Diploma Thesis

Redox state of human serum albumin in serum and cerebrospinal fluid of patients with multiple sclerosis

Submitted by

Katharina Maria Seifried

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Division of Medicinal Chemistry

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Department of Neurology, Neurology Biomarker Research Unit

under the supervision of

Ao.Univ.-Prof. Mag. Dr.rer.nat. Karl Öttl

and the co-supervision of

Assoc.-Prof. Priv.-Doz. Dr. med. univ. Michael Khalil, PhD

Graz, 26.03.2024

Declaration of academic integrity

I hereby confirm that the present diploma thesis is the result of my own independent scholarly work. I also confirm that in all cases, where material from the work of others (in books, articles, essays, dissertations, and on the internet) is acknowledged, quotations and paraphrases are clearly indicated. No material other than that cited in the reference list has been used. I have read and understood the Medical University's regulations and procedures concerning plagiarism.

Graz, 26.03.2024 Katharina Maria Seifried m.p.

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Zusammenfassung

Hintergrund und Fragestellung

Wie in vielen anderen Pathologien, einschließlich neurodegenerativer Erkrankungen, ist oxidativer Stress nachweislich in die Pathophysiologie der Multiplen Sklerose (MS) involviert. Humanes Serumalbumin (HSA) ist das häufigste Protein in menschlichem Blut sowie weiteren Körperflüssigkeiten, wie auch dem Liquor cerebrospinalis (CSF). Anhand seines Redoxstatus an der Aminosäure Cystein-34 (Cys-34) kann HSA als Indikator für oxidativen Stress dienen. Diese Diplomarbeit befasste sich mit der Forschungsfrage, ob sich der Redoxstatus von Albumin in Serum und Liquor cerebrospinalis von MS-Patient*innen von Kontrollen unterscheidet, und ob weiters Assoziationen mit Krankheitsaktivität und -schweregrad bestehen. Dies sollte als Pilotstudie im Rahmen der Suche nach MS-Biomarkern dienen.

Methoden

Wir führten eine Hochleistungsflüssigkeitschromatographie (HPLC) an gepaarten Serum- und Liquorproben von 20 MS-Patient*innen und 21 symptomatischen Kontrollpersonen durch, um die prozentuale Zuordnung von HSA zu den Fraktionen Human-Mercaptalbumin (HMA), Human-Non-Mercaptalbumin 1 (HNA1) und Human-Non-Mercaptalbumin 2 (HNA2) zu bestimmen. HMA ist die reduzierte Form des Proteins, mit einer freien Thiolgruppe an Cys-34. In HNA1 ist HSA reversibel oxidiert, wobei Cys-34 als Disulfid zusammen mit einem anderen Thiol vorliegt, während HNA2 irreversibel oxidiert ist, wobei Cys-34 als Sulfinsäure oder Sulfonsäure vorliegt. Anschließend verglichen wir die Verteilung der HSA-Fraktionen zwischen den Gruppen und zwischen Serum und Liquor, sowie suchten nach Zusammenhängen zwischen dem HSA-Redoxstatus und Krankheitsmerkmalen, welche Aktivität und Schweregrad widerspiegeln.

Ergebnisse

Wir fanden keinen signifikanten Unterschied im HSA-Redoxzustand zwischen MS-Patient*innen und Kontrollen, wobei HNA2 im Liquor einen Trend zu höheren Fraktionen zeigte. Im Liquor waren die HMA-Fraktionen signifikant höher als im Serum, während die HNA1- und HNA2-Fraktionen erniedrigt waren. Wir fanden

signifikante Zusammenhänge zwischen dem Albumin-Redoxstatus im Serum und der körperlichen Behinderung in Remission bei MS-Patient*innen, sowie signifikante Zusammenhänge zwischen dem Albumin-Redoxstatus im Liquor und Serum und der Krankheitsaktivität. Darüber hinaus gab es einige signifikante Korrelationen des HSA-Redoxzustands in beiden Kompartimenten mit dem Alter, sowie signifikante Korrelationen der HSA-Fraktionsverteilung im Serum mit Laktat im Liquor.

Schlussfolgerung

Zusammenfassend lässt sich sagen, dass unsere Daten die Beteiligung von oxidativem Stress an der Pathophysiologie von MS bestätigen. Diese Studie soll als Grundlage für weitere Forschung an HSA in MS dienen, insbesondere in größeren Kohorten und bei Patient*innen in fortgeschrittenen Krankheitsstadien. Darüber hinaus tragen unsere Ergebnisse zum Verständnis des Redox-Milieus im Liquor bei, und präsentieren HSA als interessanten Analyten für weitere Untersuchungen in diesem noch wenig erforschten Gebiet.

Abstract

Background and aim

As in many other pathologies, including neurodegenerative diseases, oxidative stress has been shown to play a role in the pathophysiology of multiple sclerosis (MS). Human serum albumin (HSA) is the most abundant protein in blood and various other body fluids, such as cerebrospinal fluid (CSF). According to its redox state in terms of cysteine-34 (Cys-34), HSA can serve as an indicator of oxidative stress. In this diploma thesis, we aimed to analyze the redox state of HSA in serum and CSF of MS patients in comparison to controls, and to further determine potential associations with disease activity and severity. This should serve as a pilot study in the search for MS biomarkers.

Methods

We performed high performance liquid chromatography (HPLC) on paired serum and CSF samples of 20 MS patients and 21 symptomatic controls, to determine the percentual allocation of HSA to the fractions human mercaptalbumin (HMA), human non-mercaptalbumin 1 (HNA1) and human non-mercaptalbumin 2 (HNA2). HMA is the reduced form of the protein, with a free thiol group on Cys-34. In HNA1, HSA is reversibly oxidized with Cys-34 as disulfide together with another thiol, whereas HNA2 is irreversibly oxidized with Cys-34 as sulfinic or sulfonic acid. We then tested for differences in HSA fraction allocation between groups and between serum and CSF, as well as for associations of HSA redox status with disease parameters reflecting activity and severity.

Results

We did not find an overall significant difference in HSA redox state between MS patients and controls, although CSF HNA2 showed a trend to higher fractions. In CSF, HMA fractions were significantly higher than in serum, while HNA1 and HNA2 fractions were lower. We found significant associations of albumin redox state in serum with physical disability in remission in MS patients, as well as significant associations of albumin redox state in CSF and serum and disease activity. Additionally, there were some significant correlations of HSA redox state in both

compartments with age, as well as significant correlations of HSA fraction allocation in serum with lactate in CSF.

Conclusion

In conclusion, our data affirm the involvement of oxidative stress in MS pathophysiology. This study should serve as a basis for further investigation of HSA in MS, particularly in larger cohorts and patients with more advanced disease stages. In addition, our findings contribute to comprehension of the redox environment in CSF, and propose HSA as an interesting analyte for further research in this still poorly understood area.

Disclosures

Parts of this study have been published as an open access article on December 13, 2022 in the *International Journal of Molecular Sciences* (Impact factor 5.6 as of 2022) under the Creative Commons Attribution 4.0 International License (CC BY 4.0):

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Further, the results of this work were presented as a poster at the *Medical University of Graz Doctoral Day* on February 9, 2023, winning the jury-award for best poster:

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Abbreviations

ACLF	acute-on-chronic liver failure
AD	Alzheimer’s disease
ATP	adenosine triphosphate
BBB	blood brain barrier
CIS	clinically isolated syndrome
CMSC	Consortium of Multiple Sclerosis Centres
CNS	central nervous system
CSF	cerebrospinal fluid
Cys-34	cysteine-34
Da	Dalton
DGN	German Neurological Society
DIS	dissemination in space
DIT	dissemination in time
DMT	disease modifying therapy
DNA	deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
EAN	European Academy of Neurology
EBV	Epstein-Barr virus
EDSS	Expanded Disability Status Scale
EMA	European Medicines Agency
FDA	Food and Drug Administration
FLAIR	fluid-attenuated inversion recovery
FLCs	free light chains

Gd	Gadolinium
GFAP	glial fibrillary acidic protein
GSH	reduced glutathione
GSH-Px	glutathione peroxidase
GSSG	oxidized glutathione, glutathione disulfide
GWAS	genome-wide association study
HLA	human leukocyte antigen
HMA	human mercaptalbumin
HNA1	human non-mercaptalbumin 1
HNA2	human non-mercaptalbumin 2
HPLC	high performance liquid chromatography
HSA	human serum albumin
IEF	isoelectric focusing
IFN	interferon
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
JC virus	John Cunningham virus
LP	lumbar puncture
MAG	myelin-associated glycoprotein
MAGNIMS	Magnetic Resonance Imaging in Multiple Sclerosis
MBP	myelin basic protein
MMP	matrix metalloproteinase
MOG	myelin oligodendrocyte glycoprotein

MOGAD	myelin oligodendrocyte glycoprotein antibody-associated disease
MPO	myeloperoxidase
MRI	magnetic resonance imaging
MS	multiple sclerosis
MSTCG	Multiple Sclerosis Therapy Consensus Group
mtDNA	mitochondrial DNA
MWU	Mann-Whitney-U test
NADPH	nicotinamide adenine dinucleotide phosphate
NAIMS	North American Imaging in Multiple Sclerosis Cooperative
NEFA	non-esterified fatty acids
NFL	neurofilament light
NMOSD	neuromyelitis optica spectrum disorder
OCB	oligoclonal band
PD	Parkinson’s disease
PE	plasma exchange
PML	progressive multifocal leukoencephalopathy
PNS	peripheral nervous system
PPMS	primary-progressive MS
Q_{Alb}	albumin quotient
$Q_{HMA/HNA1/HNA2}$	HMA/HNA1/HNA2 quotient
RDA	Research Documentation & Analysis database
RHS	reactive halogen species
RNS	reactive nitrogen species
ROS	reactive oxygen species

RRMS	relapsing-remitting multiple sclerosis
SPMS	secondary-progressive MS
T_H cell	T helper cell
T_{Reg}	regulatory T cell
VCAM-1	vascular cell adhesion molecule 1

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1 Background and aim

Multiple sclerosis (MS) is the most prevalent chronic neuro-inflammatory disease of the brain and spinal cord. It is the leading cause of chronic neurological disability in young adults, affecting more than 2 million individuals worldwide, most of which are women. MS is characterized by fully or partially reversible episodes of neurologic disability, corresponding to inflammatory demyelinating lesions in the white and gray matter of the central nervous system (CNS). After typically 10 to 20 years, the episodic disease course transitions to a continuous progression. A minority of patients present with a progressive course from disease onset on. To this day, the cause for MS remains unknown, and there is no curable therapy available. A range of disease-modifying therapies (DMTs) has been approved to reduce the frequency of clinical episodes as well as slow down disease progression, however no treatment is capable of completely inhibiting the progressive neurologic deterioration (1,2).

1.1 Epidemiology

In 2020 it was estimated that globally, 2.8 million people suffer from MS, which is equivalent to a prevalence of 36 per 100.000 people. This number has increased from 2.3 million people in 2013, with the suspected reasons being better diagnostic tools, better treatment leading to longer survival, improved national and global data collection and overall population growth (3).

However, the MS prevalence is highly variable between different regions of the world. While it is highest in Europe (133 per 100.000) and Northern America, a low prevalence of only 5 per 100.000 is found in Africa and the Western Pacific region (3). Accordingly, people of European descent are most affected, in contrast to a low prevalence among Asian, black or Native American individuals (4). The detailed global MS distribution is depicted in figure 1.

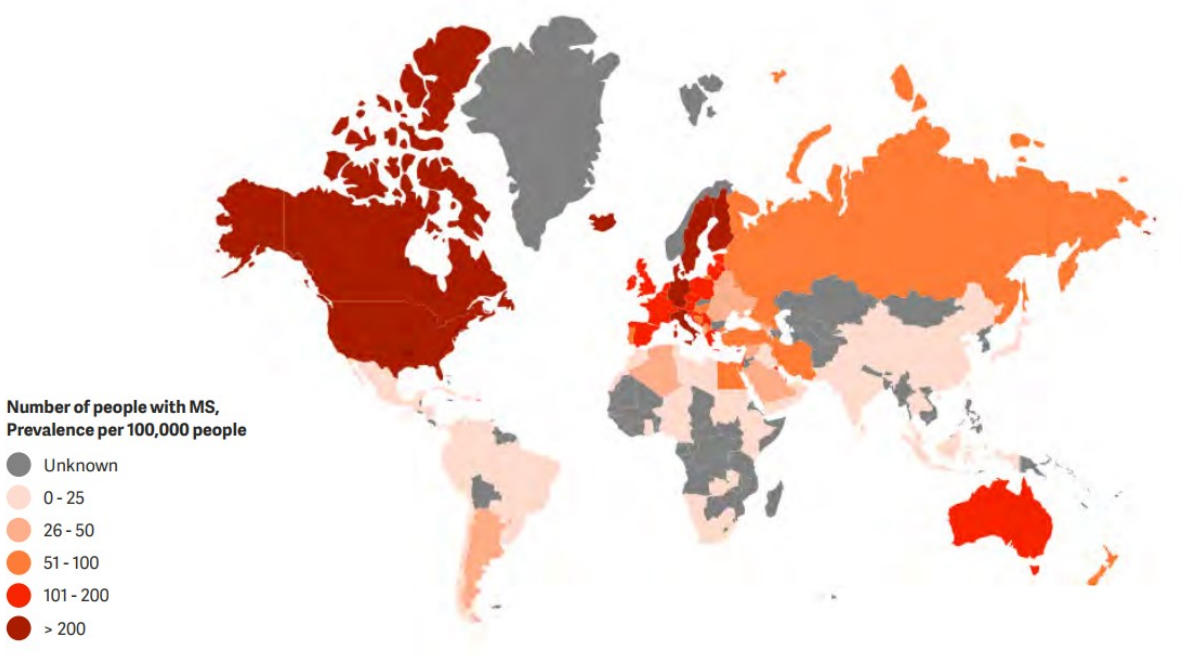

Figure 1. Number of individuals with multiple sclerosis (MS). Prevalence per 100.000 people as of 2020. Reprinted with the permission of the MS international federation (3).

MS prevalence has been linked to latitude multiple times, with greater distance to the equator being associated with a higher risk of developing MS. Suspected explanations for this are a lower exposure to sunlight and therefore lower vitamin D levels in countries located further away from the equator, as well as genetic factors, such as the distribution of the HLA-DRB1 haplotype (3–5).

MS affects women increasingly more frequently than men, with an estimated female to male ratio of 3:1 in 2010 compared to 2:1 in the 1950s. However, this sex ratio is not consistent among countries and appears to decrease with increasing latitude (4). Further, this female preponderance seems to be increasing more rapidly in certain areas, such as Egypt and the Palestinian Authority, where the proportion of affected females has doubled from 2013 to 2020. The reasons for this sex skew are still unclear and various factors could potentially be responsible, including genetic and hormonal differences, as well as differences in environmental exposures and lifestyle (3).

1.2 Etiology

1.2.1 Genetic factors

For first-degree relatives of an MS patient, the lifetime risk of also developing MS is 10 to 30 times greater than for the background population (3% vs. 0.1-0.3%). Twin studies showed that the concordance rate for monozygotic twins lies between 25% and 30%, and a positive family history is found in 15-20% of MS patients. Observances like these established a genetic component in the etiology of this disease already early on (6).

To this day, over 200 genetic variants have been linked to the development of MS, of which the majority encodes molecules involved in the immune system. The strongest association was established with the human leukocyte antigen (HLA) class I DRB1*1501 haplotype, with an odds ratio of 2.9, whereas the other risk variants increase disease probability rather weakly. In combination, multiple genetic risk variants may however increase MS susceptibility (4,7). Particularly the technology of genome-wide association studies (GWAS) enabled the identification of these risk variants. It appears that the majority lie in regulatory, non-coding regions distributed throughout the genome, and often affect regulation through cell-specific effects on gene expression and splicing. Thus, these variants presumably alter the function of both adaptive and innate immune cells in MS pathophysiology, and some of them are associated with other autoimmune and inflammatory conditions as well (8–10). Further, the first study showing an association between genetic variants and disease severity has been published recently. Again using GWAS, authors were able to identify two risk variants, namely the DYSF–ZNF638 variant (rs10191329) and the DNM3–PIGC variant (rs149097173), whose homozygous carriers displayed a significantly shorter interval between disease onset and need for a walking aid (shortened by a median of 3.7 years and 3.3 years, respectively). Additionally, this study showed that genetic susceptibility burden had an influence on age at disease onset, however apart from this affected cross-sectional and longitudinal outcomes only marginally (11).

1.2.2 Environmental factors

Nevertheless, genetic factors cannot explain disease development alone. Environmental factors have a potent effect on MS risk as well, with Epstein-Barr

virus (EBV) infection, smoking, low sunlight exposure/low vitamin D levels and adolescent obesity being the most influential ones. Some of these factors (smoking, EBV infection, obesity) can interact with HLA risk genes, suggesting an influence on adaptive immune response. Further, most environmental risk factors appear to have the strongest impact when individuals are exposed in a certain time window, i.e. adolescence, speaking for a higher susceptibility during this period (12).

Various infectious agents have been investigated as potential triggers for MS development, of which the Epstein-Barr virus (EBV) has the most robust association with the disease. Molecular mimicry causing the generation of cross-reactive T cells and antibodies has been suggested as the pathogenic mechanism behind this, however it remains unclear (4). The theory of EBV being the causative agent for MS was recently confirmed in a large-scale longitudinal study including over 10 million US military recruits. In a 20-year collaboration with the US army monitoring the EBV status of the recruits, Bjornevik et al found that MS risk increased 32-fold after EBV infection, with no increase after infection with other viruses, such as the cytomegalovirus. The EBV-seroconversion rate of recruits developing MS was 97%, in contrast to 57% among those without disease development during follow-up (13).

Another important environmental risk factor for MS is smoking. In multiple studies, an odds ratio of around 1.5 was established for smoking, with a clear dose-dependent risk relationship, and also second-hand exposure to smoke being associated with a higher MS risk. Interestingly, a Swedish case-control study found that long-term oral intake of tobacco products (snuff) decreased the probability of developing MS. Therefore, lung irritation appears to be the driving risk factor behind smoking rather than nicotine exposure (14).

1.3 Disease courses

In 2013, the International Advisory Committee on Clinical Trials of MS published an updated version of the initial descriptions of MS disease types based on a survey by international MS experts dating back to 1996. This first version was solely based on clinical phenotypes due to a lack of imaging and laboratory correlates, whereas the 2013 adaptation incorporates better understanding of the underlying MS pathology as well as imaging advances. Accordingly, four MS phenotypes are being distinguished (15):

- Clinically isolated syndrome (CIS)
- Relapsing-Remitting MS (RRMS)
- Primary Progressive MS (PPMS)
- Secondary Progressive MS (SPMS)

Figure 2 gives an overview of the different disease courses over time.

1.3.1 CIS

CIS was not included in the original 1996 disease course descriptions but was incorporated in the 2013 update. It refers to the first clinical episode appearing characteristic of a CNS demyelinating disease, but not yet fulfilling the criteria for dissemination in time (DIT) (15). A CIS is monophasic by definition and needs to last for at least 24 hours in the absence of fever or infection, with no signs of encephalopathy. Studies have reported highly variable conversion rates from CIS to MS, depending on the type of CIS studied (optic neuritis, spinal cord CIS, brainstem symptoms etc.), and the study population (16).

1.3.2 RRMS

RRMS is the most common phenotype of MS, with approximately 85% of MS patients suffering from it. It is characterized by periods of neurological impairment (relapses) that regress completely or partially, and are alternated with clinically stable periods without new symptoms (remission) (17). The definition of a relapse is a monophasic episode of clinical signs and symptoms reflecting ongoing CNS demyelination that lasts for at least 24 hours and occurs in the absence of fever or infection (18).

1.3.3 SPMS

The majority of untreated RRMS patients eventually transition to a progressive disease course, SPMS, with ongoing disease progression, potentially superimposed by further relapses. Various long-term follow-up studies estimated a median time of 19 years between onset of RRMS and progression to SPMS (19).

1.3.4 PPMS

About 10-15% of MS patients suffer from ongoing increase in neurological impairment starting from the onset of disease, called PPMS. The age of onset of this phenotype lies around 40 years, similar to the transition to SPMS from RRMS,

suggesting common underlying pathomechanisms of the progressive MS forms (20).

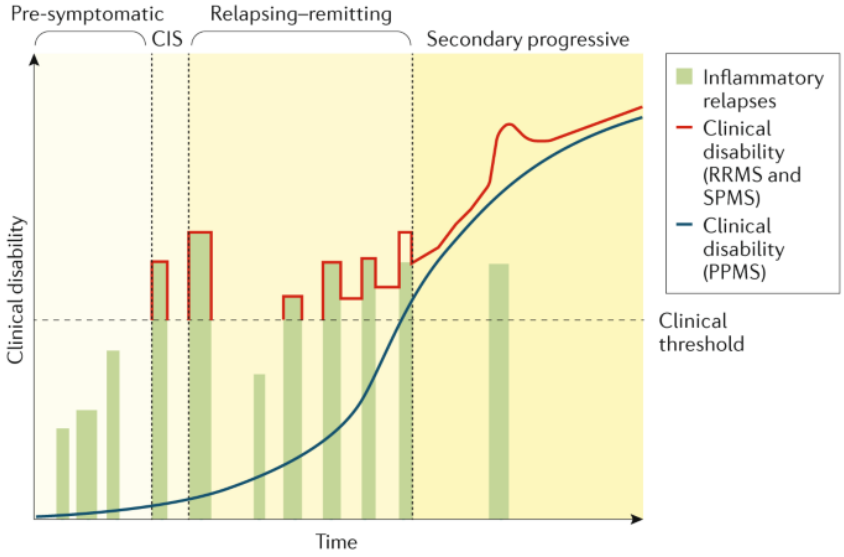

Figure 2. Disease courses of multiple sclerosis (MS). CIS: clinically isolated syndrome, RRMS: relapsing-remitting MS, SPMS: secondary progressive MS, PPMS: primary progressive MS. Reprinted with the permission of Springer Nature (4).

1.4 Pathogenesis and pathology

The hallmark of MS pathology is the presence of demyelinated lesions, also called plaques, in the white and gray matter of the CNS. Demyelination occurs on a background of inflammation containing T cells, B cells and plasma cells, which is typically initiated around postcapillary venules and veins. Subsequent features of MS plaques are oligodendrocyte loss, neuroaxonal degeneration and reactive gliosis (4,21).

MS lesions can be divided into active and inactive plaques. Inactive MS lesions are sharply demarcated and display demyelination, partial axonal preservation, reactive gliosis and a variable degree of microglia activation in the periplaque. Active lesions show a high density of phagocytic cells (microglia and macrophages), of which microglia are most abundant in the rim of active demyelination, but are also present in the periplaque and distant normal-appearing white matter. In contrast, phagocytic cells in the center of the active plaques typically present as macrophages, containing residues of the destroyed myelin sheaths (21). In 2000, Lucchinetti et al presented a further classification of active MS lesions into four different patterns of

demyelination. According to them, pattern I and II lesions present with a T cell- and macrophage-dominant phenotype located around small veins and venules, with pattern II lesions further showing prominent antibody and complement deposition. Lesions of pattern III are also infiltrated with T cells, macrophages and activated microglia. Antibody and complement deposition are absent, but a pronounced loss of myelin-associated glycoprotein (MAG) is found. The plaques are not centered around small blood vessels, contrary to pattern I and II. The borders of pattern III lesions are diffuse, and pronounced oligodendrocyte destruction often extends into the normal-appearing periplaque, with lesion centers often completely lacking oligodendrocytes. Lastly, pattern IV lesions are sharply demarcated areas of oligodendrocyte death, with T cell and macrophage infiltrates, and lacking antibody and complement deposition (22). However, this classification is based on biopsy and autopsy material of patients after a short disease duration and often acute onset. Contrary, Breij et al could not detect the same lesion heterogeneity in an unselected collection of autopsy material from MS patients with an established diagnosis (23).

1.4.1 Inflammation and autoimmunity

The concept of MS being an autoimmune disease largely originates in its similarities with its animal model, experimental autoimmune encephalomyelitis (EAE). This model is induced by immunization of animals with myelin-specific proteins or peptides, such as myelin oligodendrocyte glycoprotein (MOG) or myelin basic protein (MBP), leading to an immune response predominantly driven by myelin-specific CD4+ T cells (24). While activated myelin-specific T cells are also found in peripheral blood of MS patients, the pathophysiology of human MS however is substantially more complex, involving B cells, antibodies and components of the innate immune system, and the question whether autoimmune activation is triggered in the periphery or the CNS has not been fully answered. Moreover, the mechanism of the suspected peripheral activation of autoreactivity in MS remains unresolved (1,25).

Disruption of the blood brain barrier (BBB) is an important feature in the development of demyelinating lesions, and is represented by the extravasation of Gadolinium (Gd) in contrast-enhanced magnetic resonance imaging (MRI). Migration of immune cells across the BBB is a process involving adhesion molecules, chemokines, and matrix metalloproteinases (MMPs). For instance, the

α4β1 integrin is an adhesion molecule expressed on the surface of activated lymphocytes, and is fundamentally involved in BBB transmigration in MS. It interacts with the vascular cell adhesion molecule 1 (VCAM-1) on capillary endothelial cells, and therapeutic blockade of the α4-integrin subunit by the humanized monoclonal antibody natalizumab results in a significant reduction of both clinical and radiological disease activity (1,24).

As mentioned above, the similarities between MS and EAE, alongside the detection of T cells in early demyelinating lesions and the genetic association of MS with HLA haplotypes support a substantial pathogenic involvement of T cells in MS. Generally, the IFNγ- and IL-17A-producing T_H1 and T_H17 cells are the main CD4+ T cell subtypes involved in disease, and steering T cell differentiation towards T_H2 cells instead is thought to be a mechanism of action in the disease-modifying therapies IFNβ, glatiramer acetate and dimethyl fumarate. However, investigation of the exact involvement of these T cell subtypes has so far only yielded conflicting results. Additionally, the monoclonal antibody ustekinumab, which blocks the cytokines IL-12 and IL-23 involved in T_H1 and T_H17 cell differentiation, proved to be unsuccessful in reducing the number of new Gd-enhancing lesions on T1 MRI in a clinical phase II trial (25,26). Besides CD4+ T cells, CD8+ T cells have also been found to play a role in EAE studies, and in cortical demyelinating lesions, their numbers even exceed those of CD4+ T cells and correlate with axonal damage (25).

The involvement of autoreactive B cells in MS pathophysiology has been established, however, in contrast to other CNS demyelinating diseases, such as neuromyelitis optica spectrum disorders (NMOSD) or myelin oligodendrocyte glycoprotein antibody-associated diseases (MOGAD), it has not been possible to identify a specific autoantibody causing MS. Conflicting results of various studies moreso suggest autoantibody-mediated pathology in a subset of MS patients, namely those with prominent ‘tumefactive’ lesions (27). A controversial recent study found that clonally expanded B cells in CSF are the main source of oligoclonal bands (OCBs), a diagnostic hallmark for MS, and was able to identify a single monoclonal antibody showing cross-reactivity between the EBV transcription factor EBNA1 and the CNS glial adhesion molecule GlialCAM, suggesting a molecular mimicry underlying MS pathology (28).

Besides an over-activation of effector immune cells, defects in immune cell regulation may additionally contribute to autoimmunity. Both a decreased frequency as well as a reduced suppressive capacity of regulatory T cells (T_{Regs}) has been reported in MS, with the reasons behind these findings still under speculation. Overall, an imbalance in immune activation and regulation leads to the development of autoreactive adaptive immune cells able to infiltrate the CNS and promote inflammation (25). Figure 3 gives a simplified overview of CNS immune cell dysregulation in early and late disease.

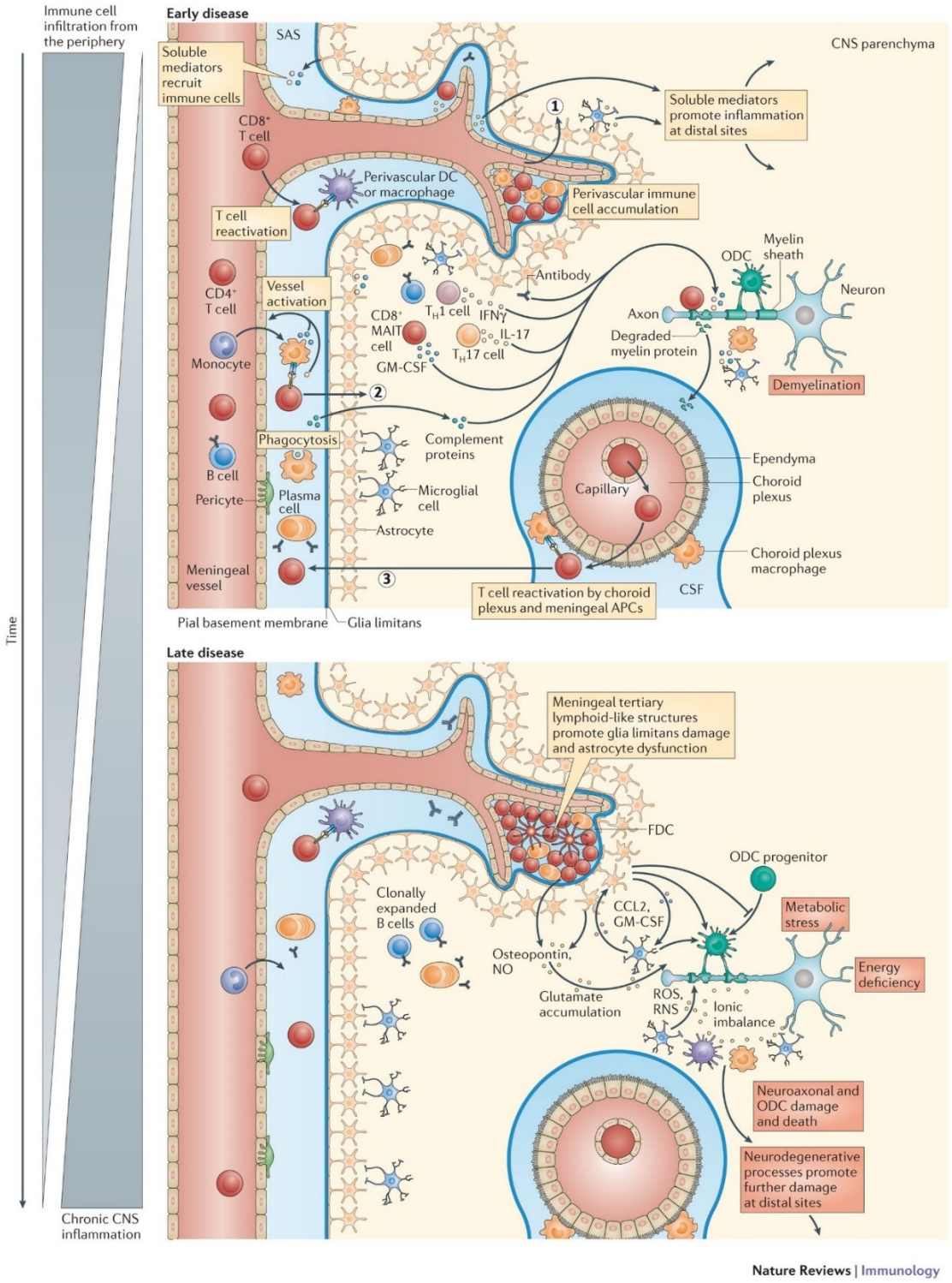

Figure 3. Multicellular immune system dysregulation inside the central nervous system (CNS) in early and late multiple sclerosis (MS). Top panel: Immune cell infiltration into the CNS parenchyma from the periphery through direct crossing of the blood-brain barrier (BBB) (1), from the subarachnoid space (2) or from the choroid plexus across the blood-cerebrospinal fluid (CSF) barrier (3). Together with CNS-resident microglia and astrocytes, infiltrating cells promote demyelination, oligodendrocyte (ODC) and neuroaxonal injury, both through direct cell contact-dependent mechanisms and through soluble mediators. Bottom panel: Waning of immune cell infiltration in later disease stages, while inflammation and neurodegeneration continue under contribution of meningeal tertiary lymphoid-like structures, CNS-resident innate immune cells and astrocytes. APC: antigen-presenting cell; CCL2: CC-chemokine ligand 2; CD8+ MAIT cell: CD8+ mucosa-associated invariant T cell; FDC: follicular dendritic cell; GM-CSF: granulocyte-macrophage colony-stimulating factor; IFNγ: interferon-γ; IL-17: interleukin-17; NO: nitric oxide; RNS: reactive nitrogen species; ROS: reactive oxygen species; TH1 cell: T helper 1 cell. Reprinted with the permission of Springer Nature (25).

1.4.2 Neurodegeneration and axonal loss

While the pathophysiological mechanisms underlying inflammation in MS have been under close investigation, the mechanisms of the accompanying neurodegeneration causing progressive disability are still not fully understood. It is hypothesized that the stressors of ongoing inflammation lead to disturbances in complex neuroaxonal metabolic pathways. Neurons react to these disturbances with the induction of compensatory mechanisms, which provide transient protection, but promote degeneration in the long run. In closer detail, increased oxidative stress due to ongoing inflammation can lead to accumulation of mutations in mitochondrial DNA (mtDNA) and mitochondrial collapse through mitochondrial permeability transition, compromising adenosine triphosphate (ATP) synthesis through oxidative phosphorylation and resulting in chronic hypoxia and neuronal energy deficiency. In parallel, multiple mechanisms lead to a pathological increase in neuroaxonal Ca^{2+} levels, namely entry through redistributed Ca^{2+} channels along demyelinated axons and through glutamate-gated receptors, and potentially release from intra-axonal Ca^{2+} storages, such as mitochondria and the endoplasmatic reticulum. Additionally, Na^{+}/K^{+}-ATPase activity is reduced due to lower ATP levels, causing elevated intracellular Na^{+} levels and a reverse conveyance of the Na^{+}/Ca^{2+} exchanger NCX, all resulting in intracellular Na^{+} and Ca^{2+} overloading. Altogether, the described mechanisms of neurodegeneration seem to propagate neuronal and axonal extinction, likely through induction of apoptosis and Wallerian degeneration, a dying-back process of axons in the CNS and peripheral nervous system (PNS) (29).

1.4.3 Oxidative stress and its role in MS

1.4.3.1 Fundamentals of oxidative stress

The term oxidative stress is defined as “an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage”, and was initially introduced in 1985. In aerobic organisms, so-called reactive oxygen species (ROS) are continuously being created, influenced by metabolic processes as well as external factors. These ROS are also referred to as oxidants due to their high reactivity, through which they easily cause oxidation of biomolecules in their environment. The most relevant ROS include the superoxide anion ($\bullet O_2^-$), the hydroperoxyl radical ($\bullet O_2H$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\bullet OH$) (30). In addition to ROS, other

groups of reactive species can cause oxidation of biomolecules as well, such as reactive nitrogen species (RNS) or reactive halogen species (RHS) (31).

Their reactivity causes oxidation and, through this, oxidative alterations in various surrounding biomolecules. In a lipophilic environment (e.g. cell membranes) for instance, oxidants lead to a chain reaction called lipid peroxidation, which is initiated by a reactive molecule oxidating an unsaturated fatty acid. This reaction creates new reactive species that then continue the oxidative chain reaction. Carbohydrates, particularly those in the extracellular matrix such as hyaluronic acid, are susceptible to oxidation as well, which typically occurs as a splitting off of hydrogen from one of the many C-H bonds, under the creation of an α-hydroxyalkyl radical. The resulting structural changes can cause functional implications. Further, oxidation of nucleic acids (both in mitochondria as well as the nucleus) can directly cause apoptosis, cell aging or malign transformation. Lastly, proteins are also vulnerable to oxidation, particularly in the sulfurous amino acids methionine and cysteine. Cysteine contains a free thiol group, which is typically oxidized by forming a disulfide bond with another free thiol. If this occurs with a second cysteine molecule, the amino acid cystine is created, which often contributes to forming the tertiary structure in proteins. Enzymes from the class of disulfide reductases can dissolve these disulfide bonds again, making this oxidation process reversible. However, free thiol groups can be higher oxidized as well, creating sulfinic or sulfonic acids in an irreversible reaction. If this results in functional impairment of the protein, it is broken down by proteases (32–36). Chapter “albumin and its redox states in serum and CSF” further elaborates oxidation of albumin.

In order to prevent these potential damages through oxidants, aerobic organisms are equipped with various antioxidant molecules and mechanisms. Overall, this protection can be categorized into an enzymatic and a non-enzymatic defense. Molecules of the non-enzymatic defense directly react with oxidants, and therefore through their action as “radical scavengers” render them harmless and stop chain reactions. Examples of this are vitamins E, A and C, or molecules with a free thiol group, such as glutathione (GSSG). Molecules of the enzymatic defense on the other hand either catalyze the conversion of oxidants to less harmful products (e.g. superoxide dismutase, glutathione peroxidase (GSH-Px), catalase), or regenerate

non-enzymatic antioxidants (e.g. glutathione reductase). Overall, antioxidants thus form a complex, closely interacting defense network (33,37).

Recently, the concept of oxidative stress has developed into the idea of an underlying steady-state redox homeostasis between oxidants and antioxidants, from which deviations are considered oxidative stress, leading to a stress response. Within a physiological range, these deviations make up oxidative eustress, which is an essential part of physiological redox control and redox signaling. However, exceeding a certain limit, oxidative processes result in disrupted redox signaling and oxidative distress, which is involved in the pathogenesis of various diseases (38).

1.4.3.2 Oxidative stress in MS

Regarding the CNS, oxidative stress was found to play a driving role in brain aging as well as in the pathophysiology of various neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD) and MS. In the CNS, macrophages and microglia upon activation express enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH)-oxidases, myeloperoxidase (MPO) and inducible nitric oxide synthase (iNOS), functioning as the predominant production source of ROS and RNS. These ROS and RNS impair the mitochondrial oxidative phosphorylation as described in chapter "Neurodegeneration and axonal loss", which in turn further elevates ROS levels in a vicious cycle. Additionally, demyelination causes the release of iron stored in oligodendrocytes and myelin. Divalent iron can catalize the formation of the highly reactive hydroxyl radical through the Haber/Weiß/Fenton reaction, further magnifying oxidative stress (29,39,40).

These ongoing oxidative processes cause tissue injury through multiple mechanisms, which seems to primarily affect neurons and oligodendrocytes in the CNS. Firstly, highly active species such as the hydroxyl radical or peroxynitrite can directly oxidize lipids, proteins and DNA, impairing the function of these biomolecules and thus leading to their degeneration. Oxidized lipids and DNA are highly prominent in active MS lesions, and the extent of lipid and DNA oxidation was found to correlate with inflammation. The oxidation of nuclear DNA can also induce degeneration through apoptosis. Further, mitochondria are highly susceptible to oxidative injury, both through interference with the respiratory chain as well as

through accumulation of mutations in the mtDNA. This leads to cellular energy deficiency, which is particularly fatal in demyelinating diseases, as demyelinated axons demand higher energy to function. As described above, mitochondrial dysfunction is believed to play a major role in neurodegeneration, and the count of respiratory-deficient neurons with extensive mtDNA deletions was strikingly elevated in progressive MS brains (39,41,42).

Altogether, signs of oxidative injury can be found in acute and progressive MS, in the white and gray matter and in highly active as well as slowly expanding lesions. However, it is likely that different mechanisms account for this in different disease stages. While microglia-mediated oxidative burst and inflammation are more prominent in early and acute MS, mitochondrial injury and amplification through iron release from demyelinated lesions seem to become more important with progression and age (39).

1.5 Diagnostics

As of today, MS diagnosis follows the 2017 revision of the McDonald's criteria, which is summarized in table 1. It is based on the detection of demyelinating CNS lesions that are disseminated in space and time (DIS, DIT). DIS refers to multifocal occurrence of lesions in typical anatomical localizations, whereas DIT relates to the development of new lesions over time. The diagnostic process for MS therefore rests on clinical examination and MRI, and can further be supported by CSF and neurophysiological testing (18,43).

Table 1. 2017 revision of the McDonald's criteria for the diagnosis of multiple sclerosis (MS) (18).

	Number of lesions with objective clinical evidence	Additional data needed for a diagnosis of multiple sclerosis
≥2 clinical attacks	≥2	None
≥2 clinical attacks	1 (as well as clear-cut historical evidence of a previous attack involving a lesion in a distinct anatomical location)	None
≥2 clinical attacks	1	Dissemination in space demonstrated by an additional clinical attack implicating a different CNS site or by MRI
1 clinical attack	≥2	Dissemination in time demonstrated by an additional clinical attack or by MRI OR demonstration of CSF-specific oligoclonal bands
1 clinical attack	1	Dissemination in space demonstrated by an additional clinical attack implicating a different CNS site or by MRI AND Dissemination in time demonstrated by an additional clinical attack or by MRI OR demonstration of CSF-specific oligoclonal bands

CNS: central nervous system; CSF: cerebrospinal fluid; MRI: magnetic resonance imaging.

1.5.1 Clinical presentation

Clinical signs and symptoms of MS are heterogeneous and vary according to lesion location in the CNS. However, for 43% of patients, sensory impairment is the first clinical manifestation, and 25% initially present with optic neuritis, causing visual disturbances. Further manifestations over the disease course include motor symptoms, sphincter and sexual dysfunction, cognitive impairment, fatigue and depression (4).

The most widely used standardized scale for evaluating neurological impairment in MS is the Expanded Disability Status Scale (EDSS). It classifies disability by rating the impairment in 8 functional systems, and then comprising these ratings in an overall score, ranging from 0 (normal neurological status) to 10 (death due to MS) in 0.5-interval steps (starting from a score of 1) (44).

1.5.2 MRI

As MRI is highly sensitive to MS CNS lesions, it is now firmly implemented in the diagnostic process of suspected MS patients as well as the monitoring of the disease course. In 2021, a revision of previous guidelines on the usage of MRI in management of MS patients was published as consensus recommendations between the Magnetic Resonance Imaging in Multiple Sclerosis (MAGNIMS) study group, Consortium of Multiple Sclerosis Centres (CMSC), and North American Imaging in Multiple Sclerosis Cooperative (NAIMS). According to these, T2-weighted 3D-fluid-attenuated inversion recovery (FLAIR), axial T2-weighted, and T1-weighted with gadolinium are the core sequences for establishing MS diagnoses (45).

MS lesions appear as focal hyperintensities on T2-weighted MRI, which are typically round to ovoid in shape, with a size of a few millimeters to even over 2 centimeters. They occur asymmetrically in both hemispheres. They can develop in any CNS region; however, certain regions have been identified as typical locations for MS lesions: the periventricular and juxtacortical white matter, infratentorial areas and the spinal cord. On postcontrast T1-weighted images, Gd-enhancement indicates disruption of the BBB in acute inflammatory lesions. This Gd-uptake typically persists for <4 weeks (46). Further, some T2 hyperintensities correspond to dark lesions on T1-weighted images. These so-called black holes usually disappear after

around 6 months (acute black holes). However, some remain (persistent black holes), and are believed to show irreversible axonal loss (47). Typical lesions on MRI scans are shown in figure 4.

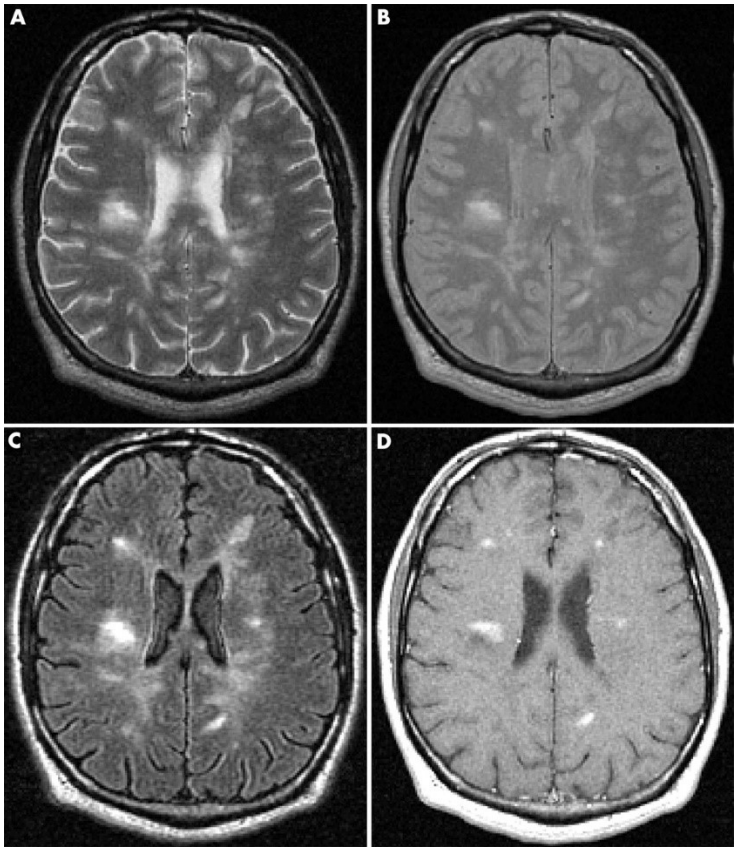

Figure 4. Typical multiple sclerosis (MS) lesions of a 30 year old patient on axial magnetic resonance imaging (MRI) scans. A: T2 weighted image, B: proton densitiy (PD) weighted image, C: fluid attenuated inversion recovery (FLAIR) image, D: T1 weighted image after administration of Gadiolinium (Gd). Reprinted with the permission of BMJ Publishing Group Ltd. (48).

MRI is widely used in the establishment of a diagnosis of MS according to the McDonald's criteria. To standardize the use of MRI for the necessary verification of DIS and DIT, the network MAGNIMS published updated consensus guidelines in 2016, based on the 2010 McDonald's criteria. However, since in 2017 updated revisions of the McDonald's criteria were published, current recommendations for MS MRI diagnostics follow these revisions. Accordingly, DIS is given in the presence of one or more characteristic T2-hyperintense lesions in two or more of the four typically affected CNS regions, that are periventricular, cortical/juxtacortical, and infratentorial brain regions, as well as the spinal cord. DIT is fulfilled either by the simultaneous occurrence of Gd-enhancing and non-enhancing lesions at any time,

or by appearance of a new T2-hyperintense or Gd-enhancing lesion of a follow-up MRI with reference to a baseline scan (irrespective of its timing). Contrary to the 2010 revisions of the McDonald's criteria and hence the 2016 MAGNIMS guidelines, there is no more distinction made between symptomatic and asymptomatic lesions (18,45,49).

1.5.3 CSF analysis

Besides clinical and MRI examination, CSF analysis remains a routinely used and valuable tool in MS diagnostics. The detection of intrathecal antibody synthesis, either qualitatively through presence of OCB in isoelectric focusing (IEF) or quantitatively through calculation of the IgG index, supports the diagnosis. Conversely, a CSF profile atypical of MS or the absence of oligoclonal bands points towards differential diagnoses (18,50). Typical routine CSF findings of MS patients are mild leukocyte pleocytosis <50 cells/µl, of which >90% are lymphocytes, normal glucose ratios and usually normal total protein or albumin quotients, as the BBB leakages in MS are focal and transient (51).

In the 2017 revisions to the McDonald criteria CSF analysis gained value in the diagnostic process of MS. Since then, the presence of CSF OCB allows the diagnosis of MS in a typical CIS patient with proven DIS in clinical or MRI examination, and absence of atypical CSF findings, even if the DIT criterion is not fulfilled clinically or radiologically (18).

Detection of OCB that are present in CSF but not in serum through IEF is a qualitative method to test for intrathecal production of antibodies. In normal CSF, all IgG stems from the blood and enters the CSF space through passive diffusion. In healthy conditions, IgG in CSF and serum is polyclonal, and if oligoclonal IgG occurs in serum, the pattern is mirrored in CSF due to diffusion. Thus, if oligoclonal IgG bands can be identified in CSF that are not present in serum, it proves the (pathological) synthesis of antibodies in the CSF space. A meta-analysis of 49 studies comprising > 11.000 patients found a diagnostic sensitivity for MS of 93% and a specificity of 94% for this method. However, this specificity dropped to 61% if only patients with inflammatory conditions were considered (52). Possible OCB patterns are shown in figure 5.

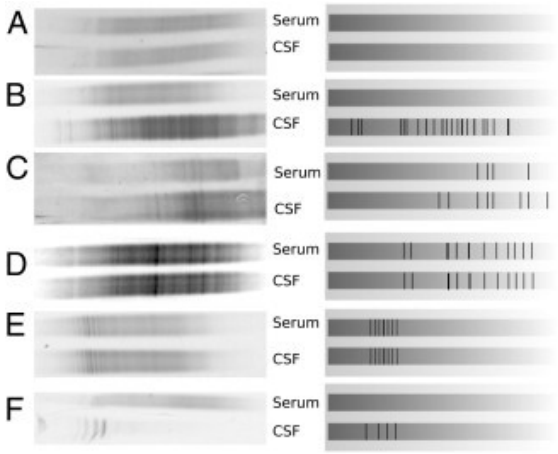

Figure 5. Patterns of oligoclonal bands (OCB) in isoelectric focusing (IEF). A: normal (no evidence for intrathecally-produced oligoclonal IgG, Type 1). B: local synthesis (Type 2). C: a mirror plus pattern (more bands in the cerebrospinal fluid (CSF) compared to the serum, Type 4). D: a mirror pattern (equal number of matched bands in CSF and serum, Type 3). E: mirror steps (monoclonal bands, Type 5). F: an artefact. Reprinted with the permission of Elsevier (52).

Alternatively to IEF, intrathecal antibody synthesis can also be demonstrated quantitatively through calculation of the IgG index. This is done by dividing the CSF/serum IgG ratio by the CSF/serum albumin ratio. An index > 0.7 speaks for intrathecal antibody production. However, this method possesses a considerably lower diagnostic sensitivity compared to CSF OCB (60-70%), and the IgG index is rarely elevated in patients without OCB (51,53).

Additionally, through advances in laboratory methods, κ-free light chains (FLCs) recently emerged as a new biomarker for MS diagnostics. These κ-FLCs stem from B cells producing antibodies by merging light chains and heavy chains via disulfide bonds and non-covalent interactions. In this process, light chains are produced in excess of 10%–40% over heavy chains, and are then secreted into the surrounding environment as κ-FLCs. Hence, κ-FLCs are elevated in the CSF in case of ongoing neuroinflammatory processes, such as in MS. These FLCs were discovered long ago, however it has only recently become possible to detect them in CSF through specific detection antibodies. A new systematic review and meta-analysis study found a diagnostic specificity and sensitivity for the κ-FLC index comparable to that of CSF OCBs (in detail: specificity of on average 89% and 92% and sensitivity of on average 88% and 85% for κ-FLC index and OCBs, respectively). As κ-FLCs have certain advantages such as a fast, easy, and cost-effective detection and the production of quantitative results, an international expert panel recently published a

consensus statement recommending the inclusion of intrathecal κ-FLC synthesis in the next revision of MS diagnostic criteria (54,55).

1.6 Therapy

Despite intensive research in the field, no causal therapy is available for the treatment of MS. The therapeutic strategy for MS patients rests on the treatment of acute relapses on the one hand, and long-term disease-modifying therapies (DMT) to reduce the number of relapses and prevent disability worsening on the other hand. Additional symptomatic therapy is used to manage the variety of clinical signs and symptoms that can occur along the disease course, such as spasticity, fatigue or sexual dysfunction (56,57).

1.6.1 Treatment of acute relapses

As stated in the McDonald's criteria, a relapse is defined as an acute or subacute monophasic clinical episode characterized by MS-typical signs and symptoms, which lasts for at least 24 hours, in the absence of fever or infection (18). Administration of high-dose intravenous corticosteroids is the established therapeutic standard in the management of acute MS relapses. According to the German Neurological Society (DGN), 500-1000 mg of methylprednisolone per day should be induced as soon as possible after symptom onset and continued over a course of three to five days. Oral administration of high-dose corticosteroids can work as an alternative, as there is no evidence for therapeutic superiority or inferiority of oral versus intravenous application (57–59). In case of insufficient symptom release, an additional high-dose methylprednisolone therapy of up to 2000 mg per day over three to five days can follow. As escalation therapy, the DGN recommends the initiation of plasmapheresis or immunoadsorption (57).

1.6.2 Disease-modifying therapies (DMTs)

The goal of DMT is the reduction and prevention of clinical relapses and disease progression as well as the preservation of quality of life. Further, subclinical disease activity detected in MRI should be reduced. Therefore, various immunomodulators and immunosuppressants have been approved for MS by the European Medicine Agency (EMA) and the US Food and Drug Administration (FDA) (57,60).

For RRMS, the approval of the first treatments (the injectables interferon beta and glatiramer acetate) in the 1990s changed the perspective on the condition, and consequently, decades of intensive therapeutic research and advances followed. Ten years after the first injectables, approval of the first monoclonal antibody in MS, natalizumab, marked another landmark in treatment. However, awareness of the risks associated with DMTs also substantially increased, when the first two cases of natalizumab-related progressive multifocal leukoencephalopathy (PML) occurred, which is a potentially fatal CNS infection with the opportunistic John Cunningham virus (JC virus). The next milestone in MS therapy was the introduction of the first oral DMT fingolimod in 2010, followed by teriflunomide and dimethyl fumarate. More recently, further monoclonal antibodies have been introduced to the market after the initial success of natalizumab, starting with alemtuzumab in 2014 (61).

Contrary to the wide selection of DMTs approved for RRMS, the choices for treating patients with a progressive disease course (PPMS and SPMS) are more limited. For SPMS, two formulations of injectable interferon beta as well as the immunosuppressant cancer drug mitoxantrone are available. Additionally, in 2020, the oral S1P receptor modulator siponimod was approved for the treatment of SPMS by the EMA (61–63). For PPMS, the monoclonal antibody ocrelizumab remains the only DMT option after its approval in 2018 (61).

Figure 6 gives an overview of developments in the treatment of MS between 1993 and 2018.

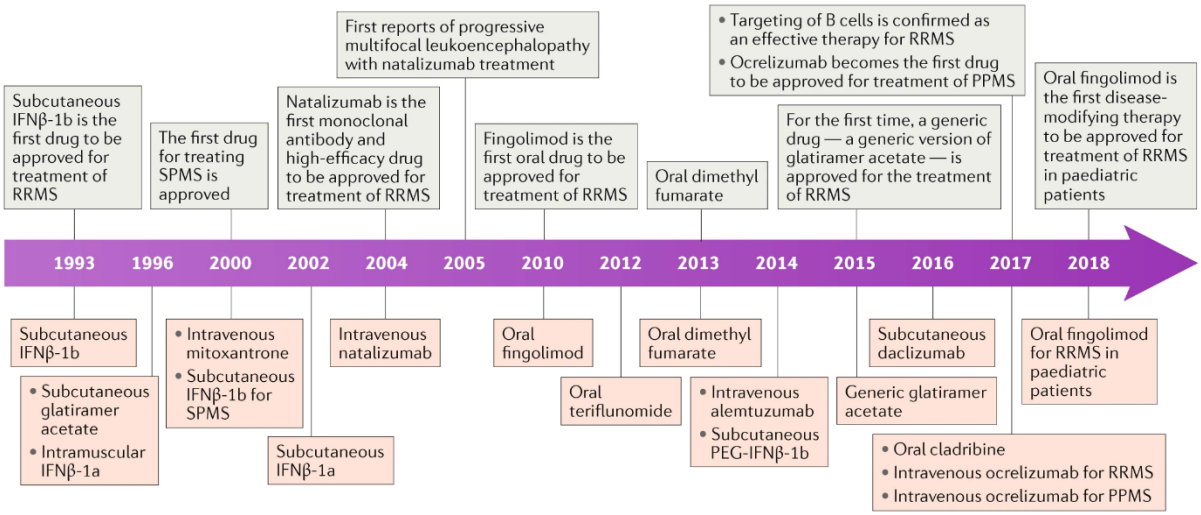

Figure 6. Timeline of developments in the treatment of multiple sclerosis (MS) from 1993 to 2018. IFNβ: interferon beta. PEG: polyethylene glycol; PPMS: primary progressive MS; RRMS: relapsing-remitting MS; SPMS: secondary progressive MS. Reprinted with the permission of Springer Nature (61).

According to the DGN, DMTs can be classified into three categories according to their efficacy in reducing the relapse rate (57):

- Category 1: relative relapse rate reduction of 30-50% compared to placebo
    - interferon beta including peginterferon
    - dimethyl fumarate / diroximelfumarate
    - glatirameroids
    - teriflunomide
- Category 2: relative relapse rate reduction of 50-60% compared to placebo
    - cladribine
    - S1P receptor modulators
- Category 3: relative relapse rate reduction of >60% compared to placebo or >40% compared to category 1 substances
    - alemtuzumab
    - CD20-antibodies (ocrelizumab, ofatumumab, rituximab)
    - natalizumab

These categories however should not be understood as a stepwise therapy recommendation. Besides efficacy, clinicians need to consider drug safety and tolerability, patient characteristics and comorbidities, disease activity and potential adverse drug reactions and complications. Based on this, a decision should be made together with the patient after thorough education on the benefits and risks of DMT (57,60,64).

1.6.3 Treatment strategies

Generally, two therapeutic approaches can be distinguished in MS: escalation vs induction therapy. In the concept of escalation therapy, the patient initially receives a low-risk DMT, which is typically associated with lower efficacy (category 1 according to the DGN). In case of insufficient response, treatment is switched to a more aggressive, higher-risk drug. Contrary, in induction therapy, treatment is initially started with an aggressive high-efficacy DMT, aiming to “reset” the immune system and prevent early structural damage, however taking into account the potential more serious side effects of these drugs. Once satisfactory disease control is achieved, a switch to a lower-efficacy drug should follow (65,66). Most traditional treatment algorithms recommend the escalation concept, reserving induction

therapy for patients with an aggressive disease activity at onset. However, some recent cohort studies support the general application of the induction approach in order to achieve better long-term disability outcomes. Nevertheless, these analyses were all done retrospectively (67–69). Currently, two large prospective randomized studies are being conducted (DELIVER-MS and TREAT-MS), which will hopefully further elucidate this controversy (70,71).

The guidelines of the DGN as well as the European Academy of Neurology (EAN) both support the escalation therapy approach, arguing that studies in favor of the induction concept are to be critically viewed due to their retrospective designs. Initial treatment with high-efficacy DMTs should only be applied in patients with a presumably highly active disease course (57,72).

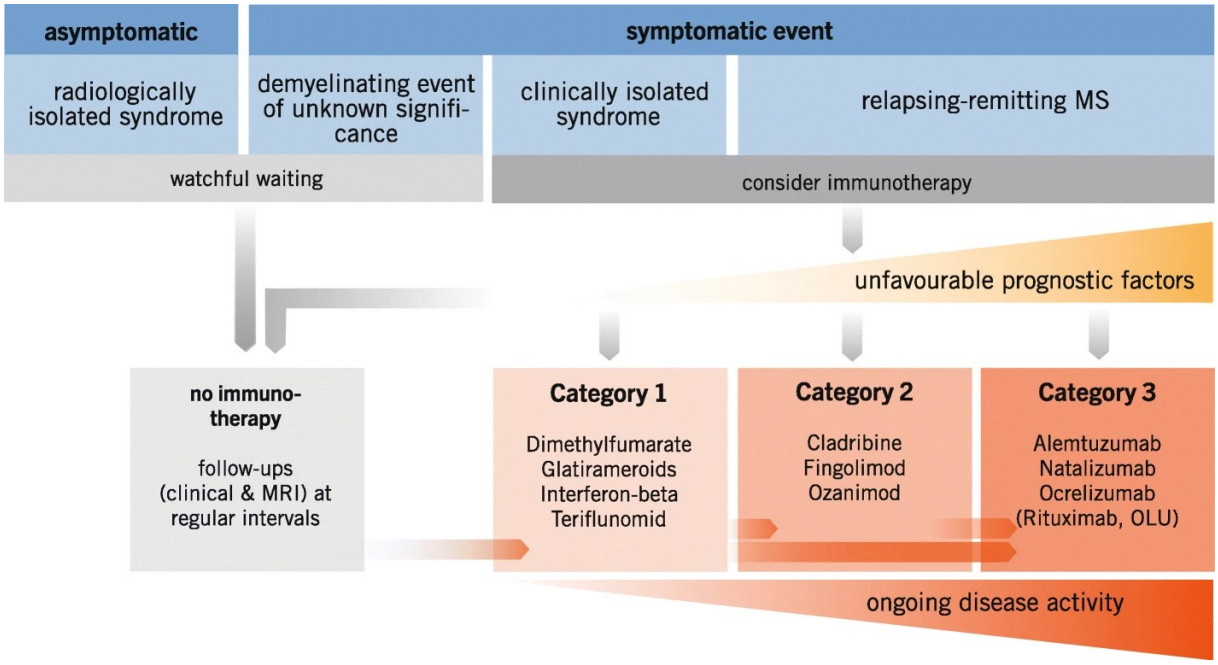

Figure 7. Therapeutic algorithm for relapsing-remitting multiple sclerosis (RRMS) by the German Neurological Society (DGN). MS: multiple sclerosis, MRI: magnetic resonance imaging, OLU: off-label use. Reprinted with the permission of Springer Nature (73).

On the contrary, the Multiple Sclerosis Therapy Consensus Group (MSTCG) published a white paper on therapeutic recommendations, which incorporates both the escalation as well as the induction concept. In this, they advocate individual decisions between the two regimens in accordance with the patients' wishes (74).

1.7 Biomarkers

A biomarker is defined as “A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (75). It should ideally be little invasive, easily detectable by an accurate and reproducible method, and fast and cost-effective for widespread application. At best it is a binary characteristic, meaning that it is present in a certain pathologic condition and absent otherwise or vice versa, and its level should correspond to the condition’s severity (76).

1.7.1 Biomarkers in MS

As MS is a highly complex and heterogenous disease in regard to clinical, radiological and pathological aspects as well as therapeutic response, there is a need for biomarkers reflecting these features. This could be beneficial in diagnostic and prognostic assessment, therapeutic decision-making and development of new therapies. Consequently, the MS biomarker research field is highly active, however, very few body fluid biomarkers have undergone rigorous validation and clinical implementation, as this is a long and laborious process (77,78). Figure 8 gives an overview of the steps involved in this process.

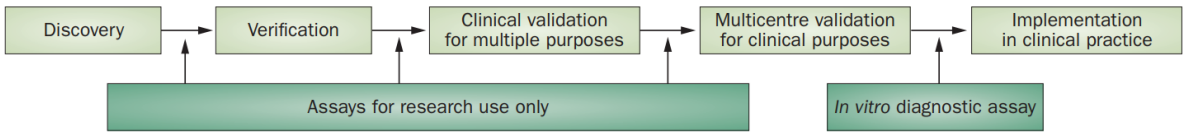

Figure 8. Schematic overview of the process of biomarker development. The first three steps can be performed rapidly (a few years), but the complete process to clinical implementation usually takes >20 years. Reprinted with the permission of Springer Nature (78).

According to Comabella et al, molecular biomarkers in MS can be divided into four categories: predictive biomarkers, that help in identifying individuals at risk of developing the disease, diagnostic biomarkers distinguishing MS patients from patients with other neurological disorders, disease activity biomarkers, that correspond to the different pathophysiological mechanisms involved in MS, and treatment-response biomarkers, which assist in monitoring an individual’s response to a DMT or identifying patients developing adverse drug reactions. Some biomarkers can also serve multiple of these functions (77). The presence of IgG-OCBs is an example for a routinely implemented diagnostic biomarker that also has

prognostic value in the prediction of conversion from CIS to MS. However, there is a lack of validated biomarkers for disease subtyping and staging, and treatment-response biomarkers are also scarce, despite an increasing variety in DMTs (78).

Nevertheless, technological advances, for instance the development of the ultra-sensitive digital immunoassay SIMOA™, also allow for progress in biomarker research (79). Consequently, neurofilaments (particularly neurofilament light (NFL)) as markers for neuroaxonal damage are currently promising candidates as disease activity and treatment-response biomarkers. Large multicenter studies are being conducted to affirm their ability to reflect brain tissue damage and to eventually result in clinical implementation (80). Additionally, glial fibrillary acidic protein (GFAP) reflects astrocyte injury and activation in CNS diseases, and is under evaluation for indicating and predicting long-term disability worsening particularly in MS patients with progressive disease (81).

One point to consider in MS biomarker research is the respective body fluid, from which the biomarker is derived. Both CSF and serum have benefits and limitations, while biomarkers obtained from other body fluids, such as urine or saliva, are scarce in MS. CSF is a highly informative fluid due to its proximity to the target organ, and it can directly mirror ongoing processes in the CNS because of its high turnover dynamics (complete renewal approximately every 6 hours (82)). However, it is collected by lumbar puncture, an invasive procedure, which impedes repeated sampling and therefore makes it unattractive in the search for monitoring biomarkers. Peripheral blood on the other hand can be obtained less invasively and repeatedly, and the development of new ultra-sensitive assays enables the detection of CSF-derived molecules in serum, that could previously only be measured in CSF. Nevertheless, various biomarkers that are now being investigated in serum were originally discovered in CSF, which to this day remains a highly interesting and relevant body fluid in MS research (50,77).

1.7.2 Albumin and its redox states in serum and CSF

Albumin is the most abundant protein in human blood. Human serum albumin (HSA) consists of 585 amino acids and has a molecular mass of 66.438,41 Dalton (Da). It is synthesized in hepatocytes and has a half-life of 19 days, before degrading in various sites including the liver, kidneys, and gastrointestinal tract. It amounts to a

total of approximately 360 g in a body of 70 kg, of which however only a third is circulating in the bloodstream. The rest is found in various body fluids and tissues in different concentrations, such as in CSF, saliva, aqueous humor, or the skin (83).

HSA fulfills multiple functions in the human body. While making up only 60% of the mass of plasma proteins, it accounts for 80% of the plasma's oncotic pressure. It is also the most important acid/base buffer plasma protein. Further, HSA functions as an essential transport vehicle for various agents, including fatty acids, bilirubin, copper and calcium ions, hormones, and vitamins, as well as pharmaceuticals (83).

Additionally, albumin has an antioxidant role, particularly due to the free thiol group of cysteine on position 34 (Cys-34). According to the binding state of this thiol group, HSA can exist in three redox states. In its reduced form, called human mercaptalbumin (HMA), Cys-34 exists as a free thiol. It can be reversibly oxidized with another free thiol, such as another cysteine, homocysteine or glutathione (GSH), creating human nonmercaptalbumin 1 (HNA1). With Cys-34 as sulfinic or sulfonic acid, HSA is irreversibly oxidized to human nonmercaptalbumin 2 (HNA2) (36,83,84). Figure 9 shows the structure of HSA in these redox states.

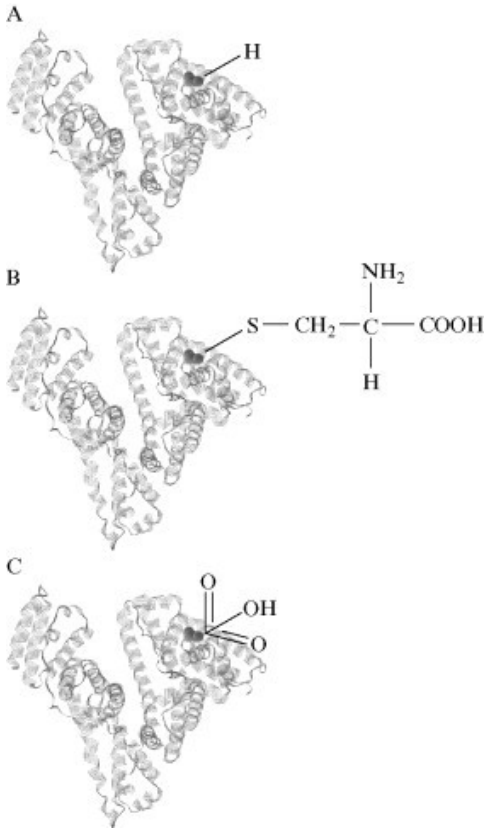

Figure 9. Structure of human serum albumin (HSA) in its redox states according to cysteine-34 (Cys-34). A: Human mercaptalbumin (HMA) with a free thiol group. B: Human nonmercaptalbumin 1 (HNA1) as disulfide with cysteine. C: Human nonmercaptalbumin 2 (HNA2) with a sulfonic acid group. Reprinted with the permission of Elsevier (36).

The allocation of HSA to these fractions can serve as a marker for oxidative stress. In healthy young individuals, HMA makes up 70-80% of total HSA in plasma, while 20-30% are oxidized to HNA1 and only 2-5% are irreversibly oxidized to HNA2. This allocation shifts physiologically towards more oxidized forms during exercise as well as with aging. As various diseases are also accompanied by a state of oxidative stress, altered HSA redox fraction allocation has been found in multiple pathophysiologic conditions. This is particularly striking in patients with liver diseases such as liver cirrhosis and acute-on-chronic liver failure (ACLF). Additionally, oxidative alterations on HSA were also reported in diabetes patients and patients undergoing hemodialysis, as well as in AD patients (36,85).

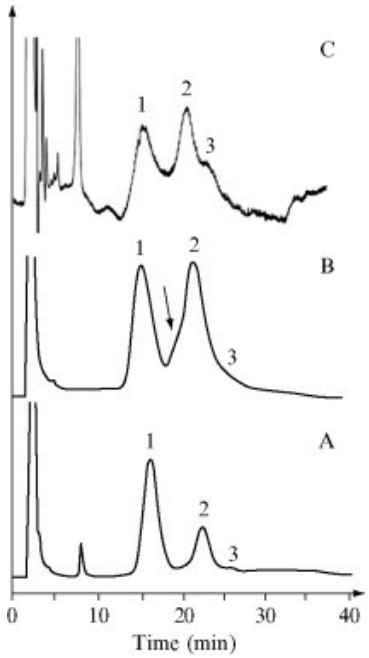

Figure 10. Representative high performance liquid chromatography (HPLC) chromatograms of plasma samples. A: Plasma of a young healthy control. B: Hemodialysis (HD) patient. C: Acute-on-chronic liver failure (ACLF) patient. In A and B fluorescence detection and in C UV detection was used (Peak 1, human mercaptalbumin (HMA); Peak 2, human non-mercaptalbumin 1 (HNA1); Peak 3, human non-mercaptalbumin 2 (HNA2)). The arrow shows an additional peak in the chromatogram of HD plasma. Reprinted with the permission of Elsevier (36).

HSA is also the main protein in CSF. Its physiological concentration in CSF (between 150 and 350 mg/l) lies below one percent of HSA serum concentration (35-55 g/l). The ratio of albumin CSF vs. serum concentration (the albumin quotient Q_{Alb}) is used as a measure for disruption of the BBB, as CSF albumin is thought to originate from the serum, despite some reports of albumin synthesis in human microglial cells (86,87). However, there are little to no studies available investigating the redox state of albumin in CSF. Based on the little data at hand, in healthy individuals, albumin in CSF appears to be more reduced than in plasma. One study including 42 patients with orthopedic disorders found a CSF albumin redox fraction allocation of >90% for HMA, 5-7% for HNA1 and around 1% for HNA2. Another study investigating HSA redox status in serum and CSF of AD patients found a CSF fraction allocation in the control group of 86.4%, 10.0% and 3.8% for HMA, HNA1 and HNA2, respectively. In the AD group, albumin in CSF was considerably more oxidized, with a fraction allocation of 9.6%, 33.2% and 52.8% to HMA, HNA1 and HNA2, respectively. This drastic shift speaks for the significance of oxidative stress in AD pathophysiology (85,88).

1.8 Aim

The involvement of oxidative stress in MS pathophysiology has been established, and oxidative processes are believed to facilitate CNS tissue damage and neurodegeneration. Various markers for oxidative stress have been found altered in MS, however, the redox state of albumin in MS has not been explored before (39,89). Thus, the aim of this pilot study is to investigate the redox state of HSA in allocation to the fractions HMA, HNA1 and HNA2 in serum as well as CSF of MS patients. Further, it intends to explore possible correlations of HSA redox state with disease activity and/or progression. In line with potential further follow-up studies, this aims to contribute to the search for novel biomarkers for MS.

2 Materials and Methods

Our investigation was approved by the ethics committee of the Medical University of Graz, Austria (ethical approval number: 31-432 ex 18/19).

2.1 Patients

We included a total of 20 MS patients and 21 controls in our study. MS patients all had a diagnosis of MS according to McDonald's 2017 criteria (18). Subjects in the control group were classified as so-called symptomatic controls according to Teunissen et al (90). They present with neurological symptoms, such as paresthesia, headache, or signs of paralysis, where further diagnostic workup (CSF diagnostics, MRI etc.) cannot find any pathological correlates. For inclusion, paired serum and CSF samples had to be stored at ≤-70°C for no longer than 16 months until HPLC analysis for patients and controls.

Exclusion criteria for both, patients and controls, were: administration of corticosteroids within the last 28 days, erythrocyte concentration in CSF greater than 500 per µL, and presence of other inflammatory neurological diseases or active liver or kidney diseases, with known influence on the albumin redox state. For MS patients, a further exclusion criterium was usage of DMT prior to sampling.

The control group was selected in accordance with a consensus paper by Teunissen et al, defining guidelines for control groups in MS CSF biomarker studies (90). To identify eligible patients, a patient export file from the database RDA (Research Documentation & Analysis, Medical University of Vienna), which is used in the Neurology Biomarker Research Unit at Medical University of Graz, was created. We then screened the exported patient files for inclusion and exclusion criteria, and excluded samples older than 16 months at planned HPLC analysis.

For the control group, we screened the CSF sample files in RDA from the same time frame and identified symptomatic controls matching our inclusion and exclusion criteria. The groups were then tested for comparability in age and sex distribution using unpaired Student's t test and Fisher's exact test, respectively. Both tests found no significant difference ($p = 0.70$ and $p = 0.52$, respectively).

Demographic and clinical data of subjects in the patient and control groups are summarized in table 2.

2.2 Samples

Paired serum and CSF samples were obtained at the Department of Neurology, Medical University of Graz, between 11.05.2020 and 06.07.2021. 8 mL of peripheral blood were collected by venipuncture in parallel to retrieval of 6-10 mL of CSF by lumbar puncture (LP). After routine diagnostic workup, samples were either stored at -80 °C at the Biobank Graz, or at -70 °C at the Department of Neurology, Medical University of Graz.

2.3 Routine diagnostic workup

Routine assessment of diagnostic CSF parameters was performed by experienced personnel in the Department of Neurology, Medical University of Graz, as recommended in international consensus guidelines (91,92). Total albumin and immunoglobulins (Ig) were assessed by nephelometry on a Beckman Coulter Image 800 analyzer (Beckman Coulter Inc, Brea USA), and lactate was measured on an ABL800 Flex analyzer (Radiometer Medical ApS, Brønshøj, Denmark). White cell count and erythrocyte count were assessed in a Fuchs-Rosenthal counting chamber. CSF/serum ratio for albumin (Q_{Alb}) was calculated as a measure for blood CSF barrier integrity (cutoff at Qalb = 4 + age / 15). Determination of intrathecal IgG production was done quantitatively by calculation of the IgG index (IgG index = (CSF/serum IgG)/(CSF/serum albumin), cutoff < 0.7) and qualitatively by testing for the presence of OCBs under IEF (93,94).

Routine CSF parameters are summarized in table 3.

2.4 Retrieval of clinical and laboratory parameters

We then retrieved necessary information from the hospital databases openMEDOCS and RDA. For MS patients, we retrieved disease-related information, such as disease course (RRMS, PPMS, SPMS), DMT, EDSS values at the last relapse and in remission, and number of relapses before sampling. We also looked up images and reports of MRI investigations done around the time of sampling, and recorded presence of pathological contrast-enhancing lesions. Further, we retrieved the dates of first onset of symptoms, MS diagnosis and start of the last relapse, and calculated the time differences between those dates and sampling. For both groups, we looked up administration of corticosteroids in the last 28 days before sampling,

neurological, renal and hepatic comorbidities, CSF diagnostic parameters, and serum laboratory parameters (INR, creatinine, liver enzymes, bilirubin).

The definition of a relapse is a monophasic clinical episode with the occurrence of patient-reported symptoms and objective findings typical of MS, that reflect (multi-)focal inflammatory demyelination in the CNS. Such an episode develops acutely or subacutely and lasts at least 24 hours with or without recovery afterwards, under the exclusion of fever or infection (18). The EDSS was developed by John F. Kurtzke in order to quantify physical disability in MS patients. It is a 20-step scale based on the neurological examination of 8 functional systems (44). For RRMS patients, the "EDSS in remission" was assessed at scheduled clinical control visits, with either >28 days having passed since the onset of the last relapse, or <28 days since then, but with signs and symptoms already subsided again. This assessment happened within 2 months of sampling. For PPMS patients, on the contrary, "EDSS in remission" was assessed at the closest control visit to sampling, with a maximum time difference of 177 days. In our investigation, the disease duration reflects the time in months between the occurrence of the first MS-typical signs/symptoms (=disease onset) and the date of sampling.

2.5 Assessment of albumin redox fractions

To determine the allocation of serum and CSF albumin to the fractions HMA, HNA1 and HNA2, we used HPLC as previously described by Hayashi et al (95). Serum samples were thawed and 10 µL of the samples were diluted in a buffer of 0.1 M sodium phosphate and 0.3 M sodium chloride, pH 6.87, in a ratio of 1:100. They were then filtered through a Whatman 0.45-µm nylon filter (Bartelt Labor- & Datentechnik, Graz, Austria). For CSF, 70 µL of the samples were diluted in the buffer described above, in a ratio of 1:2. They were not filtered.

The HPLC system consisted of a FLUX Rheos 4000 gradient pump (Spectronex, Vienna, Austria), a Shimadzu SIL-20AC autosampler, a Shimadzu CTO column oven (Shimadzu Austria, Vienna, Austria) and a Jasco 821-FP fluorescence detector (Spectronex, Vienna, Austria). 20 µL of the diluted samples were injected into the system and separated by an anion exchange column (Shodex Asahipak ES-502N 7C, 7.5 x 100 mm, Bartelt Labor- & Datentechnik, Graz, Austria) with a mobile phase of 50 mM sodium acetate, 400 mM sodium sulfate, pH 4.85. The column was kept

at 35 °C. Elution was performed with a gradient of 0 to 60% ethanol and a flow rate of 1 mL/min. Albumin fractions were detected by fluorescence at 280/340 nm and quantified according to the individual peak area using Peak Fit software (Version 4.12, SPSS Science, Chicago, IL, USA).

2.6 Formation of HMA, HNA1 and HNA2 quotients

HMA, HNA1, and HNA2 quotients (Q_{HMA}, Q_{HNA1}, Q_{HNA2}) were determined in order to evaluate the ratios of the respective albumin redox fractions in CSF to serum. For this, we estimated the concentration of the fractions by multiplying the total albumin concentration in serum or CSF with the respective fraction shares measured by HPLC. With those values, we then formed the CSF to serum quotients.

2.7 Assessment of non-esterified fatty acids (NEFA) and bilirubin content

For measurement of NEFA in serum samples, we used an in vitro enzymatic colorimetric method (FUJIFILM Wako Diagnostics, Neuss, Germany). Therefore, 10 µl of the samples were mixed with 150 µl of the kit reagent 1 and incubated at 37°C for 10 minutes at 400 rpm. Then the first measurement followed, and afterwards 75 µl of the kit reagent 2 were added. After another incubation period of 10 minutes at 37°C at 400 rpm, the second measurement followed.

HSA-bound bilirubin was determined using an in vitro colorimetric test assay (Human Biochemica und Diagnostica GmbH, Wiesbaden, Germany). Therefore, 20 µl of the serum samples were mixed with 200 µl of sample reagent (DCA reagent + NIT reagent 1+1), and incubated protected from light at room temperature for 10 minutes. Extinction measurement was done at 546 nm.

2.8 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics (Version 27, Armonk, NY, USA).

Differences in age and sex in MS patients vs. controls were assessed using unpaired Student’s t test and Fisher’s exact test, respectively. Metric variables were tested for normal distribution through Shapiro-Wilk test, and then confirmed graphically in histograms.

Differences in metric variables between groups were tested for significance using unpaired Student's t-test (for variables in normal distribution) or Mann-Whitney-U (MWU) test (for variables not in normal distribution). This was done for measured HPLC albumin redox fractions in the groups: patients vs. controls, male vs. female, RRMS vs. PPMS (patients only), relapse within 14 and 28 days before LP - yes vs. no (patients only), pathologic contrast enhancing lesions on MRI – yes vs. no (patients only), BBB dysfunction – yes vs. no (patients only). Additionally, we further divided all measured HPLC variables by their median values into two groups (e.g. serum_%HMA_≤median vs. serum_%HMA_>median). In these respective groups, we then compared the metric variables age, age at disease onset (patients only), lactate in CSF, number of relapses before LP (patients only), time between last relapse and LP (patients only), disease duration (patients only), EDSS (patients only).

We assessed correlations between metric variables calculating either Pearson's correlation coefficients (for variables in normal distribution) or Spearman's correlation coefficients (for variables not in normal distribution). Partial correlations were assessed with non-parametric partial correlation.

Categorical variables were compared with either Chi square test or Fisher's exact test.

A p-value of ≤0.05 was considered statistically significant for all analyses.

3 Results

3.1 Demographic and clinical data

20 MS patients and 21 controls were enrolled in our study. The MS group consisted of 17 RRMS and 3 PPMS patients. Further relevant demographic and clinical data are summarized in table 2.

Table 2. Demographic and clinical characteristics of MS patients and controls at time of sampling.

	Patients (n=20) RRMS/PPMS (n=17/3)	Controls (n=21)
n female	10 (50.0%)	13 (61.9%)
Age at sampling (years)	33 (27-45)	36 (29-40)
Age at disease onset (years; MS only)	31 (25-43)	n.a.
Disease duration (months; MS only)	1 (0-23)	n.a.
EDSS in remission (MS only)	1.0 (0.0-2.0)	n.a.
n relapse within 14 days prior to sampling (MS only)	6 (35.3%)	n.a.
n relapse within 28 days prior to sampling (MS only)	9 (52.9%)	n.a.
Number of relapses prior to sampling (MS only)	1 (1-2)	n.a.
n pathologic contrast-enhancing lesions on MRI (MS only)	6 (35.3%)	n.a.

Data are given as numbers (%) or medians (25th-75th percentile). Differences in sex (p = 0.536) and age distribution (p = 0.7) between patients and controls were not significant, as assessed by Fisher's exact test and unpaired Student's t-test, respectively. MS: multiple sclerosis, RRMS: relapsing-remitting multiple sclerosis, PPMS: primary progressive multiple sclerosis, EDSS: expanded disability status scale, n.a.: not applicable.

3.2 Routine CSF diagnostics

Routine diagnostic tests were performed on all CSF samples of patients and controls. The results of these analyses are summarized in table 3.

Table 3. Routine CSF diagnostics of patients' and controls' CSF samples.

	Patients (n=20) RRMS/PPMS (n=17/3)	Controls (n=21)	p-value
White cell count (n/µL)	7 (3-12)	1 (1-1)	**<0.001**
Erythrocyte count (n/µL)	0 (0-4)	5 (0-43)	0.088
CSF lactate (mmol/L)	1.5 (1.4-1.6)	1.5 (1.3-1.5)	0.295
Total CSF protein (mg/dL)	33 (27-38)	28 (24-32)	0.100
Total CSF albumin (mg/dL)	21.6 (17.0-25.2)	21.7(19.1-24.4)	0.925
Q_{Alb} (*10^{-3})	4.50 (3.66-5.69)	5.04 (3.93-5.46)	0.758
n BBB disruption	4 (20%)	0 (0%)	**0.048**
n oligoclonal bands positive	20 (100%)	n.a.	n.a.

BBB disruption was defined by an elevation in Q_{Alb}. Data are given as numbers (%) or medians (25th-75th percentile). Tests for significance were performed using Mann-Whitney-U test or Fisher's exact test. Significant results are highlighted in bold. RRMS: relapsing-remitting multiple sclerosis, PPMS: primary progressive multiple sclerosis, CSF: cerebrospinal fluid, Q_{Alb}: albumin quotient, BBB: blood brain barrier.

3.3 Redox state of albumin in serum and CSF of patients and controls

We measured HSA allocation to the fractions HMA, HNA1 and HNA2 in serum and CSF of all patients and controls. The percentual fraction allocation is compiled in table 4.

Table 4. Percentual redox fraction allocation in patients and controls.

	MS Patients	Controls	p-value
HMA Serum	69.8 ± 4.2	70.1 ± 4.8	0.917
HNA1 Serum	26.8 ± 4.0	26.5 ± 4.6	0.896
HNA2 Serum	3.4 ± 0.6	3.4 ± 0.8	0.675
HMA CSF	85.7 ± 5.5	86.3 ± 5.2	0.639
HNA1 CSF	11.4 ± 3.6	12.1 ± 3.5	0.497
HNA2 CSF	2.9 ± 2.7	1.6 ± 2.2	0.076

Numbers are given as mean percentages ± standard deviations. Tests for significance were done with Mann-Whitney-U test. MS: multiple sclerosis, HMA: human mercaptalbumin, HNA1: human non-mercaptalbumin 1, HNA2: human non-mercaptalbumin 2, CSF: cerebrospinal fluid.

We did not find significant differences in the percentual fraction allocation between the patient and the control group in either compartment. However, all fractions were significantly different between serum and CSF (HMA: $p < 0.001$, HNA1: $p < 0.001$,

HNA2: p = 0.002), as assessed by Wilcoxon signed-rank test. Albumin redox fraction allocation in both compartments in patients and controls is depicted in figure 11.

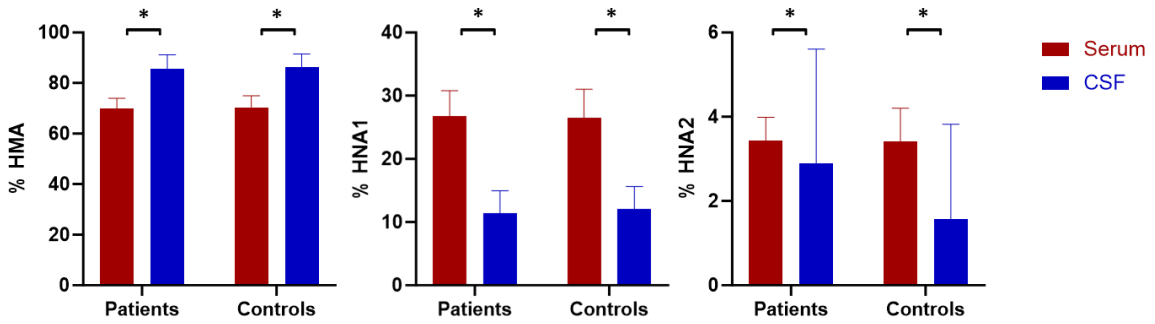

Figure 11. Redox fractions of multiple sclerosis (MS) patients and controls in serum and CSF. Data are presented as means and standard deviations. HMA: human mercaptalbumin, HNA1: human non-mercaptalbumin 1, HNA2: human non-mercaptalbumin 2, CSF: cerebrospinal fluid.

3.4 Association of albumin redox state with age

We observed some associations of HSA redox state and age at sampling. In the combined analysis group (patients and controls taken together), HNA2 in serum and HMA in CSF correlated significantly with age. In the control group, HNA2 both in serum and CSF correlated with age. There were no significant associations between either fraction and age in the MS only group. Detailed correlation coefficients and p-values are summarized in table 5.

Table 5. Correlations of albumin redox fractions with age at sampling using Spearman's correlation coefficient.

	MS + CO		MS only		CO only	
	Spearman's r	p-value	Spearman's r	p-value	Spearman's r	p-value
HMA Serum	-0.231	0.146	-0.311	0.183	-0.140	0.544
HNA1 Serum	0.213	0.182	0.329	0.157	0.064	0.784
HNA2 Serum	0.417	**0.007**	0.241	0.307	0.605	**0.004**
HMA CSF	-0.324	**0.039**	-0.289	0.216	-0.399	0.073
HNA1 CSF	0.276	0.081	0.298	0.203	0.299	0.187
HNA2 CSF	0.237	0.136	0.029	0.903	0.524	**0.015**

Significant results are highlighted in bold. MS: multiple sclerosis, CO: controls, HMA: human mercaptalbumin, HNA1: human non-mercaptalbumin 1, HNA2: human non-mercaptalbumin 2, CSF: cerebrospinal fluid.

3.5 Association of albumin redox state with lactate in CSF

For HMA, HNA1 and HNA2 in serum, we found significant correlations with lactate concentration in CSF. This was observed both in the combined analysis group and in the MS only group, but not in the controls only group. There were no correlations with albumin fractions in CSF with lactate in CSF. Detailed correlation coefficients and p-values are summarized in table 6.

Table 6. Correlations of albumin redox fractions with lactate concentration in CSF using Spearman's correlation coefficient.

	MS + CO		MS only		CO only	
	Spearman's r	p-value	Spearman's r	p-value	Spearman's r	p-value
HMA Serum	-0.504	**<0.001**	-0.630	**0.003**	-0.411	0.064
HNA1 Serum	0.466	**0.002**	0.563	**0.010**	0.377	0.092
HNA2 Serum	0.498	**<0.001**	0.659	**0.002**	0.336	0.136
HMA CSF	0.037	0.817	0.045	0.852	0.067	0.772
HNA1 CSF	-0.078	0.629	0.063	0.792	-0.173	0.454
HNA2 CSF	-0.068	0.671	-0.339	0.143	0.141	0.542

Significant results are highlighted in bold. MS: multiple sclerosis, CO: controls, HMA: human mercaptalbumin, HNA1: human non-mercaptalbumin 1, HNA2: human non-mercaptalbumin 2, CSF: cerebrospinal fluid.

Additionally, we performed a partial correlation analysis corrected for age at sampling, to rule out potential confounding. However, the correlations between serum HMA, HNA1 and HNA2 and CSF lactate in the combined and MS only group remained significant (table 7).

Table 7. Partial correlations of albumin redox fractions with lactate concentration in CSF corrected for age at sampling.

	MS + CO		MS only		CO only	
	Spearman's r	p-value	Spearman's r	p-value	Spearman's r	p-value
HMA Serum	-0.468	**0.002**	-0.577	**0.010**	-0.399	0.081
HNA1 Serum	0.432	**0.005**	0.489	**0.034**	0.373	0.105
HNA2 Serum	0.432	**0.005**	0.638	**0.003**	0.319	0.171
HMA CSF	0.147	0.364	0.219	0.368	0.136	0.568
HNA1 CSF	-0.174	0.284	-0.096	0.697	-0.228	0.335
HNA2 CSF	-0.149	0.358	-0.403	0.087	0.080	0.737

Analyses were done using non-parametric partial correlation. Significant results are highlighted in bold. MS: multiple sclerosis, CO: controls, HMA: human mercaptalbumin, HNA1: human non-mercaptalbumin 1, HNA2: human non-mercaptalbumin 2, CSF: cerebrospinal fluid.

3.6 Association of albumin redox state with EDSS in remission

We found various associations between albumin oxidation and EDSS in remission in patients. Firstly, we divided all samples into two groups, according to whether their albumin redox fractions were ≤ or > the median in the respective fraction. When we then tested for differences in EDSS in remission in these groups, we found a significantly higher EDSS in patients with a serum HMA ≤ median and a serum HNA1 > median ($p = 0.012$, $p = 0.009$, respectively). These associations are depicted in figure 12. Further, we found a significant correlation between EDSS in remission and serum HMA and HNA1 (Spearman's $r = -0.447$, $p = 0.048$, and Spearman's $r = 0.452$, $p = 0.045$, respectively; figures 13 and 14). However, these correlations were lost when correcting for age at sampling in Spearman's partial correlation analysis (HMA: $r = -0.339$, $p = 0.155$; HNA1: $r = 0.331$, $p = 0.166$).

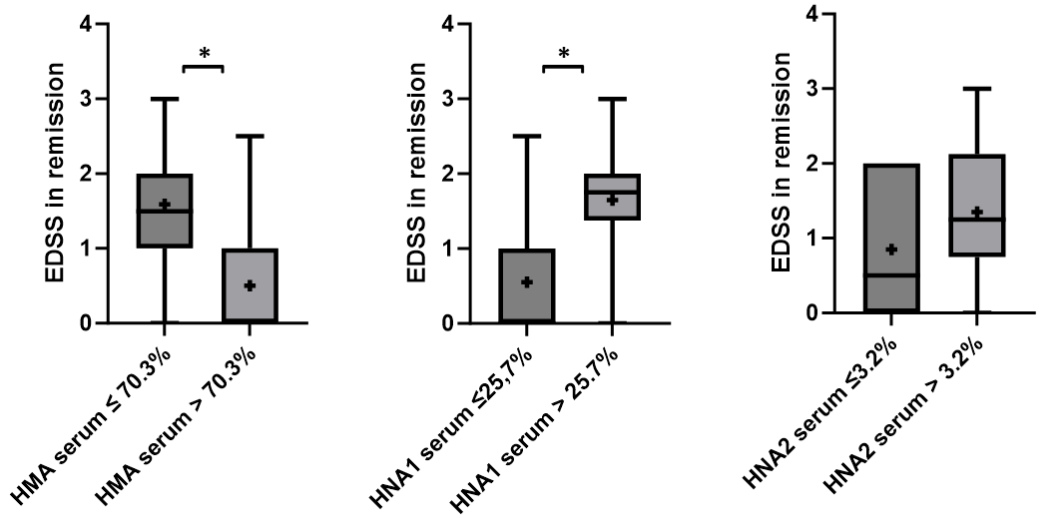

Figure 12. EDSS in remission in groups divided by serum fraction medians. Number of patients in groups ≤/> median: HMA (green): 11/9; HNA1 (orange): 10/10; HNA2 (purple): 10/10. +: mean, dots: outliers. EDSS: expanded disability status scale. HMA: human mercaptalbumin, HNA1: human non-mercaptalbumin 1, HNA2: human non-mercaptalbumin 2.

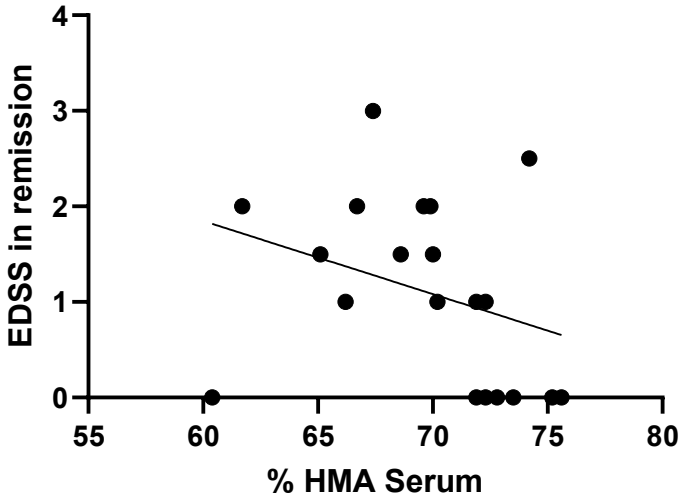

Figure 13. EDSS in remission in relation to serum HMA. Spearman's r = -0.447, p = 0.048. EDSS: expanded disability status scale, HMA: human mercaptalbumin.

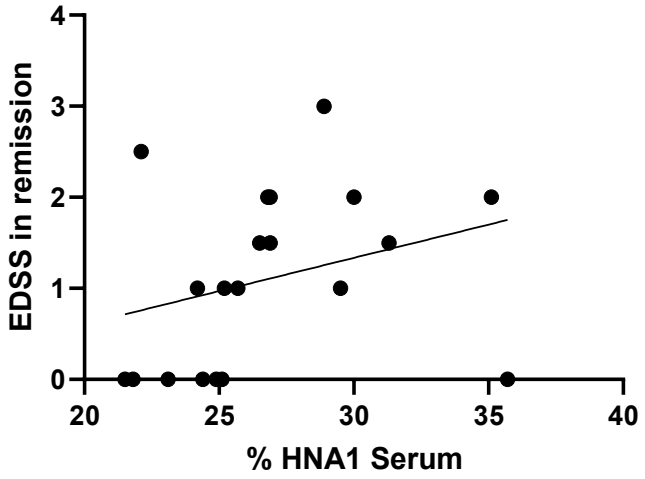

Figure 14. EDSS in remission in relation to serum HNA1. Spearman's r = 0.452, p = 0.045. EDSS: expanded disability status scale, HNA1: human non-mercaptalbumin 1.

3.7 Association of albumin redox state with relapses

HNA2 fractions were related to relapses in the MS group. We divided RRMS patients into two groups according to whether they had suffered from a relapse in the 14 days prior to sampling (n=6) or not (n=11). While HNA2 in serum was significantly lower in the patients with a recent relapse, in CSF it was significantly higher in this group (p = 0.048, p = 0.048, respectively; figure 15). The HNA2-Quotient, Q_{HNA2}, which is the ratio of total HNA2 in CSF to serum, was also elevated in this subgroup (p = 0.01; figure 16). Additionally, the time between the onset of the last relapse before sampling and sampling was significantly shorter in patients with serum HMA ≤ median and serum HNA1 and HNA2 > median, when dividing samples into two groups according to the respective fraction median (p = 0.031, p = 0.007, p = 0.038, respectively). However, when excluding outliers of this time period (n=2), only the association with serum HNA1 remained significant (p = 0.029; figures 17 and 18). Further, HMA and HNA2 in serum correlated significantly with the time between the onset of the last relapse before sampling and sampling (Spearman's r = -0.512, p = 0.043, Spearman's r = 0.613, p = 0.012, respectively). Again, these correlations were however lost when excluding outliers. Nevertheless, Q_{HNA2} significantly correlated with this time period excluding outliers (n=2), with a shorter distance to

the last relapse being associated with a higher CSF to serum HNA2 ratio (Spearman's r = -0.623, p = 0.017; figure 19).

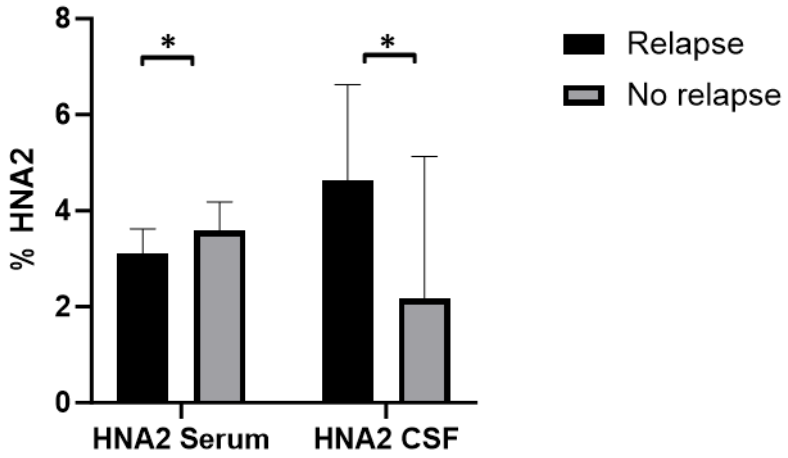

Figure 15. HNA2 in serum and CSF in patients with vs. without a relapse in 14 days prior to sampling. Patients with a relapse: n=6, patients without a relapse: n=11. Data are presented as means and standard deviations. HNA2: human non-mercaptalbumin 2, CSF: cerebrospinal fluid.

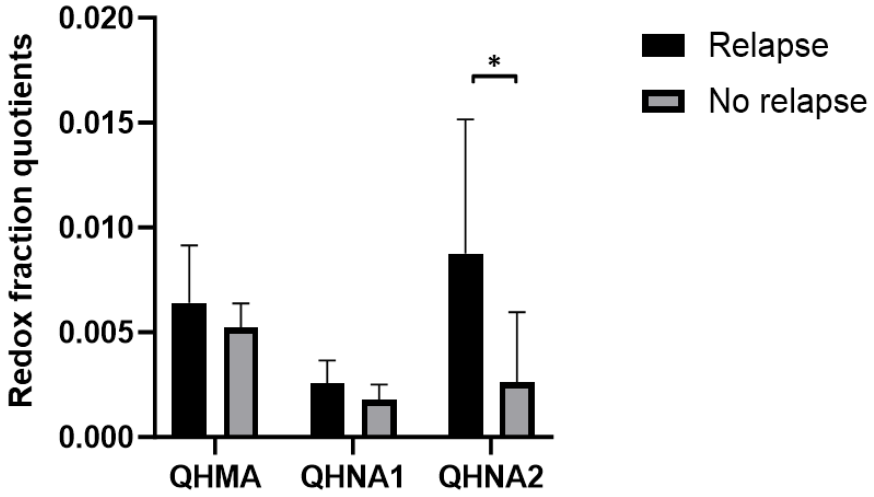

Figure 16. Redox quotients in patients with and without a relapse in 14 days prior to sampling. Calculation of redox quotients: e.g. HMA in CSF / HMA in serum. Patients with a relapse: n=6, patients without a relapse: n=11. Data are presented as means and standard deviations. HMA: human mercaptalbumin, HNA1: human non-mercaptalbumin 1, HNA2: human non-mercaptalbumin 2, CSF: cerebrospinal fluid

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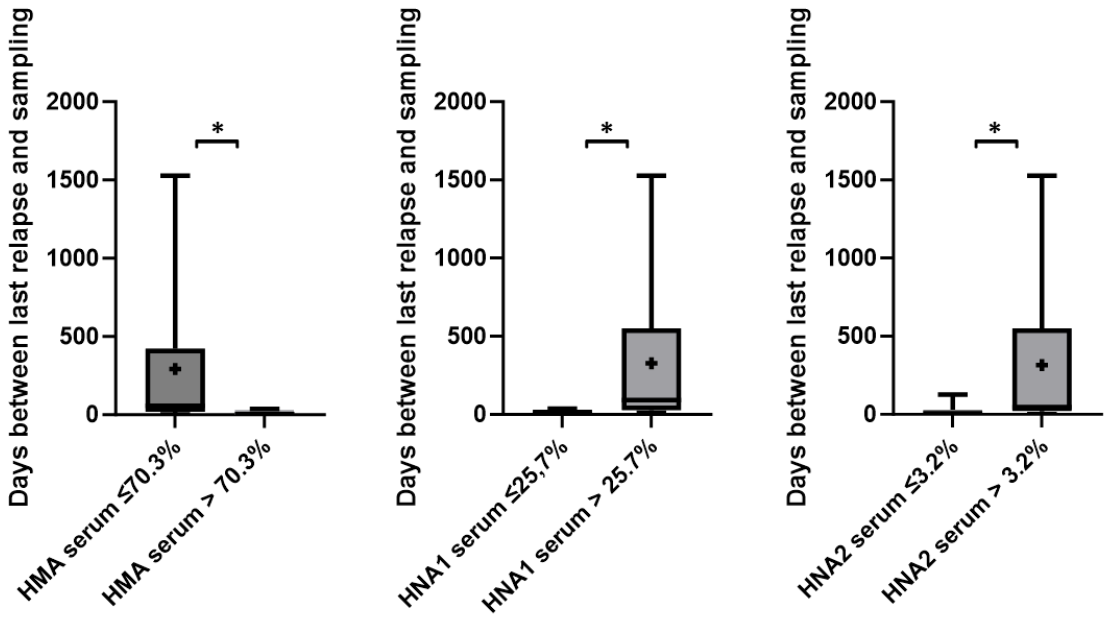

Figure 17. Time between last relapse and sampling in groups divided by serum fraction medians. Number of patients in groups ≤ / > median: HMA: 9 / 7; HNA1: 8 / 8; HNA2: 8 / 8. +: mean, dots: outliers. HMA: human mercaptalbumin, HNA1: human non-mercaptalbumin 1, HNA2: human non-mercaptalbumin 2.

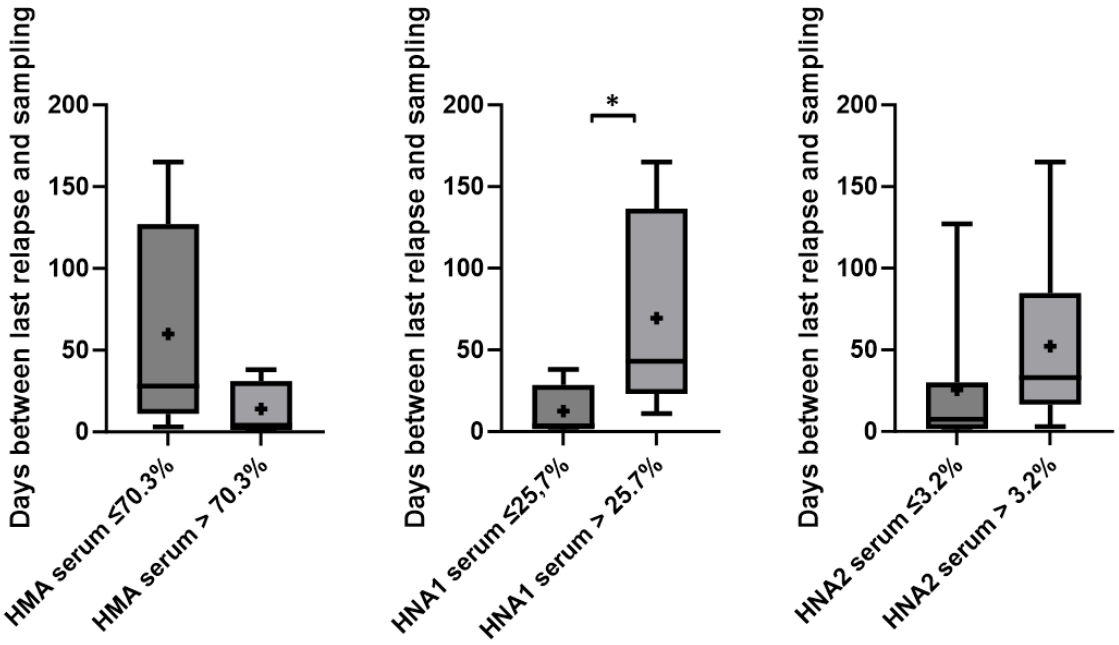

Figure 18. Time between last relapse and sampling in groups divided by serum fraction medians, excluding outliers. Outliers: n=2. Number of patients in groups ≤ / > median: HMA: 7 / 7; HNA1: 8 / 6; HNA2: 8 / 6. +: mean, dots: outliers. HMA: human mercaptalbumin, HNA1: human non-mercaptalbumin 1, HNA2: human non-mercaptalbumin 2.

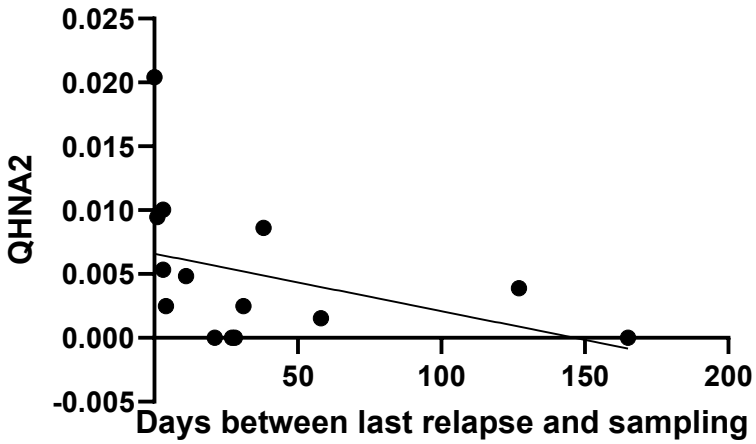

Figure 19. Correlation between time between last relapse and sampling and QHNA2, excluding outliers. Outliers: n=2. Spearman's r = -0.623, p = 0.017. HNA2: human non-mercaptalbumin 2.

3.8 Other demographic and clinical parameters

Aside from EDSS in remission, relapse within 14 days prior to sampling, and time between last relapse and sampling, we performed various other statistical tests to investigate associations of albumin redox state with demographic and disease-related parameters. We did not find any significant relations of albumin redox state to sex, MS disease course, pathological contrast enhancement on MRI, relapse within 28 days prior to sampling, total number of relapses or age at disease onset (tables 8-13).

Table 8. Percentual redox fraction allocation in females and males.

	Females	Males	p-value
HMA Serum	69.7 ± 4.4	70.3 ± 4.7	0.554
HNA1 Serum	26.9 ± 4.1	26.3 ± 4.5	0.470
HNA2 Serum	3.4 ± 0.6	3.4 ± 0.8	0.702
HMA CSF	86.6 ± 4.0	85.3 ± 6.6	0.752
HNA1 CSF	11.6 ± 2.8	11.9 ± 4.4	0.875
HNA2 CSF	1.8 ± 1.9	2.8 ± 3.1	0.453

Females: n=23, males: n=18. Numbers are given as mean percentages ± standard deviations. Tests for significance were done with Mann-Whitney-U test. HMA: human mercaptalbumin, HNA1: human non-mercaptalbumin 1, HNA2: human non-mercaptalbumin 2, CSF: cerebrospinal fluid.

Table 9. Percentual redox fraction allocation in RRMS and PPMS patients.

	RRMS	PPMS	p-value
HMA Serum	70.0 ± 4.2	68.7 ± 4.9	0.616
HNA1 Serum	26.6 ± 3.9	27.8 ± 5.0	0.546
HNA2 Serum	3.4 ± 0.6	3.5 ± 0.3	0.479
HMA CSF	85.3 ± 5.6	88.4 ± 3.9	0.546
HNA1 CSF	11.7 ± 3.7	9.5 ± 2.2	0.305
HNA2 CSF	3.0 ± 2.9	2.0 ± 1.8	0.765

RRMS: n=17, PPMS: n=3. Numbers are given as mean percentages ± standard deviations. Tests for significance were done with Mann-Whitney-U test. RRMS: relapsing-remitting multiple sclerosis, PPMS: primary progressive multiple sclerosis, HMA: human mercaptalbumin, HNA1: human non-mercaptalbumin 1, HNA2: human non-mercaptalbumin 2, CSF: cerebrospinal fluid.

Table 10. Percentual redox fraction allocation in patients with and without pathological contrast-enhancing lesions on MRI.

	Pathological contrast enhancement on MRI	No pathological contrast enhancement on MRI	p-value
HMA Serum	70.8 ± 4.8	69.7 ± 3.3	0.256
HNA1 Serum	26.0 ± 4.7	26.7 ± 3.1	0.256
HNA2 Serum	3.2 ± 0.5	3.5 ± 0.6	0.180
HMA CSF	85.2 ± 6.8	86.1 ± 4.3	1,000
HNA1 CSF	11.5 ± 5.0	11.2 ± 3.0	0.884
HNA2 CSF	3.3 ± 2.0	2.7 ± 2.7	0.404

Patients with pathological contrast enhancement: n=6, patients without pathological contrast enhancement: n=11. Numbers are given as mean percentages ± standard deviations. Tests for significance were done with Mann-Whitney-U test. MRI: magnet resonance imaging. HMA: human mercaptalbumin, HNA1: human non-mercaptalbumin 1, HNA2: human non-mercaptalbumin 2, CSF: cerebrospinal fluid.

Table 11. Percentual redox fraction allocation in patients with and without a relapse within the last 28 days prior to sampling.

	Relapse within the last 28 days before sampling	No relapse within the last 28 days before sampling	p-value
HMA Serum	70.3 ± 4.1	69.6 ± 4.5	0.541
HNA1 Serum	26.4 ± 3.9	26.9 ± 4.2	0.673
HNA2 Serum	3.3 ± 0.6	3.6 ± 0.6	0.277
HMA CSF	85.0 ± 6.1	85.5 ± 5.5	1.000
HNA1 CSF	11.9 ± 4.2	11.5 ± 3.4	0.963
HNA2 CSF	3.1 ± 2.8	3.0 ± 3.1	0.888

Patients with a relapse: n=9, patients without a relapse: n=8. Numbers are given as mean percentages ± standard deviations. Tests for significance were done with Mann-Whitney-U test. MS: multiple sclerosis, HMA: human mercaptalbumin, HNA1: human non-mercaptalbumin 1, HNA2: human non-mercaptalbumin 2, CSF: cerebrospinal fluid.

Table 12. Correlations of albumin redox fractions with total number of relapses using Spearman's correlation coefficient.

	Spearman's r	p-value
HMA Serum	-0.310	0.227
HNA1 Serum	0.274	0.287
HNA2 Serum	0.299	0.243
HMA CSF	0.029	0.911
HNA1 CSF	-0.041	0.876
HNA2 CSF	0.106	0.685

HMA: human mercaptalbumin, HNA1/2: human non-mercaptalbumin 1/2, CSF: cerebrospinal fluid.

Table 13. Correlations of albumin redox fractions with age at disease onset using Spearman's correlation coefficient.

	Spearman's r	p-value
HMA Serum	-0.275	0.240
HNA1 Serum	0.277	0.236
HNA2 Serum	0.117	0.624
HMA CSF	-0.276	0.240
HNA1 CSF	0.278	0.235
HNA2 CSF	-0.019	0.935

HMA: human mercaptalbumin, HNA1: human non-mercaptalbumin 1, HNA2: human non-mercaptalbumin 2, CSF: cerebrospinal fluid.

3.9 Storage period

To rule out potential influence of the time between sampling and HPLC analysis, we performed a correlation analysis between storage period of serum and CSF samples and the respective redox fractions. We did not find significant correlations.

3.10 NEFA and bilirubin content

There was no significant difference in NEFA content or HSA-bound bilirubin in serum samples between MS patients and controls. Interestingly, NEFA were significantly higher in MS patients, who had suffered from a relapse in the last 14 days prior to sampling ($p = 0.007$; figure 20). There were no other significant associations between NEFA content or HSA-bound bilirubin and clinical or demographic parameters.

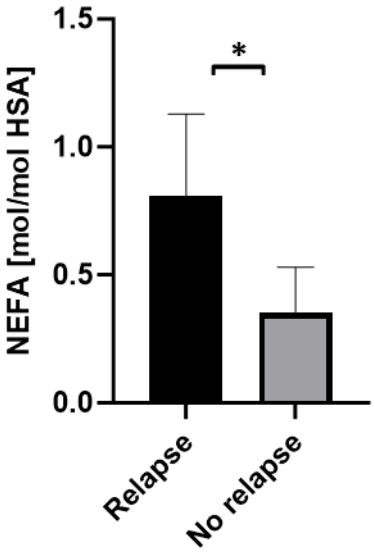

Figure 20. NEFA in patients with vs without a relapse within the 14 days prior to sampling. Relapse: n=6, no relapse: n= 11. Data are presented as means and standard deviations. NEFA: non-esterified fatty acids.

4 Discussion

The aim of this pilot study was to investigate the redox state of HSA in serum and CSF of MS patients in comparison to controls. Overall, it has been confirmed in multiple studies that oxidative processes play a role in MS pathophysiology, and various markers for oxidative stress have been found altered in MS. However, before our study, the redox state of albumin in serum and CSF had not been investigated in MS.

We did not find an overall difference in the albumin redox state between MS patients and controls in either compartment. Given the established role oxidative stress plays in neurodegenerative diseases, this was somewhat surprising at first. Numerous studies have reported altered markers of oxidative stress in MS patients compared to controls before (96–101). However, MS is a highly heterogenous disease, and characteristics such as disease course, duration, relapse activity, DMT or recent corticosteroid treatment can influence the outcome of body fluid biomarker studies. Thus, the patient cohorts are considerably diverse between as well as within these studies, and in comparison to our study. For instance, in Haider et al's patient group, two thirds of the patients presented with a progressive disease course (PPMS or SPMS) with a considerably longer disease duration than in our cohort. Tasset et al, Ortiz et al, Aydin et al and Wang et al on the other hand only included RRMS patients, which were additionally all newly diagnosed in Wang et al's group (96,98,100,101). Study cohorts also differ in regard to treatment: in Tasset et al's group, sampling was performed just before the initiation of natalizumab treatment whereas most patients in Aydin et al's group were under DMT and therefore presented with a lower EDSS at time of sampling (96,100). In our cohort, we only included patients that had not yet undergone DMT, which in turn almost only applied to patients with a relatively short disease duration (median: 1 month). This is to show that in a disease as complex as MS, countless factors that can potentially influence the outcome need to be considered in the study design, and it becomes very difficult to create comparable cohorts. Consequently, this also needs to be kept in mind when analyzing and comparing existing data on the subject.

In addition to heterogeneity of the patient cohorts, different studies examined different markers to assess ongoing oxidative stress, in part producing contradictory

results. For instance, while Wang et al and Tasset et al observed lowered total antioxidative capacity in RRMS patients, Aydin et al did not find a significant difference in total antioxidant status (96,100,101). Regarding the enzyme GSH-Px, an important regulator of intracellular oxidative stress, Wang et al and Ortiz et al found increased GSH-Px activity, while Tasset et al's and Socha et al's studies yielded exactly opposite results, namely lowered GSH-Px activity in MS patients (98,100–102). These are only two examples for incongruous results. Ibitoye et al published an extensive review article in 2016 on studies examining various oxidative stress biomarkers in serum and CSF of MS patients, summarizing consistent and contradictory results. Again, the authors highlight heterogeneity in study design, patient selection and disease characteristics, and emphasize how this poses a challenge in biomarker research in this field (89).

Although we could not find a significant difference in albumin redox state between MS patients and controls overall, we were able to find significant associations between serum and CSF albumin redox fractions and disease characteristics. For instance, serum albumin was shifted towards oxidized fractions in patients presenting with a higher EDSS in remission, a measure for functional disability in MS. Further, HNA2, the irreversibly oxidized state of albumin, was associated with recent relapses in patients. HNA2 in CSF was significantly elevated in those patients, that had suffered from a relapse in the 14 days prior to sampling. Interestingly though, in serum, HNA2 was significantly lower in these patients. We also found correlations between HNA2 in serum, as well as the HNA2 CSF-to-serum ratio, and the time between the last relapse and sampling. These results indicate associations between albumin redox state and various clinical disease parameters. Further, specifically targeted studies are necessary to precisely unravel these connections.

In AD, another neurodegenerative condition, Costa et al found a shift towards the oxidized albumin redox fractions, in both serum and, more drastically, CSF. In this disease, oxidative stress appears to induce the dysfunction of various proteins in the brain and blood, and different studies have found oxidative alterations on different proteins in CSF. However, albumin takes a particular stance because of its sheer abundance (85). It is further noteworthy that plasma exchange (PE) therapy and replacement with therapeutic albumin has been tested in AD in a clinical phase

IIb/III study (AMBAR trial, NCT01561053). PE-treated patients performed significantly better in multiple clinical and neuropsychological assessments than patients in the placebo group. Authors hypothesized that this effect could be attributable to more than one mechanism, including amyloid clearance, but also changes of inflammatory and oxidation status (103).

Previous studies report associations between serum HMA and HNA1, but not HNA2, and age of study subjects (36,88). Matsuyama et al additionally investigated albumin redox state in CSF but did not find a correlation with age. Contrary to these results, we found a significant correlation of HNA2 in serum and HMA in CSF and age, when analyzing MS patients and controls together. In the control group alone, HNA2 in serum and CSF significantly correlated with age, however in the MS group alone, all correlations with age were lost. This could be an indicator for an altered redox state in serum and CSF of MS patients, masking the association with age observed in the control group. It is unclear whether this would apply to other neuroimmunological or generally neurological diseases as well, as the study subjects in Oettl et al's and Matsuyama et al's cohorts were not suffering from neurological disorders.

Additionally, we found an unexpected significant correlation between albumin redox state in serum and lactate concentration in CSF in MS patients, which is included in routine CSF workup (93). This applied to all three albumin redox fractions. To rule out potential confounding by age at sampling, we performed a partial correlation analysis, however the correlation remained significant. The reason for this finding is yet unclear and requires further investigation.

Further, we found NEFA content to be significantly higher in those MS patients, that had suffered from a relapse in the 14 days prior to sampling, than in those without a recent relapse. We did neither find a difference in NEFA content between MS patients and controls, nor any other associations of clinical or laboratory parameters with NEFA content. Again, the reason for this finding remains unclear.

Previously to our study, the redox state of albumin in CSF had only been investigated twice: in a cohort of mild-moderate AD patients and controls, as well as in a cohort of patients with orthopedic disorders (85,88). In their control group, Costa et al found a percentual redox fraction allocation of 86.4/10.0/3.8 to HMA, HNA1

and HNA2, respectively (in the AD group, it was shifted drastically towards the oxidized forms – see above). Matsuyama et al divided their orthopedic cohort into a younger and older group, that produced very similar results for CSF albumin fraction allocation (93.0/6.7/0.3 %HMA/%HNA1/%HNA2 in the younger group). Overall, these results are in line with our findings.

CSF originates from blood as a filtrate through the choroid plexus of the ventricles of the brain. Total CSF volume is around 150 ml in adults, however 500 ml are continuously produced and resorbed every day. Likewise, albumin in CSF is thought to stem from blood simply by diffusion (50,104). Therefore, the shift of its redox state towards the reduced form compared to albumin in serum is surprising. The conversion of the reduced HMA to the oxidized HNA1 is a reversible reaction, with the ratio depending on the environment of the albumin molecule, particularly concerning the concentration of thiols (e.g. cysteine, GSH) and disulfides (e.g. cystine, glutathione disulfide (GSSG)). Nevertheless, it is important to note that the rate constant for the reduction of HNA1 to HMA through reaction with a small thiol is higher than the rate constant for the formation of disulfide through reaction with cystine or other disulfides (105). There is only sparse data available on disulfide content in CSF of MS patients, and while more studies have been performed investigating total thiol content, diverse methods were used, and data regarding the rate of thiols to disulfides is limited. Arslan et al report higher levels of CSF disulfides than native thiols, both in MS patients and healthy controls. Calabrese et al on the other hand found a higher concentration of GSH than GSSG, which is in agreement with a high HMA fraction, although the GSH/GSSG ratio was significantly lower in MS patients than in controls. In both studies, the levels of thiols and disulfides were lower in CSF than in serum (106,107).

We found a significantly lower fraction of HNA2 in CSF than in serum. This is puzzling as the oxidation of albumin at Cys-34 to sulfinic or sulfonic acid, producing HNA2, is believed to be irreversible. Matsuyama et al report on astrocytes in cell culture to reduce HNA2, however this does not appear as a sufficient explanation for the relative loss of HNA2 (88). Potentially, other cell types of the CNS are able to reduce or degrade HNA2 in vivo, perhaps through expression of receptors for modified albumin, as GP18 or GP30 (108). Alternatively, oxidized albumin is possibly unable to diffuse into CSF in the first place due to a currently unknown

reason. Further research is needed to better understand this surprising finding. In general, it can be said that the redox state and the molecules involved in it, including albumin, are only sparsely explored in CSF. Given the nature and dynamics of this body fluid, this leaves potential for further compelling research.

Our study has several limitations. Firstly, including 20 MS patients and 21 control subjects, our cohort was relatively small. This is due to various reasons: In addition to being planned as a pilot study with a typically sparse patient count, it was difficult to recruit samples that met the conditions for HPLC analysis. For our study, samples must not be stored for longer than 16 months at time of analysis, since a prolonged storage period can influence albumin redox state (109). In order to rule out that this had an effect on our outcome, we performed a correlation analysis between the storage time of our samples and the resulting albumin redox fraction allocation, which did not reveal any significant correlations. Nevertheless, this limited pre-analytical storage period restricted our choice of study subjects, that additionally had to meet further inclusion criteria, such as absence of DMT or corticosteroid administration within 28 days before sampling. Moreover, we wanted to analyze albumin redox state in both serum and CSF, meaning that only those patients and controls could be included of whom paired serum and CSF samples were available. Whereas most MS patients managed at the Department of Neurology, Medical University of Graz undergo repeated serum sampling, CSF is typically only obtained once, as lumbar puncture is an invasive procedure and not suitable for repeated disease monitoring in MS. Lumbar puncture is usually done during diagnostic workup at disease onset, therefore most patients eligible for our study had only a short disease duration and as a result a low EDSS. Nevertheless, we think that the results of our pilot study provide a good basis for further research in this field.

Additionally, the suitable sampling window coincided with the first year of the Covid-19 pandemic. Multiple studies found a drop in diagnoses of other diseases during this time, presumably due to various reasons such as patients' hesitancy to seek out healthcare services. Data on this is available for numerous disorders, including different cancer types, diabetes, heart attacks or depression. Regarding neurology, this diagnostic decline was observed for stroke, dementia, or less acute conditions such as benign headaches, as well as a general decrease of neurological emergency department presentations (110–116). There is no data available

specifically for MS, however, it is conceivable that this general phenomenon also affected MS diagnoses, and therefore further restricted the number of potentially eligible patients for our cohort.

Further, our results indicate an association of albumin redox state with functional disability as assessed by EDSS in remission in MS patients. Nevertheless, as stated above, most of the patients in our study had a rather short disease duration at time of sampling due to sample requirements. Their EDSS in remission was accordingly relatively low, with 3 being the highest score among our cohort. It would hence be interesting to apply our analysis to a cohort comprising patients in a more advanced disease stage, with higher functional disability. Additionally, nearly all patients in our cohort had a relapsing-remitting disease course, whereas only three patients suffered from PPMS. As oxidative stress appears to play a particular role in neurodegeneration, which is in turn more pronounced in progressive stages of the disease (see chapters “Neurodegeneration and axonal loss” and “Oxidative stress in MS”), a longitudinal study design following patients with a progressive disease course could further elucidate this aspect.

Part of the results of our study have already been published (117). However, as this was only designed as a pilot study, applying our findings to larger patient cohorts overall, as well as in cohorts with more homogenous patient and disease characteristics, would bring more clarity to the questions our results brought about. As CSF is of limited access, serum appears to be the more applicable body fluid for larger investigations. Additionally, it would be interesting to follow albumin redox state throughout disease progression in a longitudinal setting. Further research into albumin redox state in MS could be valuable against the background of the lack of diagnostic and monitoring biomarkers, but also in light of new therapeutic approaches with therapeutic albumin in neurodegenerative studies.

In summary, while we could not find an overall difference in albumin redox state between MS patients and controls, our data showed associations with disease activity and physical disability. This confirms the involvement of oxidative processes in MS pathophysiology and paves the way for future investigations in larger cohorts and more advanced disease stages. Additionally, our results contribute to

comprehension of the still poorly understood redox environment in CSF, and propose albumin as an interesting analyte for further research in this area.

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