Biological reflections in body fluids of multiple sclerosis progression and multiple sclerosis-related fatigue

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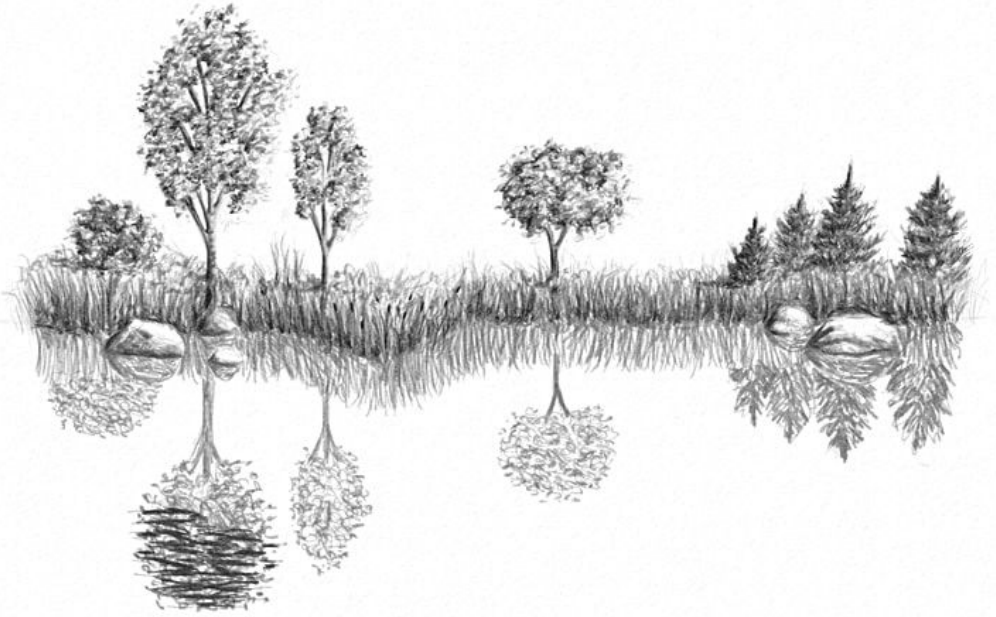
General summary, discussion and recommendations



Multiple sclerosis (MS) is the most common cause of non-traumatic neurological disability in young adults. Being diagnosed with MS not only has a major effect on patient's health, it has major complications on patient's social life, work, study and family planning. This huge burden is often exacerbated because of the unpredictable nature of disease progression.

One of the symptoms that early-on restricts social and economic participation, is the experience of MS-related fatigue. MS-related fatigue is one of the most reported symptoms and one of the more acknowledged symptoms, however no consensus on the definition of fatigue in MS exists [1]. The subjectively perceived nature and lack of pathophysiological understanding of MS-related fatigue have made it difficult to treat or alleviate MS-related fatigue. Most likely MS-related fatigue is multifactorial and several pathophysiological mechanisms have been proposed; a) imbalance in pro- and anti-inflammatory cascades, b) dysregulation of the hypothalamus-pituitary-adrenal (HPA) axis, c) impaired nerve conduction, d) impaired mitochondria energy balance, e) neuro-endocrine and or neurotransmitter dysregulation [2],[3].

The definite MS etiology and progression mechanisms remain unidentified, consequently there are no treatments for curing MS patients. Although, genetic susceptibility (pre-dominantly in immune-system associated genes) together with several environmental factors are considered to be associated with MS pathogenesis [4],[5]. Therefore, most of the current medications target the immune response, with moderate effectiveness in slowing the disease course or reducing disease severity.

Furthermore, no clinically validated biomarkers exist that reflect MS progression or MS-related fatigue mechanisms. Discovery of these biomarkers could give insight into MS progression and fatigue mechanisms associated with MS and potentially yield novel therapeutic targets. Therefore, in this thesis we had two different aims: 1) to discover blood biomarkers that can be used as prognostic biomarkers and that can discriminate different progression types of MS, and 2) determine whether HPA-axis and inflammatory cascades are related to MS-related fatigue.

In this last chapter, we will summarize and discuss our results, and put them into perspective (figure 1).

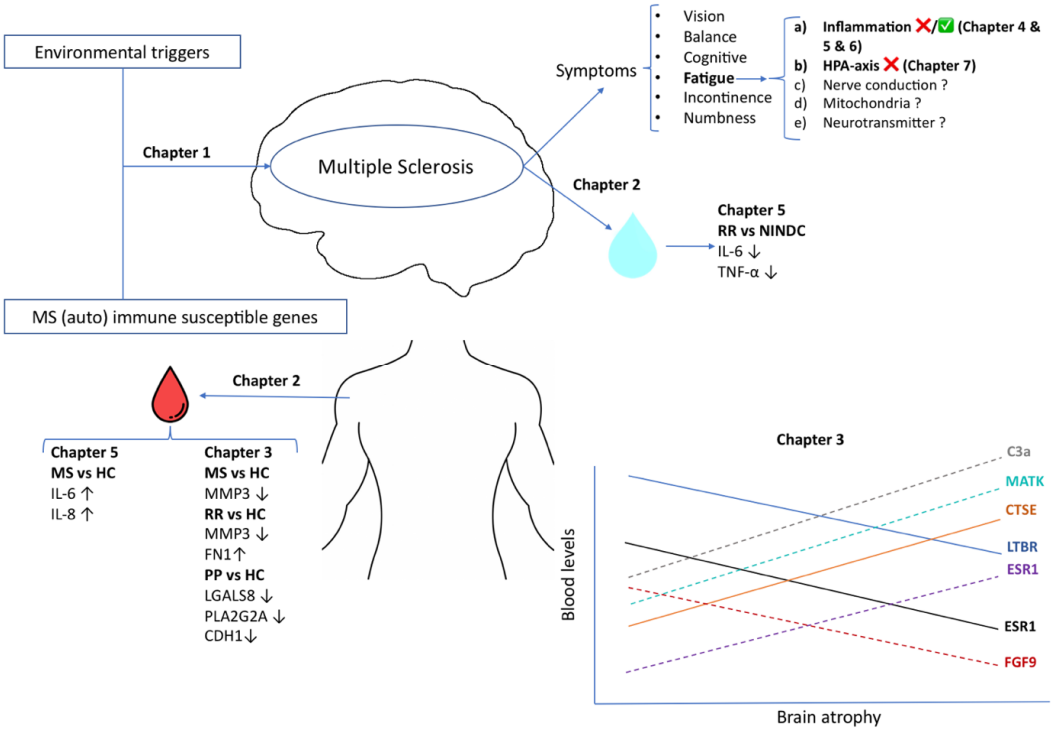


Figure 1. Schematic overview of obtained and discussed results in different chapters

Omics era and biological markers for MS

In the last decades, we have seen a tremendous development in hypothesis-free approaches for MS biomarker research. Proteins are the functional working units inter- and intra-cellularly and are most likely to be affected by disease mechanisms, such as the inflammatory response, and recovery mechanisms. Most of the MS biomarker studies have focused on MS genomics and proteomics, while transcriptomic and metabolomics are now gaining more interest and offer new candidate biomarkers [6],[7]. In **chapter 1,** we reviewed data derived from hypothesis-free omics studies (genomics, transcriptomics and proteomics) from a systems biology point of view. This to gain insight into MS pathophysiology by identifying unifying pathways [6]. In **chapter 1,** we primarily focused on non-HLA gene polymorphisms to gain insight in other underlying pathophysiological MS mechanisms. Several interesting immune system-associated loci are; IL-7RA, IL-2R, TNFRSF1A, CD40, CD6, CD58 and PTGER4. Interestingly, most of these genes encode transmembrane receptors that are involved in T- and B cell activation and homeostasis [4],[6]. Moreover, a recent study on quantitative expression on polymorphisms associated with neurodegenerative and autoimmune diseases showed predominant altered expression in T cells for MS [8]. Indicating that genes associated with MS, pre-dominantly affect T cell homeostasis, and therefore T cells could be involved in MS onset mechanisms.

One of the well-known environmental factors associated with MS is vitamin D [9],[10]. Both geographical and biological data suggest that low vitamin D levels are associated with a high risk of developing MS [9],[10]. Much of the vitamin D signaling occurs through the binding of vitamin D to vitamin D receptor (VDR). Then subsequent binding of VDR to specific genomic sequences, known as vitamin D response elements (VDRE), which are often located near gene promoter regions, affects gene transcription [11]. A putative VDRE has been shown to be near the promoter region of the HLA-DRB gene, however the exact role of VDRE near HLA-DRB gene is unknown. Other epigenetic post-transcriptional mechanisms, are binding of microRNAs to mRNA [6],[7]. The only consistent discovered miRNA that is differentially expressed in blood of MS patients compared to controls is miR-20a [6],[7]. MiR-20a is involved with T-cell activation genes, and lower blood levels of miR-20a were observed in MS compared to healthy controls [12],[13]. Despite the strong potential for biomarker discovery of the

epigenetic and non-coding RNA field, these relative novel fields have been suffering from inconsistencies due to; pre and post-analytic sample handling for RNA analyses, lack of consensus guidelines, small studies without independent validation and replications [7],[14],[15]. A recent study on MS and miRNAs, implemented multiple validation cohorts and discovered novel miRNAs associated with MS, however miR-20a was not reproduced, confirming the observed inconsistencies within the miRNA field [15].

Additionally, in **chapter 1,** we have divided protein biomarkers into immune and neuro-glial homeostasis associated proteins. Interestingly, in agreement with others, we observed a significant increase of C3 in plasma of MS patients (**chapter 3**), with EDSS progression and increase in brain atrophy [16],[17]. Complement activation is a well-known feature in grey matter lesions, where lesions are surrounded by complement receptor-positive microglia, and contribute to irreversible MS progression [18]. Furthermore, differential C3 blood levels were observed for pre-symptomatic and symptomatic MS patients compared to healthy controls [17]. Indicating the activation of C3 at lesion environment and systemic activation may reflect disease progression, however validation in larger and independent cohorts is required.

In **chapter 2,** we reviewed and summarized promising biomarkers that have been replicated in independent studies and cohorts. Interestingly, a major role of humoral response in CSF for MS diagnosis and prognosis is observed with the presence of, immunoglobulin G (IgG) oligoclonal bands (OCB), IgM OCB, antibodies against measles, rubella, varicella zoster (MRZ-reaction). Other immune-associated biomarkers of interest are chitinase-3 like protein 1 (CHI3L1) and C-X-C motif chemokine 13 (CXCL13). CHI3L1 is putatively involved with T-helper cell homeostasis and tissue remodeling, and high levels of CHI3L1 in CSF and serum have been associated with CIS patients and faster conversion rate to RRMS [19]. CXCL13 is a chemokine involved in attracting B cells and B cell activation [20]. Higher CXCL13 CSF levels were observed in CIS, RRMS and progressive MS patients in comparison with non-inflammatory neurological controls [21]. For both CXCL13 and CHI3L1 the lack of specificity, i.e. levels of these markers are often elevated in other inflammatory and infection diseases, tempers their usefulness as diagnostic and or prognostic biomarkers. Axonal and neuronal injury are hallmarks of MS progression, and therefore structural building blocks of neuronal/axonal integrity might

be of interest as diagnostic and or prognostic biomarkers. Neurofilaments are expressed in axons and dendrites, and the light (NfL) and heavy (NfH) have emerged as promising biomarkers in multiple proteopathy neurodegenerative diseases and MS [22],[23]. Most of the previously motioned protein biomarkers have been detected in CSF, although recent technical improvements for measuring NfL in serum, have been very promising [24]. Combination of inflammatory markers with axonal biomarkers could provide an MS specific profile that can be used for disease monitoring and therapeutic response.

In **chapter 3**, we performed hypothesis-free proteomics in blood of MS patients, by applying a novel aptamer technology [25]. Until now, most of the focus of MS protein biomarker discovery was on cerebrospinal fluid (CSF) proteomics, often using liquid chromatography-mass spectrometry (LC-MS) [7]. LC-MS for biomarker discovery for blood biomarker research has proven to be challenging due to pre-analytical and analytical factors, including the interference of abundant proteins e.g. albumin, low sensitivity and low dynamic range within blood [26]. The novel aptamer proteomics binds conformational protein epitopes with high specificity and sensitivity [25]. This array is able to detect >1000 different proteins with a wide dynamic range (>8 logs of concentration difference) in blood. Since the peripheral blood immune-system is involved in the hypothesized autoimmune pathology of MS , we aimed to discover protein biomarkers in plasma of MS patients with different rates of disease progression. Moreover, we had access to baseline and follow-up values of different MRI progression parameters (T1-black hole volume, T2-lesion volume, percentage brain volume change (PBVC)). Several interesting markers that significantly associated with EDSS progression were; LGALS8 (Lectin, Galactoside-Binding, Soluble 8), CCL3 (Macrophage Inflammatory Protein 1-Alpha), TNFRSF13B (TNF Receptor Superfamily Member 13B), RGMA (Repulsive Guidance Molecule Family Member A) and CDH1 (E-Cadherin). For increasing T1- black hole volume and T2-lesion volume, increased levels of plasma PIK3CA/PIK3R1complex were observed. Lastly, eight plasma proteins were significantly associated with PBVC; C3 (Complement C3 fragment a and d), FGF9 (Fibroblast Growth Factor 9), MATK (Megakaryocyte-Associated Tyrosine Kinase), LTBR (Lymphotoxin Beta Receptor), ESR1 (Estrogen Receptor 1), CTSE (Cathepsin E) and EHMT2 (Euchromatic Histone Lysine Methyltransferase 2). Increasing blood

PIK3CA/PIK3R1 complex levels were observed for both the MRI lesion parameters. The PIK3CA/PIK3R1complex is involved with activation of T and B-cell and regulation of self-antigen recognition [27]. PIK3CA/PIK3R1 blood levels could be an indication of an imbalance of T- and B-cell activation and altered self-recognition mechanisms and reflect MS progression. In addition, we observed a strong association of C3 and EHMT2 with brain atrophy, with higher C3 and lower EHMT2 plasma levels related to increasing brain atrophy. Additionally, the presence of C3 together with other complement factors, and Fibronectin aggregates at lesions have been shown to prevent remyelination and shown to be differentially regulated in serum of pre-symptomatic and symptomatic MS patients [17]. EHMT2 is a methyltransferase and is involved with demethylation of histone H3 at lysine 9. This results in recruitment of other transcription regulators and results in repression of transcription [28],[29]. *In vitro* studies show that EHMT2 promotes neuronal and immature oligodendrocyte differentiation and is required for oligodendrocyte maturation [30]. It is likely that with ongoing MS progression EHMT2 reflects altered epigenetics mechanisms caused by pro-inflammatory lesion environment, and gives insight into altered epigenetic pathways associated with MS.

Part 1. Conclusions & recommendations

Human plasma proteome is a representation of all body tissues, physiological and potentially pathophysiological processes. Discovery of a single protein marker specifically associated with MS within the sea of human plasma proteome is very unlikely. It is therefore more advantageous to use a panel of different biomarkers that are reflective of different biological processes associated with MS onset and progression; 1) (auto) immune activation, 2) immune reaction 3) BBB breakdown, 4) epigenetic alterations, 5) neuro-axonal damage, 6) neuronal repair and remyelination (Table 1). A panel of biomarkers associated with different biological pathways associated with MS pathology could serve as a biomarker profile that is specific for MS and allows monitoring of disease progression and therapeutic response.

Despite the discovery of a large number of candidate biomarkers, few biomarkers for monitoring have been rigorously validated or replicated, and are therefore not applicable for MS monitoring. Lack of replication and

validation of these markers is due to several reasons; 1) the analyses of relatively low abundance proteins in plasma requires in-depth proteome analyses, often performed with liquid chromatography-mass spectrometer (LC-MS), which is inherently a low-throughput approach [31]. Most of MS biomarker proteomic studies have been performed on a limited number of samples, without performing multiple testing corrections for the discovery set, potentially resulting in discovery of false-positive markers. Novel methods are emerging, and are combining in-depth proteome analyses with high-throughput methods [25],[32], allowing quantification of larger numbers of patients. This allows stringent multiple testing corrections, reducing the chances of false-positive markers and discovery of true-positives. This merits downstream efforts and expenses for a selection of candidate biomarkers to replicate in larger independent cohorts. 2) Discovery of peptides with LC-MS could offer new candidate markers, however lack of biomarker verification could also be attributed to reagent and antibody limitations [33]. The biomarker verification phase is of importance for analyzing the discovered candidate markers in a larger sample size than the initial discovery phase, often applying immunoassays [7],[33]. Although there is an increase in availability of antibodies targeting human proteome, antibodies targeting some of the proteins in body fluids, especially different isoforms are not available [33]. Before any of the candidate markers are excluded or included for downstream clinical validations, several technical and optimization challenges should be addressed for the specific body fluid of interest; specificity, sensitivity, reproducibility, precision and inter and intra-assay variability [33]. 3) Clinical validation of candidate markers requires a large amount of MS samples (n>1000), therefore international collaboration between MS centers is of importance. Therefore, standardization of collection, handling and storage protocols to decrease sample variability between the different centers are of importance and these guidelines have recently been published [34],[35]. 4) Lastly, the potential biological variability between patients can be reduced, with the incorporation of a systems biology point of view. For example, MS onset mechanisms in HLA-DRB polymorphism (C → T) patients could be different from patients who do not have this polymorphism. Polymorphisms in promoter regions could affect the downstream gene expression and therefore result in lower or higher expression of the gene. Therefore, genetic endo-phenotyping should be considered. Genetic endo-

phenotyping of MS patients into genetic subgroups could reduce biological variability and result in discovery of different molecular pathways associated with different genetic subgroups.

In conclusion, reducing the technical, sample and biological variation by implementing novel high throughput proteomics, stringent statistics, together with standardized guidelines and MS endo-phenotypes, could result into an MS specific biomarker profile, which aids both the clinician and MS patients.

Table 1. Overview of blood-based biomarkers associated with MS associated biological process

Immune activation	Immune reaction	BBB breakdown	Epigenetic	Neuro-axonal damage	Repair and remyelination
LGALS8 ↓	**IgM OCB ↑**	CDH1 ↓	**miR-20a↑**	**NfL↑**	*RGMA*↓
TNFRSF13B ↑	**CXCL13↑**	*MMP3*↓	EHMT2↓		
PIK3CA/PIK 3R1 ↑	*C3↑*	*FN1* ↓			
LTBR ↓	**CHI3L1 ↑**				
	CCL3 ↓				

The arrows indicate expression patterns in MS subtypes compared to controls, or indicate down and upregulation with MS clinical and MRI parameter progression. Markers shown in bold, are reported, validated and replicated by others [7]. Other markers are reported in **chapter 3**, while marker in cursive have been reported by us and in other biomarker studies, though require replication. Abbreviations; BBB (blood-brain barrier), LGALS8 (Lectin, Galactoside-Binding, Soluble 8), TNFRSF13B (TNF Receptor Superfamily Member 13B), PIK3CA (Phosphoinositide-3-Kinase, Catalytic, Alpha complex), LTBR (Lymphotoxin Beta Receptor), IgM (Immunoglobulin M oligoclonal bands), CXCL13 (chemokine (C-X-C motif) ligand 13), C3 (Complement 3), CHI3L1 (Chitinase-3-like protein 1) (E-Cadherin), CCL3 (Macrophage Inflammatory Protein 1-Alpha), CDH1 (Cadherin-1), MMP3 (matrix metalloproteinase-3), FN1 (Fibronectin), EHMT2 (Euchromatic Histone Lysine Methyltransferase 2), NfL (Neurofilament light chain), RGMA (Repulsive Guidance Molecule Family Member A),

Part 2. Multiple sclerosis-related fatigue

One of the most prominent and disabling MS symptoms is the experience of fatigue. The importance of MS-related fatigue as a disabling symptom is well-known, however the underlying mechanisms of MS-related fatigue remain unknown. MS-related fatigue can be divided into primary fatigue or secondary fatigue. Primary fatigue is considered to be a consequence of specific MS pathophysiology. Whereas secondary fatigue can be attributed to factors not unique for MS but are symptoms that are accompanied with MS, such as; sleep-disorder due to spasms or incontinence, depression and lack of physical conditioning [36]. The exact pathophysiology of MS-related fatigue remains unknown [3],[37].

In this thesis we hypothesized that imbalance in the inflammation system and or hypothalamus- pituitary adrenal (HPA)-axis mechanisms cause MS-related fatigue. We assessed whether the balance within these physiological mechanisms would be restored upon fatigue alleviation with the use of different non-pharmacological therapies: Aerobic Training (AT), cognitive behavioral training (CBT) and energy conservation management (ECM).

MS-related fatigue

The association of the immune system with different neurological pathologies is becoming more evident [38],[39]. Whether the involvement of the immune system is the root cause or consequence of MS pathophysiology and MS-related fatigue, the quantification of pro and anti-inflammatory cytokines could give insight into disease progression and therapeutic response. MS is considered to be a T-cell induced auto-immune pathology, with the involvement of B-cells and macrophages [4],[5]. Cytokine and chemokine profiles are therefore interesting for monitoring MS progression. So far limited studies have addressed cytokine profiles as potential biomarkers for MS and related MS symptoms. This could be because most of the earlier studies have used so called mono-plex ELISA assays, targeting a single cytokine at a time. Moreover, cytokines are expressed at a low baseline level and have relatively short half-life, therefore sensitivity of immune-assays is of importance [40]. Last decade has seen an increase in the use of multiplex immunoassays. Multiplex immunoassays allow quantification of multiple targets in a single aliquot of specimen.

In **chapter 4** we performed a literature study to compare technical characteristics (sensitivity, specificity, reproducibility and accuracy), of two widely used multiplex arrays, Luminex xMAP and Meso Scale Discovery (MSD) for cytokine quantification in body fluids. We especially wanted to address the technical challenges accompanied with multiplex platforms in comparison with the golden-standard ELISA for cytokine quantification in body fluids. In **chapter 4**, we observed that the multiplex platforms are able to quantify cytokines comparable with ELISA when used for cell culture supernatants. However, for blood only the abundantly expressed cytokines were reliably quantified for the multiplex platforms. These results indicate possible sensitivity and specificity issues with the multiplex platforms for quantifying cytokines in blood. Moreover, using cytokine and chemokine profiles for MS is challenging due to the inconsistent reports regarding different cytokine and chemokines associated with MS. In conclusion, multiplex platforms offer novel ways to detect multiple cytokines that can be used for MS monitoring, however technical challenges regarding the sensitivity for low abundant cytokines in blood, possible cross-reactivity in multiplex setting, should be addressed before multiplex immunoassay can be used for reliable cytokine quantification in blood.

In **chapter 5**, we experimentally compared the technical characteristics of the Luminex and MSD platform for quantification of multiple pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α) in serum and cerebrospinal fluid (CSF). Overall, the assay validation characteristics of the MSD array were better, for quantification of IL-6, IL-8 and TNF-α in serum and CSF, however this assay requires technical improvements, before using the MSD pro-inflammatory assay in the clinic. Next (**chapter 5**), using the MSD assay, the levels of the pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α) were determined in paired serum and CSF samples of MS patients and compared to healthy controls. We observed a significant increase of IL-6, IL-8 for all MS subtypes in comparison to healthy controls, whereas for TNF-α serum levels were increased only for PPMS subtype in comparison to healthy controls. Moreover, assessment of these pro-inflammatory cytokines with EDSS showed a significant negative correlation with EDSS, indicating that with EDSS progression less IL-1β is observed in serum of MS patients.

The multiplex quantification of multiple cytokines and chemokines in body fluids, offers a new approach to assess specific panels of cytokine and chemokines associated with MS. However, these multiplex platforms need to address the sensitivity, specificity and reproducibility challenges associated with quantification of these cytokines and chemokines in body fluids.

In chapter 6 we gained insight into the pro-and anti-inflammatory cytokines serum expression levels in MS patients with fatigue. Therefore, we explored the relationship between pro-inflammatory cytokines (IL-1□, IL-2, IL-6, IL-8, IL-12p70, IL-17, TNF□, and IFN-□) and anti-inflammatory cytokines (IL-4, IL-5,IL-10, and IL-13) in serum of MS patients with and without fatigue. Using the subscale fatigue from the questionnaire Checklist Individual Strength (CIS20r), we distinguished MS patients with and without fatigue [41]. Serum cytokines were quantified using the MSD multiplex platform. Similar serum cytokine levels were observed between MS patients with and without fatigue. Interestingly, we observed a significant correlation for IL-6 with CIS20r fatigue scores. However, we did not perform multiple testing due to explorative nature of this study. Therefore, the association of IL-6 with CIS20r fatigue scores requires validation in a larger group. However, due to the low prevalence of MS patients without fatigue, inclusion of these patients to generate a larger study group is very challenging. In conclusion, the assessed pro-and anti-inflammatory cytokines are likely not associated with MS-related fatigue, although other cytokines and chemokines reported to be associated with MS, such as CXCL13 and CCL3 should be assessed for a complete overview regarding cytokines and chemokines and MS-related fatigue [20],[21].

Earlier studies show hyper-active HPA-axis or HPA-axis components in MS patients with fatigue compared to MS patients without fatigue [2],[42]. In **chapter 7**, we explored the longitudinal effect of HPA-axis function on MS-related fatigue, by assessing diurnal cortisol saliva levels in patients that participated in the TREating FAtigue in MS (TREFAMS) research program that consisted of three randomized controlled trials to study the effects of aerobic training (AT) [43], energy conservation management (ECM) [44] and cognitive behavioral therapy (CBT) [45]. Moreover, we investigated whether specific treatments affect diurnal cortisol saliva secretion.
We found no association between diurnal cortisol parameter with MS-related fatigue scores. Interestingly, neither of the treatments influenced diurnal cortisol parameters, with the exception of a long-term effect in the ECM treatment group on the cortisol secretion upon awakening. This

indicates that MS-related fatigue cannot be attributed to HPA-axis diurnal secretion and is likely caused by other disease mechanisms.

Part 2. Conclusions & recommendations

We observed differential cytokine expression in MS patients compared to healthy controls, but most of these pro-inflammatory cytokines were not associated with fatigue scores. Moreover, similar serum levels of a larger subset of pro and anti-inflammatory cytokine and chemokines were observed between MS patients with fatigue and without fatigue. Lastly, we did not observe any association of HPA-axis diurnal secretion parameters with MS-related fatigue. In conclusion, we did not observe any association for MS-related fatigue with pro-and anti-inflammatory cytokines and chemokines. Moreover, no associations between MS-related fatigue and HPA-axis diurnal cortisol secretion was observed.
Future research regarding the pathophysiology of MS-related fatigue should explore other mechanisms such as: mitochondrial dysfunction and impaired nerve conduction.

In chronic fatigue syndrome (CFS) lower plasma levels of Coenzyme Q10 (CoQ10) were observed [46]. CoQ10 is primarily located in mitochondria and is a component of the electron transport chain, ATP production and energy metabolism [46]. Interestingly, in a double-blind study on MS patients with fatigue, supplementation with CoQ10 reduced fatigue in the intervention group [47]. This indicates that altered mitochondrial homeostasis and ATP production could be the underlying cause of MS-related fatigue and that CoQ10 could be a potential marker for MS-related fatigue. Future studies should address the CoQ10 levels in blood of MS patients with and without fatigue, and whether CoQ10 blood levels increase upon CoQ10 supplementation and fatigue alleviation.
Other possible mechanism associated with MS-related fatigue could be impaired nerve-conduction. In **chapter 3**, we showed that lower plasma EHMT2 levels are associated with increasing brain atrophy. It has been shown EMHT2 demethylates histone H3 lysine 9 in promotor regions of different potassium (K^+) channels, prolonging the axonal depolarization phase and therefore hypersensitivity to pain [29]. It is possible that differential EHMT2 expression in MS progression reflects increased K^+ channel expression and imbalance in nerve-conduction, which could result

into MS-related fatigue. Future studies should explore and validate EHMT2 candidate maker in an MS cohort with available fatigue scores, in comparison with MS patients without fatigue.

In conclusion, we did not observe significant associations of serum cytokines with MS related fatigue. Also, no association of diurnal cortisol secretion with MS-related fatigue was observed. Therefore, other pathophysiological pathways, such as mitochondrial dysfunction, and impaired nerve conduction should be assessed in a MS-related fatigue cohort.

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