gray matter	-0.135	0.07
Thalamus	-0.036	0.18
White matter	0.386	0.1

T1+ denotes individuals with increasing T1-lesion volumes over time, while T1- denotes individuals with stable volumes. pNfL levels were stratified into two groups: values below the lower quartile (≤ 375 pg/mL) and values above the upper quartile (≥ 1335pg/mL) in the cohort. The models were adjusted for age at MRI scan, MRI scanner, sex, disease duration, the time difference between the first and last MRI, and the time difference between blood sampling and MRI. Abbreviations: FDR, false discovery rate, pNfL, plasma neurofilament light.

4.3 Study II

In Study II, we investigated the associations of baseline MRI T1- and T2-lesion volumes, whole-brain, cortical and subcortical grey matter, white matter and thalamic fractions with partially overlapping EDSS, MSIS-29 and SDMT data over time and across different ages of a total of 989 persons with relapsing- and progressive-onset MS. These individuals were followed in our study for a median of 9.3 (IQR 6.2 – 13.7), 9.3 (IQR 5.4 – 13.8) and 10.1 (IQR 6.5 – 14.0) years for the EDSS, MSIS-29 and SDMT scores, respectively. As fixed effect covariates, we used baseline age at clinical examination, baseline age at MRI, sex, age at disease onset, clinical course (relapsing-onset vs. progressive-onset MS), total number of registered scores (in the SDMT analysis), type of FLAIR sequence (2D vs. 3D) in the T2 lesion volume analyses, platform DMT exposure and/or highly potent DMT exposure, depending on the parsimony of the models. Using multivariable rolling regressing analysis, we showed that the associations with the MRI variables increased rapidly in strength after approximately 40-50 years of age

for the EDSS as well as the physical and psychological MSIS-29 scores. The EDSS and T2-lesion volume rolling regression estimates are shown in Fig. 6 as an example of these age-varying associations. These findings remained in sensitivity analyses where persons with PPMS and/or recent relapses were removed. For SDMT, the associations across different ages did not show any obvious pattern. In separate longitudinal analyses using linear mixed-effects models, the baseline MRI volumetric predictor variables were dichotomized into below/equal to and above the median of the study cohort. We showed that low cortical/subcortical grey matter and thalamic volume fractions at baseline were associated with annual increases of 0.059, 0.047 and 0.053 EDSS scores, respectively. Low baseline whole-brain volume, cortical and subcortical grey matter and thalamic volume fractions were associated with annual decreases of 0.56, 0.47 and 0.53 SDMT scores, respectively ($p < 0.01$ for all). Furthermore, low subcortical grey matter volume fractions at baseline were associated with an annual increase of 0.021 MSIS-29 physical z-scores. These brain volume fraction measures were overall stronger predictors of the clinical scores than the T1- and T2-lesion volumes.

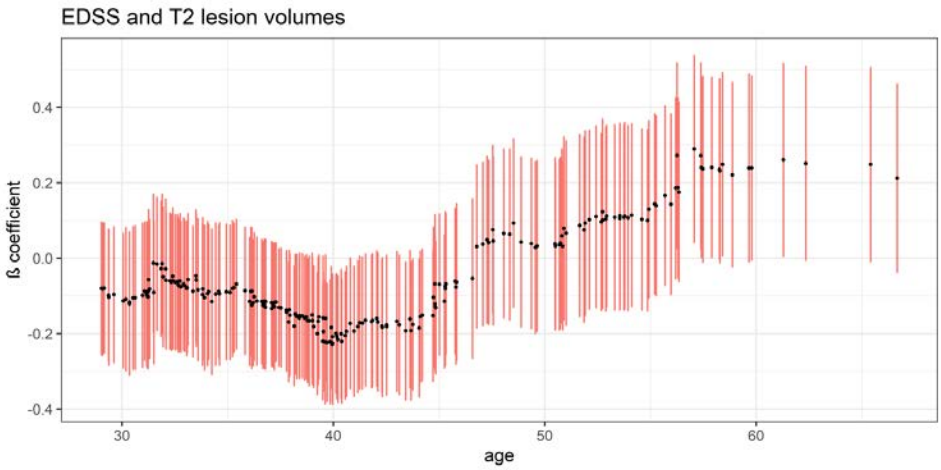

Fig. 6. *Rolling regression analysis of EDSS and T2-lesion volumes across age. The red bars represent 95 % confidence intervals. Abbreviations: EDSS, expanded disability status scale.*

4.4 Study III

In Study III, we assessed whether a weighted risk score comprising 12 previously well-established MS susceptibility HLA alleles were associated with baseline and longitudinal volumetric MRI measures: T1- and T2-lesion volumes, whole-brain, white matter and cortical and subcortical grey matter volume fractions. The study comprised 731 persons with relapsing- and progressive-onset MS. These individuals were followed with MRI for a median of 4.1 (IQR 1.2 – 8.0) years and underwent a total of 2546 scans. While this HLA risk score was not significantly associated with baseline MRI measures, we found that a high score was associated with lower cortical grey matter volume fractions longitudinally after adjustment for age at baseline, disease duration at baseline, clinical course (relapsing onset vs. progressive onset MS), sex, MRI scanner, type of FLAIR sequence (2D vs. 3D) for the T2-lesion volumes, exposure to platform and highly potent DMTs, exposure to corticosteroids and the first 11 genetic principal components. (Table 5). This association was robust for various sensitivity analyses, including MAF thresholding and adjustments for DMT exposure and potential confounding effects of

the values of the MRI measures at baseline. A further analysis showed that this effect was primarily driven by the *HLA-DRB1*15:01* allele.

Table 5. *Associations between HLA genetic burden and longitudinal MRI measures*

MRI metrics	Time interaction standard beta	CI (95%)	p-value
Whole-brain volume fractions	-0.0049	-0.010 – 0.00039	0.07
Subcortical grey matter volume fractions	-0.0041	-0.0086 – 0.00052	0.08
Cortical grey matter volume fractions	-0.011	-0.017 – -0.0044	0.0009
White matter volume factions	0.0059	-0.000023 – 0.012	0.05
T_1-lesion volumes	0.0047	-0.0014 – 0.011	0.13
T_2-lesion volumes	-0.0071	-0.020 – 0.0053	0.26

Linear mixed-effects models with random intercepts and slopes over time were used. Abbreviations: CI, confidence interval; HLA, human leukocyte antigen; MRI, magnetic resonance imaging; MS, multiple sclerosis.

4.5 Study IV

In Study IV, a GWAS was performed on pNfL levels separately in 3706 persons with MS (including both relapsing- and progressive-onset) and 829 population-based controls – and thereafter jointly in MS and controls using a case-control interaction term – to gauge the genetic regulation of this biomarker in a hypothesis-free manner. The analyses were adjusted for age, disease duration (for the MS analysis), sex, use of DMTs within 30 days before blood sampling (for the MS analysis), relapses recorded within 120 days of blood sampling (for the MS analysis), a modified Charlson comorbidity score (for the MS analysis; using the latest score prior to blood sampling), BMI (for the control and combined MS-control analyses) cohort (EIMS vs. IMSE for the MS and combined MS-control analyses) and the first 10 genetic principal components.[143] While no genome-wide associations were found in either MS cases or controls after having meta-analyzed the GSA and OE genotyping cohorts, we identified a total of 52 SNPs in 21 different loci that were suggestively significant in MS and 36 SNPs in 20 different loci in controls, which did not overlap with the SNPs associated in MS cases (Fig. 7). These SNPs were mapped to 16 and 20 non-overlapping protein-coding genes in cases and controls, respectively, using positional, 3D chromatin interaction and expression quantitative trait loci (eQTL) mapping. These genes were related to a wide range of cellular processes, some of which were related to neuronal and immunological functions. There were no significant differences in the strength of the associations between cases and controls using a case-control interaction term. Gene set analyses utilizing the entire distribution of the GWAS summary statistics from the MS analysis showed an association with an odontogenesis pathway implicating several neural crest-related genes, while no significant associations were observed in the controls. An MS susceptibility polygenic risk score was found to be significantly associated with higher pNfL levels in MS cases (beta = 143, p = 0.0035) using the same covariates as in the GWAS, and this association was primarily driven by non-HLA variants.

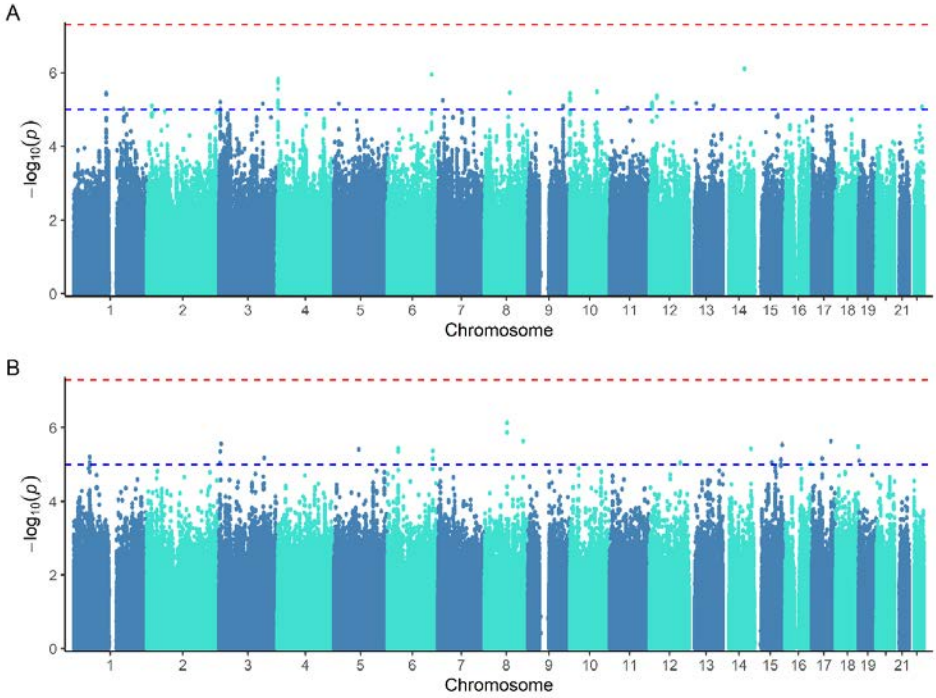

Fig. 7. *Manhattan plots of a genome-wide association study of plasma neurofilament light levels in multiple sclerosis* ***(A)*** *and population-based controls* ***(B)****. Dashed red line = genome-wide significance ($p < 5 \times 10^{-8}$). Dashed blue line = suggestive significance ($p < 1 \times 10^{-5}$). The genomic position is shown on the x-axis while the negative log base 10 of the p-value is shown on the y-axis.*

4.6 Study V

In Study V, we performed a GWAS on normalized baseline whole-brain volume fractions in 644 persons with relapsing-onset MS, similar to the analysis in Study IV. The analysis were adjusted for age and disease duration at the MRI scan, sex, MRI scanner and the first eight genetic principal components. After having meta-analyzed the GSA and OE genotyping cohorts, we identified a genome-wide significant locus with six intergenic SNPs in high linkage that were located 46 kBP upstream of the glycerol kinase 2 (GK2) protein-coding gene on chromosome 4, with rs3920463^{A} as the lead SNP (beta = -0.32, p = 1.23 x 10^$^{-8}$ [Fig. 8 A and B]). Each copy of the rs3920463 A allele conferred a 1.2 percentage unit decrease of non-transformed whole-brain volume fractions. The effect estimates were similar in the OE and GSA genotyping cohorts. A further 100 variants in 22 loci were suggestively significant. Gene set analyses applied to the GWAS summary statistics revealed significant associations with biological pathways pertaining to Hypoxia Inducible Factor-1 (HIF1), Fibroblast Growth Factor Receptor 2 (FGFR2) ligand binding and activation, and anatomical branching morphogenesis. Given the associations between NfL levels and brain volume fractions that we observed in Study I, we wanted to assess whether there was co-heritability between these traits using polygenic score analyses. A polygenic score with weights derived from the pNfL GWAS in Study IV was not significantly associated with the baseline whole-brain volume fractions (beta = -9.5, p = 0.66). Neither was there any overlap between variants that reached at least suggestive significance in the brain volume fraction GWAS and the pNfL GWAS.

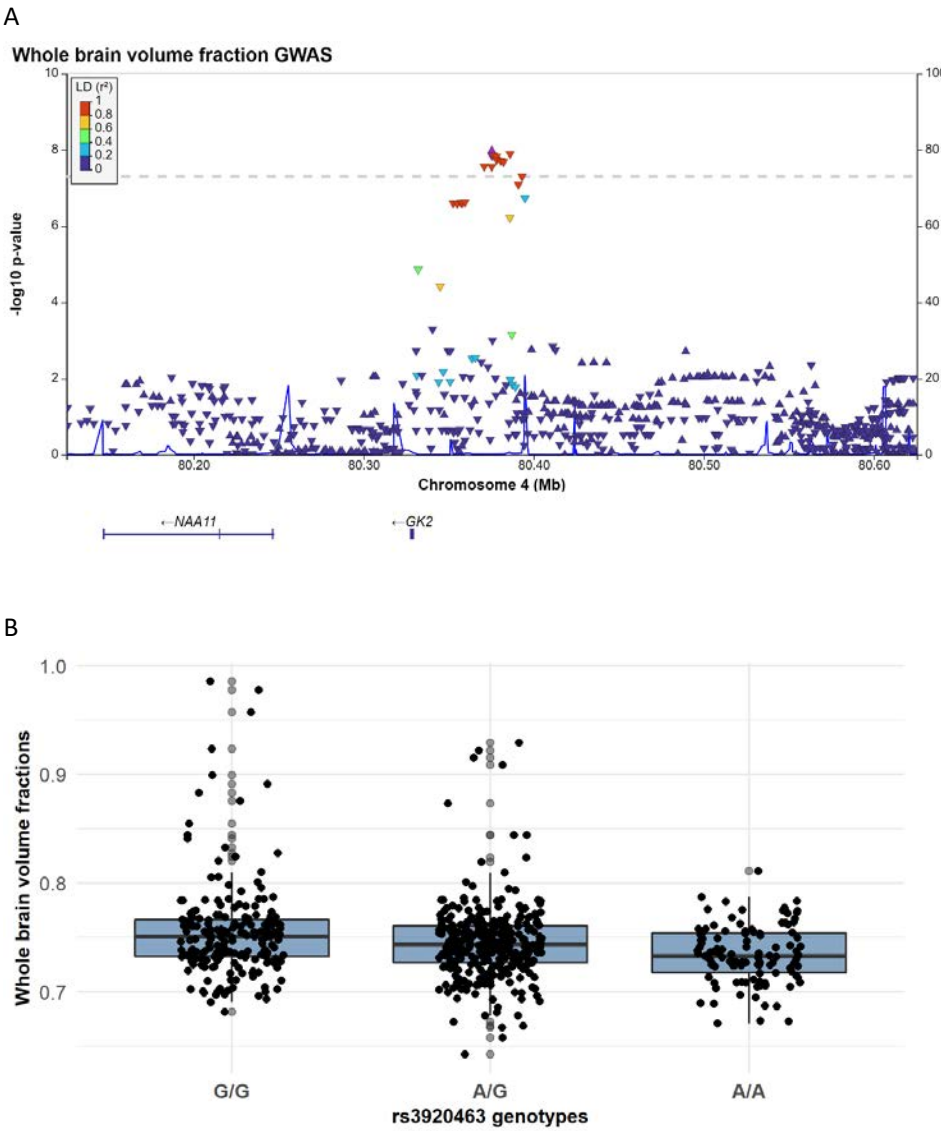

Fig. 8 A) *A Locus Zoom plot of an intergenic locus on chromosome 4 that is associated with whole-brain volume fractions in MS with genome-wide significance (dashed grey line). The lead SNP is colored in purple, while the surrounding SNPs are color-coded with respect to their LD with the lead SNP.* ***B)*** *A boxplot of whole-brain volume fractions for different rs3920463 genotypes with overlaid jittered data points. For illustration purposes, subjects with an rs3920463^{A} dosage of <0.3 were classified as non-carriers (G/G); subjects with a dosage between 0.7 and 1.3 were classified as heterozygous (A/G) while subjects with a dosage of > 1.7 were classified homozygous for the A allele. Abbreviations: LD, linkage disequilibrium; MS, multiple sclerosis; SNP, single nucleotide polymorphism.*

5 Discussion

While significant progress has been made in recent decades to elucidate the risk factors for developing MS, knowledge of the risk factors of severity and progression of the disease has been lacking.[2] This has largely been due to a lack of sensitive and specific severity biomarkers and insufficient sample sizes. In the current research work, we have investigated how different genetic, body fluid and imaging biomarkers associate with both clinical and paraclinical severity outcomes in large real-world cohorts of persons with MS.

It has become evident that lesion volumes only represent a fraction of all ongoing MS pathology, as disability can progress even in the absence of new or enlarging lesions, most notably in the primary and secondary progressive phenotypes of the disease.[63] This has prompted the search for new biomarkers, which can provide additional information to the clinician regarding the severity and prognosis of the disease. In Study I, we were able to confirm previously reported associations of CSF and pNfL levels with whole/regional brain volumes and lesion volumes.[31] In the longitudinal analysis, the associations were generally stronger for the brain volumes than the lesion volumes, which corroborates the growing body of evidence that brain volume loss is a better biomarker of MS disability than lesion volumes.[66, 70] To our knowledge, we were for the first time able to show that the association between high pNfL and low cortical grey matter fractions was independent of radiological disease activity as measured by increased T1-lesion volumes. A further topographical analysis of the cortex showed that the temporal and frontal lobes were primarily affected. This finding is corroborated by a recent longitudinal cohort study of relapsing-remitting MS showing that sNfL is associated with PIRA[144]. Taken together, our results suggest that NfL levels can be a complement to lesion metrics in the assessment of the severity and potentially treatment response for individuals with MS.

In Study II, we showed that the associations of the EDSS and the physical/psychological MSIS-29 with the MRI variables rapidly increased in strength after approximately 40-50 years of age. Interestingly, this occurred at approximately the age that most study participants transitioned into a secondary progressive phase in our cohort. Since the plasticity and recovery potential of the CNS after structural damage is known to decrease with age, it can therefore be hypothesized that this increased strength of the association represents the transition to the secondary progressive phenotype, as it was independent of recent clinical relapses and other potential confounders.[145] For SDMT, the associations across different ages did not show any obvious pattern, potentially indicating a more complex association with age. While many previous studies have investigated the associations between MRI-based brain volumetry/lesions and clinical disability, our results provide a more detailed understanding of these associations with regards to the dynamics across age and different brain regions.[63, 66, 70] In the longitudinal analysis, we were able to confirm the notion that brain volumes – especially grey matter volumes – are stronger predictors of clinical disability than lesion volumes, as these brain volumes reflect the accumulated neurodegenerative processes to a higher degree than lesions. Interestingly, these results are in line with our finding from Study I that brain volumes are generally more strongly associated than lesion volumes with NfL, which has been shown to be associated with clinical disability measures in previous studies.[31] The sample sizes, especially for the MSIS-29 and SDMT analyses, were among the largest to date, and our data confirm the results from previous smaller exploratory studies.[40, 67, 85-89] Interestingly, our reported association between low baseline subcortical grey matter volume fractions and worsening of physical MSIS-29 is, to the best of our knowledge, the first observation of

brain volume being associated with self-reported impact of MS, which is gaining increasing attention as a means to better gauge the impact of the disease on the individual level[40, 88].

In Study III, we showed that MS susceptibility HLA alleles, in particular the main risk allele *HLA-DRB1*15:01*, are associated with a reduction of the cortical grey matter fraction longitudinally, which indicates that there is some co-heritability between MS risk and cortical atrophy. This finding is consistent with previous reports of *HLA-DRB1*15:01* being associated with low age at onset, higher incidence of MS among females compared to males, treatment response, and low cross-sectional whole-brain volume and subcortical grey matter fractions.[112-117, 121] It is also consistent with the notion that MS affects the cortex already in the earliest phase of the disease, and with histopathological data showing that among persons with MS, carriers of the *HLA-DRB1*15:01* allele exhibit a higher degree of demyelination and T-cell infiltration in the CNS than non-carriers.[146, 147] This suggests that the pathomechanisms of brain atrophy in MS may involve inflammatory activity via the adaptive immune system leading to demyelination and neurodegeneration. As a result, this also indicates that the mechanisms behind MS severity and susceptibility are at least partially overlapping. However, it is unclear whether it would be the same type of immune activity in both instances, as the immunological profile in the CNS is known to change over the disease course.[10, 23]

Interestingly, while an HLA-based MS risk risk score was associated with brain atrophy in Study III, we did not observe an association between an HLA-based MS risk risk score with high pNfL levels in Study IV, even though pNfL has been consistently shown to (moderately) correlate with both global and regional brain atrophy as shown in Study I and other cohorts.[31] A genetic risk score comprising both HLA and non-HLA MS was associated with high pNfL levels in Study IV, but in fact, the non-HLA variants in the risk score were driving this association. Together, these findings suggest that there is some overlap in the genetic regulation of MS susceptibility and end-organ injury measured with pNfL levels, but that pNfL levels may reflect different neurodegenerative processes than the ones that MS susceptibility HLA variants modulate. This notion is corroborated by our post-GWAS bioinformatical functional analyses of pNfL in Study IV showing that the NfL levels may be regulated by a wide range of cellular processes, of which only some are immune-related and that previously have not been directly linked to MS susceptibility.

In Study V, we identified a genome-wide significant locus associated with whole-brain volume fractions near the GK2 gene, which previously has not been known to be directly implicated in MS risk/severity or brain volumes of healthy individuals. However, in a previous large-scale GWAS, other SNPs (rs28459916^{A} and rs17003752^{G}) linked to this gene have been associated with propensity for tobacco smoking, which is known to increase MS risk, physical disability and brain atrophy.[2, 96, 148, 149] Interestingly, the associated variants in Study V were in positional proximity and LD with these smoking-associated variants. This suggests that the effect of the SNPs that were associated with whole-brain volume fractions may be partially mediated by smoking, albeit the LD with the smoking-associated variants was weak and adjustment for smoking in our analysis did not substantially change the results.

Gene set analyses particularly implicated a HIF1-related pathway affecting the whole-brain volume fractions in Study V. Interestingly, the HIF complex (comprising an alpha and a beta subunit) is a transcription factor that is known to modulate both iron metabolism and response to hypoxia (Fig. 8), both of which can be implicated in neurodegenerative processes.[150, 151] Previous reports of increased HIFA expression in MS lesions and emerging evidence that paramagnetic iron rim lesions – a type of

chronic active lesion with iron that has been accumulated in microglia at the lesion border – are a robust diagnostic and prognostic MS biomarker may provide a causal link for our reported association between the HIFA pathway and altered whole-brain volume fractions.[26, 152] An additional causal pathway may be through hypoxia-related target genes of HIFA, in particular the vascular endothelial growth factor (VEGF), which in recent studies has been shown to play an important role in angiogenesis, neurogenesis, oligodendrocyte maturation and neuroinflammation in MS.[151] The gene set analyses also showed that whole-brain volume fractions are associated with FGFR2 and branching morphogenesis pathways. FGFR2 is abundantly expressed in oligodendrocytes and has been shown to be pivotal for myelination and neuronal development/homeostasis. Interestingly, disruption in FGF2 signaling has been reported in both EAE and MS, but the effects are complex and may lead to inflammatory activity mediated by microglia and either increased or decreased myelination.[153] The potential role of branching morphogenesis in MS less clear, although the Bone Morphogenic Proteins (BMPs) 4 and 7 – which are included in this gene set – have been associated with demyelination and lesion activity in MS.[154, 155] Overall, the results from study V suggest that genetic variants affect whole-brain volume fractions in early relapsing-onset MS through other mechanisms than acute inflammation by the adaptive immunity. This is consistent with the notion that PIRA is a substantial contributor of disability even in the early phase of MS.

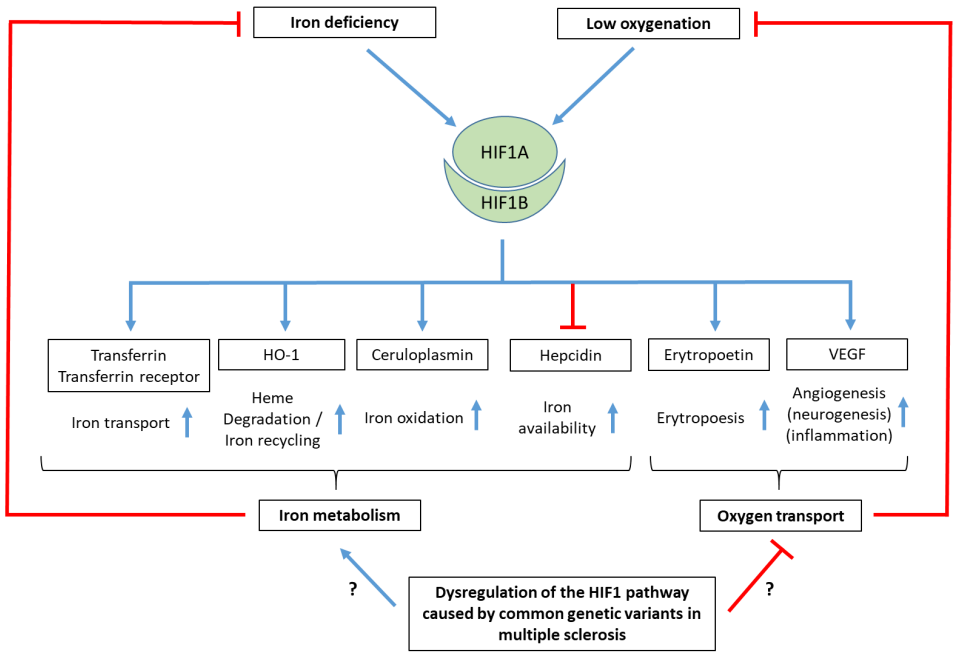

Fig. 8. *Schematic overview of the HIF1 pathway regulating iron metabolism and oxygen transport with a feedback regulation. In multiple sclerosis, this pathway may be dysregulated by common single nucleotide polymorphisms affecting iron homeostasis as well as oxygen transport and neurogenesis in the central nervous system with subsequent effects on neurodegenerative processes and brain volume loss. Abbreviations: HIF1A/B, hypoxia-inducible factor 1-alpha/beta; HO-1, heme oxygenase-1; VEGF, vascular endothelial growth factor.*

Polygenic scores weighted for the effect sizes from the pNfL GWAS in Study IV were not associated with baseline whole-brain volume fractions in Study V, suggesting that there is no substantial overlap regarding the genetic regulation of these traits. Moreover, there was no overlap in the associated SNPs or biological pathways between these studies, indicating that these severity measures at least partially reflect different neurodegenerative processes, as also suggested by results in Study I. This is further supported by the notion that NfL levels primarily reflect ongoing neurodegeneration as opposed to accumulated neurodegeneration manifested as brain atrophy.[31, 64]

One of the main strengths of the sub-studies in this thesis is the relatively large sample sizes, which is especially important in genetic studies of polygenic traits such as MS where the effect sizes are expected to be small. One possible exception is Study V (N = 644), which could be one explanation for the lack of an association in the polygenic score analysis. Another strength is the longitudinal design of Studies I-III, which allowed us to investigate the effects of the various biomarkers over long time periods and added to the robustness of our results. A limitation of all studies in this thesis is the heterogeneity of the real-world study populations. This includes differences in treatment exposure and certain technical differences in the MRI acquisition, such as the use of different MRI scanners, although these differences were controlled for in the statistical analyses. However, it should be noted that real-world data often can facilitate the study of a wider range of phenotypes and increase the generalizability of the results compared to clinical trials. Furthermore, capturing the effect of genetics on neurodegenerative and inflammatory processes in the CNS using pNfL levels as a proxy measure might be complicated by peripheral neurodegeneration, diluting effects of large BMI/blood volume as well as temporal and inter-individual differences in BBB permeability to NfL.[31, 52] However, there is still a relatively strong correlation between pNfL and CSF NfL as we showed in Study I and BMI can be statistically adjusted for[31]. There is also a question of whether the associations that we observed in our studies partially reflect neurodegeneration by normal aging and certain comorbidities (e.g. cardiovascular), as these factors are known to affect NFL levels and brain atrophy.[31, 60, 80, 156] We consider that it is unlikely that these factors substantially affected our results since the majority of study participants were relatively young, had overall low levels of comorbidity as measured with a modified Charlson comorbidity index and adjustment for these factors did not alter the results.

5.1 Points of perspective

Taking the findings from Studies I-V together, we have shown that different genetic, imaging and body fluid biomarkers of MS severity are associated with each other after correction for potential confounders, indicating that they at least partially reflecting the same underlying pathomechanisms in MS. However, the strength of these associations was at most moderate on the group level. One potential explanation for this is that these biomarkers to some degree also may reflect different pathomechanisms, which is exemplified by our finding in Study I that the associations between pNfL and cortical grey matter fractions were independent of radiological signs of disease activity in the form of increased T1-lesion volumes. Another explanation – compatible with the first one – is that there is a high degree of imprecision and technical variability in the measurement of the biomarkers and severity measures.[1] Hence, in contrast to the current diagnostic MS biomarkers – e.g. MRI lesions, presence of oligoclonal bands in the CSF and neurological symptoms – which can discriminate MS from other neurological diseases and healthy individuals with a relatively high degree of certainty, the available severity biomarkers are not yet useful to predict disease severity on the individual level and in a clinical setting.[8] Clinical implementation may be especially challenging for the MRI-based volumetric measures, due to the requirement of standardization of image acquisition parameters and the complexity and

computational demand of the image processing, although promising methods that are not as sensitive to technical heterogeneity are under development.[69, 78] Furthermore, since the differences in brain volumes – especially volumes of small subcortical regions – between different severity categories of MS oftentimes are smaller than the intra- and inter-scanner variability, these volumetric measures are generally not useful for individual-level predictions.[69, 75] For these reasons, it is important to continue the research efforts not only to discover more robust MS severity markers, but also to integrate existing clinical and paraclinical markers into more predictive risk scoring systems. For instance, one recent study showed that the inclusion of an MS polygenic risk score into a clinical risk score model could substantially increase the MS risk discriminatory power of the model compared to only using the clinical risk score.[122] Another recent study showed that serum levels of Glial fibrillary acidic protein (GFAP) – a structural intermediate filament protein expressed in many CNS cell types – is a predictor of PIRA and a stronger predictor of clinical progression than sNfL, This suggests that GFAP may be a superior marker of progressive MS than NfL, which mainly appear to reflect neurodegeneration by inflammatory processes in the early phases of the disease.[157]

Due to the somewhat limited sample size of Study V, in particular regarding the GWAS and polygenic score analysis, replication studies would be beneficial to further strengthen the evidence for our findings in that study. Longitudinal studies are also warranted to elucidate the effect of these variants on both MRI-based and clinical disease progression. Furthermore, to strengthen the evidence for the role of HIF1 in iron accumulation in the brain in MS, MRI processing techniques such as quantitative susceptibility mapping could be used for quantification of the iron content in the brain, which subsequently could be assessed in the genetic and functional analyses.[158]

It is important to note that even if the biomarkers assessed in our material may not have sufficient discriminatory effect to be clinically useful to predict clinical outcomes or guide the choice of treatment regimen on the individual level, they might give insights into the pathomechanisms of MS. The severity-associated genetic variants and biological pathways in MS that were identified in this research wor – such as the MS risk HLA variants and the HIF1 pathway for MRI-based volumetric measures – could potentially guide further research into new treatment targets or the discovery of more sensitive and specific MS biomarkers.

6 Conclusions

In this research work, I have shown that NfL, an emerging severity biomarker in MS, is generally more strongly associated with brain volumes than lesion volumes. Furthermore, NfL levels can provide information about MS severity that is not evident by the traditional lesion metrics. NfL may therefore be a complement to lesion metrics in the assessment of the severity and response to DMTs in MS. Likewise, our data show that brain volumes – in particular grey matter volumes – are stronger predictors of clinical disability than brain lesion volumes. Moreover, the strength of the associations between MRI-based volume measures and disability in MS is age-dependent, with an increased strength coinciding with clinically assessed conversion to secondary progressive MS. Importantly, this may at least on a group level constitute a neuroimaging marker of transition to secondary progressive MS. MS susceptibility HLA variants are associated with long-term cortical brain atrophy in MS, supporting the notion that there is a certain overlap in the genetic regulation of MS susceptibility and severity. These results also suggest that the neurodegenerative processes are at least partially driven by the adaptive immune system through HLA variants in MS. We also found that pNfL levels may be regulated by a wide number of genes and cellular processes, including a neural crest-related biological pathway. The differential association of MS risk HLA and non-HLA variants with brain volumes and pNfL levels suggests that the genetic regulation of MS severity is complex and not entirely driven by MS susceptibility variants. Furthermore, we showed that genetic variants, potentially related to the propensity for tobacco smoking, iron metabolism and response to hypoxia, are associated with low cross-sectional whole-brain volume fractions in MS. Studies with longitudinal designs are warranted to elucidate the effect of these variants on disease progression. Although the biomarkers we have investigated in this research work may not be useful to predict severity on the individual level, they may provide important insights into the pathomechanisms of MS.

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