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MARJA NIIRANEN

BENIGN MULTIPLE SCLEROSIS

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ABSTRACT

Multiple sclerosis (MS) is a chronic inflammatory and neurodegenerative disease of the central nervous system that leads to diverse clinical outcomes and disability. It is the most common chronic neurological disease affecting young adults. A proportion of MS patients show minimal disability even decades after the onset of MS symptoms, and this entity of so-called benign MS has been debated since the 1950s. In contrast, some MS patients have an aggressive or highly active disease course with a risk for remarkable disability. So far, there are no validated clinical prognostic markers or specific biomarkers to predict the course of MS at the disease onset.

The aim of this study was to assess disease activity and the neurodegenerative process in benign and mild relapsing-remitting MS (BRRMS) using the glial fibrillary acid protein (GFAP) and neurofilament light (NfL) novel soluble biomarkers and automated quantitative magnetic resonance imaging (MRI) techniques.

We observed elevated serum GFAP levels in BRRMS, demonstrating ongoing neurodegeneration and active glial process. Serum GFAP levels were higher in BRRMS patients without disease-modifying treatment (DMT) throughout their disease history than in those who had used DMT. Levels

of serum NfL did not differ either between BRRMS and aggressive relapsing-remitting MS (ARRMS) or between healthy controls (HC) and either of the patient groups. This indicates there was no active inflammation in either group. This study is the first to report serum GFAP and NfL levels in BRRMS.

We used a set of volumetric imaging biomarkers retrospectively extracted from routine MRI examinations in two different MS phenotypes (study II) and in BRRMS compared to HC (study III). An automated MRI quantification tool cNeuro® was used in both studies. Whole-brain and thalamic volumes were larger in BRRMS than in ARRMS. At the same time, WM lesion load was larger in ARRMS, correlating with the higher earlier inflammatory activity of the disease. Thalamic volume was the most prominent GM measure in differentiating BRRMS and ARRMS. Within the BRRMS group, patients who had never been used DMT had larger WM lesion volumes, indicating there is also subclinical inflammatory activity in seemingly mild MS. Corpus callosum index (CCI) was included in the cNeuro® analysis and was correlated with whole-brain volumes. Thus, CCI would be an easily measured brain atrophy marker. To our knowledge, our study is the first to report automated CCI measures in benign MS.

Total cortical and cerebral GM volumes were larger in BRRMS than in HC, especially in the limbic areas (i.e. the entorhinal cortex and cingulate gyrus). As expected, total brain volumes were smaller, and cerebrospinal fluid (CSF) volumes larger in BRRMS patients. In addition to CCI, the corpus callosum area (CCA) was extracted as a brain atrophy marker. Both CCA and CCI correlated positively with whole-brain volume in MS but not in HC, which is in line with an earlier report suggesting corpus callosum atrophy is an MS-specific process, including in both WM and GM pathology.

The results of this thesis strengthen the idea that benign MS as such is only a temporary description, and that the term used should be at most ‘mild MS’. The neurodegenerative component of the advanced disease can be demonstrated with elevated serum levels of GFAP, as is also the case in the mild form of the disease. There is need for more research on the consecutive measurements of different serum biomarkers in different

clinical phenotypes of MS. Automated MRI quantification methods are already feasible in clinics to detect local and minor atrophy.

Keywords: benign multiple sclerosis, biomarker, brain atrophy, GFAP, neurofilament

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TIIVISTELMÄ

MS-tauti on keskushermoston krooninen tulehduksellinen ja neurodegeneratiivinen sairaus, jossa taudinkulku ja sairauden haitta-aste on vaihteleva. Se on yleisin nuoren aikuisen neurologinen pitkäaikaissairaus. Osa MS-potilaista pärjää jopa vuosikymmeniä ilman liikuntakykyä heikentävää taudin haittaa (ns. benigni, hyvänlaatuinen tautimuoto), kun taas osalla sairaus alkaa aggressiivisesti ja oireet etenevät nopeasti. Tällä hetkellä ei kliinisessä käytössä ole vielä validoituja biomarkkereita taudin kulun ennustamiseen sen varhaisessa vaiheessa.

Tämän tutkimuksen tavoitteena oli selvittää ns. benignin ja hyvin lieväoireisen aaltomaisen MS-taudin tautiaktiivisuutta ja neurodegeneraation astetta käyttäen uusia verestä mitattavia merkkiaineita GFAP-proteiinia ja neurofilamenttia (NfL) ja, sekä uusia automatisoituja aivojen magneettikuvantamisen (MRI) menetelmiä.

Seerumin GFAP-tasot olivat koholla lieväoireista tautimuotoa sairastavilla, viitaten aktiiviseen neurodegeneraatioon. Lisäksi GFAP-tasot olivat korkeammat niillä benigniä tautia sairastavilla potilailla, jotka eivät olleet käyttäneet taudinkulkuun vaikuttavaa immunomodulatorista lääkitystä. Seerumin NfL-tasoissa ei ollut eroa lievää ja aggressiivista tautia sairastavien potilaiden välillä, eikä verrattuna terveisiin verrokkeihin.

Automatisoitua cNeuro®-työkalua käytettiin aivojen MRI-kuvien analysoinnissa retrospektiivisessä aineistossa näissä kahdessa eri MS-potilasryhmässä (osatyö II), ja benignissä MS-taudissa ja terveillä verrokeilla (osatyö III). Kokoaivo- ja talamustilavuudet olivat suuremmat benignissä ryhmässä aggressiiviseen taudinkulkuun verrattuna, kun taas valkean aineen leesiokuorma oli suurempi aggressiivisessa tautimuodossa. Talamuksen tilavuus oli vahvin erotteleva aivojen harmaan alueen mittari benignin ja aggressiivisen MS-tautiryhmän välillä. Ilman MS-taudin lääkitystä olleilla potilailla todettiin benignissä ryhmässä laajempi valkean aineen leesiokuorma viitaten hiljaiseen tulehdusaktiviteettiin. Aivokurkiaisen mitoista määritettiin corpus callosum-indeksi (CCI), joka korreloi kokoaivotilavuuteen. CCI voisikin olla helposti määritettävä aivoatrofian mittari. Tämä tutkimus on tiettävästi ensimmäinen CCI-mitat raportoiva tutkimus benignissä MS-taudissa.

Kokoaivojen ja kortikaalisen harmaan aineen tilavuus, erityisesti limbisellä alueella, oli suurempi benignissä MS-potilasryhmässä terveisiin verrokkeihin verrattuna. Oletetusti MS-potilailla oli kokoaivotilavuus pienempi ja aivonestetilojen tilavuus suurempi kuin terveillä. CCI-mitan lisäksi työkaluun kehitettiin corpus callosum area -mitta (CCA). CCI ja CCA korreloivat kokoaivotilavuuteen MS-taudissa mutta ei terveillä, mikä viittaa siihen, että aivokurkiaisen atrofia on MS-taudille spesifi prosessi.

Tuloksemme vahvistavat ajatusta siitä, että termiä ”hyvänlaatuinen” MS-tauti ei tulisi käyttää, vaikka oirekuva olisikin lievä. Koholla oleva seerumin GFAP-taso tässäkin potilasryhmässä on todiste neurodegeneraatiosta. Lisätutkimusta eri biomarkkereista toistomittauksin erilaisissa MS-taudin kliinisissä alaryhmissä tarvitaan. Automatisoidut kuvantamistyökalut ovat jo valmiita kliiniseen käyttöön aivoatrofian mittaamiseksi.

Avainsanat: hyvänlaatuinen MS-tauti, ennustetekijä, aivoatrofia, GFAP, neurofilamentti

To my family

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LIST OF ORIGINAL PUBLICATIONS

The dissertation is based on the following original publications:

I Niiranen M, Kontkanen A, Jääskeläinen O, Tertsunen HM, Selander T, Hartikainen P, Huber N, Solje E, Haapasalo A, Kokkola T, Lohioja T, Herukka SK, Simula S, Remes AM. Serum GFAP and NfL levels in benign relapsing-remitting multiple sclerosis. Mult Scler Relat Disord. 2021 Nov; 56:103280.

II Niiranen M, Koikkalainen J, Lötjönen J, Selander T, Cajanus A, Hartikainen P, Simula S, Vanninen R, Remes AM. Grey matter atrophy in patients with benign multiple sclerosis. Brain Behav 2022 Jul;12(7)

III Niiranen M, Bendel P, Koikkalainen J, Lötjönen J, Selander T, Hartikainen P, Simula S, Vanninen R, Remes AM. Cortical grey matter is locally well-preserved in limbic areas in benign multiple sclerosis. Submitted.

CONTENTS

ABBREVIATIONS

2D	two-dimensional
3D	three-dimensional
AD	Alzheimer's disease
ADL	activities of daily living
aHSCT	autologous hematopoietic stem cell transplantation
AQP4	aquaporin-4
ARRMS	aggressive relapsing-remitting multiple sclerosis
BBB	blood-brain barrier
BRRMS	benign relapsing-remitting multiple sclerosis
BVL	brain volume loss
CC	corpus callosum
CCA	corpus callosum area
CCI	corpus callosum index
CE	Conformité Européenne
CIS	clinically isolated syndrome
CNS	central nervous system
CSF	cerebrospinal fluid
DIS	dissemination in space
DIT	dissemination in time
DMT	disease-modifying treatment
EBV	Epstein–Barr virus
FDA	United States Food and Drug Administration
FS	functional system
FTD	frontotemporal dementia
Gd	gadolinium
GFAP	glial fibrillary acidic protein
HAMS	highly active MS
HC	healthy control
HLA	human leucocyte antigen
MLBG	maximal lifetime brain growth
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
MS	multiple sclerosis
NAWM	normal-appearing white matter
NEDA	no evidence of disease activity
NfL	neurofilament light chain
NMOSD	neuromyelitis optica spectrum disorder
OR	odds ratio
PIRA	progression independent of relapse activity
PML	progressive multifocal leukoencephalopathy
PMS	progressive multiple sclerosis

PPMS	primary progressive multiple sclerosis
RIS	radiologically isolated syndrome
RRMS	relapsing-remitting multiple sclerosis
SCI	spinal cord injury
sNfL	serum neurofilament light
SiMoA	single molecule assay
SPMS	secondary progressive multiple sclerosis
TBI	traumatic brain injury

1 INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory and neurodegenerative disease of the central nervous system (CNS) that leads to diverse clinical outcomes and disability. Primary symptoms are caused by the inflammatory demyelination of the CNS. A concurrent neurodegenerative process supposedly starts early in the disease. The first symptoms of MS may occur from post-pubertal time up to age of 50–60 years, but most commonly, symptoms manifest in early adulthood (20–30 years). MS presents with various neurological symptoms.

A proportion of MS patients show only minimal disability decades after the onset of MS symptoms, and this phenotype of so-called benign MS has been debated since the 1950s (G. S. Ramsaransing & De Keyser, 2006). In contrast, some MS patients have an aggressive or highly active disease course with a risk for remarkable disability (Menon et al., 2013). There are currently no validated clinical prognostic markers or specific biomarkers to predict the course of the disease at onset.

Soluble biomarkers refer to biochemical molecules drawn in blood samples. They can be used as a diagnostic tool, as predictive and prognostic markers and as disease activity and treatment markers in neurological diseases. In MS, novel soluble biomarkers may help in treatment strategy planning and follow-up (Ning & Wang, 2022; Yang et al., 2022).

Brain and spinal cord magnetic resonance imaging (MRI) is essential in the diagnosis and follow-up of MS. The level of brain atrophy and lesion volumes significantly predict long-term disability in all MS phenotypes (Eshaghi, Marinescu, et al., 2018; Eshaghi, Prados, et al., 2018; Popescu et al., 2013; Sormani et al., 2014). The development of automated and semi-automated lesion and brain volume segmentation tools aims to increase the sensitivity of MRI analysis, accuracy of results and speed of analysis (Brune et al., 2020; Zeng et al., 2020).

Current disease-modifying treatments (DMT) reduce the risk for new bouts of symptoms (relapses) and the number of new demyelinating

lesions in brain and spinal cord MRI. As early treatment decisions are crucial, more reliable paraclinical biomarkers are needed in the clinic.

The aim of this study was to assess the disease activity and especially the neurodegenerative component in benign and mild relapsing-remitting MS (RRMS).

2 REVIEW OF THE LITERATURE

2.1 DEFINITION AND EPIDEMIOLOGY OF MULTIPLE SCLEROSIS

MS is the most common chronic neurological disease affecting young adults worldwide. Globally, females are twice as likely to have MS as males (Kingwell et al., 2015; Walton et al., 2020), and in some countries, this female–male ratio is as high as 4:1 (Walton et al., 2020). The median global prevalence of MS is about 35.9 per 100,000 and an approximate total of 2.8 million people live with MS, according to the latest epidemiological registry data (Walton et al., 2020). There is a great variance in prevalence among countries, the highest being reported in those with a northern latitude (Simpson et al., 2019). The prevalence of MS rose by about 30% in the twentieth century (Walton et al., 2020). In Nordic countries, the latest epidemiological studies, from the twentieth century, reported a prevalence of 166.5/100,000 in Iceland (Elíasdóttir et al., 2018) and 189/100,000 in Sweden (Ahlgren et al., 2011). In Finland, regional differences have been reported, with the prevalence ranging from lower rates of 151/100,000 in North Karelia up to 247/100,000 in Southwest Finland (Pirttisalo et al., 2019, 2020). There are about 12,000 patients with MS in Finland (Sipilä et al., 2022).

There are several possible reasons for the increasing prevalence of MS, which include improved and earlier diagnosis; improved treatment and care, leading to prolonged survival; and increased use of national MS registries, enabling the documentation of cases (Walton et al., 2020). There also seems to have been a true increase in the incidence of MS (Grytten et al., 2015; Kingwell et al., 2015; Krökki et al., 2011)

2.2 ETIOLOGY AND IMMUNOPATOGENESIS OF MS

According to current knowledge, MS is caused by multiple environmental factors in genetically susceptible people. Population-based studies have estimated a sibling's relative risk of MS to be increased by sevenfold

(Westerlind et al., 2014). Genes within the human leucocyte antigen (HLA) complex are the strongest genetic risk factors for MS. HLA class I genes encode products for antigen presentation to CD8+ lymphocytes and HLA II genes encode for antigen presentation to CD4+ lymphocytes. The absence of the HLA-A*02 allele (HLA class I) and the presence of DRB1*15:01 (HLA II) has a combined odds ratio (OR) for the risk of MS of ~5 (Sawcer et al., 2011). Genomewide association studies have detected more than 200 other genetic variants, namely non-HLA single nucleotide polymorphisms, that regulate adaptive or innate immunity and induce a modest influence on MS risk (Canto & Oksenberg, 2018).

Migration studies have emphasised environmental factors in the aetiology of MS. The risk for MS among those moving from a low-risk country to a high-risk country before adolescence is equal to that for those who are born and living in a high-risk country (Ahlgren et al., 2012; Berg-Hansen et al., 2015).

There is already strong epidemiological evidence supporting that the strongest environmental factor in the development of MS is Epstein–Barr virus (EBV) infection in childhood and early adulthood (Jacobs et al., 2020). MS is extremely rare in EBV-seronegative people (Dobson et al., 2017). The causality of EBV theory in MS has not been completely verified, but a recent 20-year longitudinal analysis of over 10 million young adults serving in the United States military showed a 32-fold risk for MS after EBV infection (Bjornevik et al., 2022).

Higher geographical latitude and less exposure to ultraviolet radiation have been observed as risk factors for MS (Simpson et al., 2019), as has vitamin D deficiency (Munger et al., 2006, 2017). There is also a season of birth effect in MS; the mother's lower sun exposure and vitamin D in the late first trimester of pregnancy are associated with the foetus's increased risk for MS (Lucas et al., 2015).

Tobacco smoking is recognised as a risk factor for MS, and it is also associated with the progression of the disease and a worse prognosis (Arneth, 2020). Smoking also has interactions with MS-related HLA genes leading to additive interactions among risk factors (Hedström et al., 2016b). Smoking has been associated with an increased risk for developing

neutralising antibodies against certain DMT, deteriorating the treatment effect (Hedström, Alfredsson, et al., 2014; Hedström, Ryner, et al., 2014). Obesity in adolescence increases the risk for MS (Hedström et al., 2016a; Munger et al., 2013).

The cellular immunology of relapsing MS was previously thought to be driven principally by T lymphocytes. However, studies have strengthened the idea of multiple cell types involved in the pathogenesis, namely T cells, myeloid cells and B cells and their effector and regulatory cell subpopulations. The interactions and imbalances between these cells differ across patients. Inflammation is present at all stages of MS but more pronounced in the early and acute stages of the disease. Immune system dysregulation in the periphery leads to the infiltration of activated CD8+ T cells, differentiated CD4+ helper T cells, B cells and innate immune cells to the CNS. This leads to inflammation and tissue damage of the CNS. In the early stage, brain lesions can be found infiltrated with macrophages, CD8+ and CD4+ T cells, B cells and plasma cells. These cells interact with activated microglia and astrocytes and promote demyelination and neuroaxonal injury through soluble inflammatory and neurotoxic mediators (Bar-Or & Li, 2021; Dendrou et al., 2015). The key B cell role in the disease activity has been strengthened by the success of the high efficacy of B-cell selective anti-CD20 therapies (Bar-Or et al., 2018; Hauser et al., 2017). Later in the disease process, the immune cell infiltration wanes and chronic CNS-captivated inflammation and neurodegeneration take place. Meningeal lymphoid-like structures, containing mainly B cells, may contribute to the inflammation process, cortical demyelination, and tissue injury in the secondary progressive stage of the disease. Microglia can stimulate astrocytes to produce neurotoxic chemokines and other mediators that further sustain neurodegeneration (Dendrou et al., 2015). The chronic phase of non-relapsing MS is more persistent to current immunological therapies, presumably due to the compartmentalised inflammation and neurodegenerative processes of CNS.

2.3 DIAGNOSIS OF MS

The diagnostic criteria of MS have evolved from the 1960s when Schumacher and colleagues published the first diagnostic criteria that were based purely on clinical findings (Schumacher et al., 1965). Almost 20 years later, the Poser criteria were adopted, including paraclinical evidence (visual and motor evoked potentials) and cerebrospinal fluid (CSF) as well as defining definite and probable MS as different levels of diagnostic certainty (Poser et al., 1983). The first McDonald criteria, introduced in 2001, emphasised the evidence of demyelination in the brain and spinal cord with MRI (McDonald et al., 2001). Revisions to the McDonald criteria to simplify the diagnostic criteria, improve the accuracy of the diagnosis, and shorten the diagnostic delay were published in 2005, 2010 and 2017 (Polman et al., 2005, 2011; Thompson et al., 2018).

The diagnosis of MS is a clinical conclusion based on typical symptoms supported by paraclinical findings, namely brain and spinal cord MRI findings of demyelination and CSF results. Demyelination in CNS, dissemination in space (DIS) and dissemination in time (DIT) must be approved, and other possible diagnoses must be adequately ruled out. The latest McDonald 2017 diagnostic criteria (Tables 1 and 2) emphasise the brain and spinal cord imaging and oligoclonal band (OCB) findings. The main modifications to the previous criteria consisted of the demonstration that CSF OCB can substitute for a clinical second attack or DIT in MRI, that both symptomatic and asymptomatic MRI lesions are considered in the determination of DIS and DIT (except lesions in the optic nerve in a patient presenting with optic neuritis) and that cortical and juxtacortical lesions are considered equivalent to juxtacortical lesions (Thompson et al., 2018).
The first clinical episode of MS is called clinically isolated syndrome (CIS) and the symptoms may affect any part of the CNS. A clinical episode with patient-reported symptoms and objective findings is defined as a relapse (synonyms: attack, exacerbation) when it lasts at least 24 hours and is at least one month apart from another possible relapse, with or without recovery and in the absence of fever or infection. Typical symptom presentations are unilateral optic neuritis, focal supratentorial syndrome,

focal brainstem or cerebellar syndrome or partial myelopathy (Thompson et al., 2018). If the diagnostic criteria for MS are not fulfilled in a patient with CIS, a follow-up MRI and clinical assessment should be done within 6–12 months (Thompson et al., 2018).

Table 1. McDonald diagnostic criteria for relapse-onset MS, modified from Thompson et al., 2018.

	Number of lesions with objective clinical findings	**Additional data needed for a diagnosis of MS**
≥ 2 clinical attacks	≥ 2	None*
≥ 2 clinical attacks	1(as well as a clear-cut historical evidence of a previous attack involving a lesion in a distinct anatomical location**)	None*
≥ 2 clinical attacks	1	DIS demonstrated by an additional clinical attack implicating a different CNS site or by MRI
1 clinical attack	≥ 2	DIT demonstrated by an additional clinical attack or by MRI OR demonstration of CSF-specific OCB***
1 clinical attack	1	DIS demonstrated by an additional clinical attack implicating a different CNS site or by MRI AND DIT demonstrated by an additional clinical attack or by MRI OR demonstration CSF-specific OCB***

MRI criteria for dissemination in space (DIS) and and time (DIT) in a patient with clinically isolated syndrome (CIS):
DIS: one or more T2-hyperintense lesions that are characteristic of multiple

sclerosis in two or more of four areas of the CNS: periventricular, cortical or juxtacortical, and infratentorial brain regions, and the spinal cord.
DIT: simultaneous presence of gadolinium-enhancing and non-enhancing lesions at any time or by a new T2-hyperintense or gadolinium-enhancing lesion on follow-up MRI, with reference to a baseline scan, irrespective of the timing of the baseline MRI.

Table 2. McDonald diagnostic criteria for PPMS, modified from Thompson et al., 2018.

Primary progressive MS can be diagnosed in a patient with 1 year of disability progression (retrospectively or prospectively) independent of clinical relapse PLUS two of the following:
One or more T2-hyperintense lesions characteristic of multiple sclerosis in one or more of the following brain regions: periventricular, cortical or juxtacortical or infratentorial
Two or more T2-hyperintense lesions in the spinal cord
Presence of CSF-specific OCBs

2.4 CLINICAL COURSE

The clinical presentation of the disease varies between patients from the onset and over the course of the disease. The first episode of neurological symptoms is defined as CIS. The clinical course of MS is divided into RRMS and progressive MS (PMS). In most patients (about 85%), the disease starts as RRMS, in which separate attacks of new neurological symptoms, or the worsening of previous symptoms can be recognised. RRMS is characterised as active or not active within the preceding year, assessed by clinical

relapses and/or MRI activity (gadolinium (Gd)-enhancing lesions and new or enlarging T2-lesions) (Lublin, 2014). The clinical symptoms of an attack typically recover fully or at least partly in the early years of the disease, but as the disease advances, permanent neurological deficits often remain. Relapses contribute to the accumulation of disability in the early stage of MS, but later, progression independent of relapse activity (PIRA) is the main driver of permanent disability (Kappos et al., 2020; Lublin et al., 2022). PIRA is associated with significantly increased brain volume loss as a marker of neurodegeneration (Cagol et al., 2022).

The progressive state of relapsing MS is called secondary progressive MS (SPMS). Patients with SPMS often continue to have clinical relapses and subclinical MRI activity, which is called active progressive MS (Lublin, 2014). There is a need for regular evaluations of the disease activity in SPMS, since DMTs have been shown to be effective in active progressive but not in non-active progressive patients (Hawker et al., 2009; Kappos et al., 2018).

Primary progressive MS (PPMS) is far rarer than RRMS and diagnosed in only about 10%–15 % of MS patients. It is defined as the gradual onset of progressive neurological symptoms, typically spastic paraparesis (Miller & Leary, 2007). The typical age of onset is older in PPMS than in RRMS, with the mean age of onset being 40 years in PPMS and to 30 years in RRMS. The proportion of PPMS is decreasing, being about 9 % in Finland and Sweden (Laakso et al., 2019; Westerlind et al., 2016). There is also a difference in the female–male ratio, at 1:1 in PPMS versus 2–3:1 in RRMS (Miller & Leary, 2007), but there are no confirmed genetic or immunologic differences between these forms of MS (Vollmer et al., 2021). No reliable body fluid or MRI biomarkers have been recognised to distinguish between these clinical phenotypes (Lublin, 2014).

2.4.1 Assessment of clinical outcomes

Identifying and measuring clinical changes and disability in MS is challenging, since the symptoms cover the entire CNS. Some of the symptoms, such as cognitive problems, fatigue and pain, are difficult to quantify objectively, yet these often remarkably impair quality of life and

the ability to work. It is not easy to define the clinical course especially in the early stage of the disease, but there are some signs that may help in prognostication. Clinical outcome measurements in addition to the number of relapses and the recovery of relapses constitute the level of disease activity. The most widely used disability scale is the Expanded Disability Status Scale (EDSS) (Kurtzke, 1983), which is used in clinical follow-up and as an outcome measure in clinical trials, even though it has several limitations and pitfalls (Ebers et al., 2008; van Munster & Uitdehaag, 2017). EDSS is a non-linear scale from 0 to 10, with 0 being a normal neurological examination and 10 being death from MS (Figure 1). The scale includes a thorough neurological examination to assess ambulation and the following functional systems (FS): vision, brainstem, pyramidal, cerebellar, sensory, bowel and bladder, and cerebral systems. The scale places most emphasis on ambulation, and there are large functional gaps in lower EDSS scores. EDSS with low scores is subject to inter-rater errors, as the assessment of FS is a subjective interpretation, and it does not cover enough major fatigue and cognitive problems. Scores from 4.0 and 7.0 are mostly determined by ambulatory function and aid in walking, and those between 7.0 to 9.5 reflect the ability to carry out activities of daily living (ADL) (Kurtzke, 1983).

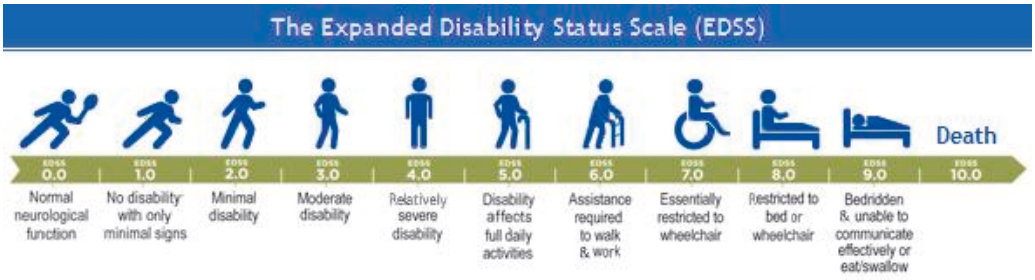

Figure 1. Extended Disability Status Scale (EDSS) (Kurtzke, 1983). Image source: https://myms.org/ms_progression.htm.

The Multiple Sclerosis Functional Composite (MSFC), which was developed to improve clinical assessment (Cutter et al., 1999), consists of the following three tested functional domains: ambulation (timed 25-foot

walk test) and hand (nine-hole peg test) and cognitive function (paced auditory serial addition task). The results form a score on a continuous scale, that has been shown to correlate with EDSS and relapse rate, and to predict the conversion of RRMS into SPMS (Cutter et al., 1999; Rudick et al., 2001).

Relapse-associated outcome measures have been used as endpoints in most phase III MS clinical trials, since the aim of the DMTs investigated is to reduce or suppress the inflammatory activity associated with relapses. The most commonly used measure is the annualised relapse rate, which refers to the number of relapses per patient year during treatment (Tur et al., 2018). To identify the severity and course of the disease, the annualized rate of severe relapses was generated. This refers to the rate of relapses demanding intravenous corticosteroid treatment or requiring hospitalisation (Comi et al., 2012). Other commonly used measures for relapse activity are the time to a confirmed relapse and relapse-free time (Tur et al., 2018).

Several patient-reported outcome measures are widely used in trials and clinical practice to assess fatigue (Fisk et al., 1994; Penner et al., 2009), depression (Beck et al., 1961), ability to carry out ADL (Hobart et al., 2001) and quality of life (Fisk et al., 2005; Sintonen, 2001).

2.4.2 Different phenotypes of RRMS

The clinical course of RRMS is variable because of variations in inflammatory activity and the progression of neurodegeneration. Some patients present early with minimal disease activity and retain a sparing disease course. The proportion of these MS patients with mild disease course varies between 5% and 64% (Benedikz et al., 2002; S. Glad et al., 2006; G. S. Ramsaransing & De Keyser, 2006). In contrast, approximately 4–15 % of RRMS patients have an aggressive or highly active course of the disease (Menon et al., 2013). There are currently no established biomarkers to determine the future disease activity in the early phase of the disease.

2.4.3 Benign MS

A proportion of MS patients show only minimal disability decades after the onset of MS symptoms, and this entity of so-called benign MS has been debated since the 1950s (G. S. Ramsaransing & De Keyser, 2006). A benign future course of MS cannot be reliably predicted based only on clinical features at the disease onset. Several different definitions of benign MS have been introduced over the decades. The oldest definitions may be the ones by McAlpine and Bauer from the 1960s, which include the ability to remain active or employed after 10 or 20 years of disease as the only criteria (Bauer et al., 1965; Mcalpine, 1961). An international survey of the United States National MS Society defined benign MS as "a disease in which patient remains fully functional in all neurological systems 15 years after disease onset" (Lublin & Reingold, 1996).

The most commonly used definition to classify a patient with benign MS is EDSS ≤ 3.0 after 10, 15 or 20 years of the disease onset (Correale, Ysrraelit, et al., 2012; G. S. Ramsaransing & De Keyser, 2006; Reynders et al., 2017; Sayao et al., 2007). None of the definitions in use address the use of immunomodulatory treatment, previously or currently; in other words, the use of DMT does not exclude a patient from the benign group. Even patients treated with mitoxantrone and cyclophosphamide were included in one study to analyse the factors associated with benign MS (Zivadinov et al., 2016).

The reported frequency of benign MS varies from 5% to 64% of all cases (Benedikz et al., 2002; S. Glad et al., 2006; G. S. Ramsaransing & De Keyser, 2006). Hospital-based cohorts tend to select more severe cases, leading to an underrepresentation of milder cases. The mildest MS cases might not be systematically followed in a neurological outpatient clinic, since previously DMT was not systematically started in most patients with a new diagnosis. Diagnostic criteria for MS have also evolved. Thus, earlier studies of patients with only restrictive clinical Poser's criteria and without MRI findings may have excluded mild cases (Poser et al., 1983),.

Benign MS usually presents as RRMS (Pittock et al., 2004). A benign disease course has also been described in PPMS (G. S. Ramsaransing & De

Keyser, 2007), but when followed for 20 years of disease duration, no benign cases with PPMS were found in population-based epidemiological studies (Benedikz et al., 2002; G. S. Ramsaransing & De Keyser, 2007). Natural history studies of MS have reported a significant association between the number of relapses within the first five years after onset and subsequent disability (Confavreux et al., 2003; Weinshenker et al., 1989). Some clinical and demographic factors have been associated with benign disease. A longer time from the onset symptom to the second attack and longer-lasting first remission have been considered to be favourable factors in benign disease (Phadke, 1990; G. Ramsaransing et al., 2001; G. S. Ramsaransing & De Keyser, 2006). The age at MS symptom onset or gender do not seem to be independent predictors for benign MS (G. S. Ramsaransing & De Keyser, 2007; Reynders et al., 2017; Sayao et al., 2007). Most studies have reported optic neuritis and sensory disturbances as the first attack to be indicators of a better prognosis, and cerebellar, brainstem or pyramidal tract symptoms to be associated with a worse outcome (Hawkins & McDonnell, 1999; Phadke, 1990; G. Ramsaransing et al., 2001; G. S. Ramsaransing & De Keyser, 2006), but no clear conclusion can be drawn only on the clinical symptoms. A British Columbia cohort of 200 benign MS patients reported that the only variable associated with disease progression at 20 years was the 10-year EDSS score. Only 52.1% of the patients defined as benign at the time of 10-year follow-up fulfilled the definition at 20 years (Sayao et al., 2007). However, a relapsing-remitting onset, only one relapse in the five first years after onset and an EDSS highest 2.0 at 5 years or EDSS highest 3.0 at 10 years of disease duration have been suggested as strong clinical predictors for having benign MS and retaining this status for another 10 years (Reynders et al., 2017). As early treatment decisions are crucial, more reliable paraclinical biomarkers are needed.

Clinical disease activity may be hidden, meaning symptoms such as fatigue and cognitive impairment being non-visible. The current definition of the treatment goal, which is no evidence of disease activity (NEDA) (Banwell et al., 2013), does not include an assessment of these symptoms. Still, a remarkable proportion of RRMS patients have been reported to

suffer from cognitive worsening despite favourable NEDA status (Damasceno et al., 2016). The prevalence of cognitive impairment and fatigue in benign MS patients has been reported with a range of 33%–50%, depending on the neuropsychological tests and scales used (Amato et al., 2006; Correale, Peirano, et al., 2012). Amato et al. studied 47 benign MS patients and found cognitive impairment to be correlated with pronounced cortical atrophy (Amato et al., 2008). Cognitive dysfunction in benign MS has been shown to associate even with severe structural brain damage resembling that of SPMS patients (Rovaris et al., 2008). Socially and economically relevant patient outcomes, such as employment, have scarcely been investigated in benign MS. Patients who remain minimally disabled are more likely to be employed, even part-time, and less likely to need disability imbursement (Sayao et al., 2011).

Controversially, a proportion of patients with a clinically benign MS have a large WM T2 lesion load (Strasser-Fuchs et al., 2008). The average T2 lesion load in benign MS can be equal to RRMS patients with a short disease duration and high EDSS scores (Filippi et al., 1996; Koopmans et al., 1989). In a prospective MRI study, new or enlarging T2 lesions, Gd-enhancing lesions and persistent black holes similar to SPMS were detected in benign MS (Correale, Peirano, et al., 2012). Brain volume loss in benign MS patients (defined as EDSS highest 3.0 and disease duration of at least 15 years) has been reported to be more profound than in healthy subjects (Rovaris et al., 2008). In benign MS the reduction of brain volume has even been comparable to SPMS (Rovaris et al., 2008). Loss of thalamic volume (Rovaris et al., 2009) and GM volumes in subcortical and frontoparietal regions (Mesaros et al., 2008) in benign MS compared to healthy controls have been reported. Most MRI data on benign MS have been from cross-sectional studies. One retrospective longitudinal study of 182 benign MS patients (EDSS highest 3.0 at 15 years from onset) and 187 non-disabling RRMS patients (EDSS highest 3.0 and disease duration less than 15 years) associated that a high T2 lesion load with a worsening of locomotor disability in the short term in benign MS (median follow-up was 29 months) (Rovaris et al., 2011). In a study investigating the evolution of newly formed lesions, the number of lesions that became designated as

black holes was lower in benign MS than in patients with SPMS (Rovaris et al., 2009).

Spinal cord damage due to lesion activity and degenerative atrophy correlate with disability accumulation in MS. In benign MS, cervical cord measures have been studied less than brain MRI measures. Cervical cord lesion load has been reported to be even somewhat similar in SPMS and benign MS, although cervical cord volume has been reported to be preserved in benign MS (Lin et al., 2003; Lycklama À Nijeholt et al., 1998).

2.4.4 Aggressive MS

Definitions of aggressive MS (AMS) or aggressive RRMS (ARRMS) are vague and ambiguous, but they share common features of repeated severe relapses and accelerated disability. Even a recent working group of the European Committee for Treatment and Research in Multiple Sclerosis failed to reach consensus on a definition because of the lack of data correlating severe disease with imaging and molecular biomarkers (Iacobaeus et al., 2020).

The term 'malignant' MS has been used in the context of aggressive MS, but it is most often used to mean fulminant forms of the disease that result in death within a timeframe of months to a few years from the onset. One example is the Marburg variant of MS, which is characterised by extensive tumefactive and necrotising lesions with mass effect in brain MRI (Mendez & Pogacar, 1988).

In a large retrospective cohort of almost 6000 patients from Canada, the following three criteria for AMS were tested: 1) reaching confirmed EDSS 6.0 or greater within five years from the onset of MS symptoms, 2) reaching confirmed EDSS 6.0 or greater by the age of 40 years and 3) reaching SPMS within three years of a relapsing-onset course. The patients with AMS were more likely to be men, older at MS symptom onset and have PPMS. This study was based on clinical details and did not include MRI data (Menon et al., 2013). The latest most commonly used term for AMS is 'highly active MS' (HAMS) (Díaz et al., 2019). There is a need for risk factors to predict the HAMS disease course, especially in assessing

treatment-naïve patients. HAMS has been defined as presenting one or more of the following characteristics in RRMS:

1. EDSS 4.0 at five years of the onset of the disease.
2. Multiple relapses (two or more) with incomplete recovery in the ongoing year.
3. More than two brain MRI studies demonstrating new lesions or an increase in the size of the lesions in T2 or Gd-enhancing lesions, despite treatment.
4. No response to treatment with one or more DMT for at least one year (Díaz et al., 2019).

In a retrospective analysis of a cohort of 401 CIS patients, a definition of EDSS 6.0 at 10 years of onset of the symptoms was proposed for aggressive MS. In this Spanish CIS cohort, a cut-off of 20 T2 lesions or at least two Gd-enhancing lesions in the baseline MRI discriminated patients with aggressive MS (Tintore et al., 2020). However, it has been argued that EDSS 4.0 is a point of no return – that is, a strong indicator for an advancing disease in which the course of progression is unlikely to change even if further relapses are prevented (Confavreux et al., 2003). Early recognition of HAMS is important because poor recovery from early relapses will develop a progressive disease course earlier than those with good recovery. The following clinical characteristics for the early recognition of a possible aggressive disease course have been recognised: poor recovery from the first two attacks (EDSS ≥ 1.5) (Scott & Schramke, 2010); brainstem, cerebellar or spinal cord syndrome (Novotna et al., 2015); and multifocal and fulminant relapse with poor recovery (Bergamaschi et al., 2001; Novotna et al., 2015). Predictors of rapid conversion to SPMS also include a short time to accumulate EDSS 3.0, a high rate of early relapses and a short interval between relapses (Novotna et al., 2015; Scalfari et al., 2010). A high brain MRI lesion load in the initial imaging has been shown to lead to greater disability in the following years, and in patients who convert to SPMS, the rate of change in the volume of lesions is three times higher than in those who do not convert (Fisniku et

al., 2008). Other early brain MRI markers that suggest HAMS are early brain atrophy (Eshaghi, Prados, et al., 2018), cortical and deep GM atrophy (Fisniku et al., 2008; Hänninen et al., 2019; Scalfari et al., 2018), infratentorial and spinal cord lesions and spinal cord atrophy (Brownlee et al., 2019).

The presence of OCB in CSF (Magraner et al., 2012) and elevated IgG index (Gasperi et al., 2019) at the time of the first demyelinating event have been connected to the HAMS disease course. Elevated serum and CSF neurofilament levels (Barro et al., 2018; Håkansson et al., 2018) and CSF C-X-C motif chemokine ligand 13 (CXCL-13) levels are also suggestive of aggressive disease course (Khademi et al., 2011). Nonetheless, none of these body fluid biomarkers have yet been established as valid markers to predict aggressive disease course.

It has been stated that these potential risk factors for an aggressive disease course should be evaluated throughout the disease course, since the disease may become active after years of stability, and in persons with a high functional reserve, it may take longer to develop disability (Krieger et al., 2016).

Since current DMTs target the inflammatory process in CNS, it is crucial to find the 'window of opportunity' of each patient and start optimal treatment (Freedman, 2008a). Patients fulfilling the HAMS criteria need highly active treatment because their window of opportunity closes rapidly. In Finland, the Current Care Guideline for MS lists the following DMTs for HAMS: alemtuzumab, fingolimod, cladribine, mitoxantrone, natalizumab and ocrelizumab (Working group set up by the Finnish Medical Society Duodecim and the Finnish Neurological Society, 2020). Any of these DMTs can be initiated as an initial medication if the patient fulfils the following criteria for HAMS:

1) One or more relapses within the previous 12 months AND either of the following MRI criteria:
2) ≥ 9 T2 lesions in the brain and/or spinal cord MRI
3) One or more Gd-enhancing lesion (Working group set up by the Finnish Medical Society Duodecim and the Finnish Neurological Society, 2020).

The definition of HAMS is an evolving concept. The proportion of patients receiving DMTs indicated for HAMS as an initial treatment strategy is increasing in many countries (Buron et al., 2020; Spelman et al., 2021). Natalizumab (United States Food and Drug Administration, FDA, approval 2004) and fingolimod (FDA approval 2010) were the first DMTs recommended for disease breakthrough use in previously treated RRMS patients. They have now been in clinical use for almost two decades and are still widely used in HAMS in Finland and globally. A recent study analysed the pooled data of 2447 patients from three earlier studies to gain a head-to-head analysis of natalizumab versus fingolimod showing the advantage of natalizumab over fingolimod in reducing the risk of relapses to be 23% and earlier recovery from neurological disability to be 40% (Andersen et al., 2021). A rebound of disease activity after treatment cessation is known for both fingolimod and natalizumab (Malpas et al., 2022; Mustonen et al., 2020; Roos et al., 2022), since these DMTs do not act to eliminate the inflammatory active cells. Cyclophosphamide (Krishnan et al., 2008; Schwartzman et al., 2009) and mitoxantrone (Edan et al., 2011; Le Page et al., 2008) have been used in the treatment of HAMS but are not recommended in the Finnish guidelines due to their possible serious adverse effects (Working group set up by the Finnish Medical Society Duodecim and the Finnish Neurological Society, 2020).

Table 3. Features of benign and aggressive MS based on a review of recent studies

	Benign MS	Aggressive MS
EDSS	At highest 3.0 at 10, 15 or 20 years after the disease onset (a, b, c, d)	4.0 or higher at 5 years of disease onset (e)
		Short time to accumulate EDSS 3.0 (d)
Time from the onset symptom to the second attack	Long (b, f, g)	Short (d)
Relapse activity in the early disease	Low (h, i)	Multiple relapses with incomplete recovery (e)
	Only one relapse in the five first years after onset (j)	
Recovery from the first attacks	Good	Poor (d, e)
Typical clinical presentation of first attack	Optic neuritis, sensory disturbances (b, g, k, l)	Cerebellar, brainstem or pyramidal symptoms (b, d, g, k, l)
Age at onset	Younger age at onset	Older age at onset (m)
Brain MRI T2 lesion load and Gd activity	Relatively high T2 lesion load despite minimal clinical disease activity (n)	More than 2 brain MRI demonstrating new or enlarging lesions or Gd enhancing lesions (e)
Brain atrophy	Loss of thalamic volume and GM volumes in subcortical and frontoparietal regions (o, p)	Early brain atrophy, cortical and deep GM atrophy (q, r, s, t)
Spinal cord MRI findings	Well-preserved spinal cord volume despite	Marked spinal cord affision, spinal cord

	moderate/marked lesion load (u, v)	atrophy (w)
Serum neurofilament levels	Not previously studied	Elevated before DMT (x, y)
DMT response	Good	Poor (e)
	No need for high-efficacy treatment	No response to treatment with one or more DMT for at least one year (e)
Clinical course	Typically RRMS (z)	RRMS, but often also PPMS (m)
Frequency in the literature	5%–64 % (c, å, ä)	4%–15% of RRMS (m)

(a) Correale, Yssraelit et al., 2012 (b) Ramsaransing G.S. & De Keyser, 2006 (c) Reynders et al., 2017 (d) Sayao et al., 2007 (e) Díaz et al., 2019 (d) Novotna et al., 2015 (f) Phadke, 1999 (g) Ramsaransing G et al., 2001 (h) Confavreux et al., 2003 (i) Weinshenker et al., 1989 (j) Reynders et al., 2017 (k) Hawkins & McDonnell, 1999 (l) Ramsaransing et al., 2001 (m) Menon et al., 2013 (n) Strasser-Fuchs et al., 2008 (o) Rovaris et al., 2009 (p) Mesaros et al., 2008 (q) Eshaghi, Prados et al. 2018 (r) Fisniku et al., 2008 (s) Hänninen et al., 2019 (t) Scalfari et al., 2018 (u) Lin et al., 2003 (v) Lycklama À Nijeholt et al., 1998 (w) Brownlee et al. 2019 (x) Barro et al., 2018 (y) Håkansson et al., 2018 (z) Pittock et al. 2004 (å) Benedikz et al., 2002 (ä) Glad et al., 2006

2.5 TREATMENT OF MS

There is no curative treatment for MS; thus, a therapeutic strategy with immunomodulatory treatment aims to reduce the risk of relapses and potential disability by relapses and progression. The first injectable immunomodulatory DMTs, interferons, were introduced in clinical practice in the early 1990s, and since then, the development of new DMTs has been revolutionary. As counted on July 2023, there were 16 different DMTs approved and reimbursed (as applies to the DMTs administered by the patient at home) for MS treatment in Finland.

2.5.1 Immunomodulatory treatment

Early treatment is beneficial, and all the approved DMTs in practice have been proven to be superior to placebo in reducing the relapse rate within two years of follow-up (Li et al., 2020). Starting a DMT has been shown in trials of CIS to delay the conversion to definite MS (Chalmer et al., 2018; Comi et al., 2017; Montalban et al., 2018). The ability to choose from a large variety of DMTs has also made it possible to plan treatment strategies more individually. The most commonly used treatment strategy has been to start with a low-efficacy DMT and switch to a more potent one in case of breakthrough disease (clinical or MRI activity). This escalation strategy aims to find the optimal treatment and to take as little risk as possible (Cree et al., 2019; Río et al., 2011; Ziemssen et al., 2016). However, recent real-world evidence has shown that the start of a highly effective DMT within two years from disease onset or as initial therapy, compared with delayed start, improves long-term disability outcome (Buron et al., 2020; Hänninen et al., 2022; He et al., 2020; Spelman et al., 2021). The decision of which DMT to choose is based on the knowledge of factors in consideration of the patient, such as disease stage and activity, earlier treatment history, possible pregnancy planning in female patients of childbearing age and prognostic indicators at the individual level, and on the other side, knowledge of DMT efficacy and risk factors for complications and the extent of safety follow-up (Cree et al., 2019; Van Wijmeersch et al., 2022). Shared decision-making with the patient is beneficial for adherence to the treatment itself and the safety follow-up protocol (Alonso et al., 2023). Financial factors may also limit the treatment.

Current DMTs for RRMS are categorised as moderate or high, according to their efficacy, although earlier terms were first-line and second-line DMTs (according to the previously prevailing treatment escalation strategy). Dimethyl fumarate, diroximel fumarate, glatiramer acetate, interferons and teriflunomide are categorised as moderate efficacy, and alemtuzumab, cladribine, fingolimod, ocrelizumab, ofatumumab and ponesimod as high efficacy (Jonasson & Sejbaek, 2020; Kappos et al., 2021; Montalban et al., 2018; Working group set up by the Finnish Medical

Society Duodecim and the Finnish Neurological Society, 2020). Ocrelizumab is the only approved DMT to be used in active PPMS (Montalban X et al., 2017). These DMTs are described in more detail in Table 4.

Autologous hematopoietic stem cell transplantation (aHSCT), a standard therapy in myeloma and lymphoma, is considered to be an experimental and rescue therapy in HAMS after failing one or two highly active DMTs. Pooled data from the published aHSCT studies have shown estimated treatment-related mortality of 2.1%, a two-year disease progression rate of 17.1%, a five-year progression rate of 23.3%, and a pooled result of NEDA at 2 years in 83% of patients (Sormani et al., 2017). Results are awaited from several ongoing multi-centre phase 2 and 3 randomised controlled trials comparing aHSCT with high-efficacy therapies such as alemtuzumab, natalizumab, ocrelizumab and rituximab.

2.5.2 Treatment of acute relapses

Acute relapses may be treated with intravenous (IV) corticosteroids (typically 10000mg of IV methylprednisolone daily for 3–5 consecutive days). An oral high dose corticosteroid treatment has also been proven to be efficient (Le Page et al., 2015; Ontaneda & Rae-Grant, 2009). Mild relapses do not necessarily need acute treatment, but if the patient suffers from worsened gait or strength, or their vision is impaired with optic neuritis, corticosteroid treatment may shorten the duration of the symptoms. Corticosteroids do not impact the overall disability outcome (Gal et al., 2015). In severe relapses that are refractory to corticosteroids, plasma exchange may be considered (Bunganic et al., 2022).

2.5.3 Symptomatic treatments

Effective MS symptom management improves quality of life, reduces the effect of disability in daily life and may help the patient to continue in work or studies. MS symptoms include mobility-related symptoms such as spasticity, ataxia and gait problems; bladder, bowel and sexual dysfunctions; fatigue; cognitive problems; mood disturbance; and sleeping problems. These symptoms often interact and as do their treatments, both

pharmacological and non-pharmacological (Dalgas et al., 2019; Thompson et al., 2010). Exercise training is safe and helps to reduce spasticity, pain, fatigue and depression (Pilutti et al., 2014). Fatigue and depression may be relieved by psychological and neuropsychological therapy (van den Akker et al., 2016).

Table 4. Immunomodulatory treatments of MS.

	Mode of action	Administration	Efficacy	Side effects
Active RRMS				
Interferon beta 1a and 1b	Anti-inflammatory modulation of different immune cell subsets	SC or IM injection	Moderate	Flu-like symptoms, elevation of liver enzymes, injection site reactions, depressive symptoms
Glatiramer acetate	Anti-inflammatory shifts of immune cell populations	SC injection	Moderate	Injection site reactions, chest pain and tachycardia
Dimethyl fumarate	Modulates immunometabolism, induces apoptosis of memory immune cells	PO tablets	Moderate	Diarrhoea, stomach pain, elevation of liver enzymes, flushing, lymphopenia (PML)
Diroximel fumarate	Modulates immunometabolism, induces apoptosis of memory immune cells	PO tablets	Moderate	Diarrhoea, stomach pain, elevation of liver enzymes, flushing, lymphopenia
Teriflunomide	Inhibits immunometabolism	PO tablets	Moderate	Diarrhoea, elevation of liver enzymes, hypertension, headache, risk of infections
Ocrelizumab	Depletes CD20-expressing B cells and T cells	IV-infusion	High	Infections, infusion reactions

Ofatumumab	Depletes CD20-expressing B cells and T cells	SC injection	High	Infections, injection site reactions
Very active RRMS				
Alemtuzumab	Depletes CD52-expressing cells	IV-infusion, cyclic dosing in two consecutive years	High	Autoimmune reactions (thyroid problems in 30%–50% of patients), infusion reactions, cerebrovascular disease
Cladribine	Accumulation of the drug leads to preferential depletion of lymphocytes	PO tablets, cyclic dosing in two consecutive years	High	Infections, teratogenity
Fingolimod	Sequesters re-circulating immune cells in lymph nodes	PO tablets	High	Bradycardia, elevation of liver enzymes, macular oedema, teratogenity
Natalizumab	Limits immune cell trafficking into CNS	IV-infusion	High	Infections, PML
Ocrelizumab	Depletes CD20-expressing B cells and T cells	IV-infusion	High	Infections, infusion reactions
Ponesimod	Sequesters re-circulating immune	PO tablets	High	Elevation of liver enzymes,

	cells in lymph nodes			macular oedema, teratogenity
Active PPMS				
Ocrelizumab	Depletes CD20-expressing B cells and T cells	IV-infusion	High	Infections, infusion reactions

2.6 MAGNETIC RESONANCE IMAGING IN MS

Brain and spinal cord MRI is essential in the diagnosis and follow-up of MS. The European Magnetic Resonance Imaging in Multiple Sclerosis (MAGNIMS) consensus provided guidelines to neurologists and neuroradiologists for the use of MRI in the diagnosis, prognosis and monitoring of MS treatment in imaging protocols and indications of MRI (Filippi et al., 2016). MRI interpretation must be done in the appropriate clinical context to avoid overreliance on MRI abnormalities in patients with non-specific symptoms. In most clinics, MRI interpretation is still done only visually by a radiologist. Focal WM hyperintensities mimicking MS lesions can be found as age-related changes and in vascular disease, in patients with migraine (Chong et al., 2022) and in several other inflammatory and antibody-mediated CNS disorders such as neuromyelitis optica spectrum disorders (NMOSD) (Geraldes et al., 2018).

A focal WM T2 hyperintense lesion of at least 3 mm in diameter in a typical location is counted as a demyelinating lesion. In the diagnosis of MS, MRI is used to show evidence for DIS and DIT. For RRMS, DIS is fulfilled with at least one T2 lesion in two of the following four areas: periventricular, cortical or juxtacortical, infratentorial or spinal cord areas. DIT requires both Gd-enhancing and non-enhancing lesions in the same scanning or a new T2 lesion in a follow-up scan (Thompson et al., 2018). A revision of the MRI recommendations by MAGNIMS, the Consortium of Multiple Sclerosis Centres and the North American Imaging in Multiple Sclerosis Cooperative in 2021 elaborated on the brain and spinal cord MRI protocols and pointed out the appropriate use of Gd-based contrast agents, and especially the avoidance of unnecessary Gd-enhancement use in follow-up imaging because of increased knowledge of Gd deposition in the CNS (Gulani et al., 2017).

The term ‘radiologically isolated syndrome’ (RIS), meaning incidental WM lesions highly suggestive of MS pathology in an individual with no clinical symptoms of MS, was first introduced in 2009 (Okuda et al., 2009). Patients with RIS should be followed up, since a substantial number of them

develop clinical symptoms typical of MS in the near future (De Stefano et al., 2018; Okuda et al., 2014).

In the DMT follow-up, it is recommended that the first reference brain MRI is performed within six months after the treatment onset and that further control MRIs are typically done within 6–2 months after that. The mechanism of action of the chosen DMT and the disease activity of the patient need to be considered in the timing of follow-up MRI (Montalban et al., 2018). NEDA is the primary goal of the MS treatment strategy. NEDA-3 includes the absence of relapses, no evidence of MRI activity (no new or enlarged T2 lesions nor new contrast-enhancing T1 lesions), and no progression of disability (no confirmed EDSS worsening) in the previous six months (Banwell et al., 2013). NEDA-3 reflects mainly focal inflammatory activity measures, while NEDA-4 includes a neurodegenerative indicator, namely disability progression unrelated to clinical relapse. In the definition of NEDA-4, yearly brain volume loss of less than 0.4% is also included (Kappos et al., 2016).

MRI follow-up is also needed for safety control of the treatment, especially in patients with a high risk for progressive multifocal leukoencephalopathy (PML), such as in patients who are John Cunningham virus (JCV)–positive and have had natalizumab treatment for a duration of over 18 months (Montalban et al., 2018).

Spinal cord MRI is important in the diagnosis of MS to show DIT and DIS in CIS patients and in differential diagnosis, such as to exclude vascular disease and spinal cord compression (Thompson et al., 2018). Spinal cord lesions are often seen in CIS patients, even those without spinal cord symptoms (Bot et al., 2002). Repeated spinal cord imaging in the follow-up of MS is helpful in patients with spinal cord symptoms or clinical disease progression that is not explained by brain MRI but not in asymptomatic patients to detect subclinical activity (Wattjes et al., 2021). Spinal cord volume can be measured, and the upper cervical cord area is the most commonly used measure. The correlation between spinal cord volume loss and clinical disability has been shown in several studies (Bernitsas et al., 2015; Lukas et al., 2015; Valsasina et al., 2013).

2.6.1 Brain atrophy analysis in MRI

As it is now recognised that MS is both an inflammatory and a neurodegenerative disease, whole-brain and regional volume measurements are essential parts of the imaging of MS to assess the neurodegenerative component. Brain atrophy has been correlated with irreversible physical and cognitive disability (Cagol et al., 2022; Popescu et al., 2013), and it can be measured using MRI. Brain atrophy is also a normal phenomenon of aging in healthy individuals, and occurs at a rate of 0.1% – 0.3% per year. In MS, this annual rate of brain volume loss (BVL) is higher compared to age-related measures: at 0.5% – 1.3% at all stages of the disease (Giorgio et al., 2010). BVL is a consequence of several factors in MS, such as myelin loss in demyelinating lesions of both WM and GM, resolution of inflammation and oedema after acute focal inflammatory events and gliosis and axonal loss in normal-appearing WM (NAWM) (Andravizou et al., 2019). Brain atrophy seems to vary between different clinical phenotypes, with ventricular enlargement and central atrophy more prominent in RRMS, while cortical atrophy appears to be more pronounced in the progressive disease (Pagani et al., 2005).

Pronounced GM atrophy is found in patients with CIS or early stages of RRMS (Bergsland et al., 2012; Calabrese et al., 2007) and PPMS (Sastre-Garriga et al., 2004). Regional deep GM atrophy, especially thalamic atrophy, has been associated with the evolution of definite MS and disability progression in early RRMS, as well as with the evolution of PPMS (Mesaros et al., 2011; Zivadinov et al., 2013, 2022).

Thalamic atrophy has been shown to evolve early in MS. It is associated with cognitive decline, fatigue and pain (Eshaghi, Prados, et al., 2018; Houtchens et al., 2007; Schoonheim et al., 2015). The thalamus is a vital relay nucleus with cortical and subcortical connections and is thus a critical location in MS. MRI studies have strengthened the previous histopathologic findings of axonal disconnection in major thalamic tracts and thalamic demyelinating lesions (Cifelli et al., 2002; Harrison et al., 2015). Thalamic volume decline has been reported to be consistently present across all MS subtypes and throughout the disease course, correlating with whole-brain

atrophy (Azevedo et al., 2018). Thus, the measurement of thalamic atrophy has been adopted as an important MRI endpoint in MS clinical trials (Ontaneda et al., 2021).

The corpus callosum (CC) is the largest and most functionally important WM fibre tract connecting the hemispheres and is known to be affected by focal demyelination and Wallerian degeneration in the pathogenesis of MS (Evangelou et al., 2000). CC atrophy is associated with the level of disability and fatigue and correlates with GM atrophy in MS (Klawiter et al., 2015; Vaneckova et al., 2012; O. Yaldizli et al., 2010). CC seems to be resistant to age-related changes in healthy individuals, making it a relevant candidate for a brain atrophy marker (Pozzilli et al., 1994; Sullivan et al., 2001). Corpus callosum index (CCI) and corpus callosum area (CCA) seem to be reliable methods for assessing CC atrophy in MRI (Granberg et al., 2015; Klawiter et al., 2015; O. Yaldizli et al., 2010).

2.7 MRI VOLUMETRIC TOOLS

The level of brain atrophy and lesion volumes significantly predict long-term disability in all MS phenotypes (Eshaghi, Marinescu, et al., 2018; Eshaghi, Prados, et al., 2018; Popescu et al., 2013; Sormani et al., 2014). These volumes measured using image segmentation have become established biomarkers in estimating treatment efficacy in research studies and clinical trials (Branger et al., 2016; Sormani et al., 2014) and been incorporated into the treatment goal by the definition of NEDA-4, which also includes brain volume loss (Kappos et al., 2016). Manual segmentation of whole-brain and regional brain volumes and lesion volumes is time-consuming and prone to rater-related errors, and visual rating scales in quantifying global brain atrophy are relatively coarse (Ashton et al., 2003; Filippi et al., 1995). Automated measurements are fast to implement and thus save time and cost and minimise operator-dependent errors. Robustness to image quality would result in more reliable and comparable results between subject and imaging sites (Anderson et al., 2006). An ideal brain atrophy measure in an automated analysis would be sensitive to brain atrophy, allowing subtle pathological changes to to be detected.

Reproducibility of the measure would enable the avoidance of measurement errors. An ideal measure would accurately detect actual tissue loss and repeated measurements of the same volume would be of the same value.

The development of automated and semi-automated brain volume segmentation tools has increased in recent years. These quantitative segmentation tools aim to increase the sensitivity of MRI analysis and the accuracy of results and speed up the analysis (Brune et al., 2020; Zeng et al., 2020). Some of these tools also automatically compare results against relevant reference population data, offering the ability to evaluate the disease course and to decide on therapeutic strategies (Smeets et al., 2016). Three-dimensional (3D) volumetric acquisitions require less dependence on slice positioning and selection than those that are two-dimensional (2D), offering more accurate results from automated techniques (Sharma et al., 2004). They also provide good CSF/brain and GM/WM contrast, enabling the visualisation and measurement of small structures. Normalisation to intracranial volume should be performed, since small volume changes may be masked by biological interindividual variability in absolute brain volumes. In cross-sectional measurements, the rate of brain atrophy can be assessed only indirectly. Therefore, longitudinal measurements enable a more precise assessment of disease progression and the identification of the true progression both individually and between different stages and phenotypes of the disease (Anderson et al., 2006). A mix of different scanner types and field strengths in the normative reference data is a general way to master inter-scanner variability (Mendelsohn et al., 2023).

Several quantitative MRI tools have been developed for clinical use and are commercially available with regulatory approval. Automated quantitative volumetric tools have been developed for the diagnostics of dementia, and these can be used in dementing neurodegenerative diseases and MS (Hedderich et al., 2020a; Pemberton et al., 2021). A recent review paper identified ten companies that provide MS lesion and brain segmentation and volume quantification tools (Mendelsohn et al., 2023). All the companies have received regulatory approval as ‘software as a

medical device' (either by Conformité Européenne, CE, or by FDA). Most of the evidence was from the technical validation of tool performance (six tools out of ten), and four companies conducted clinical validation by clinicians or through the interpretation of results alongside clinician-rated variables. Only one in-use validation was found in the review, which was a microsimulation investigating the health-economic impact of the tool in a hypothetical cohort of RRMS patients (Sima et al., 2021).

2.8 BLOOD AND CSF BIOMARKERS FOR MONITORING DISEASE ACTIVITY

There is a need for soluble biomarkers reflecting neuroaxonal injury in all neurological acute and chronic disorders. Soluble biomarkers refer to biochemical molecules drawn in blood samples. An ideal biomarker would separate affected patients from healthy individuals; in other words, it would act as a diagnostic tool. It could be used as a predictive and prognostic tool and as a disease activity marker. Biomarkers are also needed to measure treatment responses and possible disease progression. At present, only CSF immunoglobulin index (IgG index) and OCB are in clinical diagnostic use for MS (Freedman et al., 2005; Thompson et al., 2018), and there are no validated soluble biomarkers for clinical prognostic use or to monitor DMT efficacy.

2.8.1 MS biomarkers in clinical use

Assessment of CSF IgG OCB and IgG index is important in the diagnosis of MS. The presence of CSF OCBs or an increased IgG index (>0.7) is evidence for an abnormal intrathecal B cell response and supportive for MS diagnosis (Freedman et al., 2005). According to the latest 2017 McDonald diagnostic criteria of MS, the presence of CFS OCBs can substitute for a clinical second attack or DIT in MRI and verify a diagnosis in a CIS patient (Thompson et al., 2018).

Some other neuroinflammatory diseases, especially NMOSD, have clinical and radiological features similar to MS. Distinguishing NMOSD is especially important because its the prognosis and treatments differ from

those of MS. At present, anti-AQP4 antibody, a specific autoantibody against the astrocytic water channel aquaporin, is a useful diagnostic biomarker of NMOSD (Wingerchuk et al., 2006). Patients with anti-MOG antibodies are further distinguished from MS and NMOSD (Jarius et al., 2023).

Neutralising antibodies (NAb) against natalizumab are not only associated with reduced levels of natalizumab and a further reduction in the therapeutic efficacy of the drug, but also an increase in infusion-related adverse events (Calabresi et al., 2007). Up to 40% of patients treated with interferon beta generate NAbs (Sibley, 1996). A switch to another DMT is recommended in patients receiving interferon beta with persistent high titer NAbs (Polman et al., 2010).

Biomarkers for treatment safety include testing for JC virus in patients with highly effective immunosuppressive MS treatments (especially natalizumab), to assess the risk for JCV activation leading to PML (Bloomgren et al., 2012). Highly effective immunosuppressive DMTs increase the risk of herpetic infections. Thus, patients should be screened for antibodies against varicella zoster virus, and seronegative patients should be vaccinated before initiating immunosuppressive DMT (Otero-Romero et al., 2023).

2.8.2 Neurofilaments

Neurofilaments (NF) are cytoskeletal structure proteins of the neuron. The function of neurofilaments is thought to be essential for the radial growth and stability of the axon and to further enable effective nerve conduction (Rao et al., 2003). NF are classified according to their molecular weight as neurofilament heavy chain (NfH), medium chain (NfM) and light chain (NfL) and alpha-internexin and peripherin (Yuan et al., 2017). For any reason in neuroaxonal damage, NF are released to CSF and further to peripheral blood in smaller quantities. They are highly specific for neuronal cell death, making them a valuable candidate for a biomarker of neuroaxonal injury in neurological illnesses and conditions. NF levels were first investigated in CSF samples (Lycke et al., 1998; Petzold, 2005; Petzold et al., 2003;

Rosengren et al., 1996). Recent advances in assay sensitivity have enabled the detection of very small quantities (in concentrations in the range of picograms/millilitre) enabling measurements in peripheral blood. Current single-molecule array (SiMoA) technology offers a 25-fold sensitivity, compared to previous methods, and has made it possible to segregate NF levels in disease and in physiological conditions (Disanto, Barro, Benkert, Naegelin, Schadelin, et al., 2017; Gisslén et al., 2016; Rissin et al., 2010). NF measurements in peripheral blood (serum or plasma) have finally brought this biomarker closer to clinical practice, since a lumbar puncture is not needed to obtain a sample.

Neurofilaments are not specific for the reason behind neuronal damage. Normal ageing is associated with signs of neurodegeneration, such as loss of brain tissue volume and elevated levels of fluid biomarkers. Measured by NfL levels in CSF, the normal upper reference value has been found to increase 2.5-fold between the ages of 20 years and 50 years and to further double by the age of 70 years (Yilmaz et al., 2017). Similarly, a significant correlation between age and NfL blood levels has been observed by the SiMoA technique: in the blood of healthy controls, NfL levels increased by 2.2% per year between 18 years and 70 years of age (Disanto, Barro, Benkert, Naegelin, Schadelin, et al., 2017). A strong correlation between levels of NfL in CSF and blood has been observed in several studies, suggesting these two measures reflect similar physiological processes (Disanto, Barro, Benkert, Naegelin, Schadelin, et al., 2017; Kuhle et al., 2016; Piehl et al., 2017b).

NF levels in CSF and blood have been investigated in various neurological diseases in search of clinical applications to improve diagnostic accuracy, to rule out neurological diseases, in prognostic assessment and to monitor response to treatment. Blood NfL levels have been observed to increase in the early stage of AD (Mattsson et al., 2017) and in familial disease up to ten years before the expected onset of symptoms (Weston et al., 2017). Serum NfL seems to be a potential biomarker for discriminating FTD and primary psychiatric disorders (al Shweiki et al., 2019; Katisko et al., 2020). NfL levels both in CSF and blood have been shown to be elevated in amyotrophic lateral sclerosis (ALS), and

in familial forms of ALS the levels increase at the time of the early symptomatic phase (Steinacker et al., 2016; Weydt et al., 2016).

Elevated NfL levels have been found in acute stroke and traumatic brain injury (TBI). In acute ischemic stroke, blood NfL has been studied as a predictive biomarker, partly with contradictory results. In a recent study, patients who had higher levels of NfL before endovascular thrombectomy of a large vessel occlusion had an unfavourable outcome right after the procedure and 24 hours later (Chen et al., 2021). In another study, the levels of serum NfL correlated positively with the size of the infarct but not with the later disability (Onatsu et al., 2019). Elevated serum NfL levels were found in patients with recent clinically silent small subcortical infarcts in MRI (Gattringer et al., 2017). CSF and blood NfL levels are increased not only after TBI (Shahim et al., 2016) but also in mild TBI (Shahim et al., 2017). In TBI, serum NfL levels rise gradually, reaching a peak at around 7–10 days after trauma (Halbgebauer et al., 2022).

In 1998, Lycke et al showed for the first time that NF levels in CSF can act as a biomarker in MS (Lycke et al., 1998). The amount of published data on blood NfL levels in MS has increased remarkably, especially after the introduction of the SiMoA assay. Elevated levels of NfL in CSF, compared to healthy controls, have been reported in all stages and types of MS (CIS, RRMS, SPMS and PPMS) (Khalil et al., 2013; Kuhle et al., 2013; Malmeström et al., 2003; Teunissen et al., 2009). Since NfL is not specific to MS, it is not useful in diagnostics per se, but it could be used as a supplementary tool, since elevated levels of NfL in CSF have been shown to predict the conversion of RIS to clinically definite MS (Matute-Blanch et al., 2018). Levels of serum NfL have been found to be elevated as early as six years before clinical MS onset, indicating a long prodromal phase with neuroaxonal damage (Bjornevik et al., 2020). Elevated serum NfL levels have been found in CIS, compared to healthy controls (Disanto et al., 2016) and predict conversion to clinically definite MS (Dalla Costa et al., 2019).

There is increasing evidence that serum NfL can be used as a biomarker of disease activity and DMT efficacy. High baseline serum NfL levels have been shown to correlate with relapse activity in the previous year and to forecast a relapse in the near future but not later on in subsequent years

(Cantó et al., 2019). Elevated serum NfL levels have been found in patients who have Gd-enhancing lesions and large brain T2 lesion load, both in RRMS and CIS (Disanto, Barro, Benkert, Naegelin, Schädelin, et al., 2017; Siller et al., 2018; Uher et al., 2021).

Patients with ongoing DMT have been shown to have lower serum NfL levels compared to untreated patients (Disanto, Barro, Benkert, Naegelin, Schädelin, et al., 2017). Blood NfL levels decrease after the initiation of a DMT in untreated patients (Siller et al., 2018) and after therapy escalation in patients already treated with a DMT, such as after a switch from injectable therapies to a higher efficacy DMT (fingolimod) (Piehl et al., 2017a; Siller et al., 2018). High serum NfL levels within the first year of the disease have been shown to be associated with long-term disability worsening in MS, suggesting that the measurement of serum NfL level would help in identifying the right candidates for high-efficacy DMTs (Monreal et al., 2023).

The lack of reference values has decelerated the adoption of serum NfL levels in clinical use. Since NfL increases with age and decreases with body mass (Manouchehrinia et al., 2020), fixed cut-off values are not feasible. Benkert et al. collected a large control group of over 5000 healthy controls to create a reference database on serum NfL levels (over 10 000 blood samples) to consider the confounding factors of age and body mass index (Benkert et al., 2022). A conservative cut-off 10 pg/ml was used as a definition for a non-pathological concentration of serum NfL to create percentiles and Z scores, which were tested in a cohort of 1313 MS patients. A Z score of above 1.5 was associated with increased risk for future disease activity. An internet-based app was created to help clinicians determine a patient's Z score from serum NfL concentrations and demographic details (Benkert et al., 2022).

Serum NfL does not seem to be useful in differential diagnostic use between NMOSD and MS, although several studies have shown that serum NfL levels are elevated in NMOSD patients during relapses and normalised after treatment (Kim et al., 2020; Mariotto et al., 2017; Zhang et al., 2021).

Contrary to the findings in the inflammatory state of the disease, serum NfL does not seem to reflect the pathological processes in progressions of MS (Barro et al., 2022).

2.8.3 Glial fibrillary acidic protein (GFAP)

Astrocytes represent the majority of cells (20%–40%) in the brain and are essential in forming the blood–brain barrier (BBB) and the glymphatic system, and they maintain axonal metabolic homeostasis. Glial fibrillary acidic protein (GFAP) is the main cytoskeletal structure protein of astrocytes, and it also has a role in the regulation of neuronal physiology (Mccall et al., 1996). GFAP is highly brain-specific, and its levels in healthy individuals are very low (Missler et al., 1999). In neurological diseases, the damage to astrocytes leads to the leakage of GFAP into CSF and through the BBB into the blood.

Higher serum GFAP levels have been shown to predict poorer long-term outcomes after a severe TBI (Vos et al., 2004). In mild TBI, the predictive power of the serum GFAP level is more limited and needs other biomarkers in combination (Metting et al., 2012). In a large multi-centre prospective study consisting of over 1900 patients with mild to moderate TBI, Bazarian et al. measured both serum GFAP and ubiquitin C-terminal hydrolase-L1 (UCH-L1, another serum biomarker studied in TBI) with prespecified cut-off values (Bazarian et al., 2018). They found that a serum GFAP level of 22 pg/ml, in addition to serum UCH-L1 levels above 327pg/ml, could predict the presence of intracranial injuries on head CT. This study supports the potential clinical role of serum GFAP in ruling out the need for a CT scan at emergency departments in patients with TBI in whom a head CT is felt to be clinically indicated. This finding also contributed to the FDA authorisation in 2018 of the serum GFAP test in clinical use for the avoidance of unnecessary exposure to CT radiation in patients with suspected TBI. Plasma GFAP has also been shown to be useful in identifying patients with mild TBI with MRI abnormalities after a normal CT scan (Yue et al., 2019). In spinal cord injury (SCI), serum GFAP seems to predict the diagnosis of SCI prior to spinal CT scanning and to

correlate with the severity of the injury (Ahadi et al., 2015; Leister et al., 2021).

Elevated GFAP levels have been stated in several neurodegenerative disorders. It has been shown that serum GFAP can be used in discriminating AD patients from healthy individuals and behavioural variant FTD patients (Oeckl et al., 2019). A recent study by Pereira et al. showed a correlation between plasma GFAP levels and amyloid beta protein (another AD biomarker) and cognitive decline, suggesting GFAP to be a useful biomarker for the early-stage diagnosis of AD (Pereira et al., 2021). In Parkinson's disease, higher serum GFAP levels were found in patients with simultaneous dementia (Oeckl et al., 2019).

In cerebrovascular diseases, the serum GFAP level seems to be more sensitive to acute than to chronic tissue damage in cerebral small vessel disease (Gattringer et al., 2022). GFAP is released rapidly in blood after an intracerebral haemorrhage (ICH) as a result of the BBB disruption and brain injury, whereas in ischemic stroke, the release is slower (Foerch et al., 2012). In acute stroke, higher serum GFAP levels on admission predicted poorer functional outcomes one year after acute stroke in a prospective study of 286 patients (Liu & Geng, 2018). In a study of 86 patients with intracerebral and subarachnoid haemorrhage, higher serum GFAP levels predicted mortality and poor neurological outcome (Gyldenholm et al., 2022).

Compared to the extensive research on neuroaxonal marker NfL in MS, studies of astroglial markers in MS and other neuroimmunological diseases are still scarce. However, there is increasing evidence of an altered astroglial response in MS (Norgren et al., 2004; Rosengren et al., 1995). In the early 1970s, GFAP was found to be the main protein in chronic MS lesions (Eng et al., 1971). This finding was strengthened with a later histological study, in which the GFAP levels were higher in the cortical samples of MS patients compared to the control brain homogenate (Petzold et al., 2002).

The first studies exploring GFAP as a biomarker in MS were conducted with CFS samples. Malmeström et al. showed that higher CSF levels of GFAP were connected to the secondary progressive phase of MS and

increasing disability assessed with EDSS, and the idea of GFAP serving as a progression marker was introduced (Malmeström et al., 2003). In this same study, NfL levels were increased in all MS patient subgroups compared to healthy individuals, and the levels peaked up to tenfold in acute relapse. Both CSF and serum levels of GFAP have been shown to be associated with age (Axelsson et al., 2011; Högel et al., 2018). Correlation between CSF and serum levels of GFAP has been demonstrated in MS patients and healthy individuals (Abdelhak et al., 2018; Högel et al., 2018). Lately, as ultrasensitive techniques using SiMoA have been adopted, most studies in GFAP have been conducted with serum samples.

Evidence of an association between GFAP levels and acute inflammation in MS is somewhat contrasting. Kassubek et al reported a strong correlation between elevated CSF levels of GFAP and Gd-enhancing lesions, indicating that GFAP can be a marker of inflammation in patients with RRMS and CIS (Kassubek et al., 2017). However, other earlier studies on CSF levels (Norgren et al., 2004; Rosengren et al., 1995) and later ones on serum GFAP levels have not shown a correlation with inflammation (Abdelhak et al., 2018). GFAP levels have been shown not to be affected by DMT (Axelsson et al., 2014; Gunnarsson et al., 2011). Multiple studies have shown a correlation between blood GFAP level, and the level of disability measured by EDSS (Abdelhak et al., 2018; Ayrignac et al., 2020; Högel et al., 2018). A recent study by Barro et al. evaluated serum NfL and GFAP levels in MS patients with a high risk of having a progressive state of the disease to indentify the ability of these two biomarkers to discriminate between active and non-active patients and to identify disease progression. GFAP was associated with the duration of the disease, and it was prognostic for future clinically definite progression. The higher levels of serum GFAP prognosticated progression were in patients with low NfL (i.e. with low inflammation) (Barro et al., 2022). Serum GFAP levels have been found to be higher in females than in males, which may be associated with hormonal changes in aging and its effect on MS pathology (Barro et al., 2022; Giarraputo et al., 2021). Serum GFAP levels probably need both age- and sex-adjusted reference ranges.

In differential diagnostic use, blood GFAP may be useful in distinguishing between NMOSD and MS (Lee et al., 2020; Watanabe et al., 2019). NMOSD is an autoimmune inflammatory astrocytopathy, and aquaporin-4 (AQP4) antibodies found in NMOSD induce astrocytic damage. Double seronegative NMOSD patients (i.e patients with NMOSD phenotype but no antibodies against AQP4 or myelin oligodendrocyte glycoprotein MOG) are a challenge in clinical practice. CSF GFAP levels in double seronegative NMOSD patients were found to be lower than in AQP4-positive patients and similar to those in MOG-positive patients and patients with other neurological disorders. This suggests that GFAP is not useful in the diagnostics of the seronegative NMOSD phenotype and that the underlying pathogenesis is different from astrocytopathy (Hyun et al., 2022). In a multi-centre randomised controlled trial of inebilizumab (a humanised monoclonal antibody that binds to the B-cell-specific surface antigen CD19) on NMOSD, serum GFP levels were shown to be elevated within a relapse, and in the inebilizumab treatment group, the GFAP levels decreased by 12.9% from the baseline (Aktas et al., 2021).

In conclusion, it seems that blood NfL levels can serve as an inflammatory marker in MS, while GFAP is a marker of disease progression. GFAP probably indicates the shift of neuroinflammation towards a gradual chronic neurodegeneration and astrogliosis (Barro et al., 2022).

2.8.4 Other soluble biomarkers

CXCL-13, a homeostatic chemokine expressed in lymphoid organs, is involved in mechanisms of chronic inflammation and the regulation of B cell homing in MS (Legler et al., 1998). The levels of CXCL-13 have been found to be elevated not only in the CFS of MS patients, compared to healthy controls (Sellebjerg et al., 2009), but also in patients with other neuroinflammatory diseases (Alvarez et al., 2013), neuroborreliosis and infectious diseases of the CNS (Rupprecht et al., 2005). In addition, CXCL-13 may be a useful biomarker for treatment response (Novakova, Axelsson, et al., 2017; Sellebjerg et al., 2009), especially in monitoring B-cell-depleting therapies (Alvarez et al., 2015).

Osteopontin is a pro-inflammatory cytokine secreted by activated immune cells, and it is involved in MS pathogenesis in addition to a variety of other chronic inflammatory diseases, atherosclerosis and cancer (Lund et al., 2009). Plasma and CSF levels of osteopontin are elevated in MS patients, compared to healthy controls (Braitch et al., 2008; Shimizu et al., 2013), higher in RRMS during a relapse (Börnsen et al., 2011) and in PPMS the higher levels correlate with the disability (Marastoni et al., 2021). There are some reports demonstrating plasma and CSF levels of osteopontin as a treatment-response biomarker in MS (Christensen et al., 2014; Kivisäkk et al., 2014).

3 AIMS OF THE STUDY

The concept of mild, benign MS has been questioned, since most of these patients suffer from subtle, non-visible symptoms despite having well preserved motor functions. In addition, previous studies have demonstrated that these patients will have a progressive course if followed for long enough.

The aim of this study was to assess the disease activity and neurodegenerative process in benign and mild relapsing-remitting MS (BRRMS) by using novel soluble biomarkers and automated quantitative MRI techniques.

The specific aims of the study were as follows:

1. To assess the value of the serum soluble biomarkers GFAP and NfL as indicators of disease activity in BRRMS, compared to aggressive relapsing-remitting MS (ARRMS) (study I).
2. To evaluate global and regional GM volumes and WM lesion load in BRRMS using an automated MRI quantification tool (cNeuro®) compared to ARRMS (study II).
3. To evaluate global and regional GM volumes and WM lesion load in BRRMS, with or without DMT, compared to age- and gender-matched HCs, using automated and visual MRI analyses (study III).
4. To evaluate CC measures as a brain atrophy marker by using automated CC index (CCI) and CC area (CCA) measures (studies II and III).

4 SUBJECTS AND METHODS

4.1 PATIENTS

The patients in all the studies were from the Neurology Outpatient Clinics of Kuopio University Hospital and Mikkeli Central Hospital. Demographic details and MS disease history were retrospectively collected from the patient records. An experienced neurologist performed clinical evaluations, including EDSS at the time of blood sampling (study I) or at the time of MRI scanning (studies II and III). All MS patients had been diagnosed with definite MS, according to Poser (Poser et al., 1983) or McDonald criteria (McDonald et al., 2001; Polman et al., 2011). Disease duration was defined as the time passed from the onset symptoms of MS until the serum sampling (study I) or MRI scanning (studies II and III).

The definition of BRRMS was used when a patient had an EDSS score of ≤ 3 after a disease duration of ≥ 10 years. The definition of ARRMS was used when a patient had the following indicators of a highly active course of the disease: several or very disabling relapses in early disease history with MRI activity, natalizumab or fingolimod previously used as a DMT or the treatment was ongoing.

In study I, the study population consisted of 34 patients with BRRMS and 29 with ARRMS who had undergone clinical examination between February 2015 and October 2017. All MS patients were clinically stable (neither clinical relapses nor cortisone treatments) within the tree-month period prior to serum sample collection. The HC group consisted of 14 spouses of MS patients, none of whom had reported neurological signs or had a history of neurological disease. The patients and HC participated voluntarily in this study, and written informed consent was obtained from all. The study was performed according to the principles of the Declaration of Helsinki.

MRI and clinical data were collected from 2007 to 2017 in studies II and III. For both MRI studies, 35 patients with BRRMS were taken from the Neurology Outpatient Clinic of Kuopio University Hospital. In study II, 46

patients with ARRMS from Neurology Outpatient Clinics of Kuopio University Hospital and Mikkeli Central Hospital were included. In study III, 35 age and gender-matched HC were taken from an internet-based Open Access Series of Imaging Studies (OASIS; https://www.oasis-brains.org) database.

All patients were clinically stable within one month before MRI scanning (neither clinical relapses nor cortisone treatments) and had no Gd-enhancing lesions in MRI. The patients were referred to MRI with clinical indication. Thus, the time of the imaging with respect of the disease history varied due to the retrospective nature of the study (studies II and III). The latest MRI examination, including 3D T1-w images, was chosen for each patient to obtain the longest period possible counted from the onset of symptoms. The research ethics committee of Northern Savo Hospital District approved the study protocol (decision 44/2014, 29.7.2014).

Table 5. Summary of the study subjects and baseline demographics

	Number and type of study subjects	**Females/ males**	**Age at study time point (y, mean)**	**Duration of disease at study time point (y, mean)**
Study I	34 BRRMS	29/6	46.0	20.4
	29 ARRMS	17/12	54.0	12.1
	14 HC	7/7	47.7	NA
Study II	35 BRRMS	28/7	51	18.2
	46 ARRMS	33/13	43.2	12.6
Study III	35 BRRMS	28/7	51	18.2
	35 HC	28/7	51	NA

4.2 METHODS

4.2.1 Analysis of serum GFAP and NfL

Serum GFAP levels were quantified using a Simoa HD-1 GFAP Discovery Kit (Quanterix, MA, USA, REF#102336) according to the kit's instructions. All samples were analyzed in duplicate in a single assay run with eight calibrators (range 0–1000 pg/ml), two GFAP quality control samples (10 and 1000 pg/ml) and two in-house quality control serum samples. The lower limit of quantification of the assay was 0.686 pg/ml, with a maximum dynamic range of 4000 pg/mL. The intra-assay CV was 4.4% and a CV of 20% was accepted between replicates.

Serum NfL (sNfL) levels were quantified with a Simoa HD-1 NfL Advantage Kit (Quanterix, MA, USA, REF#102258) by using the standard protocol (Rissin et al., 2010). All samples were analysed in duplicate in three randomised sets with eight calibrators (range 0 – 500 pg/ml), two sNfL quality control samples (10 and 200 pg/ml) and two in-house quality control serum samples included in each assay run. Serum samples were analysed as a part of a larger study set of 223 cases. The lower limit of quantification of the assay was 0.174 pg/ml, with a maximum dynamic range of 2000 pg/ml. The intra-assay and inter-assay coefficients of variation (CV) were 7.6% and 6.7%, respectively.

4.2.2 MRI analyses

MRI data were collected from 2007 to 2017 in study II (Kuopio University Hospital and Mikkeli Central Hospital) and from 2013 to 2017 in study III (Kuopio University Hospital). Several different MRI scanner models (1.5- or 3-Tesla, Siemens and Philips) were used for the MS patients. In study II, the scanner models were evenly distributed across both ARRMS and BRRMS, with 20% of the BRRMS patients and 43.5% in ARRMS patients examined with the 3T scanners. Altogether, 41% of the 3D T1-w images appropriate for volumetric analysis were scanned with Gd enhancement. Gd-enhanced images were evenly distributed among the MS patient groups. In study III,

20% (n = 7) of the BRRMS patients were scanned with a 3-Tesla scanner, and 40% (n = 14) of the 3D T1-w images were with Gd enhancement.

The imaging protocol for the MS patients included a 3D T1-weighted gradient-echo sequence (3D T1-w), a fast fluid-attenuated inversion recovery (FLAIR) sequence and a T2-weighted sequence. The voxel size varied between 0.4–1.6 × 0.4–1.6 × 0.5–2.2 mm in the T1 images and 0.4–1.3 × 0.4–1.3 × 0.6–7.0 mm in the 2D or 3D FLAIR images.

In study III, the brain MRI data of the HC were collected from an OASIS database. In the OASIS-1 cross-sectional data, Siemens Vision 1.5 Tesla brain MRI scanners were used. The imaging protocol used included an MP-RAGE T1-weighed sequence, and the voxel size was 1.0 × 1.0 × 1.0 mm.

In studies II and III, a set of 328 different volumetry and voxel-based morphometry imaging biomarkers was originally extracted from T1-weighted and FLAIR images using the cNeuro® MRI quantification tool (Combinostics Oy, Tampere, Finland) (Lötjönen et al., 2010). Images were segmented into 102 cortical and 31 sub-cortical brain regions using the multi-atlas segmentation method (Hänninen et al., 2019; Koikkalainen et al., 2016; Lötjönen et al., 2010). The results for 27 imaging biomarkers were reported in study II and 33 in study III.

The WM lesions were segmented as described earlier (Koikkalainen et al., 2016; Wang et al., 2012) (Figure 2). Lesion volumes were reported globally and regionally for the following brain regions: the periventricular, subcortical, deep WM, pons and cerebellum regions. The method uses the state-of-the-art lesion-filling technique, removing lesions from images before T1 segmentation. All the quantified variables were normalised regarding age, gender and head size (Buckner et al., 2004; Cole & Green, 1992).

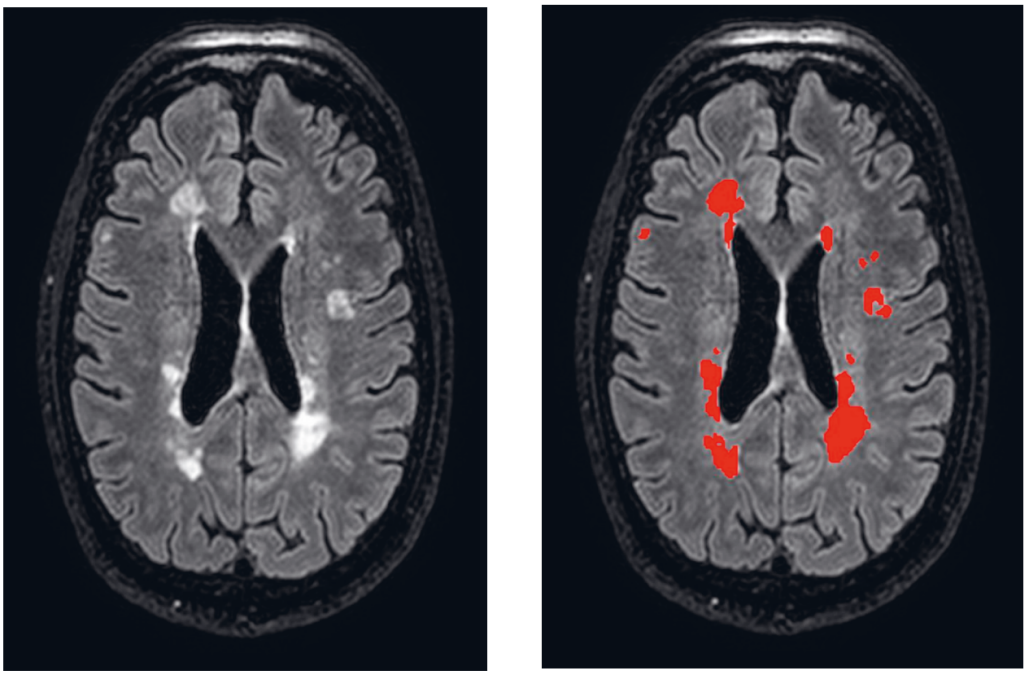

Figure 2. WM lesion segmentation using the cNeuro® tool.

Extraction of the CCI was not available with cNeuro® tool. In study II, six landmarks were first manually located on a mean anatomical template for the automated computation of the CCI (Goncalves et al., 2018; O. Yaldizli et al., 2010). The T1 image was first affinely and then non-rigidly registered with the mean anatomical template. The landmarks were then propagated accordingly to the T1 image for the automated computation of individual CCIs.

In study III, the CCI automated computation was developed further. The WM was segmented from the T1-weighted image using the cNeuro® MRI quantification tool. This segmentation was transformed into a template space using affine transformation. The template consisted of an anatomical mean image and a manually drawn mask of CC. The template was non-rigidly registered with the patient image, and the manual CC mask was propagated to the patient image. The CC mask was further dilated, and the CC segmentation of the patient image was obtained by applying the dilated mask to the WM segmentation.

The CCI (Goncalves et al., 2018; O. Yaldizli et al., 2010) is based on the distances between six CC landmarks. The following landmarks were automatically detected from the CC segmentation:

a: The most anterior point of CC

b: The most posterior point of CC

c: The point with maximal distance from the line between a and b

a', b', c': The points from the opposite border of the CC

Seven adjacent slices were analyzed independently to increase the robustness of the automatic analysis. The final CCI was defined by computing the median values for the coordinates of the six landmarks and then computing the CCI using the equation shown in Figure 3.

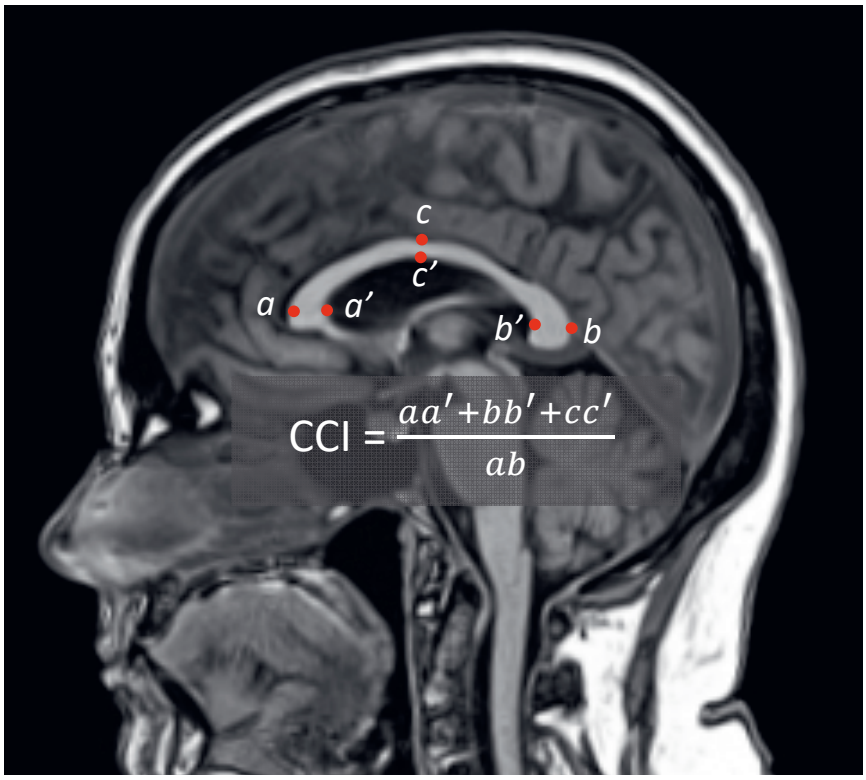

Figure 3. Computation of the corpus callosum index (CCI) in cNeuro® tool.

The CCA was computed as the mean of the areas of CC segmentation in the seven slices. As affine registration was used to normalise the template space, the size differences in the CCA between the patients were normalised. The CCI is a normalised measure as such.

In study III, an experienced neuroradiologist evaluated the MS patients' MRI images for visual atrophy rating (scale: none, mild, moderate or strong atrophy) and T2 lesion load rating (scale: lesion amount 0–9, 10–20, 21–40, or >40 lesions) regarding supratentorial, infratentorial and cortical areas. CCI was determined on a picture achieving and communication system (PACS) workstation on best mid-sagittal T1-weighted images with an established linear measurement technique for visual analysis described in earlier studies (Figueira et al., 2007; O. Yaldizli et al., 2010).

4.3 STATISTICAL ANALYSES

Statistical analyses were performed with IBM SPSS Statistics for Windows versions 24 and 27 (IBM Corp, Armonk, NY). The baseline demographics in all the studies are presented as means with ranges or frequencies with percentages. P-values <0.05 were set to indicate statistically significant results.

The results for sNfL and GFAP levels are presented as median (interquartile range, IQR). Kolmogorov-Smirnov and Shapiro-Wilks tests did not show the normality of sNfL, GFAP, the number of relapses, EDSS and time from the latest relapse at the time of blood sampling; hence, further comparisons between groups for these variables were performed with the non-parametric Mann–Whitney U test. For other continuous variables, an analysis of variance was used. The chi-square test was used for categorical variables. Correlations were calculated with nonparametric Spearman's analysis. Analysis of covariance was performed to test age-adjusted associations.

In studies II and III, demographics were tested with t-test, the Mann–Whitney U test and the chi-square test. Volumetry parameters were expressed as means with standard deviations. Brain MRI segmentation volumetric results between groups were compared by the analysis of the covariance (ANCOVA) model. In study II, in the ANCOVA model, age, the length of disease duration and Gd-enhancement (with or without Gd) functioned as adjusting variables. The regression coefficients with p-values and standardised betas were expressed to measure effect size differences between the study groups. In study III, the adjusting variables were Gd enhancement and, in the BRRMS subgroup analysis, the duration of the disease in addition. The results of the ANCOVA model are reported as adjusted mean differences with 95% confidence intervals.

5 RESULTS

5.1 CLINICAL CHRACTERISTICS OF THE STUDY SUBJECTS

In study I, the BRRMS and ARRMS patients did not statistically significantly differ in gender or age at the onset of MS symptoms, although the proportion of males was greater in the ARRMS group. Sensory paresis was more common as an onset symptom in the BRRMS group. At the time of blood sample collection, BRRMS patients were older than ARRMS patients (mean age 54 years vs. 46 years, respectively) and had a longer time since the first MS symptoms (20.4 years vs. 12.1 years) and from the latest relapse (199.4 months *vs*. 27.2 months), compared to ARMMS. The proportion of females was larger in BRRMS compared to HCs ($p = 0.010$), but otherwise the HCs did not differ from the MS patients in gender or age at the blood sampling. The BRRMS patients had used no (n = 11, 32.4%) or only first-line DMTs in their medical history. At the time of serum sample collection, 16 patients (47.1%) with BRRMS were without any DMT. In the ARRMS group, the majority of patients (20, 69%) were still using fingolimod (n = 15) or natalizumab (n = 5) at the time of blood sampling. The demographic details are given in Table 6.

Table 6. Demographic details of study I

Variable	HC	All MS patients	p*	BRRMS	ARRMS	p**	p***	p¤	BRRMS no DMT	BRRMS any DMT	p¤¤
n	14	63		34	29				16	18	
Female gender, n (%)	7 (50.0)	46 (73.0)	0.093	29 (85.3)	17 (58.6)	**0.010**	0.594	0.180	14 (87.5)	15 (83.3)	0.732
Age at onset of MS symptoms, y	–	33.7 (14–59)	–	33.6 (14–56)	33.9 (14–59)	–	–	0.900	36.9 (22–56)	30.6 (14–47)	0.095
Age at time of blood sampling, y	47.7 (31–63)	50.3 (21–78)	0.526	54.0 (32–78)	46.0 (21–68)	0.105	0.542	**0.015**	60.3 (46–78)	48.4 (32–62)	**0.003**
Duration of disease at blood sampling, y	–	16.6 (3–43)	–	20.4 (11–43)	12.1 (3–28)	–	–	**<0.001**	23.4 (13–43)	17.8 (11–31)	**0.022**
Variable	**HC**	**All MS patients**	**p***	**BRRMS**	**ARRMS**	**p****	**p*****	**p¤**	**BRRMS no DMT**	**BRRMS any DMT**	**p¤¤**
Number of relapses	–	5.3 (1–31)	–	3.8 (1–11)	6.3 (1–31)	–	–	**0.017**	3.4 (1–11)	4.2 (1–10)	0.365

before blood sampling										
EDSS at time of blood sampling, median (IQR)	–	2.2 (1–3)	–	1.75 (1–2.5)	2.5 (1.5–4.5)	–	–	0.080	1.75 (0–3.0)	1.44 (1.5–2.5)	0.484
Time from latest relapse at blood sampling, months, median (IQR)	–	44.9 (24.0–145.4)	–	119.4 (44.4–188)	27.2 (16.8–43)	–	–	**<0.001**	152 (43.4–244.7)	109.8 (42.3–163.7)	0.211

Unless otherwise indicated, the mean and range (in parentheses) are shown for the variables. Significant P-values are indicated using bold font. *HC vs. all MS patients, **HC vs. BRRMS, ***HC vs. ARRMS, ¤ BRRMS vs. ARRMS, ¤¤ BRRMS, no DMT vs. with any DMT

In study II, the patients in the BRRMS group were older (mean age 51.0 years, range 32–70) than those in the ARRMS group (mean 43.2 years, range 21- 69) at the time of MRI ($p < 0.001$). Their disease duration was longer (18.2 years vs. 12.6 years, respectively; $p < 0.001$) and they had had fewer relapses (median 4.0 versus 5.0; $p = 0.004$), than those in the ARRMS group. The onset symptoms did not differ between patient groups.

In total, 12 patients (34.3%) in the BRRMS group had never been treated with any DMT. The mean age of these patients was 54.6 years (range 46–66), compared to 49.1 years (range 32–70) for BRRMS patients with some history of DMT ($p = 0.120$). Duration of disease was slightly longer in this subgroup with no DMT (mean 20.7 years vs. 16.9 years; $p = 0.027$). The median number of relapses throughout the disease history was 3.0 (range 1–5) in patients who were without any DMT, compared to 4.0 (range 2–10) in patients with any DMT ($p = 0.050$). EDSS levels did not differ between these two BRRMS groups (median 1.75 vs. 2.00, respectively; $p = 0.861$). (Table 7).

Table 7. Patient baseline demographics in studies II and III.

Parameter (a)	All BRRMS (n=35)	ARRMS (n=46) (b)	p (BRRMS vs. ARRMS)	BRRMS any DMT (n=23)	BRRMS no DMT (n=12)	p (BRRMS any DMT vs. no DMT)
Female gender, n (%)	28 (80)	33 (71.7)	0.393	17 (73.9)	11 (91.7)	0.213
Age at onset symptoms, years (range)	32.8 (14–51)	30.5 (14–59)	0.248	32.2 (14–51)	33.9 (22–46)	0.572
Age at time of MRI, years (range)	51.0 (32–70)	43.2 (21–69)	<0.001	49.1 (32–70)	54.6 (26–66)	0.172
Duration of disease at the time of MRI years (range)	18.2 (12–33)	12.6 (0–36)	<0.001	16.9 (12–29)	20.7 (13–33)	0.031
Number of relapses at the time of MRI, median (range)	4.0 (1–10)	5.0 (1–43)	0.004	4 (2–10)	3 (1–5)	0.079
EDSS score at the time of MRI, median (range)	2.0 (0–3.0)	2.8 (0–8.5)	<0.001	2.0 (0–3.0)	1.75 (1.0–3.0)	0.905

Onset symptoms of MS, n (%) (c)	All BRRMS (n=35)	ARRMS (n=46) (b)	p (BRRMS vs ARRMS)	BRRMS, any DMT (n=23)	BRRMS, no DMT (n=12)	p (BRRMS any DMT vs no DMT)
Optic neuritis	8 (22.9)	14 (30.4)	0.448	6 (26.1)	2 (16.7)	0.529
Sensory symptoms	10 (28.6)	7 (15.2)	0.144	5 (21.7)	5 (41.7)	0.215
Motor paresis	5 (14.3)	11 (23.9)	0.281	4 (17.4)	1 (8.3)	0.467
Cerebellar/brainstem symptoms	9 (25.7)	15 (32.6)	0.501	6 (26.1)	3 (25.0)	0.944
Myelitis	11 (31.4)	7 (15.2)	0.082	6 (26.1)	5 (41.7)	0.346
DMT at the time of MRI						
None	13 (37.1)	9 (19.6)		2 (8.7)	12 (100)	
Interferon or glatiramer acetate	19 (54.3)	5 (10.9)		18 (78.3)	0	
Teriflunomide or dimethyl fumarate	3 (8.6)	3 (6.5)		3 (13)	0	
Natalizumab or fingolimod	0	25 (54.3)				
Alemtuzumab	0	4 (8.7)				

(a) Values are mean and range unless other indicated

(b) In medication history only fingolimod n=14, only natalizumab n=15, both fingolimod and natalizumab n=17

(c)Total exceeds 100% since polysymptomatic relapses occurred

Brain imaging was done after the initiation of highly effective DMT (fingolimod or natalizumab) in 41 (89.1%) patients with ARRMS. The time of MRI examination in relation to the initiation DMT varied due to the retrospective nature of the study. In five patients there were applicable MRI scans with 3D T1-w images only from the time before the start of highly effective DMT (Figure 4.) and four patients were scanned within one year of the start of the high efficacy DMT.

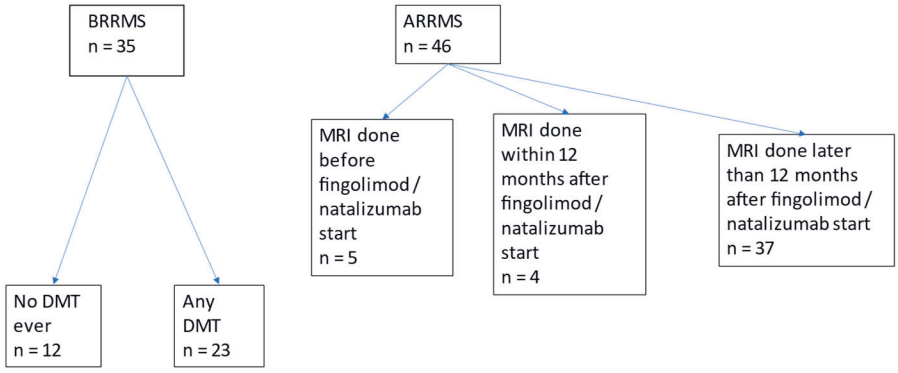

Figure 4. Patient flowchart in study II.

The majority of patients with ARRMS were still using fingolimod or natalizumab (n = 25, 54.3%) at the time of MRI examination (Table 7).
In study III, in the BRRMS and HC groups, the proportion of women was 80% (n = 28) and the mean age at the time of MRI scanning was 51 years (range 32–70). Altogether, 12 patients out of 35 (32.3%) had never been treated with any DMT from the time of the onset symptoms. There was no statistical difference in age between the patients who had not been treated with any DMT and those who had been treated (54.6 years vs. 49.1 years, respectively; p = 0.172). The duration of the disease was longer in patients without DMT (20.7 years, range 13–33) than with any DMT (16.9 years,

range 12–29, p = 0.031); thus, it was included as a covariate in further analyses between these subgroups. EDSS levels and the number of relapses did not differ between these BRRMS subgroups (Table 7).

5.2 SERUM GFAP AND NFL LEVELS AS INDICATORS OF DISEASE ACTIVITY IN DIFFERENT CLINICAL TYPES OF RRMS (STUDY I)

5.2.1 Serum GFAP and NfL levels

Serum GFAP levels in both the BRRMS (median 210.19 pg/ml, IQR 163.69–87.19) and the ARRMS groups (median 188.60 pg/ml, IQR 39.23–244.93) were significantly higher (p = 0.035 and p = 0.034, respectively) then in the HC group (median 117.93 pg/ml, IQR 60.28–183.83). No statistical difference was found in the GFAP levels between the BRRMS and ARRMS groups (Figure 5.). There were no statistical differences in sNfL levels between the BRRMS, and ARRMS groups and the HC group. In all the RRMS patients pooled, compared to the HCs, the GFAP level was significantly higher (p = 0.020) in patients with RRMS (median 14.96 pg/ml, IQR 11.29–20.91). In terms of sNfL levels, there were no differences between the RRMS patients and the HCs. The serum GFAP and NfL results are given in Table 8.

Table 8. Results of serum GFAP and NfL analysis. Unless otherwise indicated, the mean and range (in parentheses) are shown for the variables. Significant P-values are indicated using bold font.

Variable	HC n = 14	All MS patients n = 63	p (HC vs. all MS patients)	BRRMS n = 34	ARRMS n = 29	p (HC vs. BRRMS)	p (HC vs. ARRMS)	p (BRRMS vs. ARRMS)	BRRMS, no DMT n = 16	BRRMS, any DMT n = 18	p (BRRMS, no DMT vs. with any DMT)
Serum GFAP, median (IQR), pg/ml	117.93 (60.28–183.83)	198.06 (145–258.40)	**0.020**	210.19 (163.69–287.19)	188.60 (139.23–244.93)	**0.035**	**0.034**	0.976	216.04 (188.60–274.79)	196.26 (133.33–325.54)	**0.040**
Serum NFL, median (IQR), pg/ml	10.41 (8.25–15.02)	14.96 (11.29–20.91)	0.106	16.34 (11.35–23.27)	14.60 (10.81–17.77)	0.158	0.126	0.875	19.42 (11.82–28.99)	13.44 (10.75–21.29)	0.210
log (GFAP)			**<0.001**			**<0.01**	**<0.01**	0.936			0.051
log (NFL)			**0.039**			0.103	**0.033**	0.512			0.224

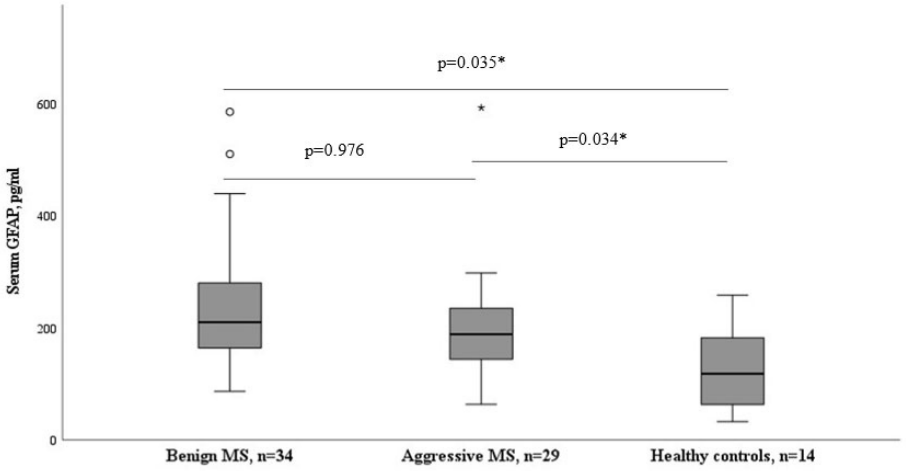

Figure 5. The serum GFAP levels of the study subjects.

5.2.2 Serum GFAP and NfL levels in correlation with age, duration of disease and relapses

There was a positive correlation between serum GFAP levels and age in the BRRMS ($r = 0.508$, $p = 0.002$) and HC- groups ($r = 0.662$, $p = 0.010$), but not in the ARRMS group ($r = 0.100$, $p = 0.605$). A positive correlation between the sNfL levels and age was also found in both the BRRMS ($r = 0.677$, $p < 0.001$) and the ARRMS groups ($r = 0.789$, $p < 0.001$) as well as in the HC ($r = 0.587$, $p=0.027$). Serum GFAP and NfL levels were strongly associated with each other in the HCs ($r = 0.574$, $p = 0.032$) and the BRRMS patients ($r = 0.407$, $p = 0.017$) but not in the ARRMS patients ($r = 0.304$, $p = 0.108$). These correlations are presented in Figure 6.

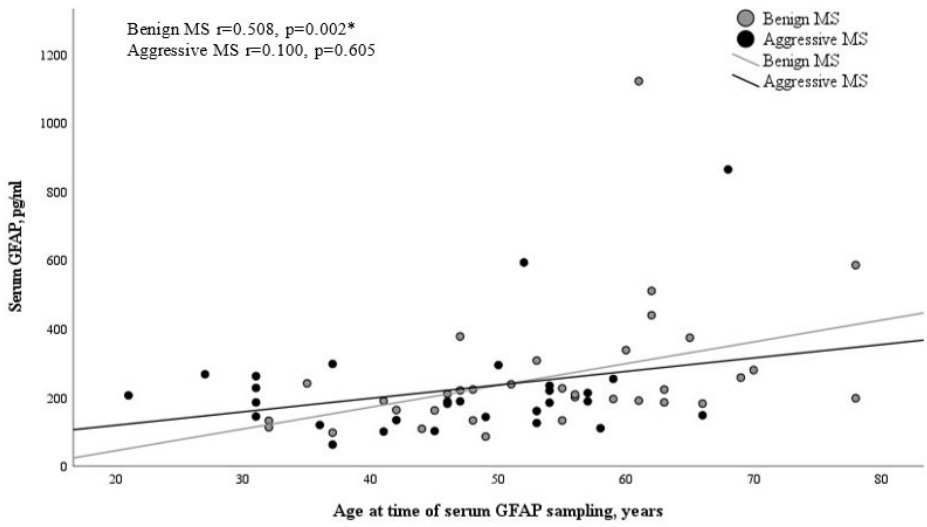

Figure 6. Correlations of serum GFAP and NfL with age.

Age correction did not change the statistical significances reported in serum GFAP and NfL levels.

There was a positive correlation between sNfL levels and the duration of the disease in the BRRMS patients as well as in the ARRMS patients (r = 0.343, p = 0.047 and r = 0.324, p = 0.087, respectively). Serum GFAP levels and the duration of the disease showed a positive correlation in the BRRMS patients (r = 0.456, p = 0.007), but not in the ARRMS patients (r = 0.031, p = 0.872). No correlation between the number of relapses in the course of the disease (r = -0.177, p = 0.316 and r = 0.133, p = 0.492) nor the time lapsed from the latest relapse (r = 0.119, p = 0.504 and r = -0.130, p = 0.500) and sNfL levels was found in either the BRRMS or the ARRMS group. The same applied to GFAP levels; the number of relapses (r = 0.055, p = 0.755 and r = 0.097, p = 0.617) and time from the latest relapse (r=-0.087, p=0.626 and r=-0.281, p=0.139) did not correlate with GFAP levels in the BRRMS or ARRMS patients.

5.2.3 Serum GFAP and NfL levels regarding DMT use in BRRMS

GFAP levels were significantly higher (p=0.040) in the BRRMS patients without DMT (median 216.04 pg/ml, IQR 188.60–274.79) compared to those who had used DMT (median 196.26 pg/ml, IQR 133.33–325.54). sNfL levels were found to be similar in BRRMS patients with or without DMT at the time of serum sampling.

5.3 GLOBAL AND REGIONAL GM VOLUMES AND WM LESION VOLUMES IN TWO DIFFERENT CLINICAL TYPES OF MS (STUDY II)

5.3.1 Whole-brain volumes, GM and WM volumes and regional GM volumes in BRRMS and ARRMS

Total brain tissue volume was larger in patients with BRRMS (mean 1098.42 ml, SD 52.82) compared to ARRMS (mean 1069.4 ml, SD 60.09; p = 0.014). Both the cerebral (mean 369.82 ml, SD 37.76; p = 0.017) and cerebellar (mean 22.12 ml, SD 3.58; p = 0.015) WM volumes were larger in patients with BRRMS. Cortical GM volumes did not differ between these groups. Thalamic volume was larger in the BRRMS group (mean 12.94 ml, SD 1.9) than in the ARRMS group (mean 11.82 ml, SD1.82; p = 0.003). The total and regional volumes are given in Table 9.

Table 9. MRI volumetry in patients with BRRMS and ARRMS.

Variable	BRRMS	ARRMS	B	p	Beta
n	**35**	**46**			
Volumes, ml (mean; SD)					
Brain tissue (total)	1098.42 (52.82)	1069.4 (60.09)	-31.01	**0.014**	-0.26
Brain WM (total)	369.82 (37.76)	351.42 (36.46)	-21.92	**0.017**	-0.29
Cortical GM (total)	493.8 (33.47)	489.14 (47.33)	-2.15	0.778	-0.03
Cerebral GM	522.05 (35.99)	515.26 (49.30)	-4.29	0.598	-0.05
Cerebellar GM	97.12 (9.54)	93.94 (7.76)	-4.32	**0.039**	-0.25
Cerebellar WM	22.12 (3.58)	20.68 (2.74)	-1.9	**0.015**	-0.30
Cerebrospinal fluid	57.87 (26.55)	63.02 (19.66)	5.63	0.306	0.12
Lobar volumes, ml (mean; SD)					
Frontal lobes	191.54 (15.48)	193 (18.64)	2.26	0.500	0.07
Temporal lobes	121.08 (7.14)	118.5 (10.9)	-2.14	0.294	-0.11
Parietal lobes	107.74 (8.2)	104.2 (11.98)	-2.48	0.252	-0.12
Occipital lobes	72.74 (7.36)	72.4 (9.94)	-0.16	0.927	-0.01
Regional volumes, ml (mean; SD)					
Postcentral gyrus	17.5 (1.86)	16.94 (2.48)	-0.32	0.481	-0.07
Postcentral gyrus (medial segment)	1.2 (0.3)	1.18 (0.28)	0.08	0.248	0.13
Precentral gyrus	22.5	23.04	0.5	0.426	0.08

	(3.04)	(2.86)			
Precentral gyrus (medial segment)	4.58 (0.64)	4.7 (0.78)	0.28	0.084	0.19
Medial temporal lobes	18.84 (1.88)	18.74 (2.02)	-0.4	0.411	-0.10
Hippocampus	6.5 (0.92)	6.4 (0.86)	-0.2	0.369	-0.11
Thalamus	12.94 (1.9)	11.82 (1.82)	-1.26	**0.003**	-0.33
Anterior cingulate gyrus	8.42 (1.38)	8.74 (1.68)	0.44	0.226	0.14
Posterior cingulate gyrus	9.56 (1.18)	9.24 (1.2)	-0.38	0.170	-0.16
CCI	0.34 (0.04)	0.32 (0.05)	-0.03	**0.011**	-0.29
Volumes of WM lesions, ml (mean; SD)					
Total	14.1 (10.73)	20.01 (11.23)	6.28	**0.020**	0.28
Periventricular	2.88 (3.6)	5.83 (5.37)	3.94	**0.001**	0.40
Subcortical	0.24 (0.49)	0.28 (0.46)	0.03	0.791	0.03
Deep white matter	8.39 (7.01)	10.67 (6.38)	1.99	0.214	0.15
Pons	0 (0.01)	0 (0.01)	0	0.207	0.15
Cerebellar	0 (0.01)	0 (0)	0	0.923	0.01

B = coefficient B in the regression analysis for group difference. Difference between ARRMS and BRRMS adjusted with the duration of disease and gadolinium enhancement.
Beta = standardised coefficient between groups.
p = p-value for group difference, adjusted with age, time from onset symptoms and gadolinium enhancement.

5.3.2 WM lesion volumes and CCI in BRRMS and ARRMS

The total WM lesion volume was larger in ARRMS patients (mean 20.01 ml, SD 11.23) than in BRRMS patients (mean 14.1 ml, SD 10.73; $p = 0.020$). Periventricular WM lesion volume in ARRMS (mean 5.83 ml, SD 5.37) was also larger than in the BRRMS patients (mean 2.88 ml, SD 3.6; $p = 0.001$). CCI was higher in the BRRMS patients (mean 0.34, SD 0.04) than in the ARRMS patients (mean 0.32, SD 0.05; $p = 0.011$) (Table 9). A positive correlation was found between CCI and whole-brain volume in both BRRMS ($r = 0.73$, $p < 0.001$) and ARRMS patients ($r = 0.80$, $p < 0.01$) (Figure 7). A negative correlation between total brain tissue volume and WM lesion volume was found in both the BRRMS ($r = -0.44$, $p = 0.008$) and ARRMS patients ($r = -0.56$, $p < 0.001$) (Figure 8).

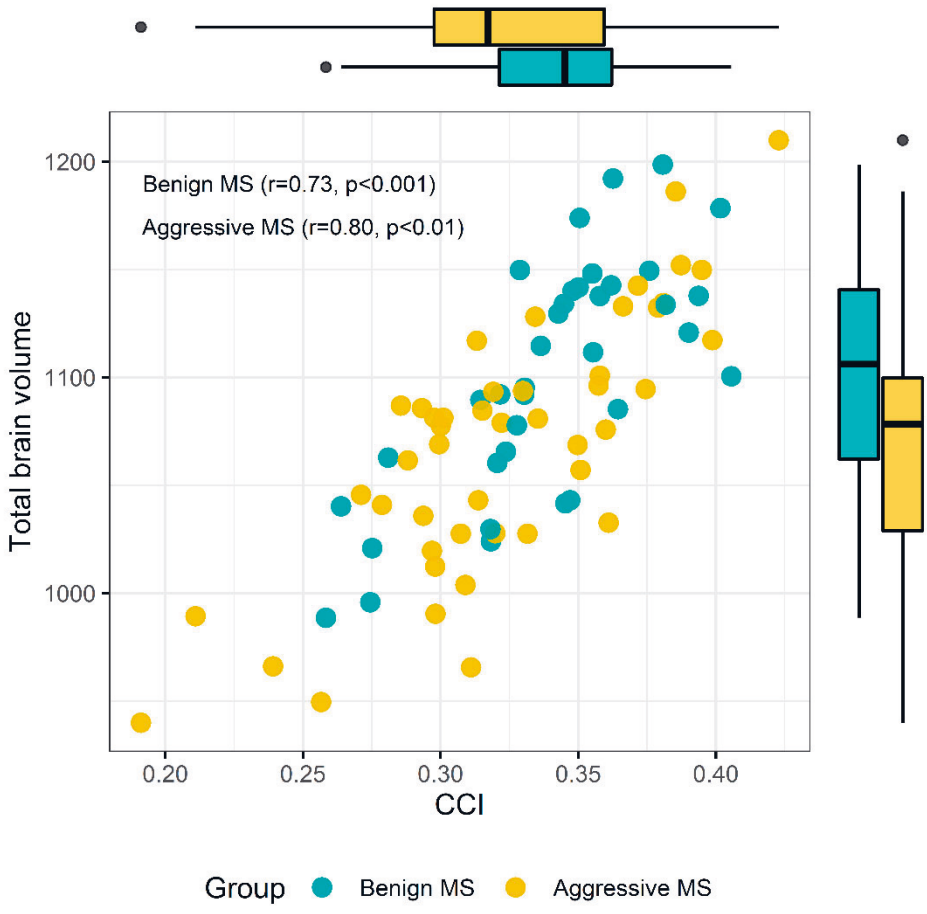

Figure 7. Correlation of CCI and total brain volumes.

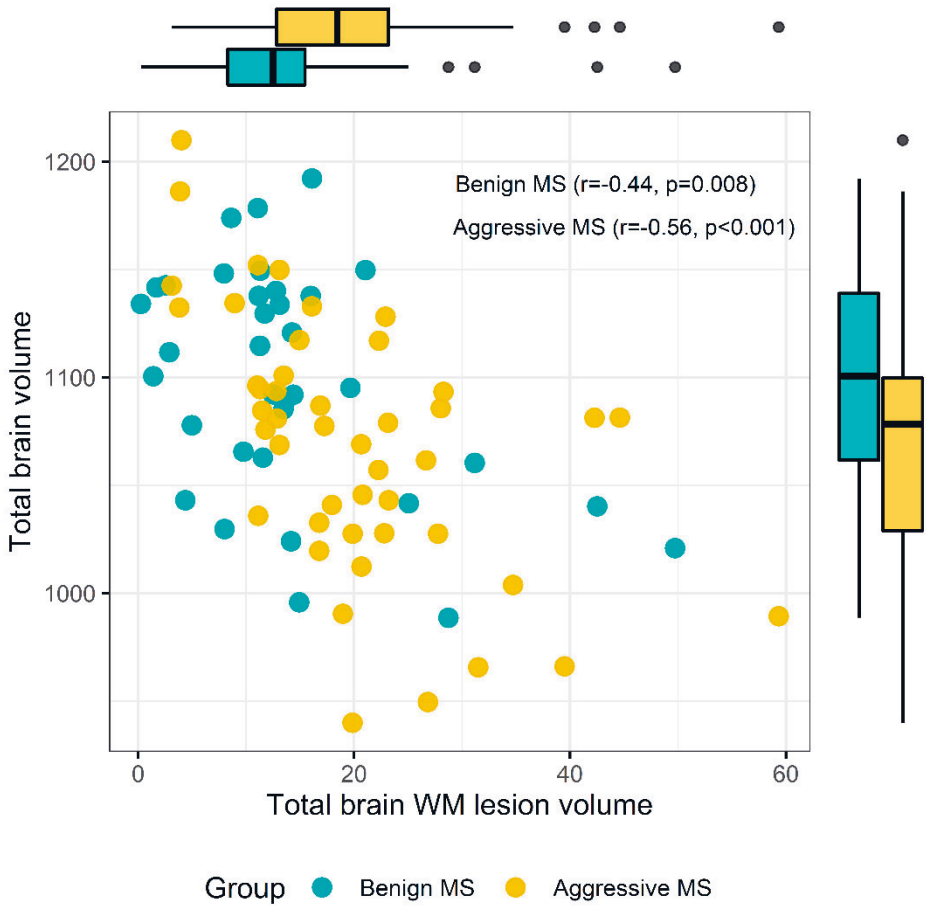

Figure 8. Correlation of WM lesion volumes and total brain volumes

5.3.3 Brain volumes and WM lesion volumes in BRRMS in relation to DMT use

There were no differences between the BRRMS patients with and without DMT in terms of total brain volume, nor were there differences in terms of regional GM volume. Also, CCI did not differ between these subgroups. In the subgroup of patients without DMT use, total WM lesion volumes ($p = 0.033$), as well as regional WM lesion volumes were found to be larger in the subcortical area ($p = 0.046$) and deep WM ($p = 0.041$).

5.3.4 Whole-brain and regional volumes, WM lesion volumes and CCI in subgroups of ARRMS compared to BRRMS

Since the time of MRI in relation to the initiation of highly effective DMT varied within the ARRMS group, a further subgroup analysis was done between the BRRMS group and the three different subgroups of ARRMS given in Figure 4. Smaller thalamic volumes and periventricular WM lesion volumes, compared to the BRRMS group, were found in ARRMS subgroups scanned before and more than 12 months after the initiation of highly effective DMT.

5.4 GLOBAL AND REGIONAL GM VOLUMES AND WM LESION VOLUMES IN BRRMS GROUP, COMPARED TO AGE- AND GENDER-MATCHED HCS (STUDY III)

5.4.1 Whole-brain, GM and lobar volumes in BRRMS and HCs

Total brain tissue volume was smaller in patients with BRRMS (mean 904.37 ml, SD 50.2) than in HCs (mean 919.47 ml, SD 23.04; $p < 0.001$). CSF ($p < 0.001$) and lateral ventricle volumes ($p < 0.001$) were larger in BRRMS patients than in HCs. Frontal ($p = 0.004$) and occipital lobe ($p = 0.020$) volumes were larger in BRRMS patients than in HCs. Both the cortical (mean 494.53 ml, SD 30.21; $p = 0.011$) and cerebral (mean 530.51 ml, SD 31.91; $p = 0.002$) GM volumes were larger in the BRRMS patients than in HCs (mean 485.86 ml, SD 19.21 and mean 525.88 ml, SD 19.76, respectively). The total and regional volumes are shown in Table 10. There was no correlation between WM lesion volumes and cortical ($r = -0.264$, $p = 0.125$) or cerebral ($r = -0.324$, $p = 0.057$) GM volumes in BRRMS.

Table 10. Volumetry in patients with BRRMS and healthy controls.

Variable	BRRMS	HC	Difference (95% CI)	p
n	35	35		
Volumes, ml (mean, SD)				
Brain tissue (total)	904.37 (50.2)	919.47 (23.04)	-37.87 (-56.48; -19.25)	**< 0.001**
Cortical GM (total)	494.53 (30.21)	485.86 (19.21)	-12.54 (-22.05; -3.03)	**0.011**
Cerebral GM	530.51 (31.91)	525.88 (19.76)	-16.99 (-27.28; -6.69)	**0.002**
Cerebral WM (total)	373.68 (31.81)	394.78 (15.46)	-22.77 (-36.63; -8.91)	**0.002**
Cerebrospinal fluid (total)	56.28 (27.73)	36.76 (11.93)	25.07 (13.53; 36.60)	**< 0.001**
Lateral ventricles	48.9 (24.69)	32.42 (10.35)	21.49 (11.27; 31.71)	**< 0.001**
Lobar volumes, ml (mean; SD)				
Frontal lobes	194.05 (14.52)	192.69 (10.26)	-7.87 (-13.20; -2.54)	**0.004**
Temporal lobes	119.23 (6.89)	115.97 (6.72)	-0.68 (-3.94; 2.57)	0.678
Parietal lobes	108.29 (8)	104.64 (5.33)	-0.29 (-3.54; 2.96)	0.859
Occipital lobes	73.35 (7.36)	73.05 (6.23)	-3.84 (-7.04; -0.64)	**0.020**
Regional volumes, ml (mean, SD)				
Postcentral gyrus	17.86 (1.89)	18.47 (1.5)	-1.49 (-2.33; -0.65)	**0.001**
Post central gyrus (medial segment)	1.18 (0.32)	1.39 (0.36)	-0.16 (-0.35; 0.03)	0.089

Precentral gyrus	22.74 (2.78)	23.93 (2.33)	-2.71 (-3.92; -1.50)	**< 0.001**
Precentral gyrus (medial segment)	4.61 (0.71)	5.09 (0.56)	-0.71 (-1.05; -0.38)	**< 0.001**
Supplementary motor cortex	9.01 (1.41)	9.64 (1.05)	-1.30 (-1.91; -0.70)	**< 0.001**
Calcarine cortex	6.44 (1.5)	6.93 (1.78)	-1.09 (-1.95; -0.22)	**0.015**
Medial temporal lobes	18.54 (1.83)	17.89 (1.28)	0.64 (-0.24; 1.51)	0.150
Hippocampus	6.54 (0.92)	6.65 (0.65)	-0.20 (-0.63; 0.24)	0.377
Thalamus	13.12 (1.81)	14.29 (0.66)	-1.77 (-2.46; -1.07)	**< 0.001**
Anterior cingulate gyrus	8.39 (1.34)	8.04 (1.06)	0.02 (-0.63; 0.67)	0.953
Middle cingulate gyrus	9.55 (1.43)	8.55 (0.85)	0.68 (0.04; 1.31)	**0.037**
Posterior cingulate gyrus	9.37 (1.17)	8.21 (0.64)	0.75 (0.26; 1.23)	**0.003**
Cingulate gyrus (total)	27.31 (2.93)	24.80 (2.09)	1.45 (0.13; 2.76)	**0.032**
Entorhinal area	4.46 (0.54)	3.96 (0.31)	0.47 (0.23; 0.71)	**< 0.001**
CCI	0.31 (0.06)	0.37 (0.03)	-0.06 (-0.09; -0.04)	**< 0.001**
CCA, mm^2	608.22 (116.51)	678.26 (87.61)	-93.21 (-149.25; -37.17)	**0.001**

p = p-value for group difference, adjusted with gadolinium enhancement

5.4.2 WM volumes and CCI and CCA results in BRRMS and HCs

Total WM volume was smaller in BRRMS patients (mean 373.68 ml, SD 31.81) than in HCs (mean 394.78 ml, SD 15.46; p = 0.002).

Both CCI and CCA were smaller in BRRMS patients ($p < 0.001$ and $p = 0.001$, respectively) than in HCs. A positive correlation was found between CCI and CCA in BRRMS patients ($r = 0.738$, $p < 0.001$) but not in HCs ($r = 0.204$, $p = 0.239$) (Figure 3). There was a positive correlation between CCI and total brain tissue volume in BRRMS patients ($r = 0.543$, $p < 0.001$) but not in HCs ($r = 0.077$, $p = 0.658$) or between total brain tissue volume and CCA ($r = 0.532$, $p = 0.001$ and $r = -0.007$, $p = 0.966$, respectively). A negative correlation was found in BRRMS patients between WM lesion volumes and CCI and CCA ($r = -0.587$, $p < 0.001$ and $r = -0.663$, $p < 0.001$, respectively).

5.4.3 Regional GM volumes in BRRMS and HC

Regional GM volumes in the postcentral gyrus ($p = 0.001$), precentral gyrus ($p < 0.001$), medial segment of the precentral gyrus ($p < 0.001$), supplementary motor cortex ($p < 0.001$), and thalamus ($p < 0.001$) were smaller in BRRMS patients than in HCs (Table 10). Cingulate gyrus ($p = 0.032$) and entorhinal area volumes ($p < 0.001$) were larger in BRRMS patients than in HCs (Table 2).

5.4.4 MRI volumetry results in relation to DMT use in BRRMS

No differences in whole-brain volumes, cortical total or regional GM volumes; WM volumes; or brain lobar volumes were found in BRRMS patients with and without DMT (Table 11). CCI and CCA were slightly smaller in patients without a history of DMT, but the results did not differ significantly between these two patient groups. The total, periventricular, juxtacortical and deep WM lesion volumes were larger in patients without a history of DMT than in those who had used DMT ($p = 0.015$, 0.010, 0.015 and 0.031, respectively) (Table 11). There were no differences in total WM lesion count groups ($p = 0.224$) or in atrophy rating ($p = 0.077$) between the treated and non-treated MS patients in visual assessment by an experienced neuroradiologist (Table 11).

Table 11. Volumetry in BRRMS, without and with DMT use.

Variable	**Without DMT**	**With DMT**	**Difference (95% CI)**	**p**
n	12	23		
Volumes, ml (mean, SD)				
Brain tissue (total)	882.93 (48.43)	915.56 (48.37)	19.31 (-15.37;53.99)	0.265
Cortical GM (total)	483.98 (21.28)	500.04 (33.03)	-0.54 (-12.81;11.73)	0.930
Cerebral GM	518.54 (23.39)	536.75 (34.37)	2.14 (-12.20;16.48)	0.763
Cerebral WM (total)	364.36 (32.77)	378.54 (30.89)	16.39 (-10.18;42.96)	0.218
Cerebrospinal fluid	65.97 (34.29)	51.22 (22.85)	-13.62 (-36.09;8.85)	0.226
Lateral ventricles	58.19 (30.87)	44.06 (19.84)	-13.42 (-33.30;6.46)	0.178
Visual atrophy rating (%)				0.077
No atrophy	6 (50)	15 (65.2)		
Mild atrophy	1 (14.3)	6 (26.1)		
Moderate atrophy	3 (25)	2 (8.7)		
Strong atrophy	2 (16.7)	0		
Lobar volumes, ml (mean; SD)				
Frontal lobe	189.44 (10.25)	196.46 (15.99)	1.30 (-5.74;8.34)	0.708
Temporal lobe	117.86 (7.09)	119.94 (6.84)	-1.31 (-5.41;2.80)	0.522
Parietal lobe	106.02 (7.18)	109.48 (8.3)	0.22 (-5.21;5.64)	0.935
Occipital lobe	70.99	74.58	-0.63 (-	0.778

	(4.78)	(8.22)	5.11;3.85)	
Regional volumes, ml (mean; SD)				
Postcentral gyrus	17.58 (1.54)	18.01 (2.07)	-0.45 (-1.75;0.86)	0.492
Postcentral gyrus (medial segment)	1.2 (0.25)	1.17 (0.36)	0.00 (-0.27;0.27)	0.991
Precentral gyrus	22.21 (1.88)	23.02 (3.15)	-0.15 (-1.80;1.49)	0.850
Precentral gyrus (medial segment)	4.73 (0.55)	4.54 (0.78)	-0.37 (-0.89;0.15)	0.154
Supplementary motor cortex	8.53 (1.03)	9.26 (1.53)	0.29 (-0.67;1.25)	0.542
Calcarine cortex	6.38 (1.49)	6.46 (1.54)	-0.61 (-1.71;0.49)	0.265
Medial temporal lobes	17.81 (1.16)	18.92 (2.01)	1.19 (-0.30;2.68)	0.115
Hippocampus	6.25 (0.63)	6.69 (1.01)	0.52 (-0.24;1.28)	0.170
Thalamus	12.48 (2.28)	13.46 (1.45)	0.57 (-0.83;1.97)	0.410
Anterior cingulate gyrus	8.09 (1.38)	8.54 (1.33)	0.30 (-0.79;1.39)	0.577
Middle cingulate gyrus	9.85 (1.13)	9.4 (1.57)	-0.73 (-1.85;0.40)	0.197
Posterior cingulate gyrus	9.21 (1.04)	9.46 (1.24)	-0.04 (-0.94;0.85)	0.921
Entorhinal area	4.2 (0.4)	4.59 (0.56)	0.33 (-0.09;0.75)	0.119
CCI	0.28 (0.07)	0.33 (0.05)	0.04 (-0.01;0.09)	0.143
CCA, mm^2	588.42 (119.39)	618.55 (116.07)	12.68 (-83.98;109.34)	0.791
CCI, visual analysis	0.30 (0.06)	0.34 (0.05)	0.03 (-0.01; 0.08)	0.161

Volumes of WM lesions, ml (mean; SD)				
Total	23.84 (18.71)	13.25 (6.47)	-12.41 (-22.27;-2.54)	**0.015**
Periventricular	5.37 (5.75)	1.52 (1.45)	-3.97 (-6.92;-1.01)	**0.010**
Juxtacortical	3.23 (3.87)	1.03 (1.14)	-2.46 (-4.41;-0.51)	**0.015**
Deep white matter	12.69 (9.49)	7.88 (4.33)	-5.97 (-11.36;-0.58)	**0.031**
Pons	0.05 (0.07)	0.05 (0.15)	0.00 (-0.10;0.11)	0.972
Cerebellar	0.04 (0.08)	0.02 (0.05)	-0.02 (-0.07;0.03)	0.409
Infratentorial	0.09 (0.11)	0.07 (0.19)	-0.02 (-0.16;0.12)	0.778
Total lesion count in numbers, visual analysis (%)				0.224
0–9	2 (16.7)	6 (26.1)		
10–20	0	5 (21.7)		
21–40	4 (33.3)	6 (26.1)		
>40	6 (50)	6 (26.1)		

p = p-value for group difference, adjusted with the duration of disease and gadolinium enhancement

5.4.5 Correlation of visual analysis with cNeuro® analysis

CCI was slightly smaller in patients without a history of DMT than in those who had used DMT, but with no statistically significant difference. Visual and automated cNeuro® CCI measures were strongly correlated ($r > 0.85$, $p < 0.001$) (Figure 9).

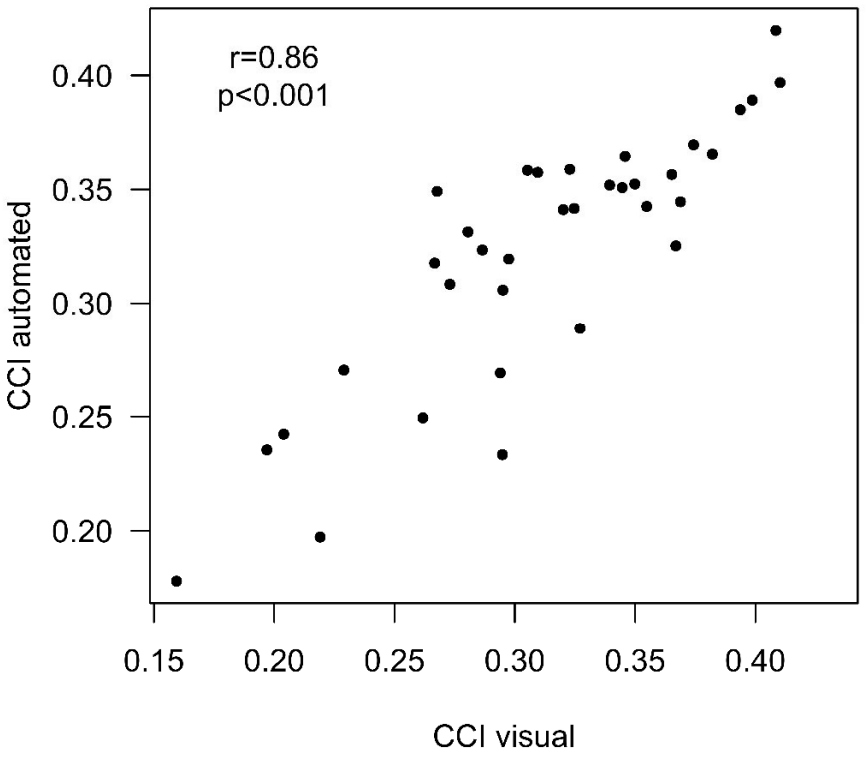

Figure 9. Correlation between visual and automated CCI analysis

6 DISCUSSION

Some MS patients present with minor clinical disability even decades after diagnosis. This study focuses on these patients, who have so-called benign MS, and especially on the neurodegenerative component of the disease in this patient group. Since the definition of benign MS is a retrospective judgement of the past disease trait, it is worth searching for the differentiating and prognostic serum and MRI biomarkers of the disease activity and neurodegeneration in patients with MS classified as benign based on clinical grounds. Still no biomarkers have been validated to define the disease course and securely choose the appropriate DMT individually.

6.1 SERUM GFAP AND NFL LEVELS AS BIOMARKERS OF DISEASE ACTIVITY IN BRRMS (STUDY I)

Our study is the first to report serum GFAP and NfL levels in BRRMS. Elevated serum GFAP levels were found as a marker of glial activation in both BRRMS and ARRMS patients compared to HCs. Also, GFAP levels were higher in BRRMS patients who were without DMT than in those who had used DMT. There have been no previous reports of DMTs showing an effect on serum GFAP levels. Few previous CSF studies have reported DMT to have no effect on GFAP levels (Axelsson et al., 2014; Gunnarsson et al., 2011). Our finding suggests that the DMTs may have an effect on the neurodegenerative component of MS in addition to a reduction in inflammation, even in the mild form of the disease. Furthermore, elevated GFAP levels in BRRMS patients refer to the assumption of an ongoing astrocytic activation and neurodegenerative process in this phenotype of MS that had earlier been thought to be mild, stable and non-progressive. The lower serum GFAP levels in the subgroup of patients who had used DMT strongly supports the use of DMT also in the mild form of the disease. A recent study by Barro et al. reported the level of serum GFAP to be prognostic for future disability progression, but not levels of sNfL, and the

prognostic value of serum GFAP to be highest in patients with low sNfL (Barro et al., 2022). Further, serum GFAP levels seem not to increase in the presence of inflammatory disease activity (Barro et al., 2022). Whether the DMTs truly have a protective effect on neurodegeneration, can only be verified by large-scale long-term follow-up studies done with different phenotypes of MS.

Serum NfL as a marker of disease activity in MS has been much more extensively studied than has GFAP. sNfL levels did not differ between BRRMS and ARRMS patients nor between HCs and either of these patient groups. This indicates there was no or only minimal subclinical inflammatory activity in BRRMS, as also was the case in ARRMS patients. A possible caveat here is the lack of information of BMI, which has been shown to significantly impact the sNfL levels. We can assume that patients in the ARRMS group were clinically stable due to earlier or ongoing highly active DMT and were in an inflammatorily stable phase of the disease, which is compatible with earlier studies of therapy initiation and escalation (Novakova, Zetterberg, et al., 2017; Piehl et al., 2017b; Siller et al., 2018). It seems to be possible to decrease neuroaxonal damage with highly effective treatment in aggressive MS. At the same time, patients with BRRMS were truly in a non-inflammatory phase with low efficacy treatment or even without DMT. Some patients with BRRMS may have an outstanding response to the first DMT. Our cohort of BRRMS included patients with only one relapse in their disease history. It is worth noting that DMT use as such has not been included in commonly used the definitions of benign MS (Amato et al., 2006; Mesaros et al., 2009).

Correlation of both GFAP and NfL serum levels and age was found in both HC and MS patients, as previous studies have also stated (Barro et al., 2018; Disanto, Barro, Benkert, Naegelin, Schadelin, et al., 2017; Högel et al., 2020; Piehl et al., 2017b; Sanchez-Valle et al., 2018). Our study also supports the previous findings that sNfL can be measured reliably in blood samples, and the results are comparable between different laboratories (Disanto, Barro, Benkert, Naegelin, Schadelin, et al., 2017; Novakova, Zetterberg, et al., 2017; Siller et al., 2018).

There is an evident need for serum biomarkers to measure both the inflammatory activity and neurodegenerative component of MS in the clinic. It seems evident that a single biomarker is not sufficient to be used as a predictor or measurement of disease activity, treatment response and neurodegenerative progression in MS. A combination of several different types of biomarkers and panels may be the conclusion.

6.2 GLOBAL AND REGIONAL GM VOLUMES AND WM LESION VOLUMES IN BRRMS AND ARRMS (STUDY II)

We used a set of volumetric imaging biomarkers extracted from routine MRI examinations in retrospective data in two different MS phenotypes. The same automated MRI quantification tool, cNeuro®, was used in studies II and III. Whole-brain volumes and thalamic volumes were larger in BRRMS patients than in ARRMS patients, while WM lesion load was larger in those with ARRMS, correlating with their higher earlier inflammatory activity of the disease. Thalamic volume was the most distinct GM measure in differentiating BRRMS and ARRMS patients.

Within the BRRMS group, those patients who had never been treated with DMT had larger WM lesion volumes, indicating there also to be subclinical inflammatory activity in seemingly mild MS, since these patients had a slightly lower number of relapses in their disease history. Our results support the idea of DMT use also in the clinically benign disease course regardless of the clinical relapse rate (Montalban et al., 2018; Ziemssen et al., 2016; Zivadinov et al., 2016). So far, evidence for the DMT's effect on GM atrophy is scarce. In a large meta-analysis of RRMS patients, lower brain atrophy was found at 24 months with second-line DMT, compared to first-line DMT, but GM atrophy was specifically not reported (Branger et al., 2016).

We developed CCI as a new parameter in the cNeuro® automated MRI quantification tool. CCI measures correlated with whole-brain volumes and thus, CCI seems to provide an easily measured structure to be used as an atrophy marker. Four patients in the ARRMS group had started highly effective DMT (fingolimod or natalizumab) within one year before MRI

scanning. Since these patients did not have smaller WM or whole-brain volumes than the other patients within the ARRMS group, and the results of the CCI and thalamic volumes were in line with the whole-brain volumes, we assume that pseudoatrophy alone does not explain the smaller whole-brain volumes in the entire ARRMS group.

To our knowledge, our study is the first to report automated CCI measures in benign MS. There have been a few reports on CCI in benign MS and a negative correlation of CCI with GM atrophy in MS, and our results are in line with these (Klawiter et al., 2015; Mesaros et al., 2009). In conclusion, CCI and thalamic volume seem to be valid MRI markers for brain atrophy in an automated MRI tool.

6.3 GLOBAL AND REGIONAL GM VOLUMES AND WM LESION VOLUMES IN BRRMS COMPARED TO AGE- AND GENDER-MATCHED HCS (STUDY III)

In study III, the focus was on brain volume measures in BRRMS patients compared to age and gender-matched HC. The automated quantification results were compared with a visual analysis made by an experienced neuroradiologist. As expected, total brain volumes were smaller and CSF volumes larger in BRRMS patients than in HCs. Interestingly, total cortical and cerebral GM volumes were larger in BRRMS patients than in HCs. Larger GM volumes in BRRMS patients were identified, especially in the limbic areas (i.e. the entorhinal cortex and cingulate gyrus), than in HCs.

This interesting finding has not previously been reported. Controversial results have been reported in cases with CIS and benign MS. In a study of 62 patients with CIS, regional atrophy in the limbic system and deep GM was demonstrated in early MS, compared to in age-matched HCs (Audoin et al., 2010). Another recent study reported in part converse results compared to our data: benign MS patients had smaller cortical and deep GM volumes and smaller normalised whole brain volumes than in HCs (Riccitelli et al., 2020). Earlier studies have also reported reduced cortical GM volumes in benign MS (Mesaros et al., 2008; Rovaris et al., 2008).

We found no correlation between WM lesion volumes and cortical total GM volumes in BRRMS patients. In earlier studies, a negative correlation between these measurements, indicating higher WM lesion volumes associated with lower GM volumes or lower cortical thickness, was most consistently reported in early relapsing MS and less in progressive disease (Charil et al., 2007; Lie et al., 2022). This again points out to the suspected progressive nature of BRRMS. However, we found a negative correlation between WM lesion volumes and CC measurements, similar to earlier reports, suggesting CC atrophy is related to WM disease and Wallerian degeneration specifically in MS (Klawiter et al., 2015).

Global and GM volume loss is known to correlate with disability progression in MS (Cagol et al., 2022; Popescu et al., 2013). It has been shown in AD studies that some elderly people with AD neuropathology do not develop dementia. These non-demented individuals with AD neuropathology have been found to have larger overall GM volume and thicker cingulate gyrus than patients with clinically demented AD (Kok et al., 2022). Possible mechanisms may not only be higher education and cognitive reserve, but also compensation for neural atrophy and reduced neuroinflammation by decreased glial activation. Similarly, the concept of a higher maximal lifetime brain growth (MLBG) has been linked to preserved cognitive and motor functions in MS patients. The brain reserve hypothesis suggests that people with larger MLBG have a better reserve against cognitive impairment (Sumowski et al., 2014). MS patients with larger MLBG were at lower risk for disability progression measured by EDSS change in a 5-year follow-up study (Sumowski et al., 2016). Functional MRI studies have also shown compensatory cortical mechanisms in benign MS (Rocca et al., 2009).

Several studies have demonstrated CCI and CCA as reliable brain atrophy markers in MS. However, the majority of the studies have been made with time-consuming visual methods, which are also subject to rater-related errors (Klawiter et al., 2015; Papathanasiou et al., 2017; O. Yaldizli et al., 2010; Ö. Yaldizli et al., 2011). Some later studies have reported automated MRI measures of CC (Granberg et al., 2015). We found that CCA and CCI both correlated positively with whole brain volume in MS but not

in HC, which is in line with an earlier report suggesting CC atrophy to be an MS-specific process including both WM and GM pathology (Klawiter et al., 2015). The results from visual and automated CCI analysis were well correlated. Unfortunately, we could not test visual and automated WM analysis correlation due to different scales.

We found similar total brain volumes, cortical and deep GM volumes, as well as WM volumes, in the treated and non-treated BRRMS patients. Also, CCA and CCI did not differ between these groups. However, WM lesion volumes were larger in patients who had never been treated with DMT, the same finding also reported in the study II. This suggests that there may be subclinical inflammatory disease activity even in seemingly mild and benign MS, supporting the use of DMT in the benign course of the disease (Montalban et al., 2018; Ziemssen et al., 2016).

6.4 STRENGTHS AND LIMITATIONS

Our study population was detailed in terms of clinical characteristics through as EDSS evaluation by an experienced neurologist. In the BRMMS group, the disease duration clearly exceeded ten years, which has been commonly accepted as a criterion for benign MS (S. B. Glad et al., 2010). Also, BRRMS patients had been treated with low-efficacy DMTs or were without any immunomodulatory drugs throughout the disease history (about one-third of BRRMS patients in all studies). A lack of cognitive testing and evaluation of fatique are clear limitations of the study. EDSS was used as the only clinical measure, and it is known to emphasise motor functions. Cognitive problems and fatigue are common in benign MS (Amato et al., 2006; Correale, Peirano, et al., 2012). Injury to the limbic system pathways, in particular, has been associated with cognitive dysfunction in MS (Keser et al., 2017); thus, it would have been informative to have also had cognitive testing included in our studies.

Patients with recent relapses and cortisone treatments were excluded from the studies, to ensure minimal possible inflammatory activity. In MRI study III, patients with Gd-enhancing lesions were also excluded to ensure that pseudoatrophy after resolution of brain oedema during inflammation

would not bias the results. A weakness of study II is that it did not include visual analysis of the MRI; in other words, we lacked information on Gd-enhancing lesions.

The sample sizes in all the studies were relatively small. Some patients with very mild benign MS may have been missing in our cohort, since those patients may not have been systematically followed in a neurological outpatient clinic, especially those who never started a DMT. This may lead to potential selection bias. However, there have been only a few recent reports on benign MS, and the results from real-world data are important.

The BRRMS cohort in study I was partly different from the cohort in studies II and III. It was not possible to combine the MRI data with serum GFAP and NfL results, since the MRI data available were retrospective and not from the time point of serum sampling. It would have been useful to have had information on possible Gd-enhancing lesions and the results of volumetry at the time of serum sampling.

Due to the nature of a retrospective real-world study, the imaging protocols, scanners and voxel sizes varied in MRI studies II and III. This may have impacted the imaging results, especially the cortical GM measures. However, the normalisation of the structures was incorporated in the cNeuro® MRI quantification tool, to minimize the risk of this bias. Also, previous studies with the same algorithm have suggested this method to be quite robust and tolerant to the variability of the imaging (Kaipainen et al., 2021; Koikkalainen et al., 2016; Lötjönen et al., 2010). Previous studies with the FreeSurfer structural tool have also shown that the use of different MRI scanners and pulse sequences does not significantly affect cortical thickness measures (Govindarajan et al., 2014; Potvin et al., 2017).

Both MRI studies II and III included only single point MRI analysis; thus, the lack of longitudinal analysis can be considered a weakness. It was impossible to achieve repeated MRI imaging with the same scanner in retrospective data.

6.5 FUTURE DIRECTIONS

The main goals in the treatment of MS are to prevent inflammatory relapses and new WM lesions and to avoid further permanent physical and cognitive disability that are correlated with brain atrophy. To reliably measure these aspects of this both inflammatory and neurodegenerative disease, we need validated soluble blood biomarkers and MRI markers in addition to the clinical parameters.

It is evident that a significant proportion of MS patients deal with the disease for decades with minimal clinical disability. Our results show that there has been clinically silent inflammatory activity in these so-called benign patients, since in BRRMS patients without DMT, the WM lesion volumes were larger. The sNfL levels were low in BRRMS patients, comparable to HC and ARRMS patients treated with highly effective DMT; in other words, there was no active inflammation at the time of blood sampling in the study. At the same time, serum GFAP levels were elevated in BRRMS patients, demonstrating an ongoing neurodegeneration and active glial process. As a sign of neurodegeneration, total brain volume was smaller in BRRMS patients than in HCs, as expected, due to the MS disease process. Still, some brain areas, such as the limbic system, were relatively well preserved. Presumably, there is a better brain reserve in this patient group, that offers a compensatory mechanism against clinical worsening.

Starting a DMT is beneficial to all MS patients, but it is still difficult to recognise patients who will get on with low-efficacy treatment. Supposedly, in the future, the milder phenotype of MS will be more common and fewer patients will enter the SPMS stage, as we alter the disease process with immunomodulatory treatments. This positive message must not be forgotten. Further, it seems beneficial to start a DMT even in patients who have been clinically stable without medication for years or even a decade but present with new T2 lesions in an incidental control MRI.

Serum NfL levels have already been widely studied in MS, and the evidence is strong in monitoring inflammation. The recent large study by Benkert et al. provided valid reference data to be used in the assessment of the sNfL results of patients (Benkert et al., 2022), and they have truly

brought this biomarker into clinical use. More research on serum GFAP as a biomarker of neurodegeneration is needed in different MS patient groups since the data so far are scarce. However, a single measurement of either serum NfL or GFAP is not useful and consecutive measurements of these, and probably other biomarkers are needed in the follow-up. Serum biomarker levels should ideally be measured in newly diagnosed patients, during a suspected relapse, after a DMT switch and in a follow-up of clinical progression.

Brain MRI is now routine during follow-up in MS clinics. We monitor the efficacy and safety of DMTs, but at the same time gather much information to be used in the assessment of brain atrophy progression. In MS clinics, patients are often scanned in recurrent follow-ups with alternating scanners, imaging protocols and field strengths due to practical circumstances. Robust automated quantification MRI tools may already partly tackle these problems with image normalisation and by using well-defined structures such as thalamus volume and CC measurements (CCA and CCI) as brain atrophy markers. However, to measure brain atrophy progression at an individual level, a similar scanner should be used in the consecutive MRIs. At the same time, we will gather more information on volumetry in specific local brain areas such as the somatosensory and motor cortical areas and limbic system in different MS phenotypes. Future research should explore whether our finding of locally well-preserved brain volumes in mild MS is connected to well-preserved cognitive functions or lesser fatigue in mild MS and if the finding persists in a longer follow-up.

Serum biomarker results and brain MRI volumetry combined with clinical parameters (EDSS, possible relapses or clinical progression, cognitive symptoms and fatigue) would lead to a more individual treatment design. Real-life data on different phenotypes of the disease are needed. As we treat increasingly aged patients whose immune system senescence exposes them to the side effects of DMTs, we also need data on when to stop the DMT safely (Hua et al., 2019; Schweitzer et al., 2019). Starting a DMT early after a definite diagnosis is crucial, even if the phenotype of the disease would seem to be mild.

7 CONCLUSIONS

As the whole concept of benign MS has been disputed for decades, this study strenghtens the idea that benign MS as such is only a temporary description. If followed for a long enough time, most patients with a seemingly benign disease will develop non-visible symptoms, overall disability and brain atrophy as a marker of neurodegeneration. The neurodegenerative component of the advanced disease can reliably be demonstrated in blood samples with levels of GFAP and NfL.

The following results of this study support idea that the term 'benign MS' should be used cautiously and, so far, the term used should preferably be, at most, 'mild MS':

1.Serum GFAP levels are elevated in a clinically mild form of MS, as well as in aggressive MS treated with highly effective DMT, reflecting astrocytic activation and disease progression (study I).

2. Serum NfL levels reflect the inflammatory component of the disease. In mild MS, sNfL levels did not differ from those of HCs in a single-point measurement. In aggressive MS with highly effective treatment and at a stable inflammatory phase of the disease, sNfL levels were similar to those of HCs (study I).

3. Thalamic volume is a distinct marker of brain atrophy and strongly differentiated patients with BRRMS and ARRMS (study II).

4. The limbic system seems to be well-preserved in mild MS despite general brain volume loss and loss of thalamic volume (study III).

5. CC measures (CCI and CCA) provide reliable brain atrophy markers in an automated quantification tool of brain MRI in MS. Automated quantification methods can already be used in clinics to detect local and minor atrophy in patients with or without DMT (study II and III).

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A proportion of MS patients show minimal disability even decades after the onset of MS symptoms, and this entity of so-called benign MS has been debated since the 1950s. This thesis provides information on the disease activity and the neurodegenerative process in benign and mild relapsing-remitting MS. The neurodegenerative component of the advanced disease can be demonstrated with elevated serum levels of GFAP, as is also the case in the mild form of the disease. Automated MRI quantification methods are already feasible in clinics to detect local and minor atrophy.

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