DISSERTATION

Development of CSF and blood biomarkers in the clinical management of multiple sclerosis

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STATUTORY DECLARATION

Herewith, I, Margarete Voortman, declare that the dissertation before you is work by my own hand in which I fully recognize the contribution every individual person and organisation made to the research represented in this work. Due acknowledgement was made throughout the text in this dissertation and the original research publications it entails. The *"Guidelines of the Medical University of Graz on Good Scientific Practice"* were followed throughout all my work.

DISCLOSURES

List of Publications

The following original research articles are presented in this dissertation. Publications are listed in order of appearance in the dissertation, including authors, their affiliations and author contribution.

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Permission for reprint

All co-authors agreed to include the content of these publications in this dissertation. Permission from third-parties was granted to reproduce the content of the published work in this dissertation.

Other contributions

I further contributed to the following publications during my PhD studies:

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ABBREVIATIONS AND DEFINITIONS

	Abbreviation	Definition
A	AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
	AE	Adverse event
	AOC	Antioxidative capacity
B	BBB	Blood-brain barrier
C	CD	Cluster of Differentiation
	CDMS	Clinically definite MS
	CIS	Clinically isolated syndrome
	CNS	Central nervous system
	CSF	Cerebrospinal fluid
D	DHR	Dihydrorhodamine
	DIS	Dissemination in space
	DIT	Dissemination in time
	DMF	Dimethyl fumarate
	DMT	Disease modifying therapy
E	EAE	Experimental autoimmune encephalomyelitis
	EBV	Epstein-Barr virus
	EDSS	Expanded Disability Status Scale
F	FLC	Free light chains
	FTY	Fingolimod
G	GA	Glatiramer acetate
	Gd	Gadolinium
	GFAP	Glial fibrillary acidic protein
H	HLA	Human leukocyte antigen
I	IFN-β	Interferon-beta
	Ig	Immunoglobulin
J	JCV	JC or John Cunningham virus
K	KFLC	Kappa FLC
L	LFLC	Lambda FLC
	LP	Lumbar puncture
M	MRI	Magnetic resonance imaging
	MS	Multiple sclerosis
	MUG	Medical university of Graz
N	NABM	Normal-appearing brain matter

	NAGM	Normal-appearing grey matter
	NAWM	Normal-appearing white matter
	NEDA	No evidence of disease activity
	NfL	Neurofilament light
	NTN	Netrin
	NTZ	Natalizumab
O	OCB	Oligoclonal bands
	OPC	Oligodendrocyte progenitor cells
	OS	Oxidative stress
P	PBMC	Peripheral blood mononuclear cell
	PEA	Proximity extension assay
	PML	Progressive multifocal leukoencephalopathy
	PPMS	Primary progressive MS
	PVALB	Parvalbumin
Q	Qalb	Albumin quotient
R	RNS	reactive nitrogen species
	ROS	reactive oxygen species
	RRMS	Relapsing remitting MS
S	Simoa	Single-molecule array
	SNPs	Single-nucleotide polymorphisms
	sNTN-1	Serum netrin-1
	SPMS	Secondary progressive MS
T	Th	Helper T cell
	Treg	Regulatory T cell
W	WHO	World Health Organization

ZUSAMMENFASSUNG

Ziel der vorliegenden Arbeit war die Identifikation und Bewertung klinisch Relevanter Biomarker in Liquor und Blut von Patienten mit Multipler Sklerose (MS) im Vergleich zu Kontrollkohorten. Die Ergebnisse der potentiellen Biomarker wurden mit longitudinalen Daten der Klinik und Magnetresonanztomographie (MRT; bei 3 T) kombiniert.

Appendix 1 – Die Eignung der **freien Leichtketten Kappa (KFLC)** und **Lambda (LFLC)** im Liquor und Serum von 48/13 oligoklonale Banden-positiven Patienten mit klinisch isoliertem Syndrom (CIS)/schubförmiger remittierender MS (RRMS) im Vergleich zu 60 Patienten mit nicht-entzündlichen neurologischen Erkrankungen (NINDC) als prognostisches Werkzeug für den Krankheitsverlauf wurde untersucht. Die Liquor-Parameter von KFLC und LFLC waren bei erkrankten Patienten erhöht. CIS-Patienten mit einem niedrigeren CSF-KFLC/LFLC-Verhältnis zeigten ein 2,89-fach höheres Risiko, eine klinisch manifeste MS zu entwickeln.

Appendix 2 – Serum-**Netrin-1** (sNTN-1) wurde bei 31/48 CIS/RRMS und 30 NINDC analysiert, und das Potenzial als Biomarker für die Krankheitsaktivität bewertet. Es zeigten sich keine Unterschiede zwischen Patienten und Kontrollen oder zwischen Patienten mit einem Gadolinium (Gd)-positiven und Gd-negativen MRT. Eine kleine Untergruppe von Gd+ Patienten mit klinischer Aktivität zeigte im Vergleich zu klinisch nicht aktiven Gd+ Probanden eine verringerte sNTN-1 Konzentration.

Appendix 3 – Es wurde die **antioxidative Kapazität (AOC)** im Liquor und Blut von 55/11 Patienten mit CIS/RRMS und 67 NINDC bestimmt. Eine reduzierte AOC konnte im Liquor von RRMS Patienten im Vergleich zu Personen mit CIS festgestellt werden. Eine verringerte AOC war desweitern mit größerer körperlicher Beeinträchtigung und erhöhtem Risiko weiterer MS Schübe assoziiert. Eine Korrelation der AOC mit MRT-Messungen war nicht ersichtlich.

Appendix 4 – Eine FACS-Analyse von **CD62L+** peripheren mononukleären Zellen wurde im Vollblut von 234 CIS/MS-Patienten mit unterschiedlichen Behandlungsschemata und 51 gesunden Kontrollen (HC) durchgeführt. Die CD62L Expression war unter Natalizumab (NTZ) und Fingolimod (FTY) verringert während die Applikation von Dimethylfumarat im Vergleich zu Interferon/Glatirameracetat, Patienten ohne Behandlung und HC einen CD62L Anstieg zur Folge hatte. CD62L war bei unveränderter Behandlung über die Zeit stabil, wurde jedoch durch Absetzung von NTZ oder FTY Einführung beeinflusst.

Insgesamt konnte der klinische Wert und Nutzen von vier Biomarkern gezeigt werden. Neben dem diagnostischen Potenzial könnten KFLC und LFLC im Liquor hilfreich bei der Prognose der Krankheitsaktivität sein. sNTN-1 gibt keine zusätzliche Information zur radiologischen Krankheitsaktivität, könnte jedoch hilfreich bei der Identifikation MS assoziierter Pathologien sein. Die AOC im Liquor spielt eine wichtige Rolle in pathologischen Prozessen und gilt als potentieller

Marker für die Krankheitsaktivität oder als Behandlungsziel selbst. Die FACS-Analyse von CD62L in Blutproben belegt das Potenzial der Methodik und zeigt den Bedarf weiterer Forschung um das Potenzial von CD62L als Marker für den Behandlungserfolg zu untersuchen.

ABSTRACT

The overall aim of the work depicted in this dissertation was the analysis of biomarkers in CSF and blood of patients with early multiple sclerosis (MS) compared to controls, and evaluating their applicability in various clinical settings. Results on body fluid biomarkers were combined with longitudinal clinical and magnetic resonance imaging (MRI; at 3 T) patient data.

Appendix 1 – We assessed the potential of **free light chains kappa (KFLC)** and **lambda (LFCL)** to serve as prognostic tool for disease progression in CSF and serum of 48/13 oligoclonal bands-positive patients with clinically isolated syndrome (CIS)/relapsing-remitting MS (RRMS) compared to 60 non-inflammatory neurological disease controls (NINDC). CSF parameters of both KFLC and LFLC were increased in diseased patients. CIS patients with a lower CSF KFLC/LFLC ratio had a 2.89-time higher risk to convert to clinically definite MS.

Appendix 2 – Serum **Netrin-1** (sNTN-1) was analysed in 31/48 CIS/RRMS and 30 NINDC to assess its potential to provide information on MS disease activity. No significant differences were found between patients and controls, or between patients with a gadolinium (Gd)-positive or -negative MRI. A small sub-group of Gd+ patients with simultaneous clinically active disease showed decreased sNTN-1 vs. clinically non-active Gd+ subjects.

Appendix 3 – We compared **antioxidative capacity (AOC)** in CSF and blood, as measured fluorometrically, between 55/11 patients with CIS/RRMS and 67 NINDC. AOC in CSF was decreased in RRMS vs. CIS. Lower CSF AOC was in turn related to greater physical disability and increased risk for future relapses in MS. AOC did not correlate to MRI measures.

Appendix 4 – FACS analysis of **CD62L+** peripheral blood mononuclear cells in whole blood was performed within one hour upon sampling in 234 CIS/MS patients under various treatments and 51 healthy controls (HC). CD62L was decreased under natalizumab (NTZ) and fingolimod (FTY), and increased with dimethyl-fumarate when compared to interferon/glatiramer acetate, no treatment and HC. CD62L was stable over time with unchanged treatment, but was affected upon NTZ withdrawal or FTY introduction.

Overall, we were able to show the potential clinical value of four body fluid biomarkers, each with a different supposed applicability. Next to their diagnostic potential, CSF KFLC and LFLC might aid in prognosticating disease activity. sNTN-1 cannot be used to detect radiological disease activity; nevertheless, it might serve a role in detecting MS pathology. AOC in CSF appears to play an important role in pathological processes related to oxidative stress, giving it potential as marker for disease activity or as treatment target itself. The direct FACS analysis of CD62L in fresh blood samples proofs the potential of the methodology and

warrants further research on the potential of CD62L as marker in treatment response.

CHAPTER 1

INTRODUCTION

Outline — In this introductory chapter, an overview is given of the state-of-the-art knowledge regarding 1) multiple sclerosis: epidemiology, aetiological and pathophysiological aspects of the disease, and current diagnostic and treatment options, and 2) biomarkers in multiple sclerosis evaluated by magnetic resonance imaging and in body fluids, relevant for the scope of this thesis. Finally, 3) the aim and outline of this dissertation are emphasised.

Multiple Sclerosis

Epidemiology

Incidence and prevalence

For over 150 years, first insights were gained into defining multiple sclerosis (MS) as a disseminated plaque-like sclerosis of the central nervous system (CNS) and a first full pathological description of the disease was provided (Brownell and Hughes, 1962; Reich, Lucchinetti and Calabresi, 2018). Today, MS is the most frequent cause of non-traumatic disability among young adults in developed countries (Lassmann, Brück and Lucchinetti, 2001; Multiple Sclerosis International Federation, 2013).

The latest global survey by the *Multiple Sclerosis International Federation* (2013) estimated that the median prevalence of MS worldwide is around 33 per 100,000 and approximately 2.3 million people worldwide are affected. Occurrence of MS varies greatly depending on latitude, and highest prevalence numbers are found in North America (140/100,000) and across Europe (108/100,000; ranging from 20/100,000 to 200/100,000 in different regions) (Multiple Sclerosis International Federation, 2013; Raggi and Leonardi, 2015). Annual incidence rates vary widely, in Europe ranging from 1 to over 10 per 100,000 (Kingwell *et al.*, 2013). Overall prevalence has been increasing over the last decades; presumably due to more advanced diagnosis and data reporting, and improved life expectancy of patients; however, e.g., dietary factors (including vitamin D and fat intake), or lifestyle changes (for instance, smoking behaviour, psychologic stress) might lay on the basis of a true increase in risk to develop MS (Multiple Sclerosis International Federation, 2013; Kamm, Uitdehaag and Polman, 2014; Goodin, 2016; Ghareghani, Zibara and Rivest, 2023; Vasic *et al.*, 2023).

Socioeconomic burden

Due to its high frequency, disease onset during young adulthood, and high-impact physical and psychological/cognitive health consequences, MS comes along with a great socioeconomic burden that has increased over time (Constantinescu *et al.*, 2011; Derfuss, 2012; Multiple Sclerosis International Federation, 2013; Raggi and Leonardi, 2015; Chen, Chonghasawat and Leadholm, 2017).

Social impact

In definition, total disease burden addresses four health variables: mortality (years of life lost), years lived with a disability, amount of disability related to the disease, and overall disability-adjusted life years (Raggi and Leonardi, 2015). MS patients have a reduced life expectancy of approximately 7 to 14 years compared to the general population, with MS being responsible as the primary cause of death in over 50 % of cases (Grytten Torkildsen *et al.*, 2008; Scalfari *et al.*, 2013). Besides, MS patients have an increased risk to develop multiple co-morbidities, both before and upon MS onset, which may affect survival (Manouchehrinia *et al.*, 2016; Chou *et al.*, 2020). The average survival time of MS patients depends on disease course, age at onset and global region, but approximates 35 years (ranging from around 25 to 45 years) (Grytten Torkildsen *et al.*, 2008; Scalfari *et al.*, 2013). MS usually starts with no or mild to moderate disability. During the course of disease, most patients experience severer forms of disability; around 60 % of those affected with MS are incapable to work and suffer from a considerable decreased quality of life.

Financial impact

Within Europe, annual costs ranged around ~30,000 euro per patient for the last two decades in 2010 (Olesen *et al.*, 2012; Raggi and Leonardi, 2015), with a total of circa 14.6 billion euro in 2010 (Olesen *et al.*, 2012). Across the United States, annual costs were extrapolated to be as high as 4.3 billion dollars in 2013 (Chen, Chonghasawat and Leadholm, 2017). A large amount of costs is due to indirect expenses, e.g., as a result of reduced productivity or work absenteeism of patients. A recent multicentre survey carried out in Germany estimated the average quarterly cost per patients at around 7,000 euro, including all medical, non-medical and indirect costs, with a significant increase in costs (total and direct medical) in patients showing disability progression compared to those who remained stable over time (Ness *et al.*, 2020).

Aetiological factors

MS is a complex disease with a multifactorial origin that is incompletely understood. It is considered an immune-mediated disease, although the self-antigen involved remains unknown. The aetiology of the disease may even vary for different subgroups of MS patients. From epidemiological data, it is suggested that complex genetic-environmental interactions might play a role (Kamm, Uitdehaag and Polman, 2014; Filippi *et al.*, 2018).

Gender influences

Gender appears to influence the susceptibility for MS. The average risk to develop MS is approximately twice as high for women as it is for men, though this ratio ranges from 1.1 to 3, depending on different geographical regions (Confavreux and Vukusic, 2006; Kingwell *et al.*, 2013; Multiple Sclerosis International Federation, 2013). Besides, the incidence increased in women over the last decades (Kingwell *et al.*, 2013; Kamm, Uitdehaag and Polman, 2014), and the mortality rate is higher for female than male patients (Manouchehrinia *et al.*, 2016).

Latitude and Vitamin D

Incidence and prevalence of MS are increased in *high-latitude* regions (north and south), which seems related to the patient's exposure to sunlight. Similar correlations are seen with a mother's sunlight exposure during pregnancy and the subsequent risk of the child developing MS. These relationships suggest that *vitamin D* (as a result to the UVB exposure from sunlight) could serve as protective agent against disease development (Kamm, Uitdehaag and Polman, 2014; Ismailova *et al.*, 2019). Increased availability of vitamin D is known to dampen the body's inflammatory response, thus lowering the risk to develop autoimmune diseases (Ismailova *et al.*, 2019).

Environmental effects

Exposure to several *microorganisms* might trigger the development of MS, e.g., as was suggested consistently for the Epstein-Barr virus (EBV) (Waubant *et al.*, 2019; Bjornevik *et al.*, 2022). A recent unique collaborative study with the US military investigated the causality of EBV seropositivity and the onset of MS using longitudinal data and, where available, serum samples from over 10 million subjects collected over a 20-year period (Bjornevik *et al.*, 2022). MS was found in 955 cases, among which only 1 out of 801 was EBV negative prior to MS onset, compared to 51 of 1,566 controls at the time of last sampling, concluding a 32-fold increased hazard of developing MS upon EBV infection. A single infectious agent has not been

found for MS though, and the likelihood for the existence of one is expected to be low (Kamm, Uitdehaag and Polman, 2014; Waubant *et al.*, 2019).

Other general *environmental and life-style* health risk factors have been associated with increased risk to develop MS or a stronger disease progression, like childhood obesity, smoking in a dose-dependent manner, diet, gut microbiota, (vascular) comorbidities, exposure to toxic environmental agents, biochemical biomarker, and traumatic accidents or interventions (Kamm, Uitdehaag and Polman, 2014; Belbasis *et al.*, 2015; Ismailova *et al.*, 2019; Waubant *et al.*, 2019). For most of these environmental factors, research was performed on small patient cohorts, results displayed great heterogeneity, or studies were suggestive of excess significance bias – factors all known to occur in observational research – making their evidence not convincing (Belbasis *et al.*, 2015; Waubant *et al.*, 2019).

Genetic effects

Even though MS is not considered a hereditary disease, genetic factors affect disease hazard and there is an increased risk of MS development in people whose family members are affected (Kamm, Uitdehaag and Polman, 2014). A great variety of genetic variations have been associated to MS susceptibility or disease course, most of which are located within or near genes involved in immunity, although none are specific for MS (Gourraud *et al.*, 2012; Waubant *et al.*, 2019).

Pathophysiology and pathogenesis of MS

Pathogenic models

Classically, MS is defined as a chronic inflammatory neurodegenerative disease of the CNS and it is known to be driven by autoreactive CD4+ helper T cells (Lassmann, Brück and Lucchinetti, 2007; Brimnes *et al.*, 2014). The pathogenesis of this neuroinflammation is complex, and involves activated microglia or macrophages, stimulated astrocytes, recruitment of adhesion molecules, pro-inflammatory cytokines and chemokines, and metalloproteases. Next to Th1 cells, also CD8+ cytotoxic T, B and antibody producing plasma cells are involved in MS pathology (Lassmann, Brück and Lucchinetti, 2001, 2007; Constantinescu *et al.*, 2011; Losy, 2013; Brimnes *et al.*, 2014; Kamm, Uitdehaag and Polman, 2014; Dendrou, Fugger and Friese, 2015; Reich, Lucchinetti and Calabresi, 2018).

What the primary and secondary events in the pathogenesis of MS are, or if non-mutual exclusive models could be both involved, is still under debate. The common model involves primary inflammation leading to secondary myelin and subsequent axonal damage (*'outside-in' model*) (Constantinescu *et al.*, 2011; Kamm, Uitdehaag and Polman, 2014). In the opposing model, primary axonal damage would be a sufficient explanation to induce demyelination and

secondary inflammation (*'inside-out' model*) (Kamm, Uitdehaag and Polman, 2014). Here, secondary inflammation might also be triggered by initial damage to neuroglial elements, as was previously shown in *in vivo* models (Constantinescu *et al.*, 2011).

MS plaques

Definition

Large confluent, multifocal **lesions** – or *plaques* – of the brain and spinal cord are a hallmark of MS and are caused by immune cell infiltration across the **blood-brain barrier** (**BBB**) and subsequent neuroinflammation, **demyelination** (destroyed myelin sheath or oligodendrocyte cell bodies), astrocytic reactive **gliosis** (glial scar formation) and neuro-axonal degeneration, leading to disruption of neuronal signalling (Lassmann, Brück and Lucchinetti, 2001, 2007; Frischer *et al.*, 2009; Constantinescu *et al.*, 2011; Büdingen *et al.*, 2012; Losy, 2013; Kamm, Uitdehaag and Polman, 2014; Grigoriadis and van Pesch, 2015; Filippi *et al.*, 2018; Lassmann, 2018). Immunopathogenesis of MS includes the occurrence of disruption and dysfunctioning of both BBB as well as blood-CSF barrier, allowing a bidirectional exchange of immune cells between the CNS and the body's periphery (see **Figure 1**).

Localisation

Initially, MS was thought to be solely affecting the **white matter**; although currently, it is well known that in all disease stages, also **grey matter** is affected in MS, including the basal ganglia, cerebral cortex, brain stem and spinal cord (Brownell and Hughes, 1962; Geurts *et al.*, 2005; Kutzelnigg *et al.*, 2005; Haider *et al.*, 2014; Calabrese *et al.*, 2015; Magliozzi, Reynolds and Calabrese, 2018; Filippi *et al.*, 2019). Though lesions can occur anywhere throughout the CNS, there are several *susceptible regions*, often seen in most MS patients: the optic nerves, periventricular and infratentorial areas, subpial/(juxta) cortical grey matter, and the spinal cord (Dutta and Trapp, 2014; Thompson *et al.*, 2018). It is debatable whether white and grey matter damage are connected to each other – i.e., white matter lesions cause linked grey matter atrophy via retrograde neurodegeneration – or exist as independent processes in MS. For both theories, accumulating evidence from various pathological and imaging studies is available (Calabrese *et al.*, 2015).

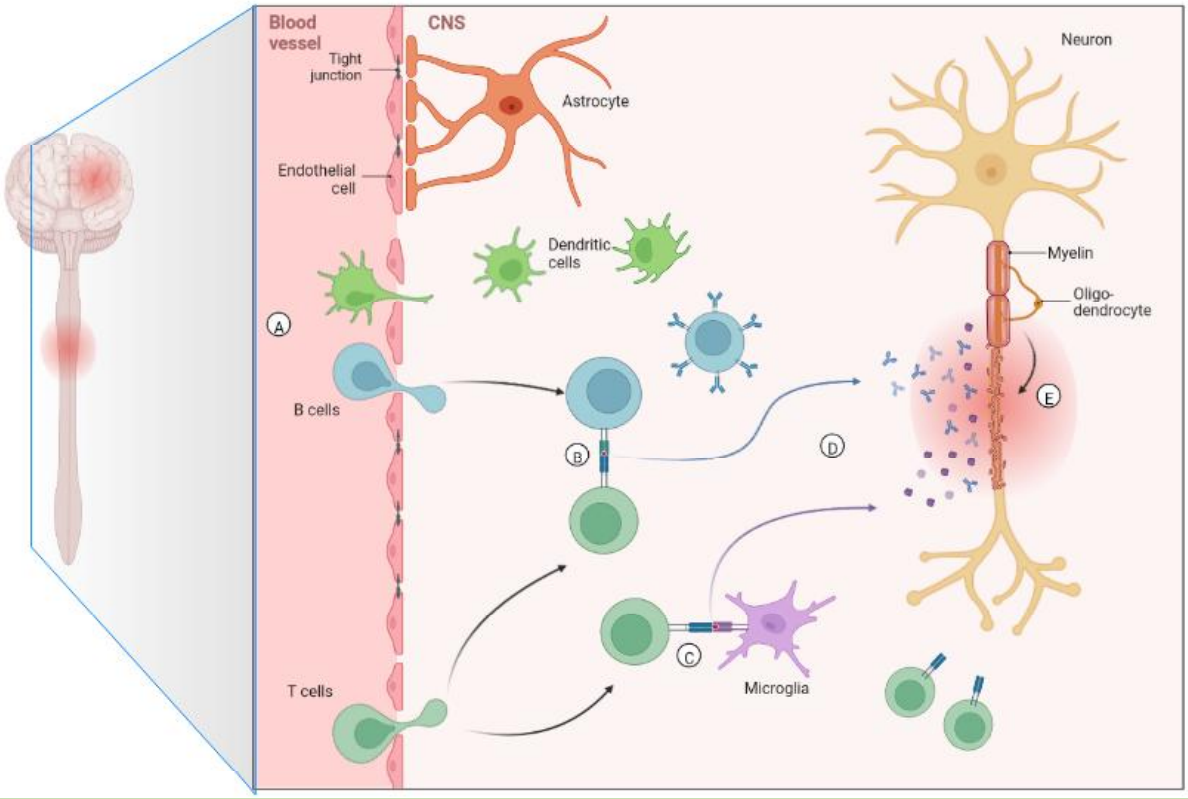


Figure 1. Schematic overview of immunopathological events in MS.

MS is an inflammatory neurodegenerative disease of the CNS – brain, spinal cord and optic nerves – with a complex pathogenesis. At sites with blood-brain barrier disruption, inflammatory lymphocytes and leukocytes infiltrate the CNS from the periphery **(A)**. In the CNS, T cells interact with B cells **(B)**, and microglia or dendritic cells **(C)**. Activated lymphocytes and microglia release antibodies and pro-inflammatory cytokines targeting the myelin sheath **(D)**. Additionally, CD8+ cytotoxic T and antibody-producing plasma cells play a direct part in targeting myelinated neurons. Together, these processes result in neural inflammation and neuronal demyelination **(E)**. (Adapted from "Pathogenesis of Multiple Sclerosis", by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates.)

The presence of leukocytes in the meninges is associated with **subpial cortical lesions**. Active demyelination and neurodegeneration affecting these parts of the cerebral cortex are *MS-specific* and not found in any other inflammatory brain disease (Lassmann, 2018). **Lymphoid follicle-like structures** are formed in the cerebral meninges in patients with more progressed disease. These structures indicate that B-cell maturation could be maintained locally in the CNS, also when the inflammation becomes compartmentalised behind a mostly closed BBB. Hereby, soluble factors contributing to a humoral immune response can be diffused from the subarachnoid space, that may lay at the basis of cortical pathology (Magliozzi *et al.*, 2007; Calabrese *et al.*, 2015).

Demyelination & Remyelination

Various types of demyelination are recognised, founded on dominating pathophysiological mechanisms. Based on myelin proteins expression, localisation and extent of plaques, pattern of oligodendrocyte injury, and deposition of activated complement components, at least four fundamentally different patterns are described (Lassmann, Brück and Lucchinetti, 2001, 2007; Lassmann, 2002; Kamm, Uitdehaag and Polman, 2014).

Initially, also **protective/repair mechanisms** and **remyelination** take place in order to restore damaged tissue, albeit insufficient to completely prevent detrimental neurodegeneration (Lassmann, Brück and Lucchinetti, 2007; Peterson and Fujinami, 2007; Zeis *et al.*, 2008; Constantinescu *et al.*, 2011; Podbielska *et al.*, 2013; Kamm, Uitdehaag and Polman, 2014). Remyelination mostly takes place either at the border or within white matter lesions during early stages of the disease; in later phases, remyelination ceases. Completely remyelinated lesions are sharply defined areas due to their lowered myelin density, known as "**shadow plaques**" (Lassmann, Brück and Lucchinetti, 2007).

Lesion activity

Depending on the activity of tissue inflammation, the degree and number of activated microglia and the presence of phagocytic cells (macrophages), generally two types of lesions can be distinguished. **Active lesions** contain a high density of intermingled pro-/anti-inflammatory cells within and around the plaque, and on larger distant in the NAWM, especially in acute and early phases of disease. During progressive phases of disease, numbers of activated microglia and phagocytic cells in the demyelinated plaque centre diminish (Zeis *et al.*, 2008). Active inflammation is further characterised by a profound disturbance of the BBB, due to which (outside the CNS activated) T and B cells from the peripheral blood can enter the CNS (Lassmann, Brück and Lucchinetti, 2007; Zeis *et al.*, 2008; Larochelle, Alvarez and Prat, 2011; Büdingen *et al.*, 2012; Brimnes *et al.*, 2014; Kamm, Uitdehaag and Polman, 2014; Pröbstel, Sanderson and Derfuss, 2015). **Inactive lesions**, on the other hand – the most common type of lesions seen in MS patients – present with moderate numbers of reactive cells, diminished acute axonal injury, and high degree of fibrillary scar tissue formation (reactive gliosis) between demyelinated axons, and are sharply demarcated (Lassmann, 2018).

Normal-appearing brain tissue

The non-lesion containing brain tissue surrounding lesions is known as the **normal-appearing *white*** and **normal-appearing *(cortical) grey* matter** (NAWM and NAGM, respectively) or normal-appearing *brain* matter (NABM). Normal-appearing tissue is defined as such, as it appears non-lesional and non-demyelinated on conventional magnetic resonance imaging

(MRI, discussed into more detail in Section *Lesional imaging* below) scans, though histopathological data reveal its complex pathology (Allen *et al.*, 2001; M Filippi *et al.*, 2016; Tavazzi *et al.*, 2020). NAWM is subjected to an overall endogenous inflammatory reaction, provoked by oligodendrocytes and microglia exerting both pro- and anti-inflammatory mechanisms (Zeis *et al.*, 2008). Immunomodulatory responses are earliest seen in white matter directly adjacent to demyelinating lesions (Zeis *et al.*, 2009). Furthermore, BBB dysfunction, activated glial cells, axonal damage and myelin loss were reported in NAWM (Allen *et al.*, 2001; Zeis *et al.*, 2008, 2009; Waller *et al.*, 2016; Tavazzi *et al.*, 2020). In the NAWM, predominantly oligodendrocytes appear to have pro-inflammatory effects (Zeis *et al.*, 2008), while astrocytes also presumably exert a neuroprotective role (Waller *et al.*, 2016).

Clinical disease course

Heterogeneity

The clinical manifestation of MS is rather heterogeneous and various clinical phenotypes and courses are distinguished (Lublin and Reingold, 1996; Multiple Sclerosis International Federation, 2013; Lublin *et al.*, 2014; Nelson *et al.*, 2016). This variability might be ascribed to differences in initial pathogenic mechanisms (Peterson and Fujinami, 2007). Disease course classification is found useful for disease management in clinical practice, especially for decision-making on appropriate treatment regimens (Nelson *et al.*, 2016).

Disease course classifications

Relapsing courses

A first single symptomatic neurological deficit that can be defined as a relapse (see definition under Section Clinical relapses) is known as a **clinically isolated syndrome** (**CIS**), which has the potential to develop into MS. Approximately 85 % of all MS patients present **relapsing-remitting MS** (**RRMS**): consecutive episodes of relapse succeeded by a remission phase with possible improvement of clinical symptoms. Already early upon disease onset, a part of patients present with discrete cognitive dysfunction (Kamm, Uitdehaag and Polman, 2014; Sorensen, 2014). An estimated 80 % of RRMS patients will develop **secondary progressive MS** (**SPMS**) within the following 20 years, a disease phase in which gradual progression of physical disability and neurological deterioration predominate, either *with or without* additional occurrence of relapses (Multiple Sclerosis International Federation, 2013; Lublin *et al.*, 2014; Dendrou, Fugger and Friese, 2015; Filippi, Preziosa and Rocca, 2018). Particularly in more progressed phases of the disease, *diffuse neurodegeneration* is seen throughout the entire

brain of MS patients – in both NAWM and grey matter – thereby aggravating both cognitive and physical impairments.

Primary progressive courses

The remaining 15 % of MS patients exhibit a progressive course from onset on: without relapses and diagnosed with **primary progressive MS** (**PPMS**, 10 %), or with relapses superimposed on the PPMS-like course as progressive-relapsing MS (PRMS, 5 %) (Multiple Sclerosis International Federation, 2013; Lublin *et al.*, 2014). Upon clinical confirmation of multiple relapses and/or disease progression, the term **clinically definite MS** (**CDMS**) may be used (Lassmann, Brück and Lucchinetti, 2007; Constantinescu *et al.*, 2011; Kamm, Uitdehaag and Polman, 2014).

Age at onset

On average, patients confronted with RRMS have an initial clinical relapse – i.e., CIS – around the age of 30, although diagnosis can follow at any age. Paediatric MS accounts for approximately 3 % of cases, the incidence of the disease increases from age 18 on, peaks between 20 and 40 years, and becomes rare above 50 years of age (Confavreux and Vukusic, 2006; Chitnis *et al.*, 2009; Ghezzi *et al.*, 2010; Multiple Sclerosis International Federation, 2013; Thompson *et al.*, 2018). PPMS typically starts later in life, at around the age of 40 (Filippi *et al.*, 2018). A schematic graphical representation of the average disease course and pathological variables associated with disease progression is given in **Figure 2**; definitions are clarified under Section Diagnosis of multiple sclerosis.

Diagnosis of multiple sclerosis

International guidelines: The McDonald criteria

In order to standardize the diagnosis of MS, the International Panel on Diagnosis of Multiple Sclerosis established the *McDonald criteria* in 2001. The criteria were revised in 2005, 2010 and lastly in 2017 (Mcdonald *et al.*, 2001; Polman *et al.*, 2005, 2011; Thompson *et al.*, 2018).

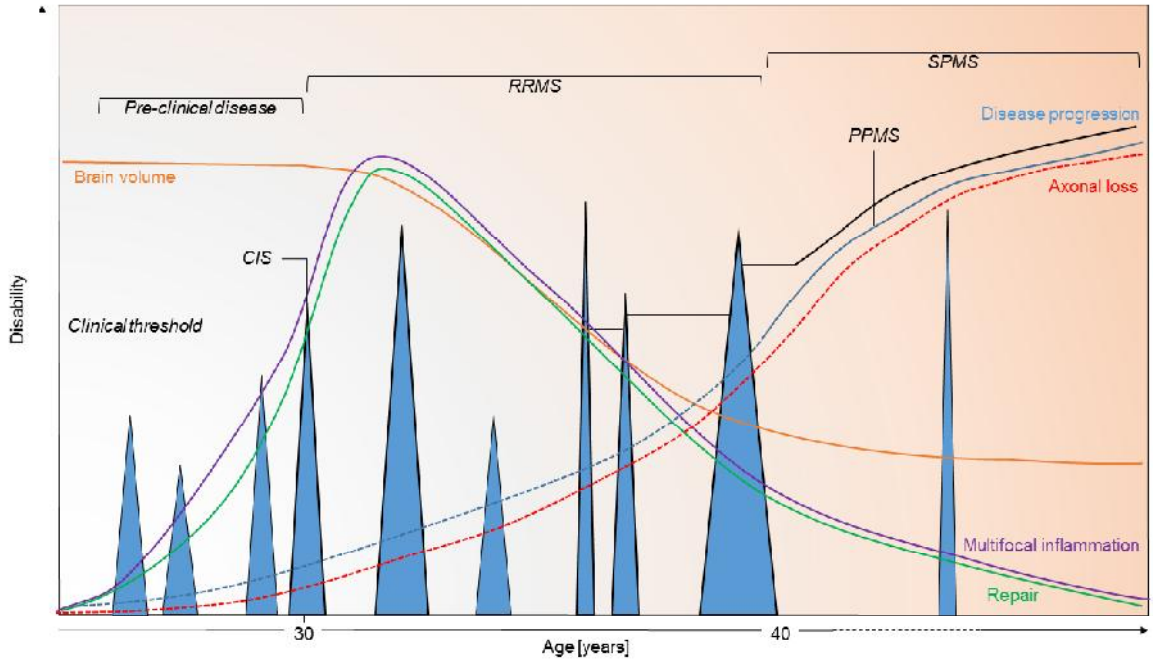


Figure 2. Average disease course in MS.

MS shows a highly variable disease course among patients. On average, patients present with first clinical symptoms around the age of 30, i.e., clinically isolated syndrome (CIS), upon which a period of multiple inflammatory relapses (**blue pyramids**) and successive remitting phases is followed in 85 % of those affected (relapsing remitting MS, RRMS). Most patients will develop secondary progressive MS (SPMS) in the following two decades, showing gradual disease progression, either with or without the occurrence of additional relapses. A minority of patients present with a progressive form from onset on, known as primary progressive MS (PPMS).

In all patients, increasing disease progression (**blue line**) is accompanied by increasing axonal loss (**red dashed line**), neurological and clinical disability (**black line**), and lesion load. Brain volume (**orange line**) is decreasing over time. Even though multifocal inflammation (**purple line**) subsides over time, also repair mechanisms (**green line**) cease.

Due to the use of the criteria, diagnosis of MS became more rapid, with optimised specificity and sensitivity (Brownlee *et al.*, 2015; Sati *et al.*, 2016), thereby allowing for better counselling of patients and appropriate disease management early on – though the McDonald criteria are not treatment guidelines (Thompson *et al.*, 2018). The latest guidelines are primarily aimed at diagnosing when disease in patients experiencing CIS converts to definite MS (see **Figure 1** for an overview of requirements). Overall, the diagnosis of MS is based on clinical neurological examination, as well as confirmation of (paraclinical) disease manifestation by magnetic resonance imaging (MRI). Laboratory assessment can support the diagnosis (Polman *et al.*, 2011; Kamm, Uitdehaag and Polman, 2014).

Table 1. Schematic overview of the McDonald 2017 criteria for the diagnosis of MS.

No. of clinical relapses	No. of lesions with objective clinical evidence	Additional requirements for MS diagnosis
≥ 2	≥ 2	None
≥ 2	1	DIS
1	≥ 2	DIT or CSF-specific OCB
1	1	• DIS and • DIT or CSF-specific OCB
0	0	**PPMS:** • 1 year disability progression and 2 of the following: • DIS by MRI with ≥ 1 brain lesion; • DIS by MRI with ≥ 2 spinal cord lesions; • CSF-specific OCB

Depending on the previous occurrence of clinical relapses and evidence of clinically objective T2-hypersensitive lesions characteristic of MS, additional information is required to make a diagnosis of definite MS according to the McDonald guidelines. In patients presenting with a clinical relapse at onset, requirements include the determination of DIS and DIT or CSF-specific OCB. Requirements for patients independent of relapses and clinically relevant lesions are relevant for the diagnosis of progressive MS. CSF = cerebrospinal fluid; DIS = dissemination in space; DIT = dissemination in time; MRI = magnetic resonance imaging; MS = multiple sclerosis; OCB = oligoclonal bands; PPMS = primary progressive MS.

Clinical relapses

A **relapse –** also called clinical attack or exacerbation **–** is defined according to the guidelines as follows.

> ***"****A monophasic clinical episode with patient-reported symptoms and objective findings typical of multiple sclerosis, reflecting a focal or multifocal inflammatory demyelinating event in the CNS, developing acutely or subacutely, with a duration of at least 24 h, with or without recovery, and in the absence of fever or infection"* (Thompson *et al.*, 2018).

The occurrence of a relapse, i.e., *clinically active disease*, can be confirmed by a clinical neurological examination.

Disease progression

Disease progression is defined as a more gradual deterioration of signs and symptoms of physical or cognitive disability confirmed over a given period of time, often minimally six months (Constantinescu *et al.*, 2011; Kamm, Uitdehaag and Polman, 2014; Filippi *et al.*, 2018).

Physical disability

A patient's **physical disability** is usually determined using Kurtzke's Expanded Disability Status Scale (**EDSS**), a distribution of disease severity ranging from 0 (normal neurological exam) to 10 (death due to MS), divided by half-point intervals, and dependent on the number of functional systems (FS) affected (total of eight systems) (Kurtzke, 1983; Kamm, Uitdehaag and Polman, 2014). In a recent study, it was confirmed that the time to reach definite EDSS progression by one step varies strongly along the EDSS scale, and further appears to be dependent on a patient's individual baseline EDSS score as well as the FS scale affected (Zurawski *et al.*, 2019). The concepts of disability and disease progression in MS are currently still under development.

Recently, upon determination of confirmed disability accumulation (CDA) by means of EDSS, it was considered relevant to make a discrepancy between relapse-associated worsening (RAW) and progression independent of relapse activity (PIRA), particularly in RRMS. This subdivision distresses the relevance and challenges to improve distinction of relapsing and progressive disease courses and to determine treatment success (Kappos *et al.*, 2020; Lublin *et al.*, 2022; Ransohoff, 2023).

Progressive disease courses

In case a **progressive disease** is concerned, it is important for disease management purposes, to distinguish between the primary and secondary disease course: **PPMS** is present from onset on and a diagnosis can be made equally to RRMS, i.e., according to dissemination revealed by MRI combined with clinical confirmation of disability progression; **SPMS** follows an initial RRMS phase as diagnosed with the given criteria in **Figure 1**, with addition of the definition for disease progression. Progressive disease can exist with or without superimposed relapses (Lublin *et al.*, 2014; Thompson *et al.*, 2018).

Disease dissemination

Upon a first relapse, i.e., in patients with CIS, **dissemination in space** (**DIS**) and **time** (**DIT**) must be demonstrated in order to diagnose MS (Thompson *et al.*, 2018).

DIS can be demonstrated either by the occurrence of an additional clinical relapse that indicates a lesion located at a distinct CNS site, or by means of MRI. Imaging requirement is the demonstration of at least one T2-hyperintense lesion, either symptomatic or asymptomatic, in minimally two out of four *MS-characteristic regions of the CNS* (i.e., periventricular, (juxta)cortical, infratentorial or in the spinal cord) (Thompson *et al.*, 2018).

DIT can be assured by confirming an additional relapse in general, or by MRI: demonstrating simultaneous gadolinium (Gd)-enhancing and non-enhancing lesions (symptomatic or asymptomatic) at any time, or the presence of new/enlarging T2-hyperintense or contrast-enhancing lesions on a follow-up scan compared to a baseline MRI at any time (Thompson *et al.*, 2018).

Cerebrospinal fluid

Oligoclonal bands

Cerebrospinal fluid (CSF) **oligoclonal bands** (**OCB**) are the gold standard to *qualitatively* indicate intrathecal immunoglobulin (Ig) synthesis, typically IgG. OCB can be seen on sample protein blots by protein electrophoresis or isoelectric focusing with subsequent immunoblotting or silver staining (**Figure 3**). OCB present only in CSF opposed to serum can identify an intrathecal B cell response indicative of CNS inflammation (Link and Huang, 2006). Even though CSF restricted OCB are not MS-specific, they can be found in 90–95 % of MS patients (Karamehic *et al.*, 2012; Stangel *et al.*, 2013; Deisenhammer *et al.*, 2019). The bands are a long-term reflection of non-specific immune stimulation and, therefore, stay stable over time, both regarding number and arrangement (Karamehic *et al.*, 2012).

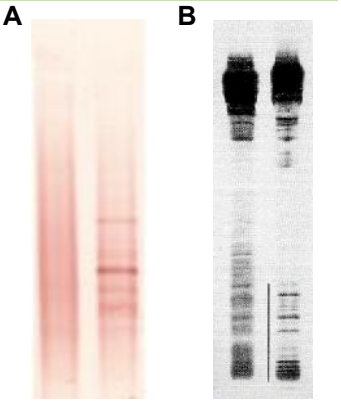


Figure 3. Oligoclonal bands.
OCB are visualised on protein blots with subsequent **(A)** immunoblotting or **(B)** silver staining. OCB are present in CSF (right blots) and not in serum (left blots).

OCB positivity varies in CIS patients (Dobson *et al.*, 2013; Petzold, 2013) depending on their baseline MRI, where it is higher in patients with high- as compared to low-risk MRI (stratified by lesion numbers or other characteristics), suggesting a prognostic value of OCB (Tintoré *et al.*, 2008). OCB positivity was also directly related to an increased risk for clinical conversion from CIS to CDMS (Kuhle *et al.*, 2015) and disease progression (Presslauer *et al.*, 2014). Since the last revision of the McDonald criteria, in the event that a second clinical relapse does not occur and the DIT requirements cannot be

met by MRI, demonstration of CSF-specific OCB positivity may officially suffice as a substitute for the diagnosis of MS (in the absence of MS-atypical CSF findings) (Thompson *et al.*, 2018).

Albumin quotient

An alternative method for demonstrating intrathecal Ig synthesis is the *quantitative* assessment of CSF and serum IgG, A and M (determined by means of nephelometry). Combined with the albumin quotient ($\mathbf{Q_{alb}}$), the resulting Ig index (see equations 1.1 and 1.2 below) may ascertain intrathecal Ig synthesis by the use of a Reibergram (normal reference value for IgG index < 0.7 (Link and Tibbling, 1977)) (Hische, Helm and Walbeek, 1982; Andersson *et al.*, 1994; Polman *et al.*, 2011; Karamehic *et al.*, 2012; Kamm, Uitdehaag and Polman, 2014). However, this result is not as sensitive as the demonstration of (two or more) CSF-specific OCB (Andersson *et al.*, 1994; Thompson *et al.*, 2018).

$$\text{Qalb} = \frac{[\text{albumin}]\text{CSF}}{[\text{albumin}]\text{serum}} \qquad [1.1]$$

$$\text{Ig index} = \frac{[\text{Ig}]\text{CSF}}{[\text{Ig}]\text{serum}} / \text{Qalb} \qquad [1.2]$$

Other CSF values

Routine diagnostic findings might further include a slightly increased white cell count in the CSF, although a deviated albumin quotient indicating BBB disturbance is not common in MS patients (Hische, Helm and Walbeek, 1982; Freedman *et al.*, 2005; Link and Huang, 2006; Polman *et al.*, 2011; Karamehic *et al.*, 2012; Kamm, Uitdehaag and Polman, 2014). In case OCB are negative or other (CSF) tests are atypical for MS, possible differential diagnoses should be considered (Thompson *et al.*, 2018).

MS treatment

Therapy availability

Immunomodulatory therapies

Depending on a patient's clinical course, disease stage, and disease activity, a variety of disease-modifying (immunomodulatory or immunosuppressive) and symptomatic therapies is available in the management of MS. The first modern **disease-modifying therapies** (**DMTs**) in MS became available in the 1990s (Compston and Coles, 2008; Hegen, Auer and Deisenhammer, 2016; Saleem *et al.*, 2019; Hemmer, 2021). Currently, a total of 17 DMTs have been approved for MS in the EU, and development towards new medicines is still ongoing.

Most treatment options exist only for relapse dominated cases (CIS, RRMS and incidental SPMS), although the number of available DMTs for progressive disease is emerging as well since the last decades (The Lancet Neurology, 2012; Hemmer, 2021; Wiendl *et al.*, 2021). For an overview of the most commonly used DMTs and their working mechanisms in MS, see **Table 2**.

Escalation therapy

Therapies are divided in first, second and third (or higher) line treatments, which guides physicians to use the least potent medication necessary to suppress the immune system, while preventing severe/serious side-effects. Upon onset of relapsing MS, **first-line** (mild) immunomodulators are the preferred choice for treatment (Hemmer, 2021; Wiendl *et al.*, 2021). In case mild to moderate therapy is proven to work insufficiently – i.e., disease activity persists or recurs despite treatment – guidelines suggest to switch to either **high-efficacy** first-line, or an even more potent **second- or third-line** therapy (**escalation therapy**). These therapeutics include immunomodulatory and -suppressive drugs that, among other effects, inhibit peripheral leukocytes from crossing the BBB and prevent lymphocytes from exerting inflammatory effects (Freedman *et al.*, 2013, 2020; Brimnes *et al.*, 2014; Kamm, Uitdehaag and Polman, 2014; Saleem *et al.*, 2019; Hemmer, 2021; McGinley, Goldschmidt and Rae-Grant, 2021; Wiendl *et al.*, 2021). Higher-efficacy DMTs also come with higher risk to develop severe and serious adverse events (AEs), e.g., the occurrence of opportunistic diseases like the JC virus-induced progressive multifocal leukoencephalopathy (PML) (Berger, 2017; Freedman *et al.*, 2020; McGinley, Goldschmidt and Rae-Grant, 2021; Wiendl *et al.*, 2021).

Supplementary therapy

Supplementary to DMTs, in the course of an acute relapse or its successive remission, **corticosteroids** can be taken temporary to counteract the acute inflammation, commonly for the duration of a number of days (1,000 mg/day methylprednisolone pulsed for 3–5 days, with or without oral tapering). **Symptomatic treatment** (medicines and/or physical and occupational therapies) can be prescribed to address a variety of neurologic sequelae occurring in all disease courses, e.g., fatigue, depression, spasticity, pain, and disorders of the urogenital and intestinal systems (Kamm, Uitdehaag and Polman, 2014; Hemmer, 2021; Wiendl *et al.*, 2021). Vitamin D is prescribed in MS patients as supplementary therapy, albeit still exploratory, as the clinical benefits have not been proven consistently (Jagannath *et al.*, 2018; Ismailova *et al.*, 2019; Waubant *et al.*, 2019; Hemmer, 2021).

Treatment response

Early vs. progressive disease

Starting appropriate treatment already in early phases of CIS and MS reduces the chance of conversion from CIS to CDMS as well as the long-term risk of developing permanent disability when compared to later onset of therapy (Kamm, Uitdehaag and Polman, 2014; Sorensen, 2014). Of importance, efficacy of *anti-inflammatory* therapies is limited to the early phases of the disease. During progressive disease, neurodegenerative processes causing disability progression become more prominent, while the extent of inflammation declines and the disease becomes compartmentalized within the CNS behind a less permeable BBB, where most current therapeutics are ineffective (Lassmann, Brück and Lucchinetti, 2007; Lassmann, Van Horssen and Mahad, 2012; Dendrou, Fugger and Friese, 2015; Cree *et al.*, 2021).

Non-consent treatment success

Since there is currently no consensus on a shared definition of treatment response, the choice of DMT in the clinic is not standardized and necessarily evidence-based. A definition of *non*-response is based on three outcome measures: disability progression, occurrence of clinical relapses, and presence of active lesions on MRI (Gasperini *et al.*, 2019). For monitoring treatment response, the current methods fall short: clinical and radiological measures lack sensitivity, have poor predictive value, and time it takes to notice definitive disease progression limits their use further (Ibitoye and Rice, 2016; Freedman *et al.*, 2020). Exact criteria for these parameters and the time frame in which they are assessed also vary widely across studies (Gasperini *et al.*, 2019). Consensus on a definition of treatment response is imperative, especially with the expanding number of therapeutics available, as it would aid the development towards precision medicine in MS.

Table 2. Summary of the most commonly used disease modifying treatments (DMTs) and other medication in MS and their main working mechanisms and (adverse) effects (Saleem *et al.*, 2019; Hemmer, 2021; McGinley, Goldschmidt and Rae-Grant, 2021; European Multiple Sclerosis Platform, 2022).

Category	Generic name	Mechanism of action & effect	Adverse effects (AE)
First-line (efficacy: relative relapse reduction compared to placebo 30–50 %)	Interferon beta (1a and 1b, incl. peginterferon) (RRMS and SPMS)	Immunomodulatory: o Inhibits T cell activation o Decreases matrix metalloproteinase activity o Decreases pro-inflammatory cytokines o Increases suppressor T cell activity → anti-inflammatory cytokines production Effect: o Improved clinical (relapse rate, disability, disease progression) and radiological findings	Common AE: o Headache, Flu-like symptoms, Injection site reaction, Leukopenia Rare serious AE: o Liver toxicity
	Teriflunomide	Immunomodulatory: o Inhibits Pyrimidine synthesis o Reduces mitochondrial enzyme dihydroorotate dehydrogenase activity → inhibition pyrimidine and T cell proliferation biosynthesis o Disrupts interaction T cells and antigen-presenting cells Effect: o Improved relapse rate, disability, MRI disease activity	Common AE: o Headache, Increased liver enzymes, Diarrhoea, Nausea, Alopecia Rare serious AE: o Hepatotoxicity, Teratogenicity
	Glatiramer acetate	Immunomodulatory/neuroprotective: o S1P receptor modulator/agonist o Binds antigen-presenting cells o Drives B cells, monocytes, dendritic cells towards anti-inflammatory responses o Induces anti-inflammatory T cells (Treg, Th2, Th3) o Downregulates pro-inflammatory T cells (Th1, Th17) o Secretes neurotrophic factors → remyelination	Common AE: o Injection site reaction, Immediate post-injection reaction Rare serious AE: o Skin necrosis

Category	Generic name	Mechanism of action & effect	Adverse effects (AE)
		Effect: o Improved clinical (relapse rate, cognition, fatigue, quality of life, stabilised disability) and radiological findings	
	Dimethyl fumarate (Diroximel fumarate)	Neuroprotective/anti-inflammatory: o Activates Nrf2 pathway Effect: o Improved relapse rate and radiologic findings	Common AE: o Skin flushing, Diarrhoea, Nausea, Abdominal pain, Vomiting Rare serious AE: o Infections, Liver toxicity, Lymphopenia, PML
Second line (efficacy: relative relapse reduction compared to placebo 50–60 %)	Cladribine (RRMS and SPMS)	Immunosuppression: o Purine antimetabolite → decreases lymphocyte count Effect: o Improved relapse rate, disability progression and MRI lesions	Common AE: o Upper respiratory tract infection, Headache, Lymphopenia, Nausea, Back pain Rare serious AE: o Malignancy, Teratogenicity, Pulmonary tuberculosis, Herpes infections, PML
	Fingolimod Ozanimod Ponesimod Siponimod (RRMS and SPMS)	Immunomodulatory: o S1P receptor modulator/agonist → decreased egression from / sequestrated lymphocytes in the lymph nodes Effect: o Improved relapse rate, disability progression, and MRI findings (number of lesions, Gd-enhanced lesions)	Common AE: o Headache, Liver enzyme elevation, Back pain, Hypertension o Ozanimod: Upper respiratory tract infections, Orthostatic hypotension, Urinary tract infections, Back pain o Ponesimod: Nasopharyngitis Rare serious AE: o Infections, PML, Macular oedema, Liver toxicity, PRES, Hypertension, Bradyarrhythmia, Heart block, Respiratory effects
Third line (efficacy: relative relapse	Alemtuzumab	Immunomodulatory: o Anti-CD52 monoclonal antibody → depletion of CD52-expressing T cells, B cells, natural killer cells, and monocytes Effect:	Common AE: o Rash, Headache, Infusion reactions, Thyroid disorder, Infection, Herpes infection Rare serious AE:

Category	Generic name	Mechanism of action & effect	Adverse effects (AE)
reduction > 60 % compared to placebo, or > 40 % compared to first-line substances)		○ Improved clinical relapse rate and time, and disability	○ Autoimmune conditions (ITP, antiglomerular basement membrane disease, hepatitis), HPV infection, Stroke, Tuberculosis, PML, Malignancy risk potential
	Natalizumab	Immunomodulatory: ○ Anti-α4 integrin receptor monoclonal antibody → decreased adhesion and migration of leukocytes (across the BBB) Effect: ○ Improved relapse rate, disability progression	Common AE: ○ Headache, Fatigue, Arthralgia, Abdominal discomfort, Urinary tract infections, Lower respiratory tract infection Rare serious AE: ○ PML, Hepatotoxicity, Herpes infections, Hypersensitivity reactions
	Ocrelizumab (Rituximab) (off-label) Ofatumumab	Immunomodulatory: ○ Anti-CD20 monoclonal antibody	Common AE: ○ Infusion reactions, Upper respiratory tract infections, Herpes infections ○ Ofatumumab: Headache Rare serious AE: ○ Hepatitis B reactivation, PML ○ Ocrelizumab: Malignancy risk potential ○ Ofatumumab: Reduction in immunoglobulins
Other	Corticosteroids (Myhr and Mellgren, 2009; Bisaga *et al.*, 2012)	○ Anti-inflammatory, anti-oedema, membrane-stabilising (including the BBB) Decreases infiltration of inflammatory cells into the CNS	
	Vitamin D (Jagannath *et al.*, 2018)	○ Inconclusive	
	Fampridine	○ Symptomatic	
	Cannabidiol / delta-9-tetrahydrocannabinol	○ Symptomatic	

BBB = blood-brain barrier; CNS = central nervous system; Gd = gadolinium; MRI = magnetic resonance imaging; Nrf2 = Nuclear factor erythroid 2-related factor 2; PML = Progressive multifocal leukoencephalopathy; RRMS = relapsing-remitting multiple sclerosis; S1P = spoingosine-1-phosphate; SPMS = secondary progressive multiple sclerosis.

Biomarkers in MS

Unmet clinical need

An interaction between multi-factorial pathophysiological mechanisms underlies the highly heterogeneous character of MS in terms of clinical and radiological aspects, and treatment response (Lublin and Reingold, 1996; Lassmann, Brück and Lucchinetti, 2001; Weiner, 2009; Grigoriadis and van Pesch, 2015). Neuropathological research has been revolutionised over the last century, and modern techniques as immunohistochemistry and MRI have strongly improved our understanding of the aetiology and pathogenesis of the disease (Lassmann, 2014; Reich, Lucchinetti and Calabresi, 2018). Still, currently used diagnostic and prognostic parameters in MS can only partially predict the long-term course or treatment response (Polman *et al.*, 2011; Kamm, Uitdehaag and Polman, 2014).

Characterising the heterogeneity of the course and symptomatology in MS with more accuracy may improve our understanding of pathophysiological mechanisms and therapeutic targeting (Dendrou, Fugger and Friese, 2015). In order to capture the complexity more comprehensively, to refine the diagnostic criteria and to enable personalized medicine in clinical practice, the availability of reliable and characteristic biomarkers is highly needed (Derfuss, 2012; Comabella and Montalban, 2014; Thompson *et al.*, 2018). Over the last years, the research towards biomarkers in MS has been highly active. Nevertheless, only very few markers have made it into the clinic yet (Comabella and Montalban, 2014). For exploratory and subsequently validated biomarkers, increasing strength of evidence needs to be collected.

Biomarker definition and classification

The *Biomarkers Definitions Working Group* has defined a **biomarker** (portmanteau of "biological marker") as follows.

> *"A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"* (Biomarkers Definitions Working Group., 2001).

Comparable definitions have been given by the *World Health Organization* (*WHO*) (Strimbu and Tavel, 2010). Thus, a biomarker is any quantifiable characteristic of biological processes intended to determine its relationship to relevant clinical endpoints, making them **surrogate endpoints** (when used in clinical trials).

Applicability

The **applicability** of biomarkers can be manifold, with specific to MS: **1)** prediction of the risk to develop MS, **2)** diagnostic purposes, **3)** identification of disease activity (purposing the distinction between disease phases, determination of disease course transition or treatment requirements), or **4)** prediction or monitoring of treatment response (Biomarkers Definitions Working Group., 2001; Comabella and Montalban, 2014). All categories suggest also a *prognostic value* of the biomarkers they contain. Alternative classifications of MS biomarkers could be applied, e.g., including subgroups of disease activity and phenotypical expression, or according to the pathophysiological implication on MS pathogenesis. The latter approach could divide biomarkers into subgroups of (immuno)genetics, all other laboratorial body fluids, and imaging (Katsavos and Anagnostouli, 2013). Overall, the use of biomarkers could be beneficial in disease management in individual patients, for example in decision-making on escalation or discontinuation of treatment (Mehr and Zimmerman, 2015).

Biomarker validation

For a biomarker to be validated and applicable as surrogate endpoint in clinical practice, its clinical relevance needs to be evaluated in a trajectory of epidemiological, therapeutic and/or pathophysiological studies, clinical trials (phase 1–4), and finally meta-analyses (Bielekova and Martin, 2004; Strimbu and Tavel, 2010). The idea that one best fitting marker exists for every single situation is improbable; ideally, clinico-radiological data and situationally appropriate biomarkers are used in combination in an integrated prognostic model of disease outcome (Bielekova and Martin, 2004; Comabella and Montalban, 2014).

Imaging biomarkers in MS

As seen from the McDonald criteria, in addition to the clinical presentation of the disease, MRI is of the utmost importance in diagnosing MS (Mcdonald *et al.*, 2001; Polman *et al.*, 2005, 2011; Thompson *et al.*, 2018), but it has also taken its place in monitoring disease activity and treatment response (M Filippi *et al.*, 2016; Filippi, Preziosa and Rocca, 2018; Gasperini *et al.*, 2019). A distinction is made between *conventional* and *non-conventional* MRI measures.

Conventional MRI

A limited but powerful number of conventional MRI metrics is available in clinical practice to detect (sub)clinical pathological processes in MS that provide a good surrogate for clinical measures like relapse rate and disability progression (Filippi, Preziosa and Rocca, 2014; Wattjes *et al.*, 2021). MRI is used in a first stage for diagnostic purposes and to set a prognosis

(Thompson *et al.*, 2018; Wattjes *et al.*, 2021). During follow-up of individual patients, imaging is used continuously in disease management decision making.

Lesional imaging

New or enlarging MS-specific **T2-hyperintense lesions** can assure disease activity (ongoing inflammation), **Gd contrast-enhanced lesions** on T1-weighted images are a sign for BBB disruption and active inflammation with immune cell infiltration at that site (Lassmann, Brück and Lucchinetti, 2007; Larochelle, Alvarez and Prat, 2011; Brimnes *et al.*, 2014; Filippi, Preziosa and Rocca, 2014; Sorensen, 2014). MRI-based evidence of *active lesions* is detected five to ten times more frequently than clinically symptomatic relapses (Gasperini *et al.*, 2019), making MRI indispensable in MS monitoring. **T1-*hypo*intense lesions**, also known as 'black holes', are considered an indicator of chronic axonal loss and neurodegeneration and used to determine disease activity as well (see **Figure 4**) (Sahraian *et al.*, 2010).

Imaging concepts of disease activity

Disease activity in MS is of interest to record over time, as it affects treatment management and prognosis (Filippi, Preziosa and Rocca, 2014; Sorensen, 2014). MRI parameters of disease activity are commonly used in clinical trials as outcome measure. As study endpoint, a variety of new definitions have been proposed over the last years, including **'no evidence of disease activity'** (**NEDA**), a concept defined by the absence of three radiological (active lesions) and clinical (relapses, disability) components of disease activity (NEDA-3), or also including the evaluation of atrophy as fourth component (NEDA-4) (Freedman *et al.*, 2005; Giovannoni *et al.*, 2015; Stangel *et al.*, 2015; Ziemssen *et al.*, 2015; Kappos *et al.*, 2016; El Najjar *et al.*, 2020; Prosperini *et al.*, 2021). In research towards the prognostic accuracy of NEDA, RAW and PIRA concepts have been taken into account as endpoints (Prosperini *et al.*, 2021). Alternative approaches like 'minimal evidence of disease activity' (MEDA), 'no evidence of progression' (NEP) or the extended 'NEP or active disease' (NEPAD) have been under investigation as well (El Najjar *et al.*, 2020; Prosperini *et al.*, 2020).

Non-conventional MRI

Advanced or non-conventional MRI techniques allow to more specifically image pathophysiological aspects of the disease; although, they are currently mainly used in research (Sahraian *et al.*, 2010; Enzinger *et al.*, 2015).

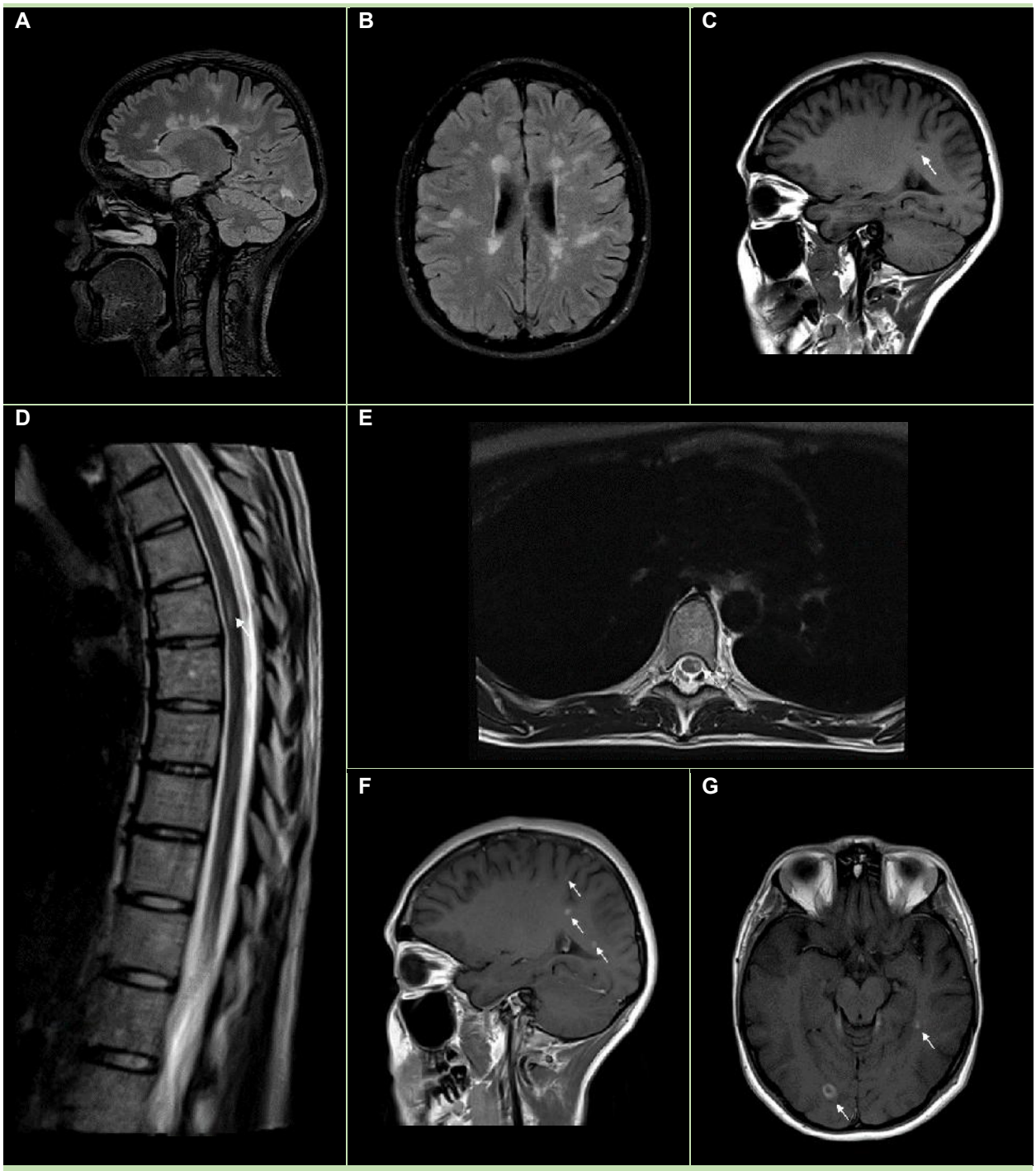


Figure 4. Typical 3T brain and spinal cord MRI features exemplary for MS.

Images were obtained from a 23-year old female patient with MS. FLAIR images demonstrate multiple juxtacortical hyperintense lesions and Dawson's fingers around the ventricles (**A**, sagittal view), multiple juxtacortical, periventricular and hemispheric T2 hyperintense lesions (**B**, axial view). T1 weighted images reveal hypointense lesions, i.e., 'black holes' on sagittal view (**C**, arrow). T2-weighted images show hyperintense thoracal spinal cord lesions (**D**, sagittal view; **E** axial view, arrow). T1-post-gadolinium images demonstrate contrast-enhancing hyperintense lesions juxtacortical frontal right and white matter lesions parieto-occipital right (**F**, sagittal view, arrow), and contrast-enhancing hyperintense lesions adjacent to the posterior horn of the left lateral ventricle and occipital right (**G**, axial view, arrow).

Measures of atrophy

Atrophy – pathologically proven, permanent tissue loss **–** is associated with (though not equal to) brain and spinal cord volume loss seen on MRI, which can be assessed in separate MS-specific regions. Atrophy occurs in all stages of disease, from early phases on, and is closely related to disability in MS (Sastre-Garriga *et al.*, 2020).

Grey matter imaging

Imaging of **grey matter pathology –** either lesions or atrophy and both cortical as well as in the deep grey matter, seen from early disease stages onwards **–** is possible by various MRI metrics and of increasing importance in disease monitoring in MS (see **Figure 4**) (Calabrese *et al.*, 2007; Jehna *et al.*, 2015; Magliozzi, Reynolds and Calabrese, 2018; Filippi *et al.*, 2019).

Normal-appearing brain matter

With the use of several explorative, novel MRI sequences, the diffuse abnormalities seen in NABM (NAWM and NAGM) could be visualised (Mangia *et al.*, 2014; Chen *et al.*, 2017; Tavazzi *et al.*, 2020).

Clinical applicability of MRI

Current clinically applied MRI metrics in MS assess **focal lesions**, i.e., the inflammatory component of the disease. Interpretation of MRI measures and application of the diagnostic criteria need to be appropriate in order to prevent misdiagnosis of MS (Thompson *et al.*, 2018; Filippi *et al.*, 2019; Wattjes *et al.*, 2021). In order to further improve the association of MRI metrics with heterogeneous clinical and prognostic measures, development of more refined techniques is ongoing.

MRI in relation to clinical data

In MS research, often there appears to exist only a weak correlation of MRI with clinical (and cognitive) outcome measures, a phenomenon known as the *(cognitive)* **clinico-radiological paradox** (Barkhof, 2002; Hackmack *et al.*, 2012; Johnen *et al.*, 2019). Several **confounders** (inappropriate clinical ratings, poor magnetic strength, neglect of spinal cord involvement, underestimation of contribution of NABT and cerebral plasticity, lack of histopathological specificity, neglect of disease burden, neglect of lesion topography) contribute to this assumed dissociation between clinical and radiological measures. In fact, if these confounders are addressed for, the lack of correlation indeed appears to be a paradox, and suitable MRI

techniques could function as surrogate outcome measure and prognosticator in MS (Barkhof, 2002; Hackmack *et al.*, 2012; Mollison *et al.*, 2017; Uher *et al.*, 2018; Johnen *et al.*, 2019; Hartmann *et al.*, 2023).

Future directions in clinical MRI

Recently, it was proposed to implement several MRI measures of **atrophy** into daily clinic to indicate diffuse neurodegeneration that is (partially) independent of inflammatory processes. Increasing evidence exists that brain and spinal cord volume measures are relevant in early treatment response-evaluation and disease prognosticating, both key issues in disease management (Sastre-Garriga *et al.*, 2020). Nevertheless, atrophy measures are not implemented in the radiological routine on an individual patient level yet, due to currently insufficient evidence and issues considering technical as well as practical recommendations (Wattjes *et al.*, 2021).

Body fluid biomarkers in MS

Choice of body fluid

Apart from clinical and radiological information, body fluids can serve as valuable source for biomarkers in MS.

Cerebrospinal fluid

In neurological disease, the **CSF** is a logical first choice for biomarker research, as it is the body fluid in closest proximity to the CNS, therefore levels of CSF biomarkers might reflect alterations of local biochemical pathology, which in turn could provide important information for disease management (Teunissen *et al.*, 2009, 2015; Katsavos and Anagnostouli, 2013). The relevance of the CSF in MS is also reflected in its use as a diagnostic tool under the latest McDonald criteria (Thompson *et al.*, 2018). Nevertheless, CSF is obtained by lumbar puncture (LP), which is more invasive and with a relatively higher safety risk to the patient than, e.g., blood or urine sampling (Teunissen *et al.*, 2009), and frequent LP is not desirable.

Blood

Blood seems to be the favoured body fluid in biomarker research in MS due to the lower safety limitations and ease of sampling, and due to the paralleled findings for many molecules in blood to CSF resulting from a direct flux between the CSF and CNS through the blood-CSF barrier and BBB, respectively (Reiber, 2003). Other fluids like urine, saliva and tears are used

in biomarker trials as well, although even scarcer in MS than in other diseases (Comabella and Montalban, 2014).

Intrathecal immune activity in diagnosis – Immunoglobulin free light chains

CSF OCB alternative

As described earlier, the presence of CSF restricted IgG OCB is considered a hallmark of MS and is currently included in the diagnostic guidelines (Thompson *et al.*, 2018). Nevertheless, the use of OCB has several drawbacks: I) analysis is quite laborious and requires experienced laboratory technicians/personnel, II) the result is a qualitative measure, and III) CSF OCB positivity is not specific for MS and can occur in several other neurological disorders (Kaplan *et al.*, 2010; Senel *et al.*, 2014). In contrast, quantification of *immunoglobulin free light chains (FLC)* is easy and yields results with comparable accuracy to those of OCB. Consequently, FLC have received great attention in recent years to serve as a possible alternative to OCB.

FLC kappa and lambda

Human IgG molecules are composed of covalently bound chains, two *heavy* and two *light* of either **kappa** or **lambda** isotype (see **Figure 5**). Immunoglobulin synthesis is performed by B lymphocytes (plasma cells). Under normal conditions, an excess of light chains is produced, of which a majority will bind to the heavy chains, and a resulting unbound fraction will circulate as so-called **free light chains** (**FLC**) (Kaplan, Livneh and Sela, 2011; Kaplan *et al.*, 2013). Renal clearance of FLC from serum is performed with a half-life of 2–6 hours (Kaplan, Livneh and Sela, 2011; Duranti *et al.*, 2013; Hassan-Smith *et al.*, 2014). Due to the small molecular weight of FLC (around 25,000 Da), usually within several hours, an equilibrium between the CSF and serum would be reached. However, resulting an excessive intrathecal synthesis of FLC, accumulation occurs, which can be detected for prolonged periods of time (Konen *et al.*, 2021). Quantification of both FLC kappa (KFLC) and lambda (LFLC) in body fluids can be performed by nephelometry (or turbidimetry), a rapid, semi-automated method that is easy to handle.

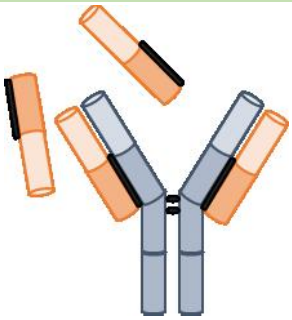


Figure 5. Immunoglobulin free light chains. Immunoglobulin G molecules are composed of two heavy (blue) and two light chains (pink). When light chains are produced in access, they exist as free light chains of either kappa or lambda isotype.

FLC in MS

Previous to the investigations as part of this dissertation, elevated CSF FLC levels were measured in MS patients with a proven comparable or superior diagnostic sensitivity and specificity to OCB (Arneth and Birklein, 2009; Kaplan *et al.*, 2010, 2013; Duranti *et al.*, 2013; Katsavos and Anagnostouli, 2013; Comabella and Montalban, 2014; Hassan-Smith *et al.*, 2014; Presslauer *et al.*, 2014, 2015; Senel *et al.*, 2014). Besides, the potential additional prognostic value of FLC in MS has been investigated (Desplat-Jégo *et al.*, 2005; Villar *et al.*, 2012; Presslauer *et al.*, 2014). Increased CSF KFLC have been related to the risk of future CIS-CDMS conversion in one trial (Villar *et al.*, 2012), though patients in the non-converter subgroup were largely OCB negative. Therefore, the apparent predictive capacity of KFLC there, may have reflected only that of OCB. Further, only a few studies included LFLC next to KFCL in their analyses (Desplat-Jégo *et al.*, 2005; Arneth and Birklein, 2009; Kaplan *et al.*, 2010; Senel *et al.*, 2014; Makshakov *et al.*, 2015). Interestingly, kappa compared to lambda FLC levels were elevated to a greater extent, i.e., the ratio KFLC/LFLC was increased in MS (Kaplan *et al.*, 2010).

From these previous results, FLC were suggested to be used as a compared to OCB non-inferior, easy to perform analysis for intrathecal Ig synthesis in MS diagnostics, with a potential role in prognostication as well. Validation of FLC as marker for both purposes has yet to be concluded before clinical application can be initiated.

BBB disruption – Netrin-1

MRI drawbacks

BBB disruption is a feature of active CNS inflammation, which can result in clinical relapses and/or sub-clinical signs by means of Gd-enhanced T1 lesions on brain and spinal cord MRI. With respect to imaging, (Gd-enhanced) MRI examinations are highly time consuming and rather costly (Mehr and Zimmerman, 2015). Moreover, Gd was found to deposit in CNS tissue, and although toxicity sequelae are uncertain, it is recommended to restrict Gd usage to what is necessary (Layne, Wood and Dargan, 2020). To resolve these drawbacks, the identification of body fluid biomarkers detecting of BBB disruption or active CNS inflammation – preferentially easily obtainable if used during follow-up, e.g., from serum samples – would be highly beneficial instead.

Netrin-1 in health and MS

Netrin (**NTN**)**-1**, part of a class of laminin-related proteins involved in axon guidance, regulates cell migration and adhesion through chemotropic features (de Castro, 2003; Shekarabi, 2005;

Jarjour *et al.*, 2008). In the CNS specifically, several processes are affected by NTN-1 that influence tissue development and repair, e.g., growth guidance and migration of axons, glial cells, neurons and oligodendroglial precursor cells (de Castro, 2003; Shekarabi, 2005; Jarjour *et al.*, 2008; Lai Wing Sun, Correia and Kennedy, 2011; Cayre *et al.*, 2013; Tepavčević *et al.*, 2014). In patients with MS, as well as in an animal model of MS (experimental autoimmune encephalomyelitis, EAE), it was demonstrated that NTN-1 is upregulated in astrocytes and macrophages. Moreover, recent cell culture and *in vivo* MS mouse model experiments demonstrated that BBB integrity and inflammation at site are importantly regulated by NTN-1, displaying a further association of NTN-1 with MS pathology (Podjaski *et al.*, 2015).
Data on NTN-1 assessed in body fluids of MS patients are scarce and contradicting; both increased (Podjaski *et al.*, 2015) as well as decreased (Mulero *et al.*, 2015) serum NTN-1 (sNTN-1) levels were found in MS patients compared to controls. Of interest, the indicated decline was even larger when patients had clinically active disease (acute and up to 60 days post-relapse) (Mulero *et al.*, 2015). If NTN-1 is sensitive to MRI-based measures of disease activity, e.g., Gd-enhancing lesions on T1 weighted images, is so far unknown.

Disease activity – Antioxidative capacity

Issues in monitoring disease activity

Currently, disease activity in MS is monitored both clinically as well as paraclinically through characterisation of subclinical disease related tissue damage by means of MRI (Kaunzner, Al-Kawaz and Gauthier, 2017), albeit with several limitations. Definite progression in clinical outcome measures (clinically symptomatic relapse, confirmed increase in EDSS score) usually is only detected after a prolonged time up to several years of disease, with a lack of sensitivity (Ibitoye and Rice, 2016). The use of increased lesion volume or atrophy seen on longitudinal MRI scans has a poor predictive value for an individual patient in a real-life setting level compared to the highly controlled trials incorporating these analyses as prognostic factor (De Stefano *et al.*, 2010). It would be a great advantage to use body fluid biomarkers related to the pathophysiology of MS purposing the detection and prediction of disease activity.

Oxidative stress & antioxidative capacity in MS

In recent years, it became increasingly evident that **oxidative stress** (**OS**) largely contributes to MS pathophysiology (Besler and Çomoğlu, 2003; Koch *et al.*, 2006; Mirshafiey and Mohsenzadegan, 2009; Haider *et al.*, 2011; Birben *et al.*, 2012; Stephenson *et al.*, 2014; Adamczyk and Adamczyk-Sowa, 2016; Ibitoye and Rice, 2016; Ohl, Tenbrock and Kipp, 2016; Filippi *et al.*, 2018; Lassmann, 2018). OS is defined as an imbalance in the body's oxidation-

reduction (redox) status resulting from an excessive production/accumulation of **oxidants** (reactive oxygen/nitrogen species) (Gray *et al.*, 2008; Mirshafiey and Mohsenzadegan, 2009; Haider *et al.*, 2011; Stephenson *et al.*, 2014; Ibitoye and Rice, 2016; Ohl, Tenbrock and Kipp, 2016; Filippi *et al.*, 2018; Lassmann, 2018) and/or a decreased production/effect of **antioxidants** (Besler and Çomoğlu, 2003; Birben *et al.*, 2012; Adamczyk and Adamczyk-Sowa, 2016). The hereby induced excess in free radicals causes damage to proteins, lipids and DNA, and finally axonal and neuronal death.

A variety of single redox parameters (or their activity) has previously been investigated for their potential role in MS; however, given the complexity of the redox system combined with conflicting results obtained with various test methods, the interpretation of data is limited (Koch *et al.*, 2006; Haider *et al.*, 2011; Ristori *et al.*, 2011; Fischer *et al.*, 2012; Ljubisavljevic, Stojanovic, Vojinovic, Stojanov, Stojanovic, Kocic, *et al.*, 2013; Ljubisavljevic *et al.*, 2014; Karlík *et al.*, 2015; Pasquali *et al.*, 2015; Adamczyk and Adamczyk-Sowa, 2016). A cumulative representation of all (anti)oxidants simplifies this approach and could be beneficial compared to single compound assessments. The body's total ability to counteract deleterious effects of oxidants can be summarised by the **antioxidative capacity** (**AOC**) of all antioxidants combined, which can be determined as such in body fluids (see **Figure 6**). MS-specific alterations of the AOC still remain inconclusive.

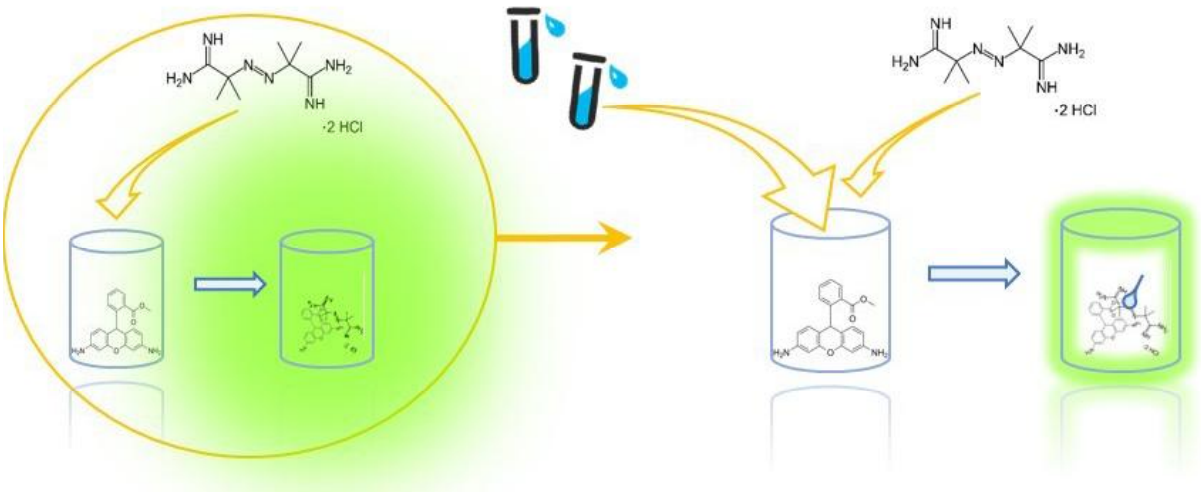


Figure 6. Schematic Antioxidative capacity (AOC) analysis

Buffer containing dihydrorhodamine (DHR) oxidises upon the addition of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), a process which, upon excitation with a wavelength of 485 nm, emits green fluorescent light (wavelength 538 nm) (left circle). AOC of a sample is determined as the sample's ability to inhibit this oxidation process by assessing the relative decrease in fluorescence intensity when the sample is added to the buffer simultaneously (right side).

Risk stratification – CD62L (L-selectin)

Aspects of treatment monitoring

As with all medicines, DMTs in MS can cause various AEs, and especially with higher efficacy DMTs – i.e., escalation therapy – the risk for serious/severe AEs to occur increases (Barbieri *et al.*, 2022). Consequently, next to treatment success with respect to prevention of disease deterioration, risk of serious AE development is important to monitor, e.g., the chance to develop opportunistic infections due to the use of immunosuppressive medication (Scutera *et al.*, 2020). Given the shortcomings of current clinical and radiological methods used in the clinical management of MS, new body fluid markers could be of great value and are highly needed (Ibitoye and Rice, 2016; Freedman *et al.*, 2020; Scutera *et al.*, 2020).

CD62L as MS treatment risk assessment

Previously, the cell adhesion molecule **CD62L** (**L-selectin**, expressed on leukocytes) was suggested to be a potential risk marker for secondary events with the use of several DMTs in MS, as its expression was shown to be decreased under natalizumab and fingolimod (Böhler *et al.*, 2004; Schwab *et al.*, 2013, 2016; Spadaro *et al.*, 2015). Decreased CD62L levels were also associated with the development of the severe demyelinating disease PML, which is caused by the human polyomavirus 2 (JC or John Cunningham virus) and a potential risk with the use of various DMTs in MS, e.g., natalizumab, fingolimod and dimethyl fumarate (Schwab *et al.*, 2013, 2016; Spadaro *et al.*, 2015; Jordan *et al.*, 2022). Currently, pre-analytical steps usually applied for the determination of CD62L in blood samples in studies towards MS are under debate. It is unclear if CD62L assessment yields relevant clinical information when performed on untreated fresh blood samples instead of isolated and cryopreserved peripheral blood mononuclear cells (**PBMCs**) (Lieberman *et al.*, 2016; Schwab *et al.*, 2016).

Data- and biobanking

The final goal of biomarker research focuses on their clinical applicability to aid in an earlier/improved, patient-individualized disease management and can be manifold: enabling earlier, substantiated diagnosis, predicting disease course and activity, ascertain treatment response, or perform risk stratification.

To achieve such ambitious goals at the *Biomarker Research Unit*, we make use of the large capacity of samples and clinical data available at the facilities of the *Medical University of Graz* (*MUG*). The *Department of Neurology* at the *MUG* accommodates a large data- and biobank containing material of over 500 MS and control patients who were regularly seen at the MS outpatient clinic. Additional CSF and serum samples are stored to a large extent in the Biobank

Graz. MRI at 3T of all MS patients is performed regularly as part of clinical management and includes several sequences and post-imaging analyses (among others, structural imaging sequences with subsequent analyses of brain regions and lesions). In addition, clinical data (e.g., disease course, treatment response, physical disability) are recorded over time in a database maintained by the *Institute for Medical Informatics, Statistics and Documentation* (*MUG*). This structured acquisition of biomaterials and data allows for comprehensive studies on the clinical value of body fluid biomarkers in MS.

Aim and outline dissertation

The aim addressed in this dissertation was the evaluation of various body fluid biomarkers in CSF and blood, and, in conjunction with longitudinal clinico-radiological data, assessment of their value in the clinical management of early stages of MS. Biomarkers of choice covered all biomarker types according to the common classification – risk to develop MS, diagnosis of MS, identification of disease activity (pathological processes as seen by clinical or radiological parameters), or treatment response (Comabella and Montalban, 2014). Findings presented in this dissertation are structured per project as follows:

Appendix 1 – Diagnostic and prognostic biomarkers: We aimed to investigate the diagnostic and prognostic value of free light chains (**FLC**) **kappa** and **FLC lambda** in CSF and blood in MS beyond IgG OCB positivity.

Appendix 2 – Disease activity biomarkers – Radiological parameters (BBB disruption): The aim was to determine the potential of netrin (**NTN**)**-1** in blood to detect BBB disruption in MS as seen by Gd-enhanced MRI.

Appendix 3 – Disease activity biomarkers – Pathological processes (Oxidative stress): We assessed the value of antioxidative capacity (**AOC**) in CSF and blood to serve as marker of OS-related pathological processes of MS.

Appendix 4 – Risk stratification biomarkers: We aimed to determine the value of **CD62L** (L-selectin) in blood as risk stratification marker considering methodological aspects and the influence of DMTs used in MS.

Chapter 3 encompasses a discussion per sub-study of this dissertation and places the results in a broader perspective to current literature. An overall conclusion of all studies combined and research outlook are given to close with.

CHAPTER 2

RESULTS

The Introduction and Discussion sections of this dissertation are based on results presented in the original research articles published as part of my PhD studies. For methods and results, please refer to the following publications, which are found in the Appendix of this dissertation:

Appendix 1 – Voortman, MM; Stojakovic, T; Pirpamer, L; Jehna, M; Langkammer, C; Scharnagl, H; Reindl, M; Ropele, S; Seifert-Held, T; Archelos, JJ; Fuchs, S; Enzinger, C; Fazekas, F; Khalil, M. Prognostic value of free light chains lambda and kappa in early multiple sclerosis. Mult Scler. 2016; 23(11):1496-1505; https://doi.org/10.1177/1352458516681503.

Appendix 2 – Voortman, MM; Pekar, T; Bachmayer D; Archelos, JJ; Stojakovic, T; Scharnagl, H; Ropele, S; Pichler, A; Enzinger, C; Fuchs, S; Fazekas, F; Seifert-Held, T; Khalil, M. Serum netrin-1 in relation to gadolinium-enhanced MRI in early multiple sclerosis. Mult Scler J Exp Transl Clin. 2017; 3(3):2055217317727294; https://doi.org/10.1177%2F2055217317727294.

Appendix 3 – Voortman, MM; Damulina, A; Pirpamer, L; Pinter, D; Pichler, A; Enzinger, C; Ropele, S; Bachmaier, G; Archelos, JJ; Marsche, G; Khalil, M. Decreased cerebrospinal fluid antioxidative capacity Is related to disease severity and progression in early multiple sclerosis. Biomolecules 2021; 11(9): 1264; https://doi.org/10.3390/biom11091264.

Appendix 4 – Voortman, MM; Greiner, P; Moser, D; Stradner, MH; Graninger, W; Moser, A; Haditsch, B; Enzinger; C; Fuchs, S; Fazekas, F; Fessler, J; Khalil, M. The effect of disease modifying treatment on CD62L expression in multiple sclerosis. Mult Scler J Exp Transl Clin. 2018; 4(3): 205521731880081; https://doi.org/10.1177/2055217318800810.

CHAPTER 3
DISCUSSION AND CONCLUSION

General objective

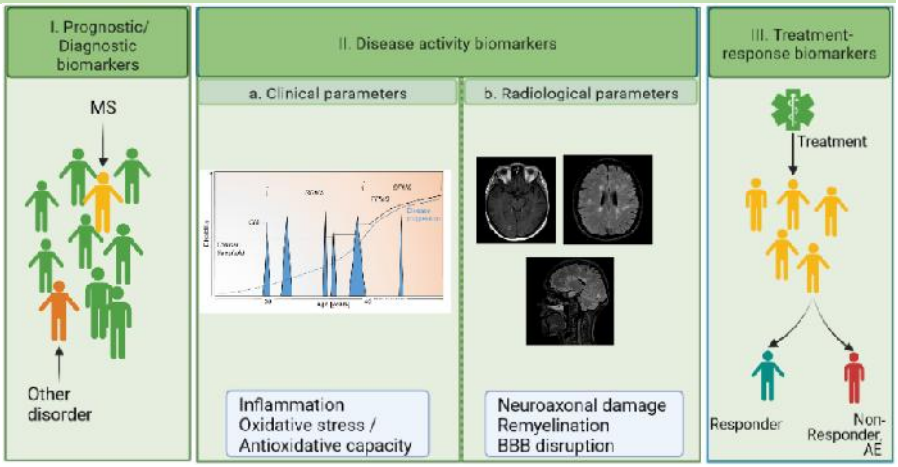


Figure 7. Biomarker types divided by applicability. (Created with BioRender.com.)

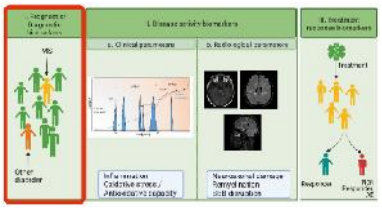
The general objective of this dissertation was the verification of the potential value of various body fluid markers in clinical practice to discriminate MS patients by disease stage and detect or predict disease progression, especially in early disease, i.e., CIS and early RRMS. Biomarkers in MS can be divided into four main groups: prognostic, diagnostic, disease activity (clinical and radiological parameters) and treatment-response biomarkers (**Figure 7**) (Comabella and Montalban, 2014). In this dissertation, we aimed for the investigation of four biomarkers, each attributed to a different biomarker subtype, and to assess their potential value in clinical practice.

1. We investigated the diagnostic and additional prognostic value of quantitative free light chains (FLC) kappa and lambda in cerebrospinal fluid (CSF) and serum beyond the presence of CSF oligoclonal bands (OCB) as marker of intrathecal immune activity in MS.
2. We explored the potential of serum netrin (sNTN)-1 to serve as marker for BBB disruption, or active inflammation, as seen on Gd-enhanced MRI images.
3. We determined to which degree oxidative stress (OS), as measured by reduced antioxidative capacity (AOC) in both CSF and serum, is related to pathophysiological processes in clinically active disease in MS.
4. We investigated methodological aspects and the effect of various treatments on the

CD62L (L-selectin) expression of peripheral blood mononuclear cells (PBMCs) to determine the value of CD62L as risk stratification biomarker for developing adverse effects with the use of disease-modifying therapies (DMTs) in MS.

To determine the potential clinical value of the above-mentioned body fluid markers in MS, we analysed them in relation to detailed longitudinal clinical data as well as conventional and non-conventional MRI metrics.

Intrathecal Immunoglobulin free light chains



Diagnostic value of FLC

In **Appendix 1** we determined CSF and serum FLC kappa and lambda in OCB-positive CIS/RRMS patients compared to controls and investigated their diagnostic/prognostic potential. We found both KFLC and LFLC levels, as well as the KFLC/LFLC ratio to be significantly increased in CSF of CIS/MS patients compared to controls. Serum levels of either FLC were comparable between patients and controls, as a result of which both quotient and index KFLC and LFLC were increased in CIS/MS patients as well. We further determined the diagnostic value of both FLC subtypes separately and combined, and found especially high diagnostic potential for KFLC by applying the threshold for KFLC index ≥ 5.9 (Presslauer's KFLC exponential function (Presslauer *et al.*, 2015)) on our data with comparable sensitivity and specificity values to the use of OCB.

Our data confirm that an increased intrathecal production of both KFLC and LFLC occurs already in early MS, and that their contribution in clinical practice may be of great diagnostic and eventually also of prognostic value (increased risk of CIS-CDMS conversion for patients with CSF KFLC/LFLC ≤ 3.38: HR = 2.89; 95 %-CI = 1.17–7.14; p = 0.016). FLC show high accuracy equal to that of OCB in diagnosing MS, and the quantitative assessment has several advantages compared to OCB determination, as it is easier to perform and less time-consuming.

Upon our work on this smaller cohort, we collaborated in a large multicentre study on both FLC isotypes including 745 patients (CIS/MS n = 242/284, various types of controls n = 219), in which the diagnostic potential of KFLC index was further corroborated (Leurs *et al.*, 2020).

In recent years, research on FLC in MS received a lot of attention and great progress was made. Especially KFLC index has been of interest and shown promising results as marker

to aid in diagnosing MS, with comparable or superior sensitivity/specificity as compared to OCB, also when considering (partly) OCB-negative patients (Hegen *et al.*, 2019; Menéndez-Valladares *et al.*, 2019; Ferraro *et al.*, 2020; Gaetani *et al.*, 2020). In solely OCB-negative MS patients, it has been suggested that KFLC quantification can be more sensitive compared to OCB to indicate an intrathecal immunoglobulin synthesis (Altinier *et al.*, 2019). Moreover, a large single-centre cohort study (CIS/MS *n* = 223, controls *n* = 101) demonstrated superior diagnostic accuracy for KFLC index compared to both OCB as well as IgG index in MS (Rosenstein *et al.*, 2021), and a prospective study on 335 patients (final diagnosis: CIS/RIS *n* = 20, MS *n* = 104, variety of controls *n* = 211) revealed excellent diagnostic accuracy of *all* KFLC metrics comparable to that of OCB (Duell *et al.*, 2020).

Others also calculated corrections of the CSF KFLC and LFLC levels other than for serum (quotient) or Q_{alb} (index): the CSF KFLC/IgG ratio (Vecchio *et al.*, 2020) and the KIgG Index ([KFLC CSF/serum quotient] / [IgG CSF/serum quotient]) (Gudowska-Sawczuk *et al.*, 2020) resulted in a high to superior diagnostic accuracy compared to other FLC measures, respectively, though lower than for OCB. Recent data showed equal diagnostic accuracy for KFLC levels compared to OCB testing in MS (both prospective and retrospective cohorts: MS *n* = 85 and *n* = 70; non-MS: *n* = 615 and *n* = 585, respectively) (Saadeh *et al.*, 2022).

Recently, so-called Reibergrams have been developed for KFLC (Q_{KFLC} vs. Q_{alb}) in order to determine cut-off values for intrathecal FLC synthesis with/without BBB disruption in various MS patient groups, which show high diagnostic performance as compared to the original hyperbolic immunoglobulin Reibergrams (Schwenkenbecher *et al.*, 2019; Rosenstein *et al.*, 2021). The use of Reibergrams was even suggested favourable over the KFLC index, as the Q_{KFLC} appeared to be less susceptible for renal function, which is relevant for FLC clearance (Konen *et al.*, 2021).

Prognostic value of FLC

Our data further indicate a potential prognostic value of FLC, for ‘high’ compared to ‘low’ KFLC/LFLC CSF ratio (median value cut-off) was related to a higher risk for CIS-CDMS conversion (follow-up 4.8, IQR 5.0 years in CIS patients).

A prognostic value of CSF KFLC/LFLC ratio has also been proven in a larger cohort of patients with CIS, RRMS and PPMS patients; although in that study, a relatively low ratio at time of diagnosis predicted higher future disability as measured by EDSS (Rathbone *et al.*, 2018). Patients in that study had a relatively higher CSF KFLC/LFLC ratio, though they were also older and had more severe disability than in our cohort, which could declare the discrepancy to our results. Even though not statistically significant, results also indicated

towards more frequent CIS-MS conversion and increased relapse rate, disability progression and need for treatment in patients with lower CSF KFLC/LFLC ratio.

Other studies reported higher CSF KFLC levels to be related to a higher CIS-MS conversion rate (Berek *et al.*, 2021) and disability progression as determined by EDSS (Vecchio *et al.*, 2019; Salavisa *et al.*, 2020), and KFLC index to be related to CIS-MS conversion (Menéndez-Valladares *et al.*, 2019; Gaetani *et al.*, 2020; Salavisa *et al.*, 2020), higher future disability progression and number of relapses (Salavisa *et al.*, 2020), and reaching an EDSS of 3 or requiring the use of high effective DMTs (Castillo-Villalba *et al.*, 2022). Results in these studies appeared to be independent of OCB status at diagnosis, although the latter group related KFLC synthesis to the presence of IgM OCB. Although these studies did not include LFLC analyses, their results are in line with our findings (high KFLC/LFLC ratio ~ high KFLC level or index). Still, the true potential of FLC to aid in disease prognosis may depend on patient characteristics and merits larger studies. In future investigations, it may also be of interest to include a measure of cognitive impairment. Even though this was not part of this work, it was recently indicated that index KFLC also relates to increased longitudinal cognitive decline (Rosenstein *et al.*, 2023).

FLC in relation to other demographic & clinical variables

We found no relations of FLC measures with demographic and most clinical variables of patients, including regular DMT usage or MRI metrics. A large cohort study demonstrated as well that KFLC index was independent of demographic factors, DMT usage or other inflammatory/degenerative processes in MS as measured by other biomarkers (Rosenstein *et al.*, 2021). Even though the DMTs used by the population were limited to fingolimod and alemtuzumab, the overall results support the assumption that KFLC index solely reflects the intrathecal immunoglobulin production rather than pathophysiological processes in MS. This equals the presence of OCB, which is rather stable throughout the course of disease (Karamehic *et al.*, 2012).

Our data did show a lowering effect of prior corticosteroid usage (within 30 days before sampling, n = 14) on CSF levels and index LFLC. Moreover, when stratifying our patients by corticosteroid usage, the relation found between KFLC/LFLC ratio and CIS-MS conversion was stronger for only patients who did not receive corticosteroids, and did not hold true for only patients who did receive corticosteroids prior to sampling. A study towards the effect of methylprednisolone on KFLC showed a decreasing effect on serum but not on CSF levels (Konen *et al.*, 2020). This implicates that studies showing increased KFLC quotient might be biased in case of corticosteroid usage in patients. For immunoglobulin quantifications (IgG,

IgM and IgA) and previously also OCB, this effect was not recognised. The decrease in serum FLC in response to corticosteroid usage might be due to various effects on either T or B cells, resulting in the reduction of circulating lymphocytes, diminished B cell stimulation or their response (Konen *et al.*, 2020). Also in saliva, FLC levels of both isotypes were decreased upon corticosteroid usage, as well as with the use of DMT (Lotan *et al.*, 2020). In that study in 55 MS patients compared to 40 controls, FLC were assessed by western blot, which also allows for detection of both monomers and dimers of FLC.

In our study, we did see significantly lower KFLC index in patients with clinically active disease, still the levels of both KFLC and LFLC were not affected by relapses prior to sampling. Interestingly, an increasing effect of acute and untreated clinical relapses on both FLC levels was seen in saliva (Lotan *et al.*, 2020).

Outlook FLC

The availability of quantitative nephelometric assays for FLC allowed for significant advances in research towards intrathecal active inflammatory responses in MS. Notwithstanding, there is a degree of variability seen among different publications on FLC in MS. It might be that pre-analytical processes are of influence (Menéndez-Valladares *et al.*, 2019), although various pre-analytical conditions like storage temperature have not shown to alter FLC results (Konen *et al.*, 2020). Moreover, especially LFLC regularly occurs as dimers or multimers, which distorts results measured by nephelometry, and for which other analytical methods may be considered (Kaplan *et al.*, 2019).

Overall, given their diagnostic performance combined with the analytical advantages regarding methodology and subject-independent interpretation, KFLC show great potential to become standard procedure and aid in clinical practice, possibly as substitute for OCB, although validation is still ongoing (Rosenstein *et al.*, 2021; Hegen, Walde, *et al.*, 2023). Two recent meta-analyses, including 32 and 116 studies, respectively, concluded that KFLC have similar sensitivity and specificity to OCB for the diagnosis of MS (Arneth and Kraus, 2022; Hegen, Walde, *et al.*, 2023). A subsequent exploratory analysis on KFLC index determined an overall discriminatory cut-off value of 6.1 (Hegen, Walde, *et al.*, 2023). A study comparing early- and late-onset RRMS and PMS showed that intrathecal KFLC synthesis is stable over the several MS types and stages, further indicating the surrogate potential of KFLC for OCB (Konen *et al.*, 2022).

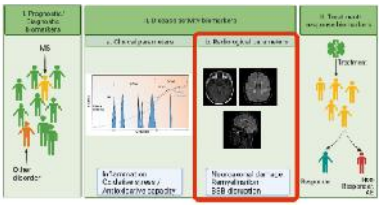
Even though for LFLC, data available seem not as promising as for KFLC, this might be due to the lower LFLC levels generally found in CSF (Voortman *et al.*, 2017; Leurs *et al.*, 2020), sometimes even below the detection range (Ferraro *et al.*, 2020). The development of

more sensitive assays might expand our knowledge and understanding of the distinction or interplay between both FLC isotypes (Ferraro *et al.*, 2020). Levels of LFLC detected might also be affected by the preanalytical treatment, as the dimer structures can be extracted from the sample by centrifugation (Arneth and Kraus, 2022). The phenomenon of increased levels or quotient/index of LFLC, but also the ratio between both isotypes might be utilised in diagnostics and potentially prognostics early on in the disease. Potentially the analysis of monomers, dimers and polymers of FLC, especially with respect to LFLC, could aid in this perspective (Kaplan *et al.*, 2019; Lotan *et al.*, 2020). Given their size, dimer/polymer structures cannot cross the BBB and would be highly sensitive to detect intrathecal inflammation (Arneth and Kraus, 2022).

Validation of FLC cut-off values will most certainly benefit from the use of the newly composed Reibergrams or validated FLC index cut-off levels. Empirical data on larger populations will be needed to establish final definitions to be used in clinical practice. In order to validate a cut-off value, different analysis platforms and assay types need to be used. Until than, the use of both OCB and KFLC are currently recommended (Hegen, Arrambide, *et al.*, 2023; Hegen, Walde, *et al.*, 2023). The use of intrathecal KFLC-synthesis – displayed as index measure with cut-off value to be determined – is further advised to be included in the next revision of the McDonald's criteria, whereby all other CSF analyses ought to be considered together with clinical and imaging data for a proper interpretation (Arrambide *et al.*, 2022; Hegen, Arrambide, *et al.*, 2023; Levraut *et al.*, 2023).

BBB disruption – Netrin-1

Serum NTN-1 levels in active MS



In **Appendix 2** we investigated the possibility to use serum netrin-1 (sNTN-1) as marker to detect BBB disruption in patients with early MS as seen by Gd-enhanced lesions (MS lesions with Gd-contrast incorporation due to BBB disruption during active inflammation) on MRI. Our results showed no differences between CIS/RRMS and controls or between Gd+ and Gd- patients (either between independent groups or longitudinal dependent groups, or when relating sNTN-1 to Gd-lesion number), indicating sNTN-1 will play no role as biomarker in the detection of MRI-based disease activity in early MS. The decreased sNTN-1 levels found in a small subgroup within the Gd+ patients with superimposed clinical relapse shortly prior to blood sampling might be an indication that this serum marker can be of value in more pregnant disease, as was also suggested in an equally small sub-analysis in a previous study (Mulero *et al.*, 2017). The only

other study also investigating sNTN-1 levels in MS patients compared to controls found contradictory results; however, this concerned data on a very small sample size and no demographic or clinical data were made available, making comparison difficult (Podjaski *et al.*, 2015).

In other diseases with respect to the CNS, sNTN-1 was suggested as valuable prognostic marker for clinical outcome. Results from studies towards traumatic brain injury (Xie *et al.*, 2021), ischemic stroke (Guo *et al.*, 2019, 2020), intracerebral (Lou *et al.*, 2020) and aneurysmal subarachnoid haemorrhage (Chen *et al.*, 2019), and cognitive impairment upon spinal cord injury (Meng *et al.*, 2022) all associated decreased sNTN-1 levels with more severe disease or worse recovery.

NTN-1 cell expression in MS

Contrary to serum levels, NTN-1 expression was shown to be elevated in astrocytes, inflammatory cells (macrophages), neurons and oligodendrocytes of demyelinated lesions in both MS patients and in human/murine MS *in vivo*/*in vitro* models (Moon *et al.*, 2011; Bin *et al.*, 2013; Cayre *et al.*, 2013; Tepavčević *et al.*, 2014). Upregulation of NTN-1 in astrocytes appeared to be especially present in chronic silent lesions (Tepavčević *et al.*, 2014).

The pathological implication of elevated NTN-1 on remyelination seems rather complex. NTN-1 was demonstrated to *promote* vascular remodelling upon demyelination, migration of neural progenitor cells, and extension of oligodendrocyte branches (Rajasekharan *et al.*, 2009; Cayre *et al.*, 2013; Birey and Aguirre, 2015), though, on the contrary, NTN-1 may *inhibit* the recruitment/migration of oligodendrocyte progenitor cells (OPC; NTN-1-receptor positive) to the lesion site, which impedes remyelination (Bin *et al.*, 2013; Tepavčević *et al.*, 2014). It was suggested that timing of elevated NTN-1 expression of lesion surrounding astrocytes affects the final repair potential of demyelinated lesions (Tepavčević *et al.*, 2014).

Dual role of NTN-1

NTN-1 is generally known to be a bifunctional molecule in the CNS that may exert both attractive and repulsive effects, depending on the effector cells and the receptors that play part (de Castro, 2003; Ly *et al.*, 2005; Meng *et al.*, 2022). In CNS development, NTN-1 is an essential chemotropic molecule in regulating axonal and neuronal growth (de Castro, 2003; Kang *et al.*, 2018), although also chemorepellent to OPC in the developing spinal cord (Jarjour *et al.*, 2003). Also in experimental setup, adult OPC were found to be chemorepelled by NTN-1 upon demyelination, although NTN-1 did increase their differentiation (Tepavčević *et al.*,

2014). Furthermore, NTN-1 promotes junctional proteins, and BBB and endothelial integrity (Wilson *et al.*, 2006; Le Noble *et al.*, 2008; Podjaski *et al.*, 2015; Xie *et al.*, 2017; Boyé *et al.*, 2022), and exerts regulatory effects on cell adhesion motility, both pro- and anti-inflammation, apoptosis and tumour growth (Lu *et al.*, 2004; Reeves, Kwon and Ramesh, 2008; Ramesh *et al.*, 2010; Lai Wing Sun, Correia and Kennedy, 2011; Ramesh, Berg and Jayakumar, 2011; Ko, Dass and Nurgali, 2012; Obermüller *et al.*, 2014; Wen *et al.*, 2014; Podjaski *et al.*, 2015; Çekmez *et al.*, 2016; Yıldırım *et al.*, 2016; Zhang *et al.*, 2022).

Under experimental conditions, NTN-1 conferred neuroprotection; murine models revealed elevated NTN-1 expression in neurons upon injury and in BBB endothelial cells upon inflammation (Wen *et al.*, 2014; Podjaski *et al.*, 2015; Xie *et al.*, 2017). NTN-1 in mice further showed to exert neuroprotective effects upon ischemic (Yang *et al.*, 2023) or traumatic brain injury (Zhang *et al.*, 2023), respectively, by activating different pathways that attenuate detrimental processes causing cell death.

To what extent NTN-1 levels in blood reflect those in tissue and vice versa is not clear. It has been suggested that the discrepancy between elevated NTN-1 expression on tissue level on the one hand, and decreased serum levels on the other, was found due to species inequality between humans and rodents (Xie *et al.*, 2021). This explanation is rather questionable, since these contradicting results were found in both humans and mice, at least in MS (models) (Bin *et al.*, 2013; Tepavčević *et al.*, 2014; Podjaski *et al.*, 2015).

Outlook NTN-1

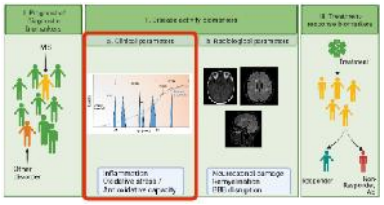
Establishing a blood biomarker to detect BBB disruption and ongoing inflammation within the CNS would be of high relevance for clinical management of MS patients. Blood sampling has several advantages over contrast-enhanced MRI scanning: it is an easy to access body fluid, less burdensome for the patient than MRI scanning, restricts toxicological risks as potentially exist with the use of GD contrast agent, is less laborious for clinical specialists, and involves overall benefits considering health care costs.

Our NTN-1 data could not distinguish subclinical disease activity in MS, although they gave the notion of decreased serum levels during clinical activity, which would be expected when comparing to literature on sNTN-1 from one larger study in MS (Mulero *et al.*, 2017) and other diseases considering CNS injury (Chen *et al.*, 2019; Guo *et al.*, 2019, 2020; Lou *et al.*, 2020; Xie *et al.*, 2021). Especially given the prognostic value that was proven for sNTN-1 in other CNS conditions, the investigation towards NTN-1 in MS still seems pertinent. In a first step, it would be of interest to analyse longitudinal clinical and imaging data of our cohort beyond the follow-up time of the median 1.2 (Gd-) or 1.8 (Gd+) years. Secondly, future studies

ought to include a larger sample size, particularly of patients with more severe disease, or patients with more prominent contrast-enhancing or tumefactive lesions.

Neuroprotective effects exerted by NTN-1 suggest a potential role for this protein in MS treatment, e.g., improved BBB integrity and anti-inflammatory effects. Two murine MS *in vivo* models investigating the effects of NTN-1 treatment resulted in improved disease severity and BBB breakdown, and promoted nerve regeneration, respectively (Podjaski *et al.*, 2015; Ji *et al.*, 2016). Nevertheless, demyelinated tissue and OPC have temporal sensitivity to NTN-1 resulting in concentration-dependent, contradictory effects (Tepavčević *et al.*, 2014). This emphasizes that more extensive research towards the exact pathological implications of NTN-1 is required in order for controlling it as an effective strategy in MS management.

Disease activity – Antioxidative capacity



CSF AOC and clinical manifestation

In **Appendix 3** we investigated the relation between total AOC in body fluids and pathological processes related to disease activity in MS as seen by clinical examination and on MRI scans. We were able to demonstrate an association between the clinical manifestation of MS and the AOC of CSF but not serum. CSF AOC was differently regulated between patients and controls, in which it was significantly lower in RRMS vs. CIS patients. Besides, in MS, lower CSF AOC was associated with higher EDSS and an increased risk of future relapses. No relation of AOC with disease characteristics seen on MRI could be detected.

Regarding CSF AOC, similar results were found in previous studies that connected an impaired defence mechanism against OS of the CNS to a more severe disease course as well as physical disability (Ljubisavljevic, Stojanovic, Vojinovic, Stojanov, Stojanovic, Cvetkovic, *et al.*, 2013; Emami Aleagha *et al.*, 2015), and increased relapses (Ristori *et al.*, 2011; Pasquali *et al.*, 2015; Ibitoye and Rice, 2016). Not only do the results underscore the relevance of OS/AOC already early in MS provoking more advanced disease, they also confirm the impact of AOC on long-term disease progression – either as a result of accumulated ROS, or decreased antioxidants (activity) (Birben *et al.*, 2012; Adamczyk and Adamczyk-Sowa, 2016).

Methodology (anti)oxidants vs. AOC analyses

Given the complexity of the body's redox system and the interdependence of the activity of all

components involved, combined with the heterogeneity of disease, it is not to be assumed that only one or a few oxidative or antioxidative compounds play a key role in MS (Ibitoye and Rice, 2016). Nevertheless, most studies towards oxidative stress and the body's protection against it (in MS) focus on the investigation of only few potential markers (Koch *et al.*, 2006; Haider *et al.*, 2011; Ristori *et al.*, 2011; Fischer *et al.*, 2012; Ljubisavljevic, Stojanovic, Vojinovic, Stojanov, Stojanovic, Kocic, *et al.*, 2013; Ljubisavljevic *et al.*, 2014; Karlík *et al.*, 2015; Pasquali *et al.*, 2015; Adamczyk and Adamczyk-Sowa, 2016; Xie *et al.*, 2022). We chose to analyse the cumulative capacity of all molecules combined to counteract controlled OS, the AOC, to gain insight into the impact that alterations in the redox balance have on disease characteristics and vice versa. The comparison to other studies aiming at analysis of the AOC is, however, still a matter of concern, as for analysis of total activity – similar to the measure of total oxidative status for oxidants (activity) – various methodologies are employed that do not all yield the same results, which might cause confounding (Güngör *et al.*, 2011; Pasquali *et al.*, 2015; Adamczyk and Adamczyk-Sowa, 2016; Oliveira *et al.*, 2017; Bizoń *et al.*, 2022; Vasic *et al.*, 2023). Future research towards AOC in larger and more diverse patient cohorts is warranted, though unity of methodology needs to be agreed upon in these studies to guarantee data are comparable and conclusions reliable. In addition to AOC, also total OS would be of interest to take into account in these cohorts.

Outlook AOC – potential therapeutic target

Our study was not designed to assess effects of DMT on AOC – no DMT were taken by our cohort at time of sampling – still, we looked into a possible predictive value of AOC on future therapy usage in patients during follow-up, but found no association. Neither did the use of corticosteroids shortly prior to sampling appeared to affect the AOC in our results. However, considering our results on the relationship between CSF AOC and physical disability and future relapses, we conclude that AOC is definitely a target of interest in the search towards new DMTs for MS, already from early on in the disease.

Even though all therapeutics currently approved in MS principally modulate the autoimmune response (Pegoretti *et al.*, 2020), few of these DMTs possess a working mechanism known to also affect the redox system. It was shown for fingolimod to decrease total OS already after a short treatment period, but not to alter the AOC (Scutera *et al.*, 2020; Yevgi and Demir, 2021), and dimethyl fumarate is known to promote cytoprotection by activation of (nuclear factor [erythroid-derived 2]-related factor 2 [Nrf2]) antioxidative pathways (Linker and Gold, 2013; McGuire *et al.*, 2016).

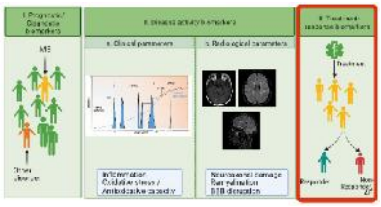
Recent (pre)clinical studies towards new therapeutic possibilities in MS included

antioxidants or compounds promoting antioxidative pathways (including non-pharmacological nutrients and synthetic antioxidants), of which several have shown promising results (Miller *et al.*, 2019; Michaličková *et al.*, 2020; Tobore, 2021; Theodosis-Nobelos and Rekka, 2022). However, it is expected that these compounds will not be used as stand-alone therapy, but rather in combination with/support of immunomodulatory therapies (Theodosis-Nobelos and Rekka, 2022). Since OS still plays a prominent role in more progressive MS, when the disease becomes compartmentalized behind a closed BBB, therapeutic applications are currently under development employing advanced drug delivery systems to, among other things, locally reduce OS (Dolati *et al.*, 2017).

As described before, OS can be provoked by either an accumulation or ROS or a decreased presence or activity of antioxidants, all of which can be induced by a multitude of possible causes. A key role in facilitating OS seems to be played by impaired mitochondria (Haider *et al.*, 2011; Mahad, Trapp and Lassmann, 2015; Islam, 2017; Adiele and Adiele, 2019; Tobore, 2021). With respect to OS, mitochondria can be the source for overproduction of reactive oxygen/nitrogen species (ROS/RNS) and disturb oxygen metabolism, thereby causing (intrinsic) cellular damage throughout the CNS. Mitochondria are highly susceptible to OS-induced damage themselves. Moreover, in demyelinated plaques, an increase in active mitochondria is found, exacerbating the effects even more (Mahad, Trapp and Lassmann, 2015). It was suggested that impaired mitochondria triggered by OS might even pre-exist before axonal damage and symptoms in MS present, at least in *in vivo* mouse studies (Nikić *et al.*, 2011). In future research, it might be of special interest to look into the particular relation of OS/AOC and mitochondria, and whether this might be a useful treatment target.

Risk stratification – CD62L (L-selectin)

CD62L in response to DMT usage



In **Appendix 4**, we presented our work on the direct analysis of CD62L expression in whole blood to use it as biomarker in the assessment of MS treatment response. We were able to show that direct analysis without pre-analytical steps gives CD62L results that are stable over time in patients under stable DMT usage, but differ among MS patients using various DMTs or within patients switching between these treatments.

Relative CD62L expression of $CD3^+CD4^+$ PBMCs decreased with the use of natalizumab and more prominently with fingolimod when compared to other, first-line DMTs, which was also found by others (Schwab *et al.*, 2013; Spadaro *et al.*, 2015). It is known that

under natalizumab usage, the number of specific lymphocyte populations in the CSF is reduced, but not the number of peripheral T cells (Stüve *et al.*, 2006). For fingolimod, it was hypothesised that the selective retention of $CD62L^+$ lymphocytes (naïve $CD4^+$ or $CD8^+$, and central memory T cells) in the lymphoid tissues, rather than $CD4^+$ T helper subsets, causes the relative decrease in CD62L expression of circulating PBMCs (Böhler *et al.*, 2004; Mehling *et al.*, 2008; Chun and Hartung, 2009; Kürtüncü *et al.*, 2019). Conversely, for dimethyl fumarate an increase in CD62L expression was seen, comparable to previous reporting (Longbrake *et al.*, 2016). It was postulated that this results from a decrease in the number of $CD62L^-$ lymphocytes (effector memory T cells), i.e., lymphopenia (Longbrake *et al.*, 2016; Mills and Mao-Draayer, 2018; Jordan *et al.*, 2022).

Longitudinal results in our cohort showed percentage CD62L expression to be stable over time within patients using various DMTs, with a rather rapid response when switching from natalizumab, or to fingolimod. These data were in line with previous findings as well (Spadaro *et al.*, 2015; Cobo-Calvo *et al.*, 2016).

CD62L as marker for PML in MS

Rationale behind the analysis of CD62L expression during DMT usage in MS is its assumed relation to the development of progressive multifocal leukoencephalopathy (PML), as a complication of various immunotherapies. PML is a severe opportunistic disease caused by reactivation of a latent JC virus infection, occurring almost exclusively in immunocompromised patients and inducing potentially severely disabling or fatal tumefactive demyelinating lesions (Schwab *et al.*, 2013, 2016; Spadaro *et al.*, 2015). In MS, most frequently, PML occurs with the use of natalizumab ('high potential risk'), although cases were described as well for fingolimod, dimethyl fumarate (both 'low potential risk'), and several more recently authorised DMTs ('no or very low potential risk') (Berger, 2017). Stratification of the potential risk to develop PML is currently only performed in natalizumab-treated patients and focuses on the presence and index of anti-JCV antibodies, combined with the previous use of immunosuppressant treatments and the duration of current natalizumab usage (Schwab *et al.*, 2016, 2017). It is mainly hypothesised that the inhibition of cell circulation migration with natalizumab alters the immune surveillance, allowing for JCV to recrudesce; although, also properties of natalizumab facilitating upregulated virus transcription and impeded virus elimination were suggested to be causative for PML development (Mills and Mao-Draayer, 2018; Khoy *et al.*, 2020). Data of the factors currently underlying the stratified risk calculations are biased, which results in an underestimation of a particular patient's true risk to develop PML (Schwab *et al.*, 2017; Hegen *et al.*, 2018). Accurate risk calculation is of great importance

in order to prevent future cases of PML with DMT usage (Schwab *et al.*, 2017).

CD62L analysis and pre-analytics

To improve the currently used risk stratification for PML development, the peripheral expression of CD62L was suggested as valuable alternative marker (Schwab *et al.*, 2013). Previous studies obtained CD62L data from isolated PBMCs, either cryopreserved before analysis (Schwab *et al.*, 2013, 2016; Spadaro *et al.*, 2015; Lieberman *et al.*, 2016) or measured directly upon isolation (Böhler *et al.*, 2004). Only in a few studies the CD62L expression was determined in whole blood. Notably, those samples were processed and stored up to 18 hours (Cobo-Calvo *et al.*, 2016) or for an unknown period of time before analysis (Longbrake *et al.*, 2016).

Not only is the direct measurement of freshly obtained whole blood samples without pre-analytical steps less laborious and more cost-effective, it is precisely the pre-analytical steps and cryopreservation implemented in other studies that previously caused debate. It was suggested that these steps might cause discrepancies between results (Lieberman *et al.*, 2016; Schwab *et al.*, 2016). CD62L expression on the surface of leukocytes was shown to be highly susceptible to *in vitro* cell stimulation and increasingly unstable under prolonged storage and freeze/thaw conditions (Weinberg *et al.*, 2009; Feuerecker *et al.*, 2012; Pignolet *et al.*, 2016). Even though immunological effects might still be detectable in samples upon long-term storage, the storage effect is visible in a time-dependent manner (Feuerecker *et al.*, 2012).

In order to prevent any potential bias to our results in this respect, we analysed whole blood samples without any pre-analytical steps outside of the regular FACS workup within one hour upon blood draw.

Outlook CD62L

Patients included in our study did not develop PML; therefore, we cannot draw any conclusions on the role of CD62L in the development of the disease. However, we were able to show that CD62L expression, measured in a non-biased manner, is affected by the use of natalizumab, fingolimod and dimethyl fumarate – DMTs for all of which PML has been reported – which warrants future research into the usability of CD62L as marker in treatment risk stratification. If CD62L expression can be validated as such marker, its direct analysis in whole blood by FACS offers a non-laborious method that could be implemented into the clinic directly. One recent study implemented the same methodology using whole blood – although analysis was performed within 24 hours upon sampling – and found CD62L to be DMT-dependent and

potentially of influence for the development of PML (Boziki *et al.*, 2020). An important caveat, however, is that the data follow from a cross-sectional study including only one PML case.

Next to CD62L, the ratio of CD4/CD8 lymphocytes was investigated for DMTs with PML risk (Spencer *et al.*, 2015; Takahashi, 2019; Kolcava *et al.*, 2021; Jordan *et al.*, 2022). In a group of almost solely NTZ-treated MS patients, high risk and one case of PML were related to increased levels of $CD8^+$ - and not $CD62L^+$ (Beldi-Ferchiou *et al.*, 2020). The involvement of $CD8^+$ T cells was also seen in several cases under the use of fingolimod where tumefactive MS lesions occurred without a diagnosis of PML (Sánchez, Meca-Lallana and Vivancos, 2018). Other studies also focused on other immune cell subsets, e.g., T/B cells expressing cell surface antigens CD3, CD4, CD11, CD19, CD34 or CD56 (Berkovich and Weiner, 2015; Assetta and Atwood, 2017; Kürtüncü *et al.*, 2019; Ghadiri *et al.*, 2020; Scutera *et al.*, 2020), or even only on lymphopenia (deficit in lymphocytes) as risk factor to develop DMT-related infection diseases (Sainz de la Maza *et al.*, 2022). Taken together with the hypothesis discussed above that the relative CD62L expression is changed under DMT usage due to unequal alterations in the numbers of $CD4^+$ and $CD8^+$ T cells, it would be of great interest to include these (and other) adhesion molecules and total number of T cells in future research as well. Employing a direct method as described in our study could be beneficial for all markers analysed in this respective. Finding new biomarkers for risk stratification – as well as treatment response in a broader sense – remains highly needed in MS in order to reduce the number of MRI scans, which is currently performed regularly in patients in order to follow clinical results (Ibitoye and Rice, 2016; Freedman *et al.*, 2020).

General discussion and future directions

Body fluid biomarkers are becoming increasingly important in neurological disorders, including MS (Lleó, 2021). The results presented in this dissertation contribute significantly to the knowledge of the biomarker field and the progress of the development of new biomarkers.

One of the main reasons for the increasing interest in biomarkers is the emergence of new or improved analytical techniques. Over the last decade, various platforms built around patented new techniques improved previous analysis methods, as well as enabled high-throughput analyses. The great advantage of these high-sensitive assays is the potential to analyse CNS-derived markers also in blood. Fourth-generation immune assays are of special interest for biomarker discovery. Interesting and promising in biomarker research is the emerging Olink **proximity extension assay** (**PEA**) (Olink Proteomics), which merges real-time PCR with multiplex immunoassays (Petrera *et al.*, 2021). The upcoming of **single-molecule array** (**Simoa**) technology improved the sensitivity of digital immunoassays

significantly already formerly (Rissin *et al.*, 2010; Kuhle, Barro, Andreasson, *et al.*, 2016).

The development of Simoa led to an enormous increase in research towards **neurofilament light** (**NfL**). Neurofilaments are major structural proteins of the neuronal cytoplasm and consist of predominantly three Nf subunits, among which is NfL. Previously, NfL was already shown to be increased in CSF of MS patients, as marker of neuro-axonal damage (Teunissen and Iacobaeus, 2009; Kuhle *et al.*, 2011; Khalil *et al.*, 2018). A recent meta-analysis conducted on CSF NfL in MS, including 14 research articles, concluded that the marker has potential in determining disease activity (Martin *et al.*, 2019). The increased sensitivity of Simoa now allows for research of NfL in serum as well (Kuhle, Barro, Disanto, *et al.*, 2016), increasing the sample availability among patients. Serum NfL was shown to increase in normal ageing (Khalil *et al.*, 2020), and it has been under investigation in several neurodegenerative diseases, like stroke (Gattringer *et al.*, 2017). In MS, serum NfL increases, which can also be related to more precise pathological processes underlying the neurodegeneration (Bsteh *et al.*, 2019, 2020). Serum NfL was even increased already in a cohort of 60 presymptomatic MS patients median 6 years prior to first clinical disease onset as compared to 60 matched controls (Bjornevik *et al.*, 2020). Serum NfL has been deployed in research towards treatment response and pharmacovigilance as well, e.g., purposing detecting the risk to delevop PML (Komori *et al.*, 2017; Fissolo *et al.*, 2021). In a recent, essential study, serum NfL was modelled in over 10,000 samples of non-neurological subjects, and subsequently validated in a large MS registry (n = 4341) to be used in prognostication of disease activity and suboptimal treatment response in the individual patient (Benkert *et al.*, 2022). Furthermore, increased serum NfL was recently related to increased brain atrophy in different brain regions upon a median time of 3.8 years as seen by MRI at 3 T, whereas sequential high NfL levels indicated an elevated risk of physical deterioration as determined by EDSS (Buchmann *et al.*, 2023).

With the arrival of Simoa, also the analysis of the astrocytic intermediate filament **glial fibrillary acidic protein** (**GFAP**) became available in blood. GFAP was discovered already in 1971 in *ex vivo* MS research, but recently more interest in MS biomarker research was gained towards this protein. GFAP is the signature intermediate filament of astrocytes, but is also expressed in other non-CNS cells. Recent Simoa data resulting from various studies showed increased GFAP serum levels in MS, especially in progressive disease (data on RRMS are still inconclusive). Serum GFAP further correlates with and may predict future physical disability, and is possibly affected by DMT usage in MS patients. Also correlation to NfL levels have been described (Magliozzi and Cross, 2020; Abdelhak *et al.*, 2022). In traumatic brain injury, the analysis of GFAP appeared to be even superior to that of NfL in detecting normal

from abnormal head CT scans (Abdelhak *et al.*, 2022).

In other recent developments towards biomarkers in MS, **parvalbumin** (**PVALB**), a protein expressed by specific inhibitory neurons vital to the CNS, was found to be increased in the CSF of patients and related to MRI findings of cortical pathology, i.e., cortical lesion number and global cortical thickness (Magliozzi *et al.*, 2021). Interestingly, the correlation between PVALB and MS-specific grey matter neurodegeneration as seen on MRI could not be found for NfL (Magliozzi *et al.*, 2019, 2021).

Future research should point out the exact value NfL will have in MS clinical practice, and whether GFAP and PVALB can support this and other new biomarkers. Numerous other biomarker assays with the various platforms are available that still deserve to be identified in MS.

Overall conclusions

Body fluid biomarkers investigated in the course of this dissertation were divided according to their supposed applicability in clinical practice – prognostic/diagnostic, disease activity (clinical and radiological parameters) and treatment-response biomarkers – a pragmatic and commonly used classification applied in biomarkers research (Comabella and Montalban, 2014). The potential clinical value of four biomarkers, each of a different subtype, was explored and discussed.

We confirmed that **FLC**, especially kappa isotype, measured in CSF and blood could aid in **diagnostics** of MS as quantitative alternative to OCB, for which validation is currently advancing in large multicentre studies. A potential role for both KFLC and LFLC in **prognostics** would necessitate further research.

Our results demonstrated that **NTN-1** blood levels cannot detect **BBB disruption** (radiological **disease activity**) in early disease. Nevertheless, given other preclinical and sparse clinical results, the role of this protein in CNS pathology, and potentially as treatment target, warrants further research.

The general measure of **AOC** appears to have potential as biomarker of various **pathological processes** related to oxidative stress in MS (**disease activity** defined by clinical relapses, physical disability), already in early disease. Also its role in treatment target deserves follow-up investigation.

Assessment of **CD62L** in fresh blood samples was proven effective and showed **treatment-effects**. To investigate the relevance of this marker in risk stratification for PML, or potentially other serious AE with MS DMT usage, research on larger cohorts is required.

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MULTIPLE SCLEROSIS JOURNAL | MSJ

Original Research Paper

Prognostic value of free light chains lambda and kappa in early multiple sclerosis

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Abstract

Background: Cerebrospinal fluid (CSF) immunoglobulin free light chains (FLC) have been suggested as quantitative alternative to oligoclonal bands (OCB) in the diagnosis of multiple sclerosis (MS). However, little is known on their role in predicting clinical and paraclinical disease progression, particularly in early stages.

Objective: To assess the prognostic value of FLC in OCB-positive patients with clinically isolated syndrome (CIS) suggestive of MS and early MS.

Methods: We determined FLC kappa (KFLC) and lambda (LFLC) in CSF and serum by nephelometry in 61 patients (CIS ($n=48$), relapsing-remitting multiple sclerosis ($n=13$)) and 60 non-inflammatory neurological controls. Median clinical follow-up time in CIS was 4.8 years (interquartile range (IQR), 1.5–6.5 years). Patients underwent 3T magnetic resonance imaging (MRI) at baseline and follow-up (median time interval, 2.2 years; IQR, 1.0–3.7 years) to determine T2 lesion load (T2LL) and percent brain volume change (PBVC).

Results: CSF FLC were significantly increased in CIS/MS compared to controls (all $p<0.001$). A lower KFLC/LFLC CSF ratio was associated with CIS-clinically definite multiple sclerosis (CDMS) conversion (hazard ratio (HR) = 2.89; 95% confidence interval (CI) = 1.17–7.14; $p<0.05$). No correlations were found for FLC variables with T2LL or PBVC.

Conclusion: Our study confirms increased intrathecal synthesis of FLC in CIS/MS which supports their diagnostic contribution. The KFLC/LFLC CSF ratio appears to have a prognostic value in CIS beyond OCB.

Keywords: Multiple sclerosis, magnetic resonance imaging, immunoglobulin kappa chains, immunoglobulin lambda chains, prognosis, cerebrospinal fluid, serum

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Introduction

The presence of immunoglobulin (Ig) G oligoclonal bands (OCB) in cerebrospinal fluid (CSF) is known to support a diagnosis of multiple sclerosis (MS) although such evidence is not included in current diagnostic criteria except for primary progressive MS.[1] Measuring the levels of CSF Ig free light chains (FLC) kappa (KFLC) and lambda (LFLC) has been proposed as a potential alternative to the qualitative assessment of OCB and showed comparable diagnostic sensitivity and specificity.[2–8] The presence of OCB has also been associated with a higher risk of conversion from a clinically isolated syndrome (CIS) to clinically definite multiple sclerosis (CDMS)[9,10] and disease progression.[3]

To what extent a quantification of FLC may also add information in predicting progression of MS was yet investigated only in a few studies.[3,11,12] In one of them, KFLC have been associated with conversion from CIS to CDMS. However, in that study a substantial number of non-converters were OCB negative.[11] Thus, the observed contribution of KFLC could have mirrored just the predictive capacity of OCB and not necessarily indicates a predictive role of KFLC on their own. Furthermore, most studies focused solely

on the levels of KFLC and only a few studies have also studied LFLC in MS.[6–8,12,13]

In addition to supporting the diagnostic potential of FLC in MS we therefore investigated whether quantitative analysis of FLC could add prognostic information. In this respect, we analysed both KFLC and LFLC and calculated the KFLC/LFLC ratio. As the presence of CSF OCB modifies the prognosis of patients with CIS, we wanted to exclude this confounding factor and determine whether quantitative assessment of FLC could add additional information on disease prognosis against the background of OCB positivity. Besides using clinical findings as a marker of disease progression, we also attempted to substantiate associations of FLC with magnetic resonance imaging (MRI) markers of disease progression.

Patients, materials and methods

This study was approved by the ethics committee of the Medical University of Graz, Austria. All participants gave written informed consent.

Patients and controls

This study included patients ($n=61$) who were seen at the MS outpatient clinic of the Department of Neurology/Medical University of Graz and fulfilled the following criteria: (1) a diagnosis of CIS suggestive of MS or relapsing-remitting multiple sclerosis (RRMS) according to available criteria;[1,14] (2) availability of CSF and serum samples from a diagnostic lumbar puncture and parallel blood sampling; (3) presence of OCB in CSF and not in serum; (4) no MS-specific treatment prior to sampling, except for corticosteroids; (5) MRI examination close to sampling; and (6) clinical follow-up.

Patients were considered to be in an active state of disease at the initial examination, that is, baseline, if lumbar puncture was performed within 14 days of a clinical attack. Patients with primary or secondary progressive MS were not considered.

Controls ($n=60$) consisted of individuals who were seen at the outpatient clinic of the Department of Neurology/Medical University of Graz and met the following profile: (1) diagnosis of a neurological disease of non-inflammatory aetiology (cranial/peripheral palsy – non-inflammatory neurological disease controls; headache or sensory disturbances – symptomatic controls),[15] (2) availability of CSF and serum samples from a diagnostic lumbar puncture and parallel blood sampling, (3) all routine-diagnostic variables measured in CSF and serum within normal range,[15] and (4) no immunomodulatory or immunosuppressive treatment prior to sampling.

Clinical assessment and follow-up

Demographic and clinical data recorded included age, gender, age at disease onset, time between the diagnosis of CIS and conversion to CDMS (upon second relapse), and degree of disability as determined by the Expanded Disability Status Scale (EDSS).[16] Subsequent to sampling and diagnosis, patients were followed by experienced neurologists during scheduled follow-up visits.

Relapses were recorded over time according to previous definition, that is, at least one neurological symptom (re)appears or an old symptom attributed to MS worsens for at least 24 hours succeeding a stable or improving neurological state during at least 30 days.[17] Upon confirmation of a relapse during neurological examination, patients were usually treated with steroid pulses of either 3- or 5-day 1000 mg/day methylprednisolone. Thus, at baseline, 14 patients received corticosteroids within 30 days prior to CSF sampling and none were on long-term disease-modifying treatments (DMTs). At the time of the last available follow-up (time since lumbar puncture median, 4.8 years; interquartile range (IQR), 1.5–6.5 years), 37 patients received DMTs, including interferon beta ($n=19$), glatiramer acetate ($n=10$), natalizumab ($n=7$) and fingolimod ($n=1$).

CSF and serum sampling and analyses

At lumbar puncture, a total volume of 6–10 mL of CSF and 8 mL of peripheral blood were obtained from each patient. After routine diagnostic work-up, excess volumes of CSF/serum pairs were stored immediately at −80°C until further analyses. All samples were handled and stored according to international consensus guidelines[18] and sample analyses were performed by trained analysts blinded to clinical information.

Routine diagnostic work-up of CSF and serum were performed as follows: CSF white cell count was determined using the Fuchs Rosenthal Counting Chamber. Levels of albumin and IgG, IgA and IgM were determined by nephelometry using the Beckman Coulter Image 800 analyser (Beckman Coulter Inc., Brea, CA, USA). The CSF/serum quotient of albumin (Q alb) was calculated in order to assess the blood CSF barrier function.[19] Intrathecal IgG synthesis was assessed

both quantitatively by calculating the IgG index ([CSF/serum quotient IgG]/Q alb; reference < 0.7)[20] and qualitatively by the determination of oligoclonal bands (OCB) using isoelectric focusing followed by immunoblotting.[21]

KFLC and LFLC were measured in serum and CSF by immunonephelometry (Freelite®, The Binding Site Group Ltd., Birmingham, UK) on a BNII analyser (Siemens Healthcare Diagnostics, Marburg, Germany).

We determined the CSF/serum quotients (Q FLC) of KFLC and LFLC and calculated the indices of KFLC and LFLC by correcting for the albumin quotient (index FLC = Q FLC/Q alb). Next, the proportion of kappa to lambda was determined in serum and CSF separately, that is, [serum KFLC]/[serum LFLC] and [CSF KFLC]/[CSF LFLC].

MRI

All patients underwent MRI of the brain at baseline on a 3 T Tim Trio system (Siemens Medical Systems, Erlangen, Germany) using a 12-element phased-array head coil. For structural imaging we used a T1-weighted three-dimensional (3D) Magnetization Prepared Rapid Acquisition Gradient Echo (MPRAGE) sequence (repetition time (TR)/echo time (TE)/inversion time (TI)/flip angle (FA) = 1.9 s/2.19 ms/0.9 s/9°; isotropic resolution = 1 mm) and a T2-weighted two-dimensional (2D) fast fluid-attenuated inversion recovery (FLAIR) sequence (TR/TE/TI = 9000 ms/70 ms/2500 ms, in plane resolution = 0.9 × 0.9 mm^2, slice thickness = 3 mm). A follow-up MRI scan using the identical imaging protocol was performed in 56 (91.8%) patients.

We determined the following morphologic markers of progression of tissue damage as previously described.[22,23] In brief, for assessing T2 lesion load (T2LL), MS lesions were outlined on a transparency overlaid on hard copies of the FLAIR sequence. Using these templates, lesion masks were then created using the DISPImage program.[24] To measure the percentage of brain volume change (PBVC), we co-registered follow-up MRI scans from each individual to baseline MRI, applying SIENA.[25]

All imaging analyses were performed by trained and experienced MRI technicians, blinded to clinical data.

Statistical analyses

Statistical analyses were performed using SPSS Statistics (version 22.0, IBM Corp. Armonk, NY, USA) and GraphPad Prism (version 5.00, GraphPad Software, San Diego, CA, USA).

All variables were tested for normal distribution using the Kolmogorov–Smirnov test. Group differences were determined by either chi-square test for categorical data or Mann–Whitney *U* test for continuous variables. Differences between more than two groups were defined by applying Kruskal–Wallis test followed by post hoc Dunn's multiple comparison test.

We performed Spearman correlations to determine the correlation coefficients for serum and CSF variables with demographic, clinical and MRI data. Pearson partial correlations corrected for time between two MR images taken were applied on longitudinal MRI data. Significance level was set at 5% ($p < 0.05$). The diagnostic value was determined for index KFLC by applying the empirically well-established threshold published for potential diagnostic use (index KFLC > 5.9[5]) on our data.

The prognostic value of FLC was determined by comparing CIS-CDMS converters versus CIS non-converters by binary logistic and Cox regression analyses, and hazard graphs.

Results

Subject description

The patient group ($n = 61$) consisted of 48 patients with CIS and 13 patients with RRMS. The control group ($n = 60$) consisted of 14 patients with cranial/peripheral palsy (non-inflammatory neurological disease controls), 31 with headache and 15 with sensory disturbance (both symptomatic controls). The demographic and clinical data of both groups are shown in Table 1. Patients and controls were comparable regarding age and gender distribution.

CSF samples were obtained at the time of diagnosis in CIS. For MS patients, median disease duration at the time of sample collection, that is, time between presentations of first symptoms and lumbar puncture, was 1.2 years (IQR, 0.8–4.2 years). Active disease (clinical attack within 14 days prior to lumbar puncture) was present in 33 (54.1%) patients. Among all CIS patients 23 (47.9%) converted to CDMS during follow-up (median, 4.8 years; IQR, 1.5–6.5 years). Active disease at the time of lumbar puncture was present in 13 of 23 converters and 14 of 25 non-converters. Diagnostic variables of CSF and serum

Table 1. Demographic, clinical and MRI data of study subjects.

	CIS/MS ($n=48/n=13$)	Controls ($n=60$)
n female	46 (75.4)	40 (66.7)
Age at LP (years)	28.8 (23.2–36.9)	30.5 (25.4–44.9)
Age at disease onset (years)	27.1 (21.8–36.9)	NA
Duration of disease at LP (years)	0.1 (0.0–0.3)	NA
EDSS at LP	2.0 (1.0–3.0)	NA
n with corticosteroids at LP[a]	14 (23.0)	NA
n with DMT at LP	0 (0)	NA
n with DMT at FU	37 (60.7)	NA
T2LL (cm^3) at LP	1.2 (0.4–3.3)	NA
T2LL (cm^3) at FU	1.0 (0.5–3.4)	NA
T2LL change LP-FU (cm^3)	−0.09 (−0.58 to 0.34)	NA
T2LL change rate LP-FU (cm^3/year)	−0.03 (−0.29 to 0.09)	NA
PBVC LP-FU (%)	−0.96 (−2.32 to −0.40	NA
PBVC rate LP-FU (%/year)	−0.56 (−0.80 to −0.21)	NA

MRI: magnetic resonance imaging; CIS: clinically isolated syndrome; DMT: disease-modifying treatment; EDSS: Expanded Disability Status Scale; FU: follow-up; LL: lesion load; LP: lumbar puncture; MS: multiple sclerosis; *n*: number of subjects; NA: not applicable; PBVC: percentage brain volume change.
Values are given as number (%) or as median (interquartile range).
[a]Number of patients who received intravenous corticosteroids within 30 days prior to LP. Differences between CIS/MS and controls regarding gender and age were not significant.

Table 2. CSF routine diagnostic parameters in CIS/MS patients and controls.

	CIS/MS ($n=48/n=13$)	Controls ($n=60$)	*p*-value
CSF white cell count (nr/µL, ref. ⩽4)	10 (5–19)	2 (1–3)	<0.001
Quotient albumin ($\times 10^3$)	4.67 (4.02–6.73)	5.10 (4.19–5.84)	NS
Index IgG	0.89 (0.66–1.39)	0.49 (0.45–0.54)	<0.001
Index IgA	0.35 (0.29–0.42)	0.28 (0.23–0.32)	<0.001
Index IgM	0.18 (0.10–0.36)	0.09 (0.06–0.13)	<0.001
n OCB positive	61 (100)	NA	NA

CIS: clinically isolated syndrome; CSF: cerebrospinal fluid; Ig: immunoglobulin; MS: multiple sclerosis; *n*: number of subjects; NA: not applicable; nr: number of cells; NS: not significant; OCB: oligoclonal bands; ref.: reference value.
Values are given as number (%) or as median (interquartile range).

samples of both patients and controls are given in Table 2. All diagnostic variables of the controls were within the normal range.

FLC in CSF and serum – group differences

Levels, quotients and indices of KFLC and LFLC, and the KFLC/LFLC ratio in serum and CSF of patients and controls are given in Table 3. KFLC and LFLC levels and KFLC/LFLC ratio were significantly increased in CSF of CIS/MS patients compared to controls (all $p<0.001$, see Figure 1), but no differences were seen in serum. Quotients and indices of KFLC and LFLC were significantly increased in patients compared to controls. In addition, FLC concentrations did not differ significantly between men and women.

Serum and CSF levels of KFLC and LFLC did not differ between CIS and MS subgroups (Figure 1). The index KFLC was significantly lower ($p<0.05$) in patients with active disease (median, 50.8; IQR, 23.5–100.4) compared to those with non-active disease (median, 106.4; IQR, 28.4–232.3), but the actual levels of KFLC or LFLC were not different in regard to disease activity. There were no significant differences in KFLC and LFLC levels between active and non-active patients, neither within CIS-CDMS converters nor non-converters.

FLC and diagnostic value in MS

Applying the previously published threshold for index KFLC on our data of CIS/MS compared to controls

Table 3. Free light chains kappa and lambda in CIS/MS patients and controls.

	CIS/MS ($n=48/n=13$)	Controls ($n=60$)	p-value
Serum KFLC (mg/L)	11.00 (8.70–13.30)	11.50 (8.78–13.50)	NS
CSF KFLC (mg/L)	4.27 (1.46–6.83)	0.07 (0.04–0.08)	<0.001
Quotient KFLC ($\times10^{-3}$)	349.03 (132.59–713.05)	5.69 (4.55–7.54)	<0.001
Index KFLC	66.54 (24.12–138.27)	1.24 (0.95–1.52)	<0.001
Serum LFLC (mg/L)	11.90 (9.49–15.60)	12.90 (10.13–16.05)	NS
CSF LFLC (mg/L)	0.59 (0.29–1.97)	0.05 (0.04–0.08)	<0.001
Quotient LFLC ($\times10^{-3}$)	48.81 (28.12–148.16)	4.68 (3.14–5.85)	<0.001
Index LFLC	10.31 (4.21–30.83)	0.96 (0.70–1.29)	<0.001
Serum ratio KFLC/LFLC	0.85 (0.73–1.06)	0.82 (0.68–1.09)	NS
CSF ratio KFLC/LFLC	3.43 (1.71–9.62)	1.00 (0.89–1.60)	<0.001

CIS: clinically isolated syndrome; CSF: cerebrospinal fluid; index: FLC quotient/albumin quotient; KFLC: free light chain kappa; LFLC: free light chain lambda; MS: multiple sclerosis; *n*: number of subjects; NS: not significant; quotient: [CSF]/[serum]. Values are given as number (%) or as median (interquartile range).

resulted in a sensitivity and specificity of 96.6% and 98.3%, respectively.

FLC in CSF and conversion to MS

In patients with CIS, the KFLC/LFLC CSF ratio was significantly lower in those patients who later converted to CDMS compared to non-converters ($p<0.05$). This association was confirmed using regression analyses, where CIS patients were allocated as 'high' or 'low' according to the median values of the respective FLC variables.

Of CIS patients with low CSF KFLC/LFLC (⩽3.38), 66.7% converted to CDMS, whereas 29.2% of patients with high CSF KFLC/LFLC (>3.38) (logistic regression of high vs low CSF KFLC/LFLC ratio on likelihood of CIS-CDMS conversion: odds ratio = 4.86; 95% confidence interval, 95% CI = 1.43–16.50; $p=0.011$). CIS patients with low CSF KFLC/LFLC had a higher risk to convert to CDMS during follow-up than those with high CSF KFLC/LFLC (hazard ratio, HR = 2.89; 95% CI = 1.17–7.14; $p=0.016$) (Figure 2(a)). When considering only CIS patients who had not received corticosteroids within 30 days prior to lumbar puncture ($n=39$), this association was even more prominent (HR = 3.94; 95% CI = 1.37–11.35; $p=0.007$) (Figure 2(b)); for CIS patients who had received corticosteroids ($n=9$), no association was found.

CIS patients with a low CSF KFLC/LFLC ratio had significantly higher CSF LFLC levels (mean 2.59 ± standard deviation (SD) 3.29) compared to patients with a high KFLC/LFLC ratio in the CSF (mean 0.60 ± SD 0.88) ($p=0.001$). CSF KFLC levels were not significantly different (mean 4.55 ± SD 6.19 and mean 6.06 ± SD 6.97, respectively). The quantitative association of both subunits with the KFLC/LFLC ratio in relation to CIS-MS conversion are visualized for each CIS patient in Figure 2(c) and (d).

FLC and the association with demographic and clinical data

FLC measurements did not correlate with demographical or clinical data, including age at lumbar puncture or disease onset, EDSS at lumbar puncture or follow-up, the change of EDSS over time and relapse rate. The CSF level and index of LFLC were significantly lower when patients had received corticosteroids 30 days prior to lumbar puncture (both $p<0.05$).

FLC and MRI metrics

All patients underwent MRI at baseline (time between lumbar puncture and scan median, 2.8 months; IQR, 1.1–7.3 months). A follow-up MRI scan was available in 56 (91.8%) patients after a median time interval of 2.3 years (IQR, 1.0–3.7 years).

No correlations were found for FLC variables with T2LL at baseline and the change over time or the PBVC.

Discussion

Our study confirms increased intrathecal kappa and lambda FLC production and a higher CSF KFLC/LFLC ratio in patients with CIS and early MS compared to controls[2–8,12,26] which attest to the high diagnostic potential of FLC in MS.[5,7] We found that a lower CSF KFLC/LFLC ratio was associated with a

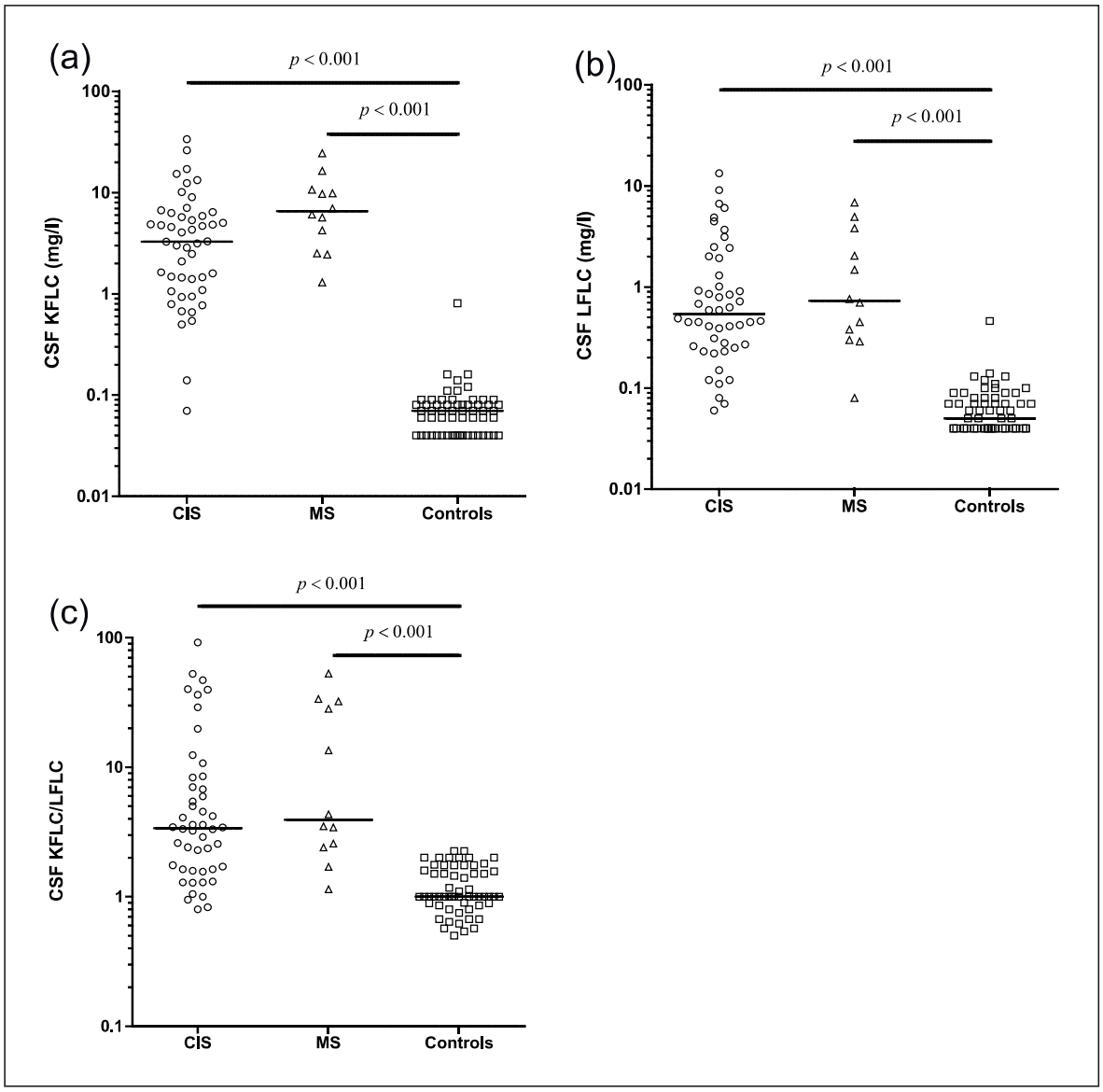

Figure 1. CSF FLC levels of CIS/MS patients and controls. Comparisons between patients with CIS ($n=48$), MS ($n=13$) and controls ($n=60$) of CSF levels of (a) KFLC, (b) LFLC and (c) the KFLC/LFLC CSF ratio.
Significantly elevated levels of KFLC and LFLC and KFLC/LFLC ratio were found for both CIS and MS patients compared to controls (all $p<0.001$) by applying Kruskal–Wallis test with post hoc Dunn's multiple comparison test. No significant differences were found between CIS and MS patients. Horizontal bars represent median values.
CIS: clinically isolated syndrome; CSF: cerebrospinal fluid; KFLC: free light chain kappa; LFLC: free light chain lambda; MS: multiple sclerosis.

higher risk of conversion from CIS to CDMS. This suggests a prognostic role of this variable despite our failure to find significant correlations of FLC metrics with cross-sectional and longitudinal MRI measures of brain damage.

There is a large body of evidence that B cell and antibodies play a central role in the immunopathogenesis of MS.[27] Detection of intrathecal Ig production either by quantitative or qualitative means is a supportive criterion in the diagnosis of MS.[1] Since years the gold standard to verify intrathecal Ig synthesis is the detection of OCB in the CSF by isoelectric focusing followed by immunoblotting.[28] The downside of this qualitative measure is that it is a rather time-consuming procedure and that the interpretation of the results is rater dependent.[28] Subsequent to B-cell activation, plasma cells not only produce intact immunoglobulins but also an excess of light chains,[29] which can be found as FLC in the circulation and in the CSF in case of intrathecal immune activation. Different from OCB, FLC can easily be determined

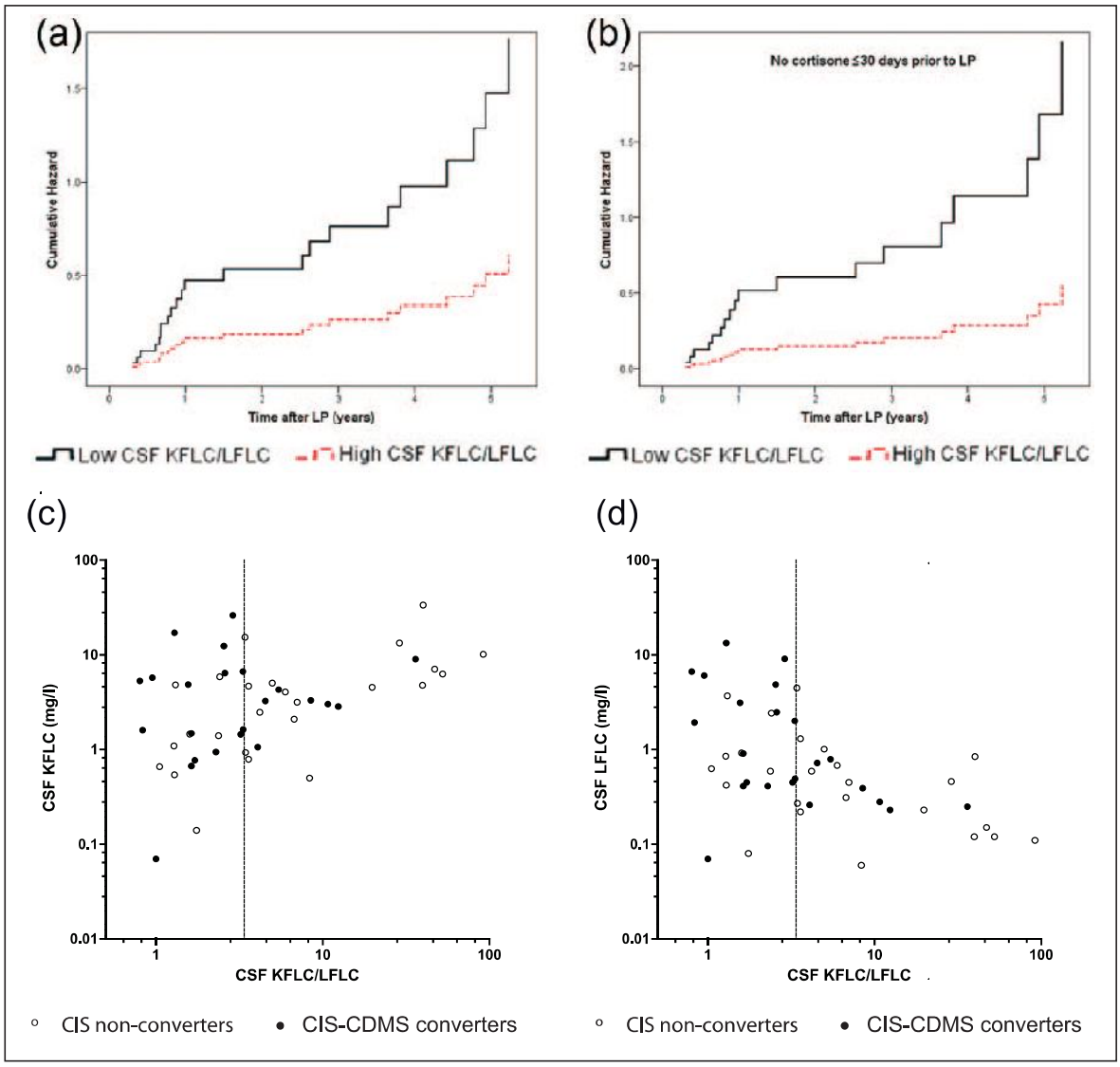

Figure 2. Predicting conversion of CIS to MS with reference to the KFLC/LFLC CSF ratio. Cumulative hazard function for CIS to CDMS conversion stratified by 'high' and 'low' KFLC/LFLC CSF ratio, plotted against time after LP. Cut-off value equals the median of CSF KFLC/LFLC in CIS patients (i.e. 3.38). (a) Cox regression analyses resulted in a significantly lower conversion risk for high compared to low values (HR = 2.89, 95% CI = 1.17–7.14, $p = 0.016$) for all CIS patients. (b) When considering only patients who did not receive corticosteroids within 30 days prior to LP ($n = 39$), this difference was more prominent (HR = 3.94, 95% CI = 1.37–11.35, $p = 0.007$). The relation between the KFLC/LFLC CSF ratio with (c) CSF KFLC and (d) CSF LFLC levels for each CIS patient is visualized. Vertical dotted lines represent the cut-off value between low and high KFLC/LFLC CSF ratio used in the prognostic analyses (i.e. 3.38). Low CSF KFLC/LFLC was mainly due to higher CSF LFLC levels ($p = 0.001$), and changes for CSF KFLC levels were not significant.

CDMS: clinically definite multiple sclerosis; CI: confidence interval; CIS: clinically isolated syndrome; CSF: cerebrospinal fluid; HR: hazard ratio; KFLC: free light chain kappa; LFLC: free light chain lambda; LP: lumbar puncture.

and quantified in a rater-independent manner.[29] The presence of CSF FLC indicates a polyspecific Ig response, which has comparable diagnostic sensitivity and specificity compared to CSF OCB.[2–5,7,8,11,12]

The presence of OCB has been associated with higher risk for conversion from CIS to CDMS[9,10] and disability progression.[30,31] In some recent studies, the prognostic value of FLC to predict conversion from CIS to CDMS has been studied solely for KFLC;[3,11,12,26] however, no relevance has been found in most of these studies.[3,12,26] In one study, higher levels of KFLC in CSF of CIS patients (values over 0.53 mg/L, defined by mean + 2 SD of non-inflammatory

neurological controls) were associated with faster conversion to CDMS.[11] Notably, the patient group designated as 'low' KFLC in this report consisted of 86% OCB-negative patients, which needs to be taken into consideration when interpreting these data. A recent study assessing CIS patients over 2 years found significantly higher CSF levels of both KFLC and LFLC in CIS-MS converters ($n=98$) compared to CIS non-converters ($n=41$).[13] Also here, the non-converter group consisted of significantly more OCB-negative patients than the converters (51% and 7%, respectively, $p<0.0001$).

Contrarily, in this study, we aimed to test whether a quantitative measure, like CSF FLC might yield additional information against the background of already existing OCB positivity, which eradicates a potential confounding effect. Thus, we here embarked on studying exclusively OCB positive CIS/MS patients.

The KFLC/LFLC serum ratio has been shown to be prognostic in several diseases, for example, monoclonal gammopathies[32] and diffuse large B-cell lymphoma.[33] Given the possible different biologic effect of each FLC subunit,[29] we hypothesized that calculating the CSF KFLC/LFLC ratio might yield additional clinical information also in MS. We found that a low ratio of KFLC/LFLC in CSF was associated with conversion from CIS to CDMS when dividing CIS patients at the median of this ratio. Quantitative comparisons show that this ratio is mainly driven by differences in LFLC levels, but also KFLC levels appear to affect the CSF KFLC/LFLC ratio. Calculating the ratio of CSF KFLC to LFLC seems therefore to be superior rather than considering each subunit alone. To what extent this reflects specific immunologic mechanisms needs further exploration. Also the definition of a clinically applicable ratio to predict disease progression will require validation in an independent cohort.

No differences in KFLC and LFLC levels were found between patients with active and non-active disease at the time of lumbar puncture and their proportions were similar in CIS-CDMS converters and non-converters. However, the interpretation of these results is limited due to the small number of patients in each subgroup.

We did not find any correlation of FLC with clinical data on disease severity, that is, EDSS at lumbar puncture and EDSS change during follow-up, although this has been reported in other studies.[34,35] However, this may be explained by the fact that we investigated primarily patients with CIS and a few with early MS. Therefore, our cohort had relatively low disability at baseline and a small range in clinical change over time, which may have hampered more robust correlations and needs to be acknowledged as a limitation of the study. Therefore, future studies should also investigate patients with more severe and progressive disease. The same, that is, no or only a very small change over time holds true for the MRI metrics investigated which also made it unlikely to observe meaningful correlations with FLC variables.

One recent study, also investigating CIS patients, found significant correlations for KFLC variables with EDSS considering CIS-MS converters and for LFLC variables with number of gadolinium positive lesions on MRI.[13] In an earlier study, weak but significant correlations were shown for CSF KFLC levels with Gd-enhanced lesion volume ($r=0.188$; $p\leqslant 0.001$) and T2 lesion volume ($r=0.164$; $p\leqslant 0.001$) in MS patients ($n=262$).[26] However, this study only included RRMS patients and may therefore not be directly comparable to our investigation.

Altogether, our study extends the current notion of the diagnostic value of FLC and provides evidence for a prognostic relevance of CSF KFLC and LFLC in CIS. CSF FLC could not be related to brain tissue damage as evidenced by MRI. Further research is needed to validate FLC as prognostic tool in MS in a multi-centre setting, including more progressive forms of MS and a longer follow-up time.

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Serum netrin-1 in relation to gadolinium-enhanced magnetic resonance imaging in early multiple sclerosis

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Abstract

Background: Netrin-1, a secreted laminin-related protein, is known to regulate not only axonal guidance and neuronal cell migration, but also blood–brain barrier integrity and inflammation. Two preliminary studies reported altered serum netrin-1 levels in multiple sclerosis; however, associations with longitudinal clinical and magnetic resonance imaging activity have not been investigated.

Objectives: We aimed to assess serum netrin-1 in multiple sclerosis and controls with respect to disease activity and its temporal dynamics.

Methods: Serum netrin-1 was assessed by enzyme-linked immunosorbent assay in 79 patients with clinically isolated syndrome or multiple sclerosis, and 30 non-inflammatory neurological disease controls. In patients, serum samples were collected immediately prior to gadolinium-enhanced 3 T magnetic resonance imaging at two time points (initial contrast-enhancing gadolinium+ $n = 47$, non-enhancing gadolinium– $n = 32$; reference gadolinium– $n = 70$; median time-lag 1.4, interquartile range 1.0–2.3 years).

Results: Serum netrin-1 levels were similar in clinically isolated syndrome, multiple sclerosis and controls, and gadolinium+ and gadolinium– patients. Among gadolinium+ patients, serum netrin-1 was decreased in clinically active ($n = 8$) vs non-active patients ($n = 39$; $p = 0.041$). Serum netrin-1 showed no temporal dynamics in multiple sclerosis and was unrelated to clinical data.

Conclusions: Serum netrin-1 levels show no multiple sclerosis specific changes and are not sensitive for detection of subclinical disease activity. Netrin-1 changes during relapses may deserve further examination.

***Keywords*:** Multiple sclerosis, netrin-1, serum, disease activity, blood–brain barrier, magnetic resonance imaging

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Introduction

Netrin-1 (NTN-1) is a secreted laminin-related protein with both attracting and repelling chemotropic effects, which regulate cell-migration and cell-cell or cell-substrate adhesion.[1–3] In the central nervous system (CNS), NTN-1 is known to affect various processes required for tissue development and repair: axonal guidance, glial cell migration, neural growth, and the recruitment and differentiation of oligodendroglial precursor cells.[1–6] A recent experimental study demonstrated, in cell culture and experimental autoimmune encephalomyelitis (EAE) models, that NTN-1 also acts as an important regulator of blood–brain barrier (BBB) integrity and inflammation.[7] Furthermore, NTN-1 has been associated with multiple sclerosis (MS) pathology, as this protein has been reported to be upregulated in astrocytes and macrophages in human and murine demyelinated lesions.[5,6,8,9] Conversely, NTN-1 levels appeared to be decreased in serum and cerebellum and spinal cord tissue of EAE versus control mice.[10] Recently, two independent studies reported NTN-1

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serum levels in MS in humans to be either elevated[7] or decreased[10] compared to controls, respectively. Moreover, the decrease in NTN-1 serum levels appeared to be more prominent in MS patients undergoing a clinical relapse, and 60 days post-relapse.[10]

In MS, active phases of the disease are associated with inflammation and BBB disruption. Magnetic resonance imaging (MRI)-based evidence of disease activity, indicated by the occurrence of new/enlarged T2 or T1 gadolinium (Gd)-enhancing lesions, provides a good surrogate for clinical measures like relapse rate and disability progression.[11] Gd-enhancing lesions on MR images display ongoing inflammation, which is related to BBB disruption and immune cell infiltration at the site.[12]

The recently described results on NTN-1 in MS are partly contradictory, and the extent to which possible alterations of soluble NTN-1 levels may reflect BBB disruption, or even be implicated in pathophysiological processes of BBB breakdown in humans, has not yet been clarified. Therefore, we were prompted (a) to reassess possible differences in NTN-1 levels between MS patients and controls with non-inflammatory neurological diseases, and between patient subgroups (i.e. clinically isolated syndrome (CIS) and relapsing–remitting MS (RRMS)), and (b) to study the association of NTN-1 levels with disease activity, evidenced by Gd-enhanced lesions seen on MRI or by a clinical relapse. Furthermore, we wanted (c) to search for longitudinal changes of this protein in relation to MS.

Patients and methods

This study was approved by the ethics committee of the Medical University of Graz, Austria. All participants provided written informed consent.

Patients and controls

According to our study objectives we searched for patients ($n = 79$) who were seen at the MS outpatient clinic of the Department of Neurology, Medical University of Graz, between 2007–2015, and met the following criteria: (a) diagnosis of clinically isolated syndrome (CIS) suggestive of MS, or RRMS, according to available criteria[13,14] at time of inclusion; (b) availability of an initial and optionally a reference Gd-enhanced MRI scan as described below, at two time-points with a minimum time interval of six months; (c) availability of serum samples at the time of both MRI scans; (d) no corticosteroid infusion within four weeks prior to sampling; and (e) availability of detailed clinical data.

Patients were considered to be in an active state of disease at the time of either examination if Gd-enhancement was present on MRI (i.e. subclinical activity), or if within 30 days prior to MRI and sampling a clinical attack had occurred (i.e. clinical activity).

As controls ($n = 30$), we included individuals who were seen at the outpatient clinic of the Department of Neurology, Medical University of Graz, between 2010–2012, and fulfilled the following criteria: (a) diagnosis of a neurological disease of non-inflammatory etiology (cranial/peripheral palsy – non-inflammatory neurological disease controls; headache or sensory disturbances – symptomatic controls);[15] (b) availability of a diagnostic serum sample; (c) all routine-diagnostic variables measured in cerebrospinal fluid (CSF) and serum at diagnosis within normal range;[15] (d) no immunomodulatory or immunosuppressive treatment prior to sampling.

Clinical assessment

The following demographic and clinical data were recorded at the time of, and in between, both MRI scans in the patients: age, gender, age at disease onset, occurrence of a relapse, and severity of disability assessed by the Expanded Disability Status Scale (EDSS).[16] A relapse was defined as the appearance of at least one neurological symptom or the reappearance of a previous symptom attributed to MS with clinical worsening for at least 24 h succeeding a stable or improving neurological state during at least 30 days.[14] Upon occurrence of a clinically documented relapse, patients usually obtained pulsed steroid therapy of either three- or five-day 1000 mg/day methylprednisolone (number of relapses in between both MRI scans: $0\,n = 49$, $1\,n = 17$, $2\,n = 8$, $3\,n = 3$, $4\,n = 2$). The time lag between the last corticosteroid infusion was at minimum four weeks prior to MRI and blood sampling (time interval last infusion prior to scan: at initial scan median 629 days, interquartile range (IQR) 258–1191 days; at reference scan median 679 days, IQR 379–1136 days). At the time of the initial scan, 36 out of 79 patients received long-term disease-modifying treatment (DMT) (interferon beta $n = 16$, glatiramer acetate $n = 10$, natalizumab $n = 8$, fingolimod $n = 1$, teriflunomide $n = 1$). At the time of the second MRI scan, 52 of 70 patients received DMT (interferon beta $n = 24$, glatiramer

acetate $n = 7$, natalizumab $n = 16$, fingolimod $n = 3$, teriflunomide $n = 1$, dimethyl fumarate $n = 1$).

Serum sampling and analyses

Blood samples were drawn at the time of the MRI examinations in patients or as part of the diagnostic evaluation in controls. 8 ml of peripheral blood was obtained from each subject; an additional CSF sample was drawn by lumbar puncture in controls. Samples were aliquoted and stored at −80°C immediately after collection for patients, or after routine diagnostic work-up for controls, until further analyses. Handling and storage of samples was performed according to international consensus guidelines.[17] For serum and CSF sample pairs of controls a routine diagnostic work-up was performed.[18] All of the control patients tested negative for inflammatory variables in serum and CSF.

Serum NTN-1 levels were measured by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (CSB-E11899h, Cusabio Biotech, Wuhan, China). For the evaluation of temporal dynamics of serum NTN-1 the relative concentration change from initial to the second MRI examination was calculated.

MRI

All patients underwent MRI examinations of the brain on a 3 Tesla Tim Trio system (Siemens Medical Systems, Erlangen, Germany) using a 12-element phased-array head coil. Intravenous Gd contrast (0.1 ml Gadovist per kg body weight) was administered subsequent to structural and T2-weighted imaging and blood sampling. Structural imaging was performed using a T2-weighted 2D fast fluid attenuated inversion recovery (FLAIR) sequence (repetition time/echo time/inversion time (TR/TE/TI) = 9000 ms/70 ms/2500 ms, in plane resolution = $0.9 \times 0.9\,mm^2$, slice thickness = 3 mm). Following Gd administration, an axial magnetization prepared rapid acquisition gradient echo (MPRAGE) sequence (TR/TE/TI = 1410 ms/2.29 ms/900 ms) matching the resolution and angulation of the FLAIR sequence was performed for the detection of active lesions. A second MRI examination, including blood sampling, was performed according to the identical protocol in 70 (88.6%) patients (time interval between both scans median 1.4 years, IQR 1.0–2.3 years).

Image analysis focused on the determination and counting of the number of Gd-enhancing lesions, and was performed by an experienced neurologist.

Statistical analyses

Statistical analyses were performed using SPSS Statistics (version 23.0, IBM Corp. Armonk, New York, USA) and GraphPad Prism (version 5.00, GraphPad Software, San Diego, USA).

All variables were tested for normal distribution using the Kolmogorov-Smirnov test. Group differences were determined by either chi-square test for categorical data or Mann–Whitney U test for continuous variables. Differences between more than two groups were defined by applying Kruskal–Wallis test, followed by post-hoc Dunn's Multiple Comparison test. Longitudinal, paired samples were compared using the Wilcoxon signed rank test or sign test as appropriate. We performed Spearman correlations to determine the correlation coefficients for serum levels with demographic and clinical data.

Significance level was set at 5% ($p < 0.05$).

Results

Subject description

Demographic and clinical data of patients and controls included in this study are listed in Table 1. Patients ($n = 79$) and controls ($n = 30$) were comparable regarding age and gender, as were patient and control subgroups.

At the time of the initial MRI scan, 47 of all patients showed Gd-enhancing lesions (Gd+) (70.2% female; age median 32.0, IQR 26.6–41.1 years; CIS $n = 13$, RRMS $n = 33$, secondary progressive MS $n = 1$) and 32 did not (Gd−) (59.4% female; age median 33.3, IQR 27.2–40.4 years; CIS $n = 18$, RRMS $n = 14$). Eight of the Gd+ but none of the Gd− patients had suffered from a clinical relapse within 30 days prior to the examination, i.e. were in an active disease stage as per our definition. In all patients with clinical activity blood sampling was performed prior to corticosteroid treatment. The control group consisted of non-inflammatory neurological disease controls (cranial/peripheral paresis $n = 9$) and symptomatic controls (headache $n = 8$, sensory disturbances $n = 13$).

Serum NTN-1 group differences

NTN-1 levels measured in serum from MS patients at time of the first MRI and from controls are given in Table 2.

Serum NTN-1 levels did not significantly differ between CIS/MS patients and controls, or between CIS and clinically definite MS patients (Figure 1(a)).

Table 1. Demographic and clinical data of study subjects.

	CIS/MS $n=31/n=48$	**Controls** $n=30$	***p*-Value**
n Female	52 (65.8)	19 (63.3)	n.s.[a]
Age (years)	32.5 (26.7–41.0)	36.5 (28.9–46.2)	n.s.[b]
Disease duration (years)	3.2 (1.0–8.1)	N/A	
Time lag first-second MRI (years)	1.4 (1.0–2.3)	N/A	
EDSS	1.0 (0.0–2.3)	N/A	
EDSS at second MRI	1.0 (0.0–2.0)	N/A	
n DMT	36 (45.6)	N/A	
n DMT at second MRI	52 (77.2)	N/A	
ARR	0.72 (0.42–1.35)	N/A	
ARR at second MRI	0.73 (0.41–1.13)	N/A	
Number of Gd+ lesions[c]	2 (1–4)	N/A	

ARR: annualized relapse rate for RRMS patients; CIS: clinically isolated syndrome; DMT: disease-modifying treatment; EDSS: Expanded Disability Status Scale; Gd+: gadolinium positive; MRI: magnetic resonance imaging; MS: multiple sclerosis; *n*: number of subjects; N/A: not applicable; n.s.: not significant; RRMS: relapsing–remitting multiple sclerosis.
Unless otherwise indicated, data are given for time at the first MRI scan. Values are given as number (%) or as median (interquartile range). Significance ($p < 0.05$) was assessed between subgroups by Chi-squared test[a] or Mann–Whitney *U* test.[b]
[c]Data are given for Gd+ patients only ($n=13/n=34$).

Table 2. Netrin-1 serum levels in clinically isolated syndrome (CIS)/multiple sclerosis (MS) patients and controls.

	CIS $n=31$	**MS** $n=48$	**CIS/MS** $n=31/n=48$	**Controls** $n=30$	***p*-Value**
Gd+ CIS/MS $n=13/n=34$	440.3 (240.9–482.7)	343.0 (267.1–537.0)	344.1 (264.5–531.8)		n.s.[a]
Gd– CIS/MS $n=18/n=14$	347.0 (290.4–429.8)	292.1 (269.0–467.5)	316.1 (279.4–438.5)		n.s.[a]
All	376.0 (280.0–471.1)	335.8 (268.0–512.3)	342.4 (272.8–482.7)	404.5 (273.9–550.1)	n.s.[b]
***p*-Value**	n.s.[a]	n.s.[a]	n.s.[a]		

Gd+: gadolinium-positive; Gd–: gadolinium-negative; *n*: number of subjects; n.s.: not significant.
Values are given in pg/ml as median (interquartile range). Significance ($p < 0.05$) was assessed between subgroups by Mann–Whitney *U* test[a] or Kruskal–Wallis test.[b]

No significant difference of serum NTN-1 levels was observed when comparing patients with initially MRI-based evidence of disease activity and non-active patients (Figure 1(b)). Within the subgroup of Gd+ patients, those patients who experienced an additional clinical attack within 30 days prior to examination (clinically active Gd+, $n=8$) showed significantly decreased serum NTN-1 levels compared to patients who did not (clinically non-active Gd+, $n=39$) ($p=0.041$, Figure 1(c)). No differences were seen between clinically active Gd+ and initially Gd– patients ($n=32$). Of the clinically active Gd+ patients, five underwent a second MRI scan, at which they were clinically inactive and showed no contrast enhancing lesions. At that time point their serum NTN-1 levels were not different from the initially Gd+ or Gd– patient groups any more. The subgroup of clinically active Gd+ patients did not differ from other patients (Gd+ and/or Gd-) with regard to any clinical or demographic data at time of either examination, or over time.

Serum NTN-1 – association with MRI data

At time of the initial scan, the Gd+ patients had a median of two Gd-enhanced lesions (IQR 1–4 Gd+ lesions). No associations between the number of Gd+ lesions and the NTN-1 levels at either time point were seen. Given the fact that serum NTN-1

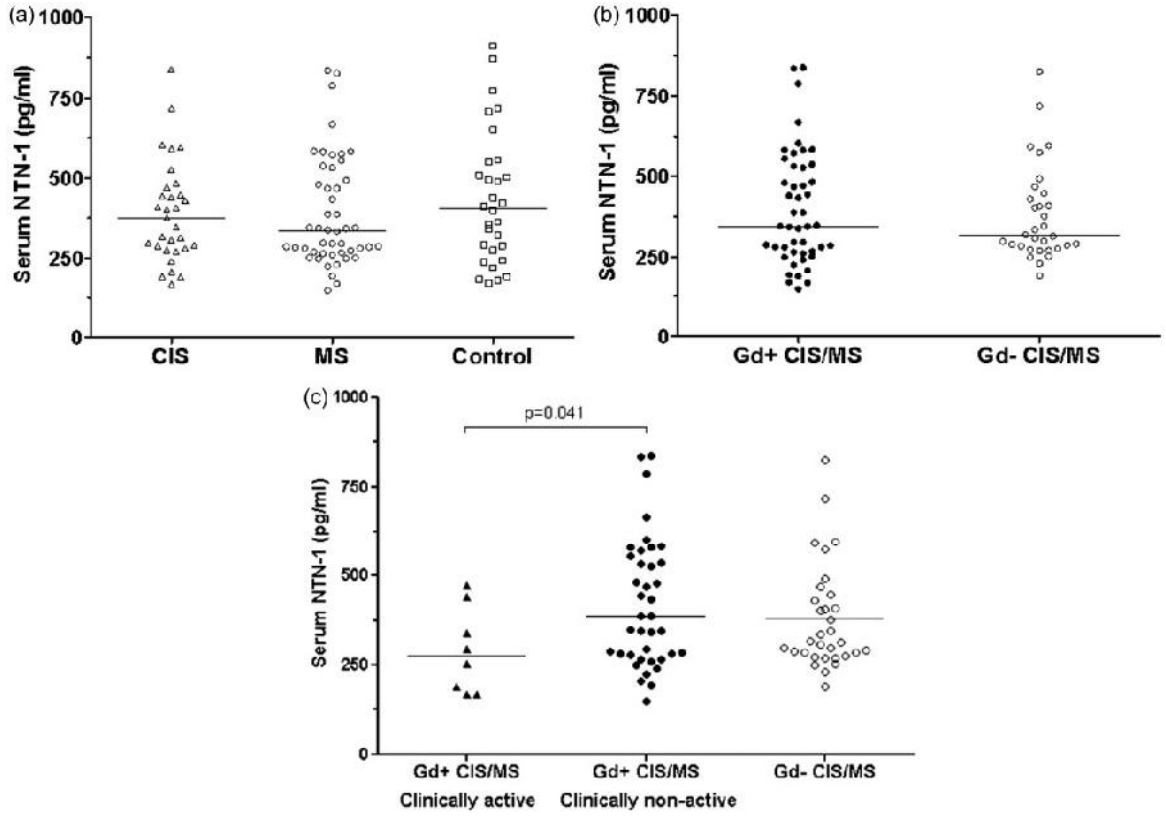

Figure 1. Serum netrin-1 (NTN-1) levels in gadolinium-positive (Gd+) and gadolinium-negative (Gd−) clinically isolated syndrome (CIS)/multiple sclerosis (MS) patients and controls.
Cross-sectional comparisons of serum NTN-1 levels were not significant between CIS patients ($n = 13$), MS patients ($n = 34$), and controls ($n = 30$) (a), and between CIS/MS patients with Gd-enhanced lesions at first magnetic resonance imaging (MRI) (Gd+ $n = 47$) and without Gd-active lesions (Gd− $n = 32$) (b). Only among Gd+ patients, those who were also in a clinically active state of disease (relapse within 30 days prior to examination, $n = 8$) had significantly lower serum NTN-1 levels compared to clinically non-active Gd+ patients ($n = 39$) ($p = 0.041$). NTN-1 levels in clinically active Gd+ were similar to NTN-1 levels in initially Gd− patients ($n = 32$) (c). Significance ($p < 0.05$) was assessed by applying Kruskal–Wallis test, or Mann–Whitney U test.
n: number of subjects.

levels did not associate with Gd+ lesions, and no serum NTN-1 changes were found for CIS/MS compared to controls, or longitudinally for both the Gd+ and Gd− patient groups, we did not perform any further MRI analyses.

Longitudinal changes of serum NTN-1 in MS

A second MRI was performed in 38 of the initially Gd+ patients (CIS $n = 10$, RRMS $n = 28$) and all of the initially Gd− patients (CIS $n = 11$, RRMS $n = 21$), and was Gd-negative in all patients. The time lag between the initial and second MRI scans was comparable between initially Gd+ (median 1.8 years, IQR 1.0–2.9 years) and Gd− (median 1.2 years, IQR 1.0–2.3 years) patient subgroups.

Serum NTN-1 levels showed no temporal dynamics in either initially Gd+ (serum NTN-1 at first MRI median 344.1, IQR 264.5–531.8 pg/ml; at second MRI median 318.5, IQR 252.0–389.7 pg/ml, Figure 2(a)) or Gd- subgroup (serum NTN-1 at first MRI median 316.1, IQR 279.4–438.5 pg/ml; at second MRI median 370.1, IQR 296.6–485.1 pg/ml, Figure 2(b)). Also, the ratio of the second to the initial serum NTN-1 level did not differ significantly between initially Gd+ (ratio second/initial NTN-1 median 0.89, IQR 0.53–1.32) and Gd− patients (ratio second/initial NTN-1 median 1.08, IQR 0.78–1.55). When considering only patients that showed either an increase or decrease in serum NTN-1 levels over time, no distinct clinical or

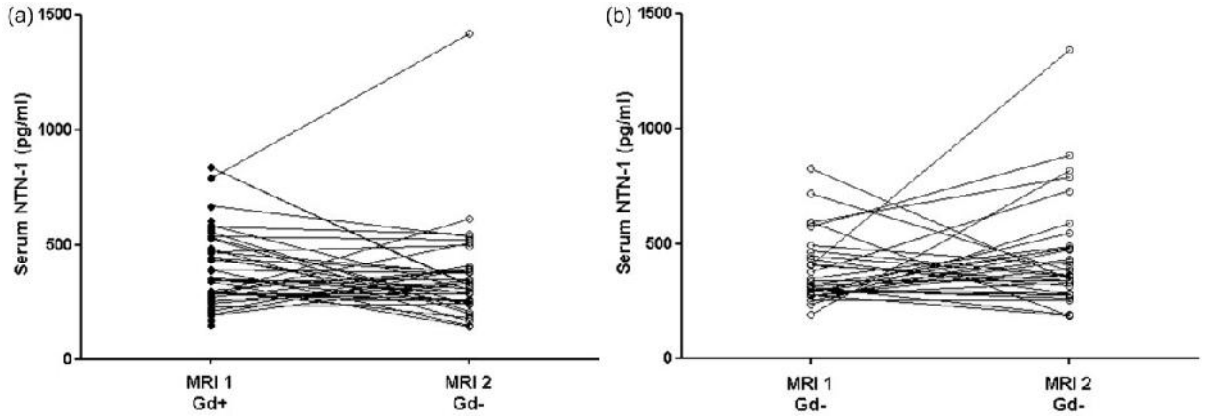

Figure 2. Temporal dynamics of serum netrin-1 (NTN-1) in gadolinium-positive (Gd+) and gadolinium-negative (Gd–) clinically isolated syndrome (CIS)/multiple sclerosis (MS) patients.
Longitudinal comparisons of serum NTN-1 between first and second scan time were not significant for either initially Gd+ (time interval magnetic resonance imaging (MRI)1–MRI2 median 1.8 (interquartile range (IQR) 1.0–2.9) years) (a) or Gd– CIS/MS patients (time interval MRI1–MRI2 median 1.2 (IQR 1.0–2.3) years) (b). Significance ($p < 0.05$) was assessed by applying Wilcoxon signed rank test.

demographic characteristics were notable between both groups.

Serum NTN-1 – association with demographic and clinical data

Serum NTN-1 levels or the longitudinal change in time (delta NTN-1) were similar for both genders. DMT usage, type, or change over time did not alter NTN-1 variables. Also, no correlations were found for NTN-1 variables with age at sampling, age at disease onset, disease duration, physical disability as determined by EDSS at sampling, EDSS change over time, annual relapse rate at time of either time point of examination (for RRMS patients: at initial scan median 0.72, IQR 0.42–1.35; at reference scan median 0.73, IQR 0.41–1.13), and the number or rate of relapses between both examinations. The presence of relapses between both examinations ($n = 29/70$) did not affect the NTN-1 serum levels.

Discussion

We aimed to investigate the role of serum NTN-1 in early MS and its relation to BBB disruption, i.e. disease activity evidenced by contrast-enhanced MRI or clinical relapse. We found no evidence for altered serum NTN-1 levels in patients with CIS or MS compared to controls, nor did NTN-1 levels differ between Gd+ and Gd– patients. Moreover, we did not find any temporal dynamics for NTN-1 in longitudinal serum samples in relation to disease activity assessed by Gd-enhancing lesions on MRI. Gd+ patients with additional clinical disease activity at the time of blood sampling had significantly lower serum NTN-1 levels compared to clinically stable patients in this subgroup.

NTN-1 is a protein known to have a varied and complex functionality within the nervous system,[1–7] and intercellular upregulation was seen at site of lesions in MS.[5,6,8,9] The functionality of NTN-1 was associated with inflammation,[7,8,19] endothelial and in specific BBB integrity,[7,20,21] and remyelination.[5,6,9,22] Along these lines, two studies have also reported on NTN-1 serum levels in MS patients with contradictory results. In a primarily experimental study, a twofold increase was reported in MS patients ($n = 7$) compared to controls ($n = 9$) ($p \leq 0.05$).[7] NTN-1 levels of the patients in that study were up to 15 times as high as in our patient groups.

Another study analyzed serum samples from a larger cohort of MS patients ($n = 90$) and found decreased NTN-1 levels compared to controls ($n = 30$) ($p \leq 0.001$). This decrease appeared to be more prominent in patients with RRMS during and until 60 days after a clinical relapse ($n = 10$), compared to non-active patients ($p \leq 0.001$), whereas solely CIS patients did not differ from controls.[10] Notably, serum NTN-1 levels of MS patients in this study were comparable to the serum levels found in both our patients and controls. However, their control group (mean 51.9 ± standard deviation (SD) 10.5 years) seemed to be considerably older than their MS patient group (mean 42.2, range 22–76 years).[10] Experimental studies have suggested that NTN-1 expression may increase with age, because

of a decreased NTN-1 responsiveness with older age.[23,24] Furthermore, we included only patients with CIS and early RRMS, whereas the previous study also investigated progressive forms of MS.[10] Notably, the same ELISA kit was used in both previous and our studies.

We tested if soluble NTN-1 would be differently regulated during active phases of the disease, but could not find any significant differences between Gd+ and Gd− patients. Only among patients with MRI-based signs of disease activity, those who additionally experienced a clinical attack within 30 days prior to sampling had decreased serum NTN-1 levels compared to those who did not. As these results are based on a very small number of subjects, the possible association of serum NTN-1 alterations with clinical relapses should be investigated in further studies including a higher number of patients.

Recently, the effect of NTN-1 on BBB regulation has been experimentally investigated in human in vitro[7] and murine in vivo models.[7,25] NTN-1 appeared to regulate junctional protein levels,[7,25] to reduce diffusion of blood-derived plasma proteins across the BBB, and to counteract inflammatory effects at the BBB.[7] Also, treatment with NTN-1 in EAE mice improved disease severity and BBB breakdown.[7] We did not find any associations of NTN-1 levels with BBB dysfunction in the human situation by correlating the serum levels with the number of Gd-enhanced lesions or by comparing patients with and without Gd-enhancing lesions on MRI. Moreover, we did not find any changes in serum NTN-1 levels when following the dynamics thereafter, in either initially Gd+ or Gd− patients.

MS lesions with only subtle breakdown of the BBB can show delayed enhancement or enhancement at higher doses only.[26] It therefore cannot be excluded that some patients with disease activity have been missed. However, to obtain consistent sensitivity, a five-minute post-injection delay was included in all examinations, which was considered to be sufficient to detect most of the active lesions.[27]

Still, the effect of BBB disruption, as evidenced by Gd-enhanced MRI in MS patients, on serum NTN-1 might have been too small to detect here. This could partially explain the fact that clinically active patients did show a significant decrease in NTN-1 serum levels in comparison to solely sub-clinically active patients. Possibly, correlations between serum NTN-1 and MRI-based evidence for disease activity might arise when considering patients with more extensive or tumefactive contrast-enhancing lesions.

Apart from BBB regulation, other functions of NTN-1 might play a role in MS as well. Multiple functions of NTN-1 have been shown not only in the CNS, but also in the periphery: anti- or pro-inflammation, cell adhesion, motility, proliferation and differentiation during development of several epithelial and endothelial tissues, and apoptosis and tumor growth mediation.[4,28–35] We further searched for a correlation of serum NTN-1 with demographic and clinical data, including disease duration, physical disability, etc. However, we found no correlations of NTN-1 levels with any clinical or demographic data.

Here, we focused on patients in early phases of the disease with relatively short disease duration and low grade of disability, which needs to be acknowledged as a limitation of the study. We therefore cannot exclude the possibility that serum NTN-1 might be altered in more advanced phases of the disease, including patients with a higher degree of disability, as well as progressive MS forms. Nevertheless, with the present study design with simultaneous collection of blood samples and MRI examinations we can conclude that at least in the early phase of the disease BBB breakdown as evidenced by MRI contrast-enhancing lesions is not reflected by altered NTN-1 levels in the serum.

In summary, serum NTN-1 is unrelated to MS-specific changes and not sensitive to subclinical disease activity. The possible association of serum NTN-1 changes with clinical relapses may deserve further examination.

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Conflicts of interest

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Decreased Cerebrospinal Fluid Antioxidative Capacity Is Related to Disease Severity and Progression in Early Multiple Sclerosis

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Abstract: Background: Oxidative stress-induced neuronal damage in multiple sclerosis (MS) results from an imbalance between toxic free radicals and counteracting antioxidants, i.e., antioxidative capacity (AOC). The relation of AOC to outcome measures in MS still remains inconclusive. We aimed to compare AOC in cerebrospinal fluid (CSF) and serum between early MS and controls and assess its correlation with clinical/radiological measures. **Methods:** We determined AOC (ability of CSF and serum of patients to inhibit 2,2′-azobis(2-amidinopropane) dihydrochloride-induced oxidation of dihydrorhodamine) in clinically isolated syndrome (CIS)/early relapsing-remitting MS (RRMS) (n = 55/11) and non-inflammatory neurological controls (n = 67). MS patients underwent clinical follow-up (median, 4.5; IQR, 5.2 years) and brain MRI at 3 T (baseline/follow-up n = 47/34; median time interval, 3.5; IQR, 2.1 years) to determine subclinical disease activity. **Results:** CSF AOC was differently regulated among CIS, RRMS and controls (p = 0.031) and lower in RRMS vs. CIS (p = 0.020). Lower CSF AOC correlated with physical disability (r = −0.365, p = 0.004) and risk for future relapses (exp(β) = 0.929, p = 0.033). No correlations with MRI metrics were found. **Conclusion:** Decreased CSF AOC was associated with increased disability and clinical disease activity in MS. While our finding cannot prove causation, they should prompt further investigations into the role of AOC in the evolution of MS.

Keywords: multiple sclerosis; cerebrospinal fluid; serum; antioxidant activity; magnetic resonance imaging

1. Introduction

Multiple sclerosis (MS) is a multifactorial, heterogeneous, chronic immune-mediated disease of the CNS, characterized by ongoing neuro-inflammation and -degeneration [1,2]. Converging evidence suggests that central features promoting the pathophysiology of MS are oxidative stress (OS) factors due to an imbalanced redox system [1–8].

Under normal conditions, oxidants are formed as a product of the aerobic cellular metabolism and exert specific intracellular functions [3,9,10]. Antioxidants, which are a wide variety of both enzymatic and non-enzymatic substances, counteract the potentially deleterious effects caused by oxidation of vital cellular components by these free radicals

and related molecules. MS-specific disease characteristics, including activated immune cells, mitochondrial dysfunction and extracellular metal ion accumulation, may cause excessive release of reactive oxygen species (ROS) [1–7]. Further imbalance between ROS production and the body's antioxidative defense causes OS and may contribute to the pathophysiology of MS through activation of inflammatory processes [11–13].

Numerous measures of ROS and antioxidants, i.e., single (metabolic) molecules, chemical elements, markers for lipid/total oxidation or antioxidant activity (of cells, tissues or fluids), have been investigated in the context of MS, predominantly in early phases of the disease (clinically isolated syndromes (CIS) and relapsing-remitting MS (RRMS)), but also in progressive stages [6,8,12,14–19]. Nevertheless, results are largely conflicting and the relation of OS parameters, i.e., (anti-)oxidative compounds, to clinical as well as subclinical measures of disease outcome remains unclear. Subclinical disease activity can be demonstrated by magnetic resonance imaging (MRI), which is of utmost importance in diagnosing as well as monitoring/surveillance of MS in clinical practice [20,21].

The cumulative activity of all antioxidants in body fluids of a patient is reflected by the total antioxidative capacity (AOC), which can be measured fluorometrically. We hypothesized that AOC may be affected in early MS and related to disease characteristics, including both clinical and subclinical measures of disease activity. Hence, we assessed AOC in cerebrospinal fluid (CSF) and serum in CIS and early RRMS compared to other neurological controls and probed its relation to longitudinal clinical and MRI data.

2. Subjects, Materials and Methods

This study was approved by the ethics committee of the Medical University of Graz, Austria (ethical approval number: 31-432 ex 18/19, 17-046 ex 05/06).

2.1. Patients and Controls

All participants included were seen at the MS outpatient clinic of the Department of Neurology, Medical University of Graz, from 2008 to 2013 and gave written and informed consent.

We included patients (n = 66, 66.7% female) meeting the following criteria: (1) diagnosis of CIS (n = 55) suggestive of MS or RRMS (n = 11), according to available criteria at time of inclusion [22,23]; (2) availability of a paired CSF and serum sample taken for diagnostic purposes; (3) no use of disease-modifying treatment prior to sampling (except for short-courses of corticosteroids); (4) available clinical follow-up data; (5) optionally available MRI examination of the brain at 3 Tesla at baseline and during follow-up.

The control group (n = 67, 67.2% female) consisted of subjects meeting the following criteria: (1) diagnosis of a neurological disease of non-inflammatory etiology (cranial/peripheral nerve palsy (n = 15, i.e., non-inflammatory neurological disease controls), headache (n = 29), or sensory disturbances (n = 23, i.e., symptomatic controls)) [24]; (2) availability of a diagnostic paired CSF and serum sample taken for diagnostic purposes; (3) routine-diagnostic variables measured in CSF and serum within normal range [24]; (4) no immunomodulatory or immunosuppressive treatment prior to sampling. Controls were matched to CIS/RRMS patients regarding sex and age.

2.2. Clinical Assessments and Follow-Up

Demographic and clinical data were recorded at time of diagnosis and during clinical follow-up (time since sampling median, 4.5 years; interquartile range (IQR), 5.2 years) in patients—age, sex, age at disease onset, time interval between the diagnosis of CIS and conversion to clinically definite MS (CDMS, upon second clinical relapse) and degree of disability as determined by the Expanded Disability Status Scale (EDSS) [25]. Upon sampling and diagnosis, scheduled follow-up examinations were performed by experienced neurologists.

Clinical relapses were recorded over time according to the following definition: at least one neurological symptom (re)appeared or an old symptom attributed to MS worsened

for at least 24 h, succeeding a stable or improving neurological state during at least 30 days [23]. Upon confirmation of a clinical relapse during neurological examination, patients were usually treated with IV steroid pulses, for either 3- or 5-days, with 1000 mg/day methylprednisolone. Patients were considered to be in an active state of disease at the time of examination if sampling was performed within 30 days of a clinical relapse.

At the time of sampling, 19 patients (28.8%) had received corticosteroids within 30 days prior to CSF sampling and no one was on long-term disease-modifying treatments (DMTs). At some time during the clinical follow-up period, a total of 47 patients (71.2%) was prescribed DMTs. At the time of the last available clinical follow-up, 33 patients (50.0%) received the DMTs interferon beta (n = 16), glatiramer acetate (n = 8), dimethyl fumarate (n = 6), or fingolimod (n = 3). During clinical follow-up, 21 out of 55 CIS patients (38.2%) converted to CDMS, i.e., experienced a second clinical relapse.

2.3. Serum and CSF Sampling and Antioxidative Capacity Analyses

As part of a diagnostic evaluation, a total volume of 8 mL of peripheral blood was obtained and 6–10 mL of CSF was drawn by lumbar puncture in all subjects. Serum and CSF samples were aliquoted and stored at −80 °C immediately after routine diagnostic workup [26] until further analyses, according to international consensus guidelines [27].

The antioxidative capacity (AOC) of CSF and serum samples was determined by assessing the sample's ability to inhibit 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced oxidation of dihydrorhodamine (DHR). Samples were pre-diluted 1:10 in phosphate buffered saline. AAPH, as well as the fluorescent DHR, were added to the assay buffer (20 mM HEPES, 150 mM NaCl, 10 g/L of Chelex-100, 1 mM AAPH, 7.5 µM DHR, pH of 7.4) in the absence or presence of the pre-diluted CSF (final dilution 1:300) or serum (final dilution 1:180) samples. Fluorescence intensity was measured. Readings (excitation wavelength, 485 nm; emission wavelength, 538 nm) were performed every 5 min for 60 min. Finally, the AOC per sample was calculated as percentage inhibition in fluorescence per minute due to oxidation of DHR after addition of the sample compared to blank assay buffer.

2.4. MRI Protocol

Imaging of the brain of CIS/RRMS patients was performed on a 3 Tesla Tim Trio scanner (Siemens Medical Systems, Erlangen, Germany) using a 12-element phased-array head coil. Structural imaging was performed using a T1-weighted three-dimensional (3D) Magnetization Prepared Rapid Acquisition Gradient Echo (MPRAGE) sequence (repetition time (TR)/echo time (TE)/inversion time (TI)/flip angle (FA) = 1.9 s/2.19 ms/0.9 s/9°; isotropic resolution = 1 mm) and a T2-weighted 2D fast Fluid Attenuated Inversion Recovery (FLAIR) sequence (TR/TE/TI = 9000 ms/70 ms/2500 ms, in plane resolution = 0.9 × 0.9 mm^2, slice thickness = 3 mm).

Normalized regional brain tissue volumes of the caudate nucleus, globus pallidus, putamen and thalamus were determined using FSL-FIRST [28]. Normalized brain volumes were determined at baseline using SIENAX and longitudinal percentage of brain volume changes (PBVC) were assessed by applying SIENA at follow-up scans [29]. PBVC was annualized (PBVC/follow-up period).

For T2 hyperintense lesion load (T2LL) assessment, MS lesions were outlined with DispImage, a semi-automatic region growing technique that is based on local thresholding [30]. T2LL was calculated by multiplying the area of all lesion masks by the slice thickness. Image analyses were performed by an experienced neurologist, blinded to clinical data.

2.5. Statistical Analyses

Statistical analyses were performed using SPSS Statistics (version 25.0, IBM Corp. Armonk, NY, USA) and GraphPad Prism (version 5.00, GraphPad Software, San Diego, CA, USA). Normal distribution was tested for all variables using the Shapiro–Wilk test. We

performed either the chi-square test for categorical data, or the independent t-test or the Mann–Whitney *U* test for dichotomous continuous or non-parametric data to determine group differences. Differences between paired samples were analyzed by the Wilcoxon signed-rank test or paired-samples sign test. Multiple comparisons were performed by using the Kruskal–Wallis test with subsequent post-hoc Dunn's multiple comparison test. A univariate general linear model (GLM) was used to determine group differences with adjustment for covariates (demographic data and sample storage time). Correlation coefficients for AOC values with demographic, clinical and MRI data were assessed by Spearman (partial) correlations. Hierarchical linear and binary logistic regression analyses were performed for longitudinal data. The significance level was defined by $p < 0.05$ (2-tailed).

3. Results

3.1. AOC in Relation to Demographic and Laboratory Data

Demographic and clinical data of patients and controls are given in Table 1. Routine diagnostic laboratory parameters, as well as AOC results, are listed in Table 2. In both patients and controls, AOC CSF and serum values were correlated to each other (both $r = 0.45$, $p < 0.001$; Figure 1A), with higher levels found in serum (both $p < 0.001$).

Table 1. Demographic and clinical data of study subjects.

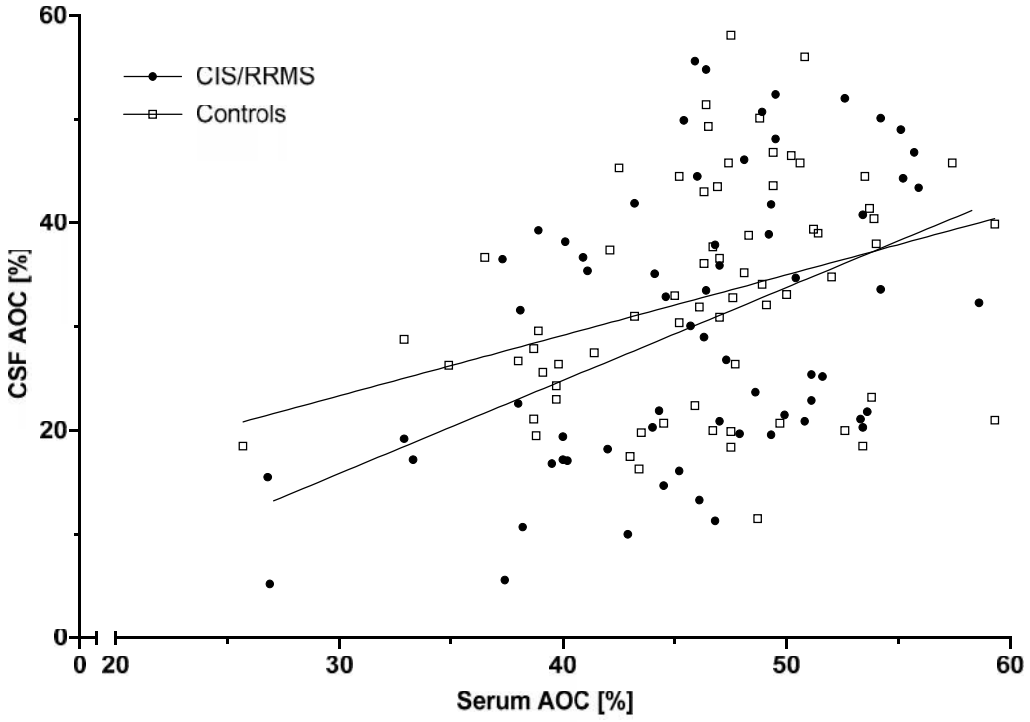
	CIS/RRMS ***n* = 55/*n* = 11**	**Controls** ***n* = 67**
n female	44 (66.7)	45 (67.2)
Age (years)	32.0 (26.4–39.1)	32.7 (25.2–44.9)
Age disease onset (years)	31.1 (25.2–39.0)	N/A
Disease duration (months)	0.5 (0.3–3.9)	N/A
Clinical FU (years)	4.5 (1.9–7.0)	N/A
EDSS	1.5 (0.0–3.0)	N/A
EDSS (in remission)	1.0 (0.0–2.0)	N/A
EDSS last FU (in remission)	0.0 (0.0–1.5)	N/A
n Active disease $\leq$ 30 days prior to sampling	45 (68.2)	N/A
n Cortisone $\leq$ 30 days prior to sampling	19 (28.8)	N/A
n DMT	0 (0)	N/A
n DMT at last FU	33 (50.0)	N/A
ARR last FU (*n* = 21/11) †	0.5 (0.3–0.9)	N/A

Unless otherwise indicated, data are given for time at sampling. Values are given as number (%) or as median (25th–75th quartile). Differences between CIS/RRMS and controls regarding sex ($p = 0.951$) and age ($p = 0.589$) were not significant. ARR = annualized relapse rate; CIS = clinically isolated syndrome; DMT = disease modifying treatment; EDSS = Expanded Disability Status Scale; FU = follow-up; *n* = number of subjects; N/A = not applicable; RRMS = relapsing-remitting multiple sclerosis. † ARR for RRMS at follow-up, disease duration $\geq$ 1 year.

Table 2. Routine diagnostic parameters and AOC in CSF and serum of patients and controls.

	CIS/RRMS ***n* = 55/*n* = 11**	**Controls** ***n* = 67**	***p*-Value**
CSF white cell count (nr/μL) (ref. ≤ 4)	9 (5–17)	1 (1–2)	<0.001 [a]
n OCB positive	64 (97.0)	0 (0)	<0.001 [b]
Q_{alb} ($\times 10^3$)	4.89 (4.19–6.96)	4.96 (4.11–5.84)	0.434 [a]
n increased Q_{alb} (BBB disruption)	17 (25.8)	0 (0)	<0.001 [b]
CSF lactate (mmol/L) (normal range < 2.1)	1.5 (1.4–1.7)	1.4 (1.4–1.5)	0.027 [a]
CSF total protein (mg/dL) (normal range < 45)	35 (27–43)	30 (27–35)	0.012 [a]
CSF AOC (%)	29.5 (19.6–40.8)	32.8 (23.0–41.4)	0.180 [a]
Serum AOC (%)	46.4 (41.1–50.4)	47.0 (43.0–50.0)	0.763 [a]

Data are given for time at sampling. Values are given as number (%) or as median (25th–75th quartile). Significance ($p < 0.05$) was assessed between subgroups by Mann–Whitney *U* test [a] or chi-squared test [b]. AOC = antioxidative capacity; BBB = blood–brain barrier; CIS = clinically isolated syndrome; CSF = cerebrospinal fluid; *n* = number of subjects; OCB = oligoclonal bands; Q_{alb} = CSF/serum albumin quotient; RRMS = relapsing-remitting multiple sclerosis.



(**A**)

Figure 1. *Cont*.

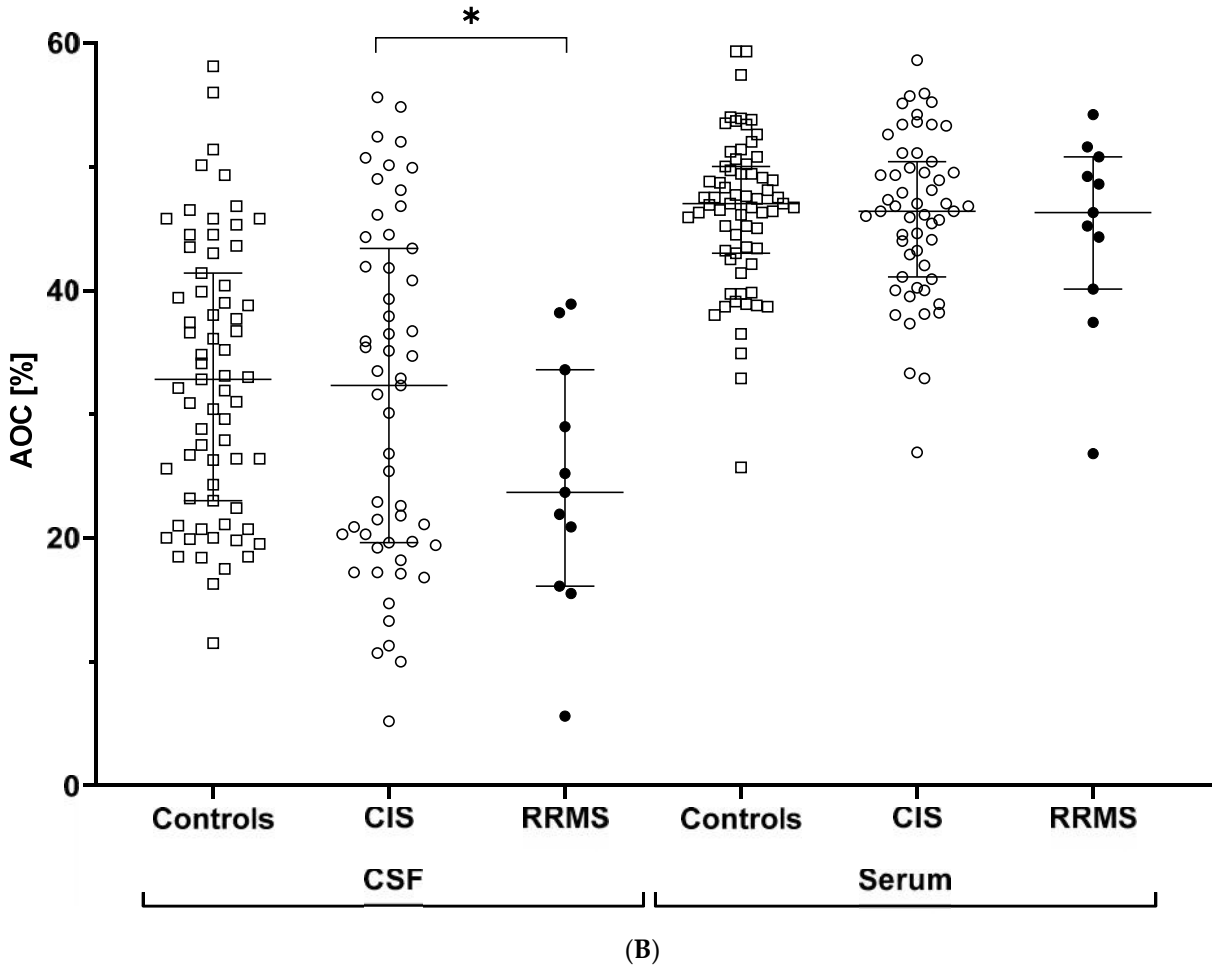


Figure 1. AOC in serum and CSF and its relation to disease course in MS. AOC values in serum and CSF in CIS and RRMS patients and controls. (**A**) CSF and serum AOC correlated mutually in both patients and controls (both $r = 0.45$, $p < 0.001$). (**B**) CSF AOC was significantly different between CIS and RRMS patients and controls ($p = 0.031$; adjusted for covariates) and showed lower values in RRMS compared to CIS patients ($p = 0.020$). Serum AOC was similar for CIS and RRMS patients and controls. AOC = antioxidative capacity; CIS = clinically isolated syndrome; CSF = cerebrospinal fluid; RRMS = relapsing-remitting multiple sclerosis. Horizontal lines represent median values. Significance ($p < 0.05$) was assessed between subgroups by univariate general linear model and Bonferroni post-hoc test. * $p < 0.05$.

CSF AOC was not associated with sex and only a moderate linear correlation was found with age in controls ($r = 0.382$, $p = 0.002$). In serum, we found lower AOC in females compared to males (both in patients (median, IQR: female 44.9, 7.8%; male 51.4, 7.3%) and controls (median, IQR: female 45.2, 8.8%; male 49.7, 6.1%); $p < 0.001$); no association was found with age at sampling.

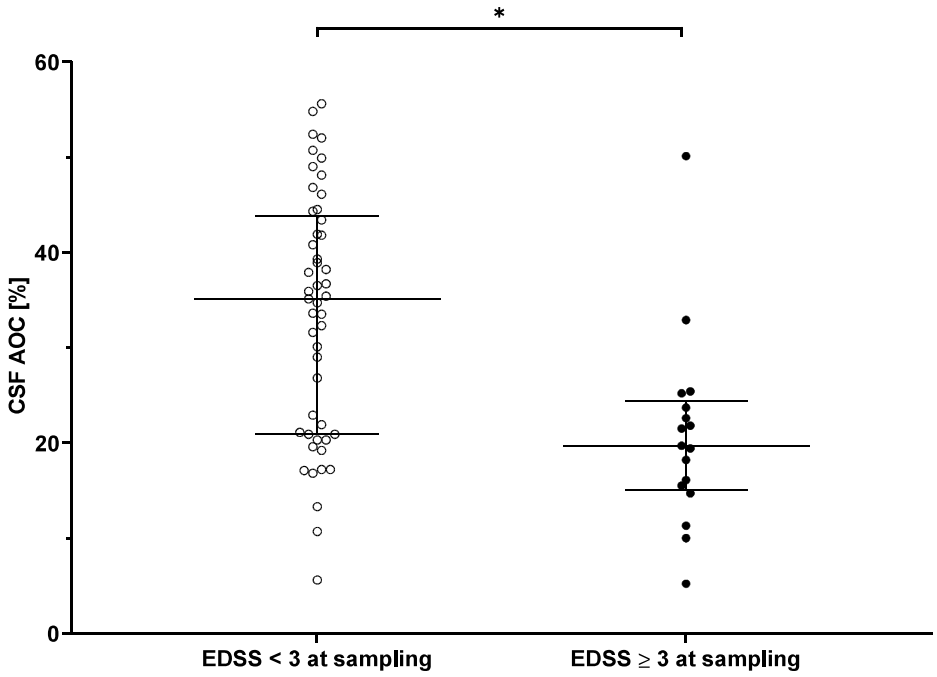
Serum but not CSF AOC was significantly lower in patients who used corticosteroids within 30 days prior to sampling (median, 44.1; IQR, 8.9%; $n = 19$) than that in those who did not (median, 47.3; IQR, 8.4%) ($p = 0.020$, corrected for multiple comparisons). No significant correlations were found between AOC levels and the time since the last corticosteroids were taken (median, 3; IQR, 6 days). AOC was not associated with the use of disease modifying treatments during follow-up.

3.2. CSF AOC in Association with Disease Severity in CIS and RRMS

AOC in CSF was differently regulated among CIS and RRMS patients and controls ($p = 0.031$; adjusted for age at sampling, sex and sample storage time). More specifically, CSF AOC in RRMS patients (estimated mean 26.2 ± SD 3.0%, adjusted for covariates)

was lower than that in CIS patients (34.0 ± 1.4%) (p = 0.020, not corrected for multiple comparisons; Figure 1B).

Lower CSF AOC levels were associated with increased disability at time of sampling. Patients with higher EDSS scores (≥3; n = 17) had significantly lower CSF AOC (estimated mean 26.0 ± 2.6 %, adjusted for covariates) compared to those with lower EDSS scores (<3; n = 49, CSF AOC 34.6 ± 1.4%) (p = 0.022; Figure 2A). CSF AOC also negatively correlated with EDSS at time of sampling (all patients, r = −0.365, p = 0.004) (Figure 2B).



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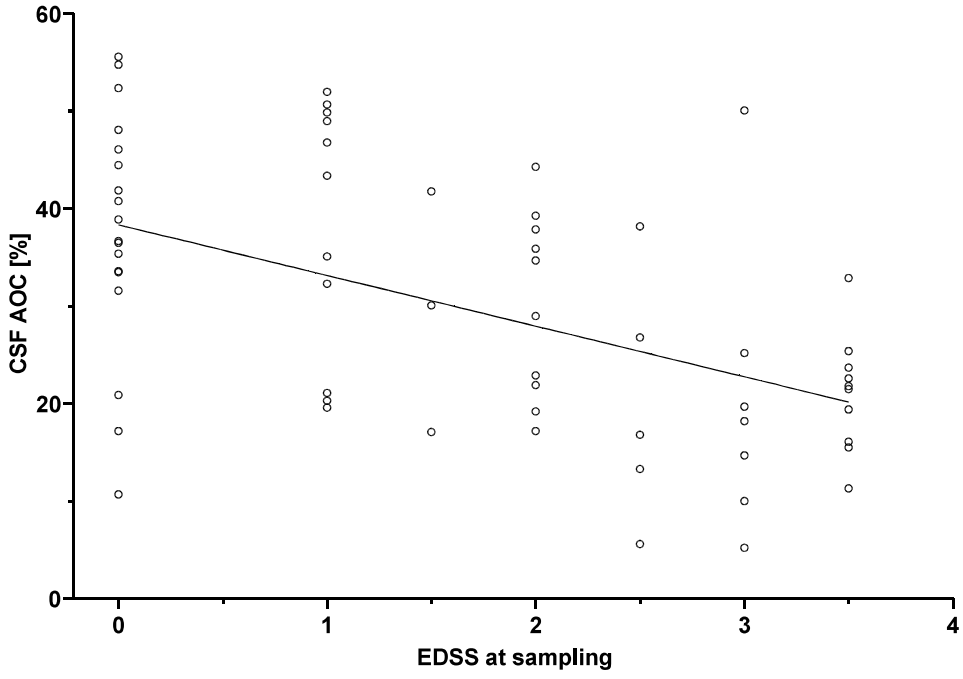
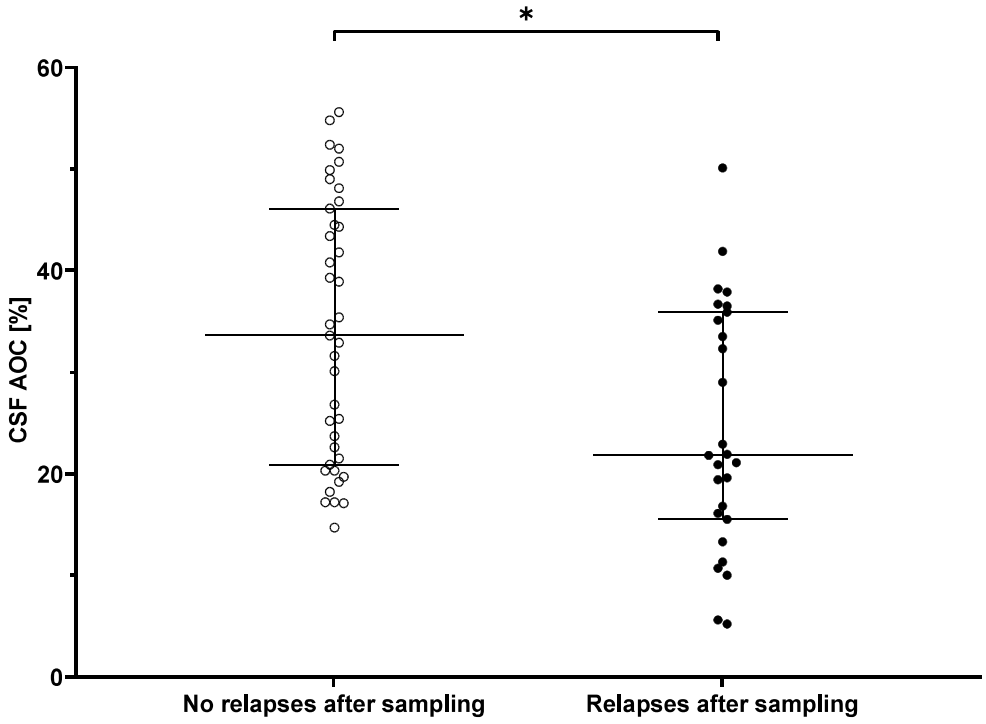


(**B**)

Figure 2. *Cont.*



(**C**)

Figure 2. Decreased CSF AOC is associated with physical disability and predicts disease progression in MS. (**A**) CSF AOC was significantly lower in patients presenting with EDSS $\geq$ 3 (n = 17) those those with lower physical disability (n = 49) (p = 0.002). (**B**) CSF AOC correlated negatively with the EDSS at time of sampling (n = 66, r = −0.365, p = 0.004). (**C**) Graphical display of the association between CSF AOC and the occurrence of relapses after sampling. A decrease in CSF AOC was significantly associated with an increased likelihood of exhibiting new clinical relapses post sampling, as shown by binary logistic regression (exp(β) = 0.929, p = 0.033). During the follow-up time, 27 CIS/RRMS patients underwent a new clinical relapse and 39 did not. AOC = antioxidative capacity; CIS = clinically isolated syndrome; CSF = cerebrospinal fluid; EDSS = Expanded Disability Status Scale; RRMS = relapsing-remitting multiple sclerosis. Significance ($p < 0.05$) was assessed by Mann–Whitney U test, partial Spearman correlation (covariates age at sampling, sex, sample storage time, time between sampling and EDSS examination), or binary logistic regression. * $p < 0.05$.

3.3. CSF AOC and Prediction of Clinical Disease Activity over Follow-Up

Binary logistic regression ascertained that a decrease in CSF AOC upon sampling was associated with an increased likelihood of exhibiting new clinical relapses (exp(β) = 0.929, p = 0.033) (Figure 2C). The model (p = 0.002; including demographic (age/sex) and time dependent covariates (sample storage time, follow-up time) and CSF AOC) explained 33.4% (Nagelkerke R^2) of variance for cases with future relapses (n = 27) and classified 77.4% correctly. We did not find any association of AOC with the time from sampling until the next relapse (median, 1.5; IQR, 3.30 years) or annualized relapse rate at follow-up in RRMS (n = 32).

3.4. Serum AOC and the Association with Clinical Disease Characteristics

AOC in serum did not differ between patient and control subgroups (Figure 1B). Serum AOC was not associated with any of the disease specific parameters, i.e., physical disability determined by the EDSS, clinical disease activity (confirmed relapses) at time of sampling or during follow-up.

3.5. AOC in Relation to MRI Metrics

One or more MRI examination(s) was/were available in 64 (97.0%) of all patients (time interval of first scan since sampling median, 6.3; IQR, 10.6 months). We included

baseline scans acquired within 1 year from serum/CSF sampling (n = 47) and follow-up scans within 2–5 years upon sampling (n = 34; time interval since sampling median, 3.5; IQR, 2.1 years). Normalized brain volumes were assessed for the entire brain, grey and white matter, cortical regions and ventricles. Global brain atrophy was examined by the PBVC and its annualized rate accounting for differences in follow-up and the T2LL was assessed at baseline and follow-up (Table 3). AOC of CSF and serum were not associated with any of the MRI metrics, neither at baseline nor at follow-up, nor did further sub-analyses (including less variable time intervals from sampling to MRI scan) yield significant correlations.

Table 3. MRI metrics of MS patients at baseline and follow-up.

	MRI BL n **= 47**	**MRI FU** n **= 34**	p**-Value** n **= 22**
Time from MRI to body fluid sampling [a] (months), [b] (years)	5.06 (1.22–7.52) [a]	3.51 (2.53–4.60) [b]	<0.001
Normalized brain volume (cm^3)	1617.9 (1566.1–1662.7)	1582.8 (1536.7–1623.2)	0.001
Normalized grey matter volume (cm^3)	832.6 (786.1–861.4)	798.4 (772.0–831.8)	0.004
Normalized white matter volume (cm^3)	779.9 (760.9–818.4)	787.7 (756.5–805.4)	0.017
Normalized cortical grey matter volume (cm^3)	674.9 (638.1–708.5)	641.1 (613.4–682.9)	<0.001
Normalized ventricular volume (cm^3)	28.6 (23.0–42.1)	35.9 (24.7–44.1)	0.004
PBVC (%)	N/A	−0.45 (−0.92–−0.02)	N/A
Annualized PBVC rate (%/year)	N/A	−0.13 (−0.46–−0.02)	N/A
T2LL (cm^3)	5.9 (2.4–10.9)	5.5 (3.3–7.5)	1.000

Values are given as median (25th–75th quartile). Significance ($p < 0.05$) was assessed between subgroups by the paired-samples sign test (n = 22). BL = baseline; FU = follow-up; MRI = magnetic resonance imaging; n = number of subjects; N/A = not applicable; PBVC = percentage brain volume change; T2LL = T2 lesion load.

4. Discussion

Oxidative stress is believed to play a central role in MS pathophysiology. The deleterious effects of reactive oxygen species leading to tissue damage are counteracted and, therefore, also in part determined by the body's ability to delay or prevent oxidation, which is subsumed under the term total antioxidative capacity (AOC). We here provide results indicating that the AOC in the CSF may be reduced in MS patients and relates to physical disability determined by the EDSS. Lower CSF AOC levels further seemed to partly predict the development of clinical relapses.

Oxidation–reduction (redox) status is an important balance between reactions causing deleterious and counteracting effects and is regulated in the body through highly complex mechanisms. The body's redox system comprises of numerous both oxidative and antioxidative compounds, of which many still remain unknown or hard (or even impossible) to measure [5,11,31]. An antioxidant has been defined as "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" [32] and can be classified as either enzymatic (e.g., superoxide dismutase and catalase) or non-enzymatic (low molecular weight elements, e.g., uric acid, albumin, vitamins and antioxidant ions) [3,12,13]. The protective relevance of a specific antioxidant depends on the type of ROS generated (e.g., superoxide, hydrogen peroxide, hydroxyl and peroxyl radicals), the target of damage and where and how it was produced [32]. Given the above-mentioned points, apparently, interpretation and comparison of studies investigating single redox parameters, also in the light of the markedly heterogeneity of MS, is limited [5]. Therefore, a representation of the in vivo balance of the redox system by the AOC, cumulatively for all (anti)oxidants, may be advantageous compared to measuring single compounds [11,31,33]. So far, it remained inconclusive

whether the AOC is altered in the CSF and/or serum in MS and if its levels are related to clinical and imaging parameters.

We found a reduced AOC in the CSF of patients with RRMS vs. CIS, suggesting a relatively decreasing capability of defense mechanisms against OS as the disease advances. This is in line with two recent studies showing decreased CSF AOC in patients with RRMS (n = 22 [34] and n = 57 [35], respectively) compared to controls (n = 20 in both studies). The latter study also found CSF AOC in RRMS to be decreased compared to CIS patients (n = 50), who in turn also had lower AOC levels compared to controls. Notably, both previous studies included patients that were older and had a longer disease duration, as well as more severe physical disability, than our patients, possibly explaining unconforming results. The finding of decreased AOC in CSF in early MS is intriguing and may reflect the predominant role of OS already early in the disease, which can emerge either through the accumulation of (excessively released) ROS, or a depletion of (the activity of) antioxidants [12,13]. AOC can be altered under the influence of several excessive redox dependent changes relevant in MS, such as mitochondrial failure, high lipid peroxidation, loss of BBB integrity, hyperactivation of oxidative compounds, or intake of dietary antioxidants [11,35,36].

Independent from disease stage, we found lower CSF AOC levels to be associated with higher EDSS scores as measure of physical disability at time of sampling. It would be likely that accrual in physical disability is provoked by the perturbation of the body's redox potential in the CNS. A recent study described higher CSF AOC in patients with lower vs. higher EDSS scores ($\leq$3 vs. >3 in CIS, $\leq$5 vs. >5 in RRMS), with a moderately to strongly negative correlation between CSF AOC and EDSS [35]. Another study showed a similar relation, although non-significant, between CSF AOC and EDSS, but with accompanying positively significant correlations of CSF AOC with the antioxidative marker Klotho and, in turn, Klotho with EDSS [34]. Analogous correlations between physical disability and other specific antioxidative markers catalase and superoxide dismutase have been reported [15]. Our data strengthen the hypothesis that perturbation of the defense mechanisms against OS may promote ongoing tissue damage and neuroaxonal loss—the latter representing the pathophysiological substrate of permanent disability—making the AOC a potential treatment target.

The longitudinal clinical data presented here, including a relatively long follow-up time ranging from 2 to 7 years, further show a significant relationship of lower CSF AOC levels at baseline with an increased risk of developing new relapses over time. A few preceding studies also reported decreased AOC parameters either during or as precursor to (clinical) relapses [5,14,18], confirming that a weakened CSF AOC could unfavorably impact the long-term clinical course in MS.

Serum AOC appeared to be decreased in patients with the use of corticosteroids prior to sampling, although this effect was marginal and results in this subgroup were still similar to the controls. Besides, AOC was not affected by the duration of the therapy, nor was the CSF AOC affected by corticosteroid usage. A previous study also found no effect of corticosteroid usage on various markers of oxidative stress (oxidants and antioxidants, including total AOC) in serum and saliva [19]. Only beneficial effects on relieving oxidative stress in MS by the use of corticosteroids have been described, e.g., by a decrease in the lipid peroxidation [37] and oxidative and nitrosative stress markers in CSF/serum of patients [38]. From this, we do not expect corticosteroids to exert a significant influence on results of AOC.

For serum AOC, no associations with other demographic, clinical and morphological data were found, which is in line with findings of other recent studies [8,31,39,40]. However, few other reports showed reduced serum or plasmatic AOC in MS, compared to healthy controls. Importantly, the patient cohorts in these studies differed from ours, as these included more advanced MS with longer disease duration, mostly DMT-treated RRMS [35,41–45], progressive forms of MS (primary or secondary) [11,40], or a combination thereof [14,18,19,46–49]. Only one study including subjects with seemingly similar demographic and clinical features to ours demonstrated serum AOC to be decreased in

CIS and even more in CDMS patients, compared to controls (all groups $n = 49$). Besides, a decrease in serum AOC was associated with an increased risk in CIS patients to convert to CDMS (i.e., to suffer from a second clinical attack, $n = 25$) during a 3-year follow-up time [14]. Future studies including larger sample sizes should clarify if serum AOC may be used as biomarker for disease severity and progression and whether alterations in serum AOC only appear in more advanced MS.

In an attempt to show a relation of AOC with subclinical disease activity, we calculated correlations with cerebral MRI metrics, including T2 lesion load and normalized brain volumes; however, these were not significant. Increasing lesion volume and atrophy are markers of subclinical disease activity and can be used as surrogates to determine treatment response [50,51], but these correlations come from controlled and adequately powered trials and most probably correlations are stage-dependent in "real-life" settings. Importantly, our study was not primarily designed to find a relationship with MRI measures; hence, the number of scans included was limited and the time between sampling and MRI scans varied over our cohort. Additionally, all patients included had a relatively short disease duration accompanied by only minor morphologic brain changes. The resulting potential to find significant associations between AOC and MRI metrics thus remains quite limited for several reasons. Only one recent study reported lower CSF AOC to be associated with higher T2 lesion number (number of lesions $\leq$9 vs. >9 in CIS, or $\leq$40 vs. >40 in RRMS, respectively), although no significant correlations with MRI metrics (absolute number of T2-weighted lesions and volume of gadolinium-enhanced lesions) were found [35]. Another study reported a positive correlation between serum AOC and T2 and gadolinium-positive lesion numbers, although this was only seen in interferon-beta-treated RRMS patients in which lesion numbers were in part also correlated to the patients' disease duration [40]. Further studies including a higher number of patients, more advanced MS disease courses and more frequent MRI scans at regular intervals aiming to capture subclinical disease activity as in phase II treatment trials are needed to draw firm conclusions on the relation of AOC to MRI metrics.

It is important to note that direct comparisons of different studies on AOC may be limited, as different analysis methods have been applied, either proposed to express the total antioxidant level or the antioxidative capacity [52]. Some assays do not measure important antioxidants adequately/efficiently, while, with others, it is unclear which antioxidants contribute to what extent to its values, which may confound results [12,18,45,52]. 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) is a free radical initiator commonly used to evaluate the antioxidant capacity of biological fluids (e.g., CSF and serum). We determined the AOC of CSF and serum samples by assessing the ability of the samples to inhibit the oxidation of dihydrorhodamine (DHR) induced by AAPH. DHR exposed to AAPH oxidizes and begins to fluoresce (at a linear rate). The rate of oxidation of DHR is significantly lower when CSF (or serum) is added. Harmonization of different analysis methods is of great importance to further study this marker in different MS cohorts.

Some limitations of the current study need to be acknowledged specifically. A relatively low number of patients was included, especially in the RRMS group. The focus of our study was further on early disease, which did not include patients with progressive disease and, thereby, not the entire MS spectrum was covered. As patients with CIS and early RRMS are still scarcely represented in the literature, we nonetheless strongly believe that our study contributes to the better understanding of redox imbalance in the pathophysiology during disease onset and early disease. Nevertheless, it would be of interest to include patients with prolonged disease duration and/or more progressed disease, in particular, since some previous studies did find disease-specific associations with the AOC both in CSF and serum that we did not.

Altogether, we here indicate that decreased CSF AOC is associated with increased disease activity and progression in CIS and early RRMS. The AOC therewith seems to be a useful factor to target in order to counteract MS pathology already in the earliest phases of the disease. Our study provides promising results that could serve as a good

basis for future research on extended cohorts to further elucidate the clinical significance of alterations in the AOC in MS. More comprehensive MRI data, as well as cognitive analyses, should be included to investigate the potential role of the AOC as treatment target, or its contribution as a prognostic tool.

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Data Availability Statement: The data presented in this study are available from the corresponding author upon reasonable request.

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Original Research Paper

The effect of disease modifying therapies on CD62L expression in multiple sclerosis

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Abstract

Background: The increasing armamentarium of disease-modifying therapies in multiple sclerosis is accompanied by potentially severe adverse effects. The cell-adhesion molecule CD62L, which facilitates leukocyte extravasation, has been proposed as a predictive marker for treatment tolerability. However, pre-analytical procedures might impact test results, thereby limiting its clinical usability. Whether the immediate analysis of CD62L expression of peripheral blood mononuclear cells can aid treatment decision making is yet unclear.

Objective: To investigate the effect of various disease-modifying therapies in multiple sclerosis on CD62L expression of $CD3^+CD4^+$ peripheral blood mononuclear cells in freshly collected blood samples.

Methods: We collected peripheral blood samples from patients with clinically isolated syndrome and multiple sclerosis (baseline/follow up $n = 234/n = 98$) and healthy controls ($n = 51$). $CD62L^+CD3^+CD4^+$ expression was analysed within 1 hour by fluorescence-activated cell sorting.

Results: $CD62L^+CD3^+CD4^+$ expression was significantly decreased in patients treated with natalizumab ($n = 26$) and fingolimod ($n = 20$) and increased with dimethyl-fumarate ($n = 15$) compared to patients receiving interferon/glatiramer acetate ($n = 90/30$) or no disease-modifying therapies ($n = 53$) and controls ($n = 51$) ($p<0.001$). CD62L expression showed temporal stability during unchanged disease-modifying therapy usage, but increased after natalizumab withdrawal and decreased upon fingolimod introduction.

Conclusion: $CD62L^+CD3^+CD4^+$ expression is altered in patients treated with different disease-modifying therapies when measured in freshly collected samples. The clinical meaning of CD62L changes under disease-modifying therapies warrants further investigation.

Keywords: CD62L, lymphocytes, multiple sclerosis, clinically isolated syndrome, disease-modifying therapies, immunology

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Introduction

An increasing number of highly effective disease modifying therapies (DMTs) are available in multiple sclerosis (MS). Nevertheless, both treatment response and the occurrence of potentially severe side effects in individual patients remain highly unpredictable.[1] Therefore, a strong clinical need exists for body fluid markers that can aid in treatment decision making in terms of efficacy and tolerability.[1]

CD62L, or L-selectin, is a cell adhesion molecule expressed on all leukocytes, which facilitates lymphocyte extravasation and homing of naive T cells to peripheral lymphoid organs. The presence of CD62L ligands on oligodendrocytes and myelin in the central nervous system (CNS) might furthermore

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mediate leukocyte targeting to myelinated axons, e.g. in demyelinating diseases.[2] Recently, it was suggested that the expression of CD62L on $CD3^+CD4^+$ peripheral blood mononuclear cells (PBMCs) is decreased with the use of different DMTs in MS, especially natalizumab and fingolimod.[3–5] It was hypothesized that as a secondary result of this dysfunctional immune status, immune cells in these patients are not able to suppress viral activity as sufficiently, allowing viruses to induce disease. On this basis, CD62L expression could potentially serve as a risk marker for the development of viral infection under the use of DMTs.[3,6] Currently, the reduction of CD62L due to DMT usage has mainly been associated with the development of progressive multifocal leukoencephalopathy (PML), a severe John Cunningham (JC) virus-induced demyelinating disease.[3,4,6–9]

Notably, most studies towards CD62L in MS were performed on cryopreserved PBMCs, for which a pre-analytical procedure is required, which has been shown to impact the test results.[1,6,8,10] Two studies postulated that the CD62L expression is solely an indicator of immune cell stress and subsequent decreased cellular integrity caused by the freeze/thaw procedure.[6,10] The clinical applicability of CD62L as a treatment risk marker may be limited due to the influence of sample quality and the potential requirement of the pre-analytical steps, which may further differ among laboratories. Nevertheless, it is not yet clear if immediate measurement of CD62L may yield comparable results in prognosticating treatment tolerability, and how expression of this surface marker in freshly collected samples may be altered by various DMTs. Therefore, we here aimed to determine longitudinal CD62L expression of $CD3^+CD4^+$ PBMCs in freshly collected whole blood in MS patients using different types of DMTs, and compare it to healthy controls.

Patients, materials and methods

This study was approved by the ethics committee of the Medical University of Graz, Austria. All participants gave written informed consent.

Patients and controls

We included consecutive patients ($n = 234$) who were seen at the MS outpatient clinic of the Department of Neurology, Medical University of Graz, during regular clinical visits between March and December 2015, and met the following criteria: 1) diagnosis with clinically isolated syndrome (CIS) suggestive of MS, or definite MS, according to available criteria at time of inclusion;[11,12] 2) availability of one or multiple fresh full blood samples; and 3) availability of detailed clinical data.

Healthy controls ($n = 51$) consisted of volunteers who were seen at the 'Area health insurance of Graz' between April and October 2015, and met the following criteria: 1) legal age and maturity; 2) no diagnosis with neoplastic disease, acute or chronic infections, autoimmune diseases, acute or chronic diseases with organ damage, pregnancy, or severe anaemia ($Hb < 9$ mg/dl); and 3) availability of a freshly taken full blood sample.

Clinical assessment and follow up

Demographic and clinical data of patients were recorded at every visit by experienced neurologists. These included age, gender, age at disease onset, occurrence of relapses, degree of disability as determined by the Expanded Disability Status Scale (EDSS) score,[13] and medication.

Blood sampling and CD62L analyses

From each subject full blood samples, i.e. PBMCs, were collected in EDTA tubes. Leukocyte cell surface antigen analysis was performed by flow cytometry within 1 hour of sampling. Samples were transferred into fluorescence-activated cell sorting (FACS) tubes and incubated with antibodies (dilution per antibody 1:20 in sample) against CD3 (BD Pharmingen APC-H7 mouse anti-human CD3, cat. no. 341110, BD Biosciences), CD4 (BD Horizon V500 mouse anti-human CD4, cat. no. 560768, BD Biosciences) and CD62L (PE mouse anti-human CD62L, cat. no. 304806, BioLegend) for 30 minutes. Red blood cells were lysed with BD FACS Lysing Solution (cat. no. 349202, BD Biosciences). Upon incubation, samples were washed and pellets were resuspended in cell wash. All steps were performed at room temperature and protected from light. Readout of CD3, CD4 and CD62L was performed with the BD FACSCanto II system (BD Biosciences).

Statistics

Statistical analyses were performed using SPSS Statistics (version 23.0, IBM Corp. Armonk, New York, USA) and GraphPad Prism (version 5.00, GraphPad Software, San Diego, USA). Data were tested for normal distribution using the Shapiro-Wilk test. Group differences were determined by either chi-square test for categorical variables, or Mann-Whitney *U* test for continuous variables. Multiple comparisons were performed using Kruskal-Wallis test and post-hoc Dunn's multiple comparison test. Longitudinal

paired data were analysed using the Wilcoxon signed rank test. Correlations were determined by Spearman's rank-order correlation. Significance was set at 5% ($p < 0.05$).

Results

Cohort description

Demographic and clinical data of all subjects included are listed in Table 1. At time of the first analysis, 181/234 patients received long-term DMT: natalizumab ($n = 26$), fingolimod ($n = 20$), dimethyl fumarate ($n = 15$), interferon beta ($n = 90$) and glatiramer acetate ($n = 30$). Longitudinal blood samples were obtained in 98 patients (up to a total of seven samples per patient; total time interval between baseline and last sample collection median 113, interquartile range (IQR) 78–163 days), see Table 2 and Table 3 for data per subgroup.

For the entire patient group ($n = 234$), the clinical follow-up time was median 9.5, IQR 6.2–13.4 months upon baseline sampling.

Table 1. Demographic and clinical data of study subjects.

	CIS	MS	Controls	*p*-value
n Patients (% female)	48 (72.9)	186 (59.7)	51 (39.2)	0.002[a]
Age (years)	33.3 (26.7–44.1)	36.1 (30.5–45.3)	49.1 (34.1–60.9)	<0.001[b]
Age disease onset (years)	30.9 (25.1–40.2)	26.1 (21.0–31.4)	N/A	0.001[c]
Disease duration (years)	2.3 (1.2–4.8)	9.2 (5.0–15.3)	N/A	<0.001[c]
EDSS	1.0 (0.0–2.0)	1.8 (0.0–3.0)	N/A	0.005[c]
n DMT	40 (83.3)	141 (75.8)	N/A	n.s.[c]
DMT duration (years)	1.8 (0.4–4.1)	2.9 (1.0–6.7)	N/A	0.008[c]
Time since last relapse (years)	2.5 (1.3–4.9)	1.9 (0.5–5.8)	N/A	n.s.[c]
Time since last cortisone (years)	2.4 (1.2–5.0)	2.0 (0.5–4.9)	N/A	n.s.[c]
Time clinical follow-up (months)	8.0 (5.2–10.8)	10.2 (6.3–14.4)	N/A	0.022[c]
Time until last sampling ($n = 12/85$) (days)	117 (56–150)	113 (83–165)	N/A	n.s.[c]

CIS: clinically isolated syndrome; DMT: disease modifying therapy; EDSS: Expanded Disability Status Scale; MS: multiple sclerosis; *n*: number of subjects; N/A: not applicable; n.s.: not significant.
Unless otherwise described, data are given for time at the first sampling. Values are given as number (%) or as median (interquartile range). Significance ($p < 0.05$) was assessed by chi-square test[a], Kruskal-Wallis test[b], or Mann-Whitney *U* test[c].

Table 2. CD62L expression of study subjects per patient subgroup at baseline and follow up.

	CIS $n_{BL} = 48$ / $n_{FU} = 12$	MS $n_{BL} = 186$ / $n_{FU} = 79$	Controls $n_{BL} = 51$	*p*-value
$CD62L^+$ (% $CD4^+$) BL	84.6 (79.5–87.2)	84.5 (79.1–89.5)	84.1 (78.3–88.2)	n.s.[a]
Time BL-FU (days)	117.0 (56.0–150.0)	113.0 (77.0–165.0)	N/A	n.s.[b]
Merged longitudinal $CD62L^+$ (% $CD4^+$)	84.5 (80.5–89.5)	81.8 (75.1–86.9)	N/A	n.s.[b]
$CD62L^+$ (% $CD4^+$) last FU	85.4 (79.3–92.4)	82.2 (73.2–87.8)	N/A	n.s.[b]

BL: baseline; CIS: clinically isolated syndrome; FU: follow up; MS: multiple sclerosis; *n*: number of subjects; N/A: not applicable; n.s.: not significant.
FU time and longitudinal CD62L expression (merged and at last FU) are given for patients who did not change their DMT regarding natalizumab or fingolimod during FU. Merged longitudinal CD62L expression is the combined data of all longitudinal measurements. Values are given as number (%) or as median (interquartile range). Significance ($p < 0.05$) was assessed by Kruskal-Wallis test[a] or Mann-Whitney *U* test[b].

Table 3. CD62L expression of study subjects per DMT subgroup at baseline and follow up.

	NTZ $n_{BL}=26$ / $n_{FU}=24$	FTY $n_{BL}=20$ / $n_{FU}=13$	DMF $n_{BL}=15$ / $n_{FU}=5$	IFN/GA $n_{BL}=90/30$ / $n_{FU}=21/7$	No DMT $n_{BL}=53$ / $n_{FU}=21$	Controls $n_{BL}=51$	*p*-value	Differences
$CD62L^+$ (% $CD4^+$) BL	80.2 (72.7–82.5)*	54.7 (44.0–63.1)*	92.3 (89.7–97.7)**	85.6 (80.6–88.9)	85.9 (81.9–90.5)	84.1 (78.3–88.2)	<0.001[a]	NTZ*, FTY*, DMF**
Time BL-FU (days)	168.5 (130.0–176.5)	96.0 (86.0–113.0)	86.0 (85.0–118.0)	115.5 (80.5–162.0)	63.0 (39.0–120.0)	N/A	<0.001[a]	NTZ > No DMT NTZ > FTY
Merged longitudinal $CD62L^+$ (% $CD4^+$)	80.2 (74.5–83.1)	56.5 (46.5–66.3)	92.4 (90.4–97.3)	86.8 (84.1–90.2)	86.1 (80.5–90.7)	N/A	<0.001[a]	NTZ*, FTY* (incl. NTZ)
$CD62L^+$ (% $CD4^+$) last FU	77.6 (73.4–82.1)	52.1 (46.2–63.4)	94.2 (92.5–97.3)	86.2 (85.1–90.0)	85.2 (80.4–90.2)	N/A	<0.001[a]	FTY < DMF / IFN/GA / no DMT NTZ < DMF / IFN/GA

BL: baseline; DMT: disease modifying therapy; DMF: dimethyl fumarate; FTY: fingolimod; FU: follow up; IFN/GA: interferon beta/glatiramer acetate; *n*: number of subjects; N/A: not applicable; NTZ: natalizumab.
FU time and longitudinal CD62L expression (merged and at last FU) are given for patients who did not change their DMT regarding NTZ or FTY during FU. Merged longitudinal CD62L expression is the combined data of all longitudinal measurements. Values are given as number (%) or as median (interquartile range). Significance ($p < 0.05$) was assessed by Kruskal-Wallis test[a] with Dunn's post-hoc test.
*Patients had significantly decreased CD62L expression compared to all other subgroups (except other * group).
**Patients had significantly increased CD62L expression compared to all other subgroups.

Group comparisons

Cross-sectional measurements of CD62L expression of $CD3^+CD4^+$ PBMCs at baseline were comparable between CIS, MS and controls (Table 2). Comparison of subgroups of patients receiving different types of DMT at the time of the first measurement showed that CD62L expression was significantly decreased in natalizumab- (median 80.2, IQR 72.7–82.5% $CD4^+$) and fingolimod-treated patients (median 54.7, IQR 44.0–63.1% $CD4^+$) compared to all other patient subgroups and controls. CD62L expression was significantly increased with dimethyl fumarate (median 92.3, IQR 89.7–97.7% $CD4^+$) compared to the use of other DMTs (interferon beta/glatiramer acetate, $n = 90/30$; median 85.6, IQR 80.6–88.9% $CD4^+$; no difference in CD62L expression was found between both DMTs), no therapy ($n = 53$; median 85.9, IQR 81.9–90.5% $CD4^+$) and controls (median 84.1, IQR 78.3–88.2% $CD4^+$) (multi-comparison model $p < 0.001$) (Figure 1a).

Longitudinal CD62L expression

CD62L expression showed no significant temporal dynamics in longitudinal samples considering all patients, and in patients without switching DMT

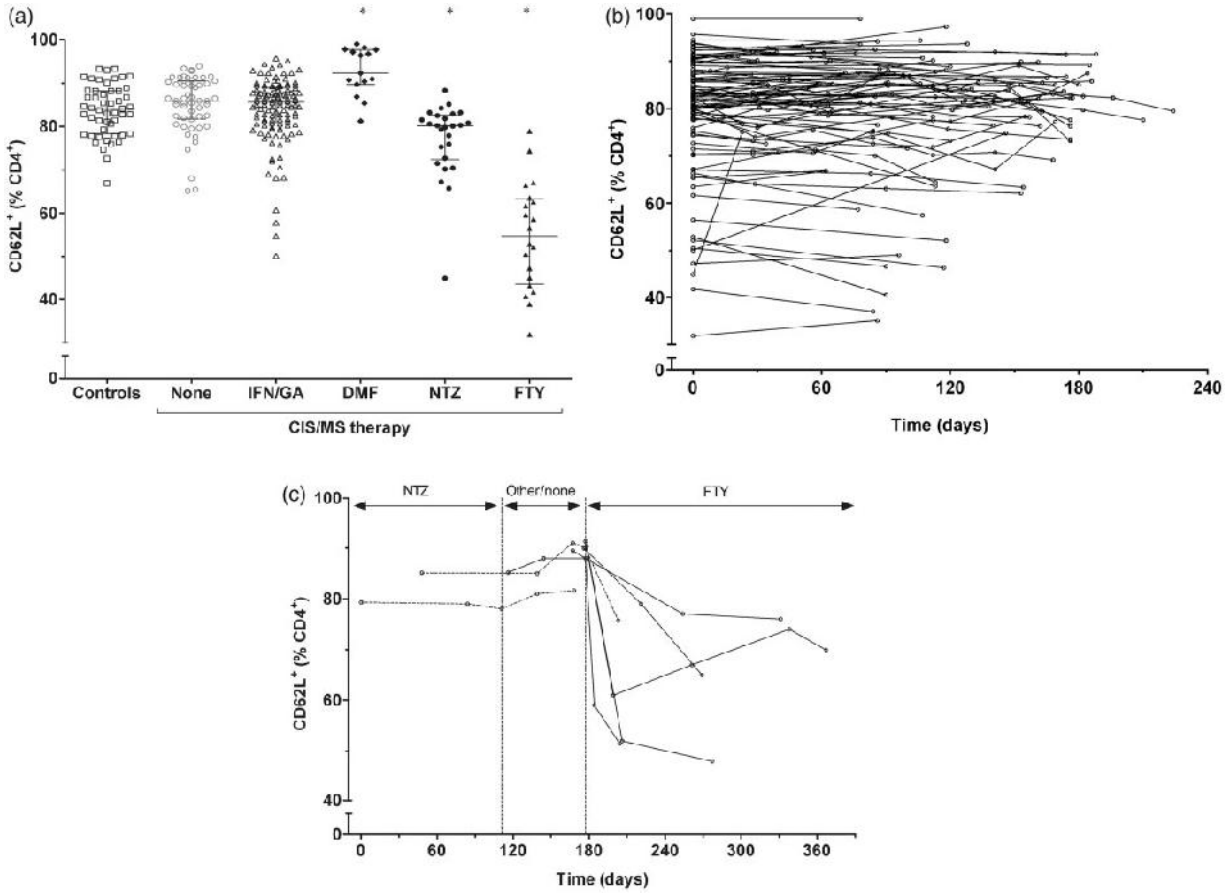

Figure 1. CD62L expression of $CD3^+CD4^+$ PBMCs in CIS/MS patients receiving different types of DMTs and healthy controls. (a) CD62L expression was significantly decreased with the use of natalizumab ($n = 26$) and fingolimod ($n = 20$), and significantly increased with the use of dimethyl fumarate ($n = 15$) compared to all other DMT subgroups and healthy controls (multi-comparison model $p < 0.001$). The data shown refer to baseline measurements. (b) Longitudinal measurements showed no overall alteration of CD62L expression in patients with stable DMT usage ($n = 91$; time interval median 113, IQR 77–163 days). (c) Effects of natalizumab and fingolimod on CD62L expression were visible in longitudinal PBMC samples of patients upon natalizumab withdrawal ($n = 2$, dotted lines; natalizumab treatment until 5.9 and 6.8 years) or after fingolimod initiation ($n = 6$, other/none DMT-treated before). In one patient, CD62L expression was measured when switching from natalizumab to fingolimod with a 2-month interim period.
CIS: clinically isolated syndrome; DMF: dimethyl fumarate; DMTs: disease-modifying therapies; FTY: fingolimod; IFN/GA: interferon beta/glatiramer acetate; IQR: interquartile range; MS: multiple sclerosis; NTZ: natalizumab; PBMCs: peripheral blood mononuclear cells.
Significance ($p < 0.05$) was analysed by Kruskal-Wallis test with Dunn's post-hoc test, or Wilcoxon signed-rank test.

during the sampling interval (Table 2 and 3, Figure 1b). Effects of DMT on PBMC CD62L expression were visible in longitudinal samples of patients who did switch from natalizumab to fingolimod therapy (Figure 1c). CD62L expression was increased upon natalizumab withdrawal in two patients (natalizumab treated until 5.9 and 6.8 years, respectively). A rapid and considerable decrease in CD62L expression was visible in six patients upon fingolimod initiation.

Associations with demographic and clinical data

No gender or age-related effects on CD62L expression were seen. Spearman correlations were not significant for baseline CD62L expression with age (CIS: $r = -0.060$, $p = 0.684$; MS: $r = 0.069$, $p = 0.348$; controls: $r = -0.243$, $p = 0.085$), age at disease onset (CIS: $r = -0.032$, $p = 0.831$; MS: $r = -0.054$, $p = 0.461$), or disease duration (CIS: $r = -0.122$, $p = 0.409$; MS: $r = 0.023$, $p = 0.754$). Mann-Whitney U test showed that CD62L expression was equal between males and females for CIS ($p = 0.602$), MS ($p = 0.198$) and controls ($p = 0.263$). Moreover, Kruskal-Wallis test showed that CIS, MS and control subjects had equal CD62L expression in both males ($p = 0.830$) and females ($p = 0.620$).

We also did not find any significant correlations of CD62L expression with other clinical data, i.e. therapy duration, EDSS at time of sampling and during follow up, and the annualised relapse rate.

None of the patients included developed PML during the study follow-up time. Considering only natalizumab-treated patients, there was no significant difference between JC virus-antibody seronegative ($n = 15$) and seropositive ($n = 11$) patients regarding CD62L expression at the time of the first measurement. A total of six natalizumab treated patients did change their therapy during the total clinical follow-up period (total treatment time natalizumab median 5.8, IQR 5.3–6.9 years; change to dimethyl fumarate $n = 1$, fingolimod $n = 1$, no DMT $n = 4$). Patients who switched from natalizumab during follow up had similar CD62L expression at baseline compared to patients who stayed on natalizumab.

Discussion

In this study we investigated the CD62L expression of $CD3^+CD4^+$ PBMCs in freshly collected whole blood during the use of various DMTs in MS, using direct FACS flow cytometry. We show that the CD62L expression of fresh $CD3^+CD4^+$ PBMCs is decreased with natalizumab and even more with fingolimod treatment, whereas increased levels are found in dimethyl fumarate-treated patients when compared to patients treated with other/no DMTs and healthy controls.

The direction of changes in CD62L expression with natalizumab and fingolimod is comparable to previous results on cryopreserved $CD4^+$ PBMCs.[3,4] Treatment with fingolimod causes a selective retention of predominantly $CD4^+$, and subsidiarily $CD8^+$, naive T cells and central memory T cells (both $CD62L^+$) in the lymphoid tissues, without affecting effector memory T cells ($CD62L^-$) or inducing lymphocyte destruction.[5,14,15] As a result, the overall count of peripheral blood lymphocytes is reduced under fingolimod, in particular that of $CD62L^+$ lymphocytes. Our results on dimethyl fumarate are in line with previous findings, showing that expanded $CD4^+CD62L^+$ expression was found in fresh blood samples of fumarate-treated MS patients compared to non-treated patients and healthy controls.[16] It is thought that this immunophenotypical shift consists of a reduction in the number of effector memory T cells ($CD62L^-$) and a relative increase of naive T cells ($CD62L^+$). The underlying mechanisms leading to alterations in CD62L expression under various DMTs are debated and further research into lymphocyte differentiation and redistribution is needed to better understand the information conveyed by this marker.

Results from a small subcohort of patients who switched their treatment during the time interval of longitudinal sampling show that CD62L expression increases after natalizumab withdrawal, and decreases rapidly upon fingolimod initiation. This indicates the hampering effect of these DMTs on CD62L expression of $CD3^+CD4^+$ PBMCs, and the ability for CD62L recovery after DMT withdrawal. Similar results were shown in a recent study on $CD4^+$ and $CD8^+$ PBMCs in freshly collected blood samples (processed within 6 hours of collection, subsequent analysis within 12 hours).[17] Our longitudinal data further demonstrate temporal stability of CD62L expression in patients who were untreated or did not change their DMT use during multiple sampling. Until now, the invariability of CD62L expression was merely indicated for long-term natalizumab treatment in MS.[4,17]

It has been suggested that CD62L expression of PBMCs might be used as a risk-stratification marker for PML.[3,4,6–8] PML is usually a fatal

opportunistic infection of the CNS caused by the JC virus, which appears most frequently in natalizumab-treated patients,[18] although cases have also been described during treatment with fingolimod[1] and dimethyl fumarate.[19] The association between CD62L and PML is not completely understood; it has been suggested that the general loss of T cells (lymphocytopenia), or the differential effect on T cell subsets by DMTs could be the most important risk factor.[1,19] Some studies showed decreased CD62L expression in pre-PML compared to non-PML natalizumab-treated patients when using cryopreserved PBMCs.[3,6,8] However, these data could not be confirmed in another study[10] and in a sub-cohort using freshly isolated PBMCs.[6] This discrepancy could be caused by assay variability due to pre-analytical procedures, i.e. the freeze/thaw procedure,[1,6,10] or the sample storage protocol handled prior to the cell surface assessment.[8] Here we solely analysed freshly collected blood samples within 1 hour of sampling to prevent any potential pre-analytical bias.

Overall, we could demonstrate feasibility to detect significant alterations in CD62L expression of $CD3^+CD4^+$ PBMCs with various DMTs when measured in fresh blood samples, without laborious pre-analytical steps. We found CD62L to be temporally stable, but differentially regulated with the use of various DMTs. The clinical significance of these findings is not yet clear. Future research is warranted to investigate if CD62L, when immediately measured, may serve as a biomarker for risk stratification of DMT side effects in MS, and as a possible marker for treatment response or disease activity/progression over a longer time. Flow cytometry is a widely accessible method, and the protocol proposed here could readily be implemented in clinical practice.

Conflict of Interests

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F Fazekas serves on scientific advisory boards for Biogen Idec, Sanofi Genzyme, Merck, Novartis, and Teva ratiopharm; serves on the editorial boards of the European Stroke Journal, Multiple Sclerosis Journal, Neurology, the Polish Journal of Neurology and Neurosurgery, and the Swiss Archives of Neurology and Psychiatry; provides consulting services for Actelion, Medday, Parexel and Teva Ratiopharm; and has received speaker honoraria from Merck, Genzyme-Sanofi and Teva Ratiopharm.

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