Biomarkers and Disease Activity in Multiple Sclerosis

A cohort study on patients with
clinically isolated syndrome and
relapsing remitting multiple sclerosis

Irene Håkansson

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To Alvar and Sara

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ABSTRACT

This thesis focuses on disease activity in clinically isolated syndrome (CIS) and newly diagnosed relapsing remitting multiple sclerosis (RRMS). The papers are based on data from a prospective longitudinal cohort study of patients with CIS and newly diagnosed RRMS. Patients were consecutively enrolled and followed-up by the same neurologist (Irene Håkansson) at the Department of Neurology, University Hospital of Linköping, Sweden. At baseline, and at one year, two years and four years of follow-up, patients underwent clinical neurological examination, sampling of peripheral blood and cerebrospinal fluid (CSF), neuropsychological evaluation and magnetic resonance imaging (MRI) of the brain. All patients were untreated at baseline. Age- and sex-matched healthy controls for blood and CSF samples were recruited from blood donors.

Paper I focused on the clinical need for prognostic biomarkers in newly diagnosed CIS and RRMS. Levels of CXCL1, CXCL8, CXCL10, CXCL13, CCL22, neurofilament light chain (NFL), neurofilament heavy chain, glial fibrillary acidic protein, chitinase-3-like-1 (CHI3L1), matrix metalloproteinase-9 (MMP-9) and osteopontin in CSF from 41 patients and 22 healthy controls were determined with multiplex bead assay and enzyme-linked immunosorbent assay methodology. The prognostic value of baseline levels of these neurodegenerative and neuroinflammatory markers in relation to disease activity during the first two years of follow-up was evaluated. Disease activity was defined as clinical relapses, new T2 lesions in brain magnetic resonance imaging and/or sustained Expanded Disability Status Scale (EDSS) progression. Absence of these three signs of disease activity was called no evidence of disease activity (NEDA-3). Logistic regression analysis showed that NFL in CSF was the best predictive marker of disease activity and correctly classified 93% of the patients with evidence of disease activity during two years of follow-up and 67% of those without. An overall proportion of 85% (33 of 39 patients that completed two year follow-up) were correctly classified. Combining NFL with a chemokine did not improve these results. This study rendered further support for NFL in CSF as a clinically relevant biomarker of disease activity in CIS and RRMS.

Paper II presented four year follow-up data from the cohort and, in addition, expanded the disease activity concept by including brain volume data. Serum levels of NFL were also included in this paper. Paper II aimed to determine the correlation between NFL in serum and CSF and to evaluate the relationship between the body fluid biomarkers, NEDA-3 and brain volume loss, respectively. NFL in serum was determined using a single molecule array (Simoa) method. Brain volume was calculated as brain parenchymal fraction (BPF) using SyMRI[®], based on quantitative MRI data. We found a fairly strong correlation between NFL in CSF and serum ($r=0.74$, $p<0.001$). However, NFL in CSF displayed stronger associations with NEDA-3 status, new T2 lesions and brain volume loss during follow-up than NFL in serum. NFL in CSF turned out to be associated with new T2 lesions as well as with brain volume loss, whereas CHI3L1 in CSF was associated mainly with brain volume loss and CXCL1, CXCL10, CXCL13, CCL22 and MMP-9 in CSF were mainly associated with new T2 lesions. This study confirmed that serum and CSF levels of NFL correlate, but that CSF-NFL predicts and reflects disease activity better than S-NFL. These findings further added to the accumulating evidence that CSF-NFL is a clinically

useful biomarker in CIS and RRMS and that NFL could be included in the expanding NEDA concept.

Paper III addressed the patients' self-reported Modified Fatigue Impact Scale (MFIS) scores in relation to other cohort study data. MFIS scores from baseline and one year follow-up was recorded in 38 patients and were analyzed in relation to other questionnaire data (Hospital Anxiety and Depression scale (HAD), Multiple Sclerosis Impact Scale 29 (MSIS-29) and Short Form 36 (SF-36)), neuropsychological test results (*e.g.* Auditory Consonant Trigram Test (ACTT)), clinical parameters (*e.g.* EDSS), MRI data and several neurodegenerative/neuroinflammatory markers in CSF and serum/plasma. MFIS scores correlated with HAD, MSIS-29 and SF-36 scores as well as ACTT results (Spearman's rho 0.45-0.78, all $p \leq 0.01$) but not with other neuropsychological test results, EDSS ratings, number of T2 lesions, total brain volume or neurodegenerative/neuroinflammatory markers, including NFL levels in CSF and serum. In conclusion, fatigue scores were associated with other self-assessment questionnaire data and to some extent with neuropsychological test performances, whereas no association with clinical, neuroimaging or CSF parameters could be demonstrated. These results indicate that subjective fatigue scores are not well reflected by some commonly used and objectively measurable disease parameters.

Paper IV aimed to compare levels of the complement factors C1q, C3, C3a and sC5b-9 in CSF and plasma from cohort patients and from healthy controls, as well as to assess associations between complement levels and disease activity and some of the biomarkers from paper I and II. C1q, C3, C3a and sC5b-9 levels were determined by nephelometry, enzyme-linked immunosorbent assay and magnetic bead sandwich immunoassay methodology, in patient samples at baseline and after one, two and four years of follow-up as well as in control samples. CSF-C1q was significantly higher in patients than in HC at baseline. The subgroup of patients with ongoing relapse at baseline also had higher levels of CSF-C3a than controls. Baseline levels of CSF-C1q and CSF-C3a correlated significantly with several pro-inflammatory chemokines as well as with MMP-9, CHI3L1 and NFL in CSF. Baseline CSF-C3a also correlated significantly with the number of T2 lesions and Gadolinium enhancing lesions in brain MRI at baseline, as well as with the number of new T2 lesions during follow-up. This study indicates that the complement system is involved already at early clinical stages of MS. It also suggests that especially CSF-C1q and CSF-C3a levels are associated with other neuroinflammatory and neurodegenerative markers and that CSF-C3a levels may carry some prognostic information.

Taken together, this thesis confirms and extends the knowledge of NFL as a useful biomarker in CIS and RRMS and suggests that NFL, rather than total brain volume loss, could be included in an expanded NEDA concept and used in clinical monitoring of disease activity/treatment effect. Although serum levels of NFL were correlated with the corresponding CSF levels, CSF-NFL showed a stronger association to subsequent disease activity. In addition to NFL, several other biomarkers showed promising results, but need confirmation. Data supporting the presence of complement activation in the central nervous system in CIS and RRMS, as well as possible associations between complement activation and disease activity, are also presented.

SVENSK SAMMANFATTNING

Denna avhandling fokuserar på sjukdomsaktivitet hos patienter med kliniskt isolerat syndrom (CIS) som kan tyda på utveckling av multipel skleros, samt nyligen diagnostiserad skovvis förlöpande multipel skleros (RRMS). Delarbetena är baserade på data från en prospektiv longitudinell kohortstudie av patienter med CIS och RRMS. Patienterna inkluderades och följdes upp av samma neurolog (Irene Håkansson), vid Neurologiska kliniken på Universitetssjukhuset i Linköping. Vid baseline och vid ett, två och fyra års uppföljning genomgick patienterna klinisk neurologisk undersökning, provtagning av blod och cerebrospinalvätska (CSF), neuropsykologisk bedömning och magnetkameraundersökning (MRI) av hjärnan. Samtliga patienter var obehandlade vid baseline. Kontrollmaterial i form av blod och CSF erhöles från ålders- och könsmatchade friska kontroller, rekryterade bland friska blodgivare.

Delarbete I utgick från det kliniska behovet av prognostiska biomarkörer vid nydiagnostiserat CIS och RRMS. Nivåer av en rad neuroinflammatoriska och neurodegenerativa markörer (CXCL1, CXCL8, CXCL10, CXCL13, CCL22, neurofilament light chain (NFL), neurofilament heavy chain, glial fibrillary acidic protein, chitinase-3-like-1 (CHI3L1), matrix metalloproteinase-9 (MMP-9) och osteopontin) bestämdes i CSF från 41 patienter med CIS eller RRMS resp. från 22 friska ålders- och könsmatchade kontroller. Delarbete I fokuserar främst på dessa markörers prediktiva värde, närmare bestämt på om nivåer vid första provtagningstillfället (baseline) predikterar sjukdomsaktivitet under de kommande två åren. Sjukdomsaktivitet definierades här utifrån det s.k. NEDA-3 begreppet, dvs. kliniska skov, MRI aktivitet och/eller försämring av EDSS. NFL i CSF visade sig vara den bästa prognostiska markören för sjukdomsaktivitet under två års uppföljning. NFL-nivåer vid baseline kunde korrekt identifiera 93 % av patienterna som uppvisade sjukdomsaktivitet under uppföljningstiden och 67 % av patienterna som inte gjorde det, med totalt 85 % korrekt klassificerade patienter (33 av 39 patienter med fullständig uppföljning efter två år). Studien gav ytterligare stöd för NFL i CSF som kliniskt värdefull biomarkör för sjukdomsaktivitet i MS-sammanhang.

Delarbete II fördjupade och breddade vissa av frågeställningarna i delarbete I samt redovisar även data från fyraårsuppföljning av kohorten, hjärnvolymdata och NFL i serum. Vi fann en tämligen stark korrelation mellan NFL i CSF och serum ($r=0,74$, $p<0,001$). NFL i CSF var dock starkare associerat med NEDA-3 status, nya T2-lesioner och hjärnvolymförlust under uppföljning än vad NFL i serum var. NFL i CSF visade sig vara associerat med både nya T2 lesioner och hjärnvolymförlust, emedan CHI3L1 i CSF var associerat med främst hjärnvolymförlust och CXCL1, CXCL10, CXCL13, CCL22 samt MMP-9 i CSF istället var associerade med främst nya T2-lesioner. Studien visade att NFL-nivåer i serum och CSF visserligen korrelerar starkt, men att NFL-nivåer i CSF har ett högre prediktivt värde och bättre reflekterar sjukdomsaktivitet än NFL i serum. Detta talar för att det kan vara av värde att bestämma NFL i CSF i vissa lägen, trots att det kräver ryggvätskeprovtagning istället för blodprovstagning. Att mäta NFL i serum kan vara av värde och är mer rimligt vid behov av tät uppföljning.

Delarbete III fokuserar på patienternas egen fatigueskattning i förhållande till andra data. Modified Fatigue Impact Scale (MFIS) poäng från både baseline och ett års uppföljning erhöles från 38 av patienterna. Eventuella samband mellan MFIS poäng och andra enkätdata (Hospital Anxiety and Depression scale (HAD), Multiple Sclerosis Impact Scale 29 (MSIS-29) och Short Form 36 (SF-36)), neuropsykologiska testresultat (bl.a. Auditory Consonant Trigram Test (ACTT)), kliniska parametrar (bl.a. EDSS), MRI data samt flera neurodegenerativa/neuroinflammatoriska markörer i CSF och serum/plasma undersöktes. MFIS poäng korrelerade tydligt med HAD, MSIS-29 och SF-36 poäng respektive med ACTT prestation (Spearman's rho 0,45-0,78, samtliga $p \leq 0,01$), men inte med övriga neuropsykologiska testresultat, EDSS poäng, antal T2-lesioner, total hjärnvolym eller neurodegenerativa/neuroinflammatoriska markörer. Studien kunde således påvisa samband mellan MFIS-poäng och främst andra subjektiva självskattningar men inte mellan MFIS-poäng och ett flertal frekvent använda och objektivt mätbara sjukdomsparametrar.

Delarbete IV jämförde nivåer av komplementfaktorerna C1q, C3, C3a och sC5b-9 i CSF och plasma från patienter och friska kontroller, samt undersökte om samband mellan komplementfaktornivåer och sjukdomsaktivitet, inkl. vissa av biomarkörerna för neuroinflammation och axonal skada från delarbete I och II, kunde påvisas. C1q, C3, C3a och sC5b-9 nivåer bestämdes i prover från baseline och från ett, två och fyra års uppföljning av patienterna. CSF-C1q var signifikant högre hos patienter än kontroller vid baseline. Subgruppen patienter som hade pågående skov vid baseline hade även högre nivåer av CSF-C3a än kontrollerna. Baselinenivåer av CSF-C1q och CSF-C3a korrelerade signifikant med flera proinflammatoriska kemokiner samt med MMP-9, CHI3L1 och NFL i CSF. Baseline CSF-C3a korrelerade även signifikant med antal T2-lesioner och Gadolinium-laddande lesioner i magnetkamerabilder av hjärnan vid baseline och med antal nya T2-lesioner under uppföljningstiden. Fynden talar för att komplementsystemet är involverat redan i tidigt kliniskt sjukdomsskede vid CIS och RRMS. Särskilt CSF-C1q och CSF-C3a förefaller vara associerade med andra neuroinflammatoriska och neurodegenerativa markörer och CSF-C3a har möjligen visst prognostiskt värde.

Sammantaget visar denna avhandling på ett betydande värde av NFL som biomarkör vid CIS och RRMS och talar för att NFL, snarare än förändring av total hjärnvolym, kan inkluderas i ett utvidgat NEDA-begrepp och användas för monitorering av sjukdomsaktivitet/behandlingseffekt. Även om NFL nivåer i serum korrelerade till korresponderade CSF nivåer så var CSF-NFL starkare associerat med efterföljande sjukdomsaktivitet. Utöver NFL så visade flera andra biomarkörer lovande resultat men dessa fynd behöver bekräftas. Resultat som stöder komplementaktivering i centrala nervsystemet vid CIS och RRMS, samt möjliga samband mellan komplementaktivering och sjukdomsaktivitet, presenteras också.

LIST OF PAPERS

The thesis is based on the following four papers, which will be referred to by their roman numerals.

I. Neurofilament light chain in cerebrospinal fluid and prediction of disease activity in clinically isolated syndrome and relapsing-remitting multiple sclerosis

Irene Håkansson, Anders Tisell, Petra Cassel, Kaj Blennow, Henrik Zetterberg, Peter Lundberg, Charlotte Dahle, Magnus Vrethem, Jan Ernerudh
Eur J Neurol 2017;24:703-712.

II. Neurofilament light chain in serum and cerebrospinal fluid in relation to disease activity and brain volume loss during follow-up in clinically isolated syndrome and relapsing remitting multiple sclerosis

Irene Håkansson, Anders Tisell, Petra Cassel, Kaj Blennow, Henrik Zetterberg, Peter Lundberg, Charlotte Dahle, Magnus Vrethem, Jan Ernerudh
J Neuroinflammation 2018;15:209.

III. Fatigue scores correlate with other self-assessment data, but not with clinical and biomarker parameters, in CIS and RRMS

Irene Håkansson, Lovisa Johansson, Charlotte Dahle, Magnus Vrethem, Jan Ernerudh
Submitted manuscript

IV. Complement activation in cerebrospinal fluid in clinically isolated syndrome and early stages of relapsing remitting multiple sclerosis

Irene Håkansson, Jan Ernerudh, Magnus Vrethem, Charlotte Dahle, Kristina Nilsson Ekdahl
Submitted manuscript

Other papers containing data from the cohort study:

Quantitative MRI for Analysis of Active Multiple Sclerosis Lesions without Gadolinium-Based Contrast Agent

Ida Blystad, Irene Håkansson, Anders Tisell, Jan Ernerudh, Örjan Smedby, Peter Lundberg, Elna-Marie Larsson

AJNR Am J Neuroradiol 2016; 37:94-100.

Dynamic Response Genes in CD4+ T Cells Reveal a Network of Interactive Proteins that Classifies Disease Activity in Multiple Sclerosis

Sandra Hellberg, Daniel Eklund, Danuta Gawel , Mattias Kopsen, Huan Zhang, Colm Nestor, Ingrid Kockum, Tomas Olsson, Thomas Skogh, Alf Kastbom, Christopher Sjöwall, Magnus Vrethem, Irene Håkansson, Mikael Benson, Maria Jenmalm, Mika Gustafsson, Jan Ernerudh

Cell Rep 2016; 16:2928-2939.

Improved Precision of Automatic Brain Volume Measurements in Patients with Clinically Isolated Syndrome and Multiple Sclerosis Using Edema Correction

Marcel Warntjes, Anders Tisell, Irene Håkansson, Peter Lundberg, Jan Ernerudh

AJNR Am J Neuroradiol 2018; 39:296-302.

Oxylipins in cerebrospinal fluid in clinically isolated syndrome and relapsing remitting multiple sclerosis

Irene Håkansson, Sandra Gouveia-Figueira, Jan Ernerudh, Magnus Vrethem, Nazdar Ghafouri, Bijar Ghafouri, Malin Nording

Prostaglandins Other Lipid Mediat 2018; 138: 41-47.

ABBREVIATIONS

ACTT	Auditory Consonant Trigram Test
AHSCT	Autologous Hematopoietic Stem Cell Transplantation
ANOVA	Analysis Of Variance
AUC	Area Under Curve
BPF	Brain Parenchymal Fraction
CCL20	Chemokine Ligand 20
CCL22	Chemokine Ligand 22
CHI3L1	Chitinase-3-Like-1
CIS	Clinically Isolated Syndrome
CMIV	Center for Medical Imaging and Visualization
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CXCL1	Chemokine Ligand 1
CXCL8	Chemokine Ligand 8
CXCL10	Chemokine Ligand 10
CXCL13	Chemokine Ligand 13
D-KEFS	Delis-Kaplan Executive Function System
DMD	Disease Modifying Drug
EDSS	Expanded Disability Status Scale
ELISA	Enzyme linked immunosorbent assay
GFAP	Glial Fibrillary Acidic Protein
HAD	Hospital Anxiety and Depression scale
HC	Healthy Control
9-HODE	9-hydroxyoctadecadienoic acid
13-HODE	13-hydroxyoctadecadienoic acid
HsCRP	High-sensitivity C-Reactive Protein
IL-1	Interleukin 1
IL-6	Interleukin 6
MFIS	Modified Fatigue Impact Scale
MMP-9	Matrix Metalloproteinase-9
MOA	Mechanism Of Action
MRI	Magnetic Resonance Imaging
MRS	Magnetic Resonance Spectroscopy
MS	Multiple Sclerosis
MSIS-29	Multiple Sclerosis Impact Scale 29
NEDA	No Evidence of Disease Activity
NFH	Neurofilament Heavy Chain
NFL	Neurofilament Light Chain
OCT	Optical Coherence Tomography
OPN	Osteopontin
PASAT	Paced Auditory Serial Addition Test
POMS	Possible Multiple Sclerosis
PPMS	Primary Progressive Multiple Sclerosis
qMRI	quantitative MRI

ROC	Receiver Operating Characteristic
RRMS	Relapsing Remitting Multiple Sclerosis
SF-36	Short Form 36
Simoa	Single Molecule Array
TMT	Trail Making Test
VFT	Verbal Fluency Test

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INTRODUCTION

Clinical presentation and disease course in multiple sclerosis

Multiple sclerosis (MS) is an autoimmune chronic neuroinflammatory and neurodegenerative disease of the central nervous system (CNS), *i.e.* the brain and spinal cord. The prevalence in Sweden is estimated to 190/100 000 and the female to male ratio is approximately 2.4:1 [1]. Disease onset age peaks in the third and fourth decades of life. MS is the most common non-traumatic cause of neurological disability in young adults [2]. MS presents with symptoms and/or signs due to involvement of the CNS. Relapsing remitting multiple sclerosis (RRMS) usually manifests itself as relapses with varying degree of recovery and accumulating loss of neurological function over time, as schematically visualized in Figure 1. A relapse is an episode of symptoms and/or signs due to MS that has a duration of at least 24 hours. Common symptoms and/or signs in MS are visual loss due to optic neuritis, diplopia, ataxia, sensory loss or paresthesias, affected motor function, neurogenic bladder dysfunction, cognitive impairment, fatigue, pain and depression. Sooner or later most patients with RRMS enter a progressive phase where neurological deterioration occurs with or without further relapses. A first clinical episode suggestive of MS, but not fulfilling the diagnostic criteria for MS, is called a clinically isolated syndrome (CIS) or possible MS (POMS). With time, a majority of CIS patients convert to MS. Approximately 10-15% of MS patients have primary progressive multiple sclerosis (PPMS), where the condition is progressive from disease onset. Disease activity and long term neurological outcome vary a lot between individuals with MS.

Figure 1. Schematic visualization of the disease course in relapsing remitting multiple sclerosis. CIS: clinically isolated syndrome, RRMS: relapsing remitting multiple sclerosis, MS: multiple sclerosis, MRI: magnetic resonance imaging

Diagnostic criteria for multiple sclerosis

In this cohort study, the 2010 revised McDonald diagnostic criteria [3] for MS were used. A diagnosis of MS requires a clinical picture that is compatible with MS and that diagnoses that could better explain the clinical picture have been ruled out. A classic hallmark in the process of diagnosing MS is the demonstration of dissemination of lesions in time and space, separating MS from monofocal and monophasic neurological conditions. A diagnosis of MS still requires that classic dissemination in time and space, but nowadays MRI lesions can fulfil dissemination criteria, allowing for earlier diagnosis. Demonstration of dissemination in space and/or time with MRI results in conversion from CIS to MS before a second clinical episode has occurred in some CIS patients. A simplified overview of the 2010 revised McDonald criteria for RRMS is presented in Table 1.

Table 1. A simplified overview of the 2010 revised McDonald criteria for RRMS

Relapses	Lesions	Additional evidence needed
≥ 2	Objective clinical evidence of ≥2 lesions OR objective clinical evidence of 1 lesion combined with historical evidence of a prior relapse.	None needed (but desirable)
≥ 2	Objective clinical evidence of 1 lesion	Demonstration of dissemination in space ^a OR await new relapse implicating new lesion location
1	Objective clinical evidence of ≥2 lesions	Demonstration of dissemination in time ^b OR await new relapse
1	Objective clinical evidence of 1 lesion	Demonstration of dissemination in space ^a AND time ^b OR await new relapse providing clinical evidence of dissemination

^a: Dissemination in space can be demonstrated by at least 1 T2 lesion in at least 2 of 4 locations considered characteristic for MS (juxtacortical, periventricular, infratentorial and spinal cord), with lesions within the symptomatic region excluded in patients with brainstem or spinal cord syndromes.

^b: Dissemination in time can be demonstrated by a new T2 and/or gadolinium-enhancing lesion on follow-up MRI or the simultaneous presence of asymptomatic gadolinium-enhancing and non-enhancing lesions at any time.

The 2017 revisions of the McDonald criteria [4] included the following changes: In patients with a typical clinically isolated syndrome and clinical or MRI demonstration of dissemination in space, the presence of CSF-specific oligoclonal bands allows a diagnosis of multiple sclerosis. Symptomatic lesions can be used to demonstrate dissemination in space or time regardless of localization, optic nerve lesions being the only exception.

Cortical lesions can also be used to demonstrate dissemination in space. At the time of diagnosis, the disease course should be specified (relapsing-remitting, primary progressive, or secondary progressive). Also, whether the disease course is active or not, and progressive or not, based on the previous year's history, should be specified and periodically re-evaluated.

A comment on diagnostic criteria in relation to the cohort patients in this thesis is that some of the patients that were classified as CIS patients at baseline using the 2010 McDonald criteria would instead have been classified as RRMS patients if the 2017 McDonald criteria had been used, due to the presence of CSF-specific oligoclonal bands.

Immunopathology in relapsing remitting multiple sclerosis

MS is an autoimmune disease where both neuroinflammatory and neurodegenerative processes result in demyelination and axonal damage in the CNS [5]. Neuroinflammation is considered to be more prominent early in the disease course and neurodegeneration is more prominent in later stages, although present early as well.

It is still unknown exactly what causes MS. Several factors are known to increase the risk of developing MS; (1) genetic factors (*e.g.* the HLA haplotype DRB1*15:01 [6, 7] and specific polymorphisms of many other genes related to immune function, as demonstrated in genome wide association studies [8-10]); (2) environmental factors like low sunlight exposure/low levels of vitamin D, smoking and Epstein-Barr virus/infectious mononucleosis are known to increase the risk of developing MS [11-13]. The classic neuropathology finding in RRMS is demyelinating lesions in the CNS, and the focal white matter lesions that they cause are the lesions being most easily demonstrated with conventional MRI. However, there is also a more generalized pathology in the CNS in RRMS, in addition to focal pathology. There is marked clinical heterogeneity; some patients recover well from their relapses, whereas others don't; some become progressive early and others don't. There is marked heterogeneity in MS lesion pathology as well, with varying types of focal lesion pathology. Some have suggested that the same type of lesions dominate within individual patients, whereas lesion pattern type vary between patients [14, 15]. Others believe that different types of lesions exist in the same individual [16]. Still others think that the immunopathological appearance of active demyelinating lesions in long standing MS is uniform, with no lesion heterogeneity between patients [17].

Most autoreactive T cells are deleted during their development in the thymus, constituting an important step in the establishment of central tolerance. However, some autoreactive T cells escape into the periphery, where peripheral tolerance mechanisms, for example regulatory T cells, often prevent the development of autoimmune diseases. If peripheral tolerance mechanisms fail, for example due to regulatory T cell dysfunction or failure of T cells or B cells to suppress, CNS-directed autoreactive T cells or B cells can be activated in the periphery. Activation may be initiated by molecular mimicry, recognition of sequestered CNS antigen released into the periphery, novel autoantigen

presentation or bystander activation, especially in genetically and environmentally predisposed individuals. It has been hypothesized that RRMS starts in the periphery, *i.e.* outside the CNS, by activation of autoreactive CD4+ T cells with specificity for CNS autoantigens, perhaps by antigen presenting cells presenting processed oligodendrocyte antigens in the deep cervical lymph nodes [18]. These autoreactive CD4+ Th1 and Th17 cells then enter the CNS in a process that involves up-regulated expression of adhesion molecules on the lymphocytes, release of chemoattractants, interactions with endothelial cells and proteolytic effects on the intercellular matrix. The autoreactive CD4+ Th1 and Th17 cells are subsequently re-activated in the CNS by local antigen presenting cells like microglia and B cells. This leads to the production of pro-inflammatory cytokines that recruit/activate CD8+ T cells, monocytes/macrophages, microglia and astrocytes. It is possible that epitope spreading and bystander activation play an important role in aggravating the autoimmune inflammation. Astrocytes are involved in leucocyte migration from blood into the CNS and have immunological functions like antigen presentation and cytokine production. CD8+ T cells secrete cytokines and cause demyelination and axonal damage by direct contact/attack. B cells contribute to MS pathophysiology by autoantibody production, by activation of T cells through antigen presentation and by cytokine production that activates other immune cells and microglia. B cells leaving the CNS can undergo affinity maturation in cervical lymph nodes and then re-enter the CNS where they, or memory B cells recruited by other pathways, have been shown to activate brain-homing autoreactive T-cells [18]. In the inflammatory milieu in the CNS in early RRMS, further recruitment and activation of both innate and adaptive immune cells occurs and with time the inflammation becomes compartmentalized in the CNS and change character. Dysfunctional downregulation of inflammation contributes to the development of chronic inflammation, again involving both innate immune cells like microglia/macrophages and regulatory NK cells as well as adaptive immune cells like regulatory T cells.

In MS, various effector mechanisms are involved in the attack on oligodendrocytes and neurons, resulting in demyelination, axonal damage, gliosis and atrophy. These effector mechanisms include damage caused by reactive oxygen and nitric oxide radicals and proteases produced by activated macrophages and microglia, NK cell and CD8+ T cell direct cytotoxicity, and phagocytosis by microglia/macrophages. Phagocytosis can be stimulated by cell to cell contact with Th1 cells, by cytokines like interferon gamma produced by Th1 cells, NK cells and CD8+ T cells and by opsonization by antibodies and complement proteins. There are also protective, inflammation down-regulating and repairing mechanisms at work in the CNS in MS and here too, heterogeneity is present at the clinical as well as the neuropathology level. The details of the inflammatory processes in MS are considered to vary between individuals as well as over time in the same individual. For reviews on MS pathogenesis, see Dendrou *et al* [5] and Massey *et al* [19]. Early and late stage immunopathology in the CNS in MS is illustrated in Figure 2 [5].

Figure 2. Schematic presentation of the immunopathology in the central nervous system in multiple sclerosis. Reprinted from “Immunopathology of multiple sclerosis”, Dendrou *et al*, Nature Reviews Immunology 2015, 15:545-558, with permission from the publisher Springer Nature.

Disease modifying drugs for relapsing remitting multiple sclerosis

There has been a treatment revolution in the MS field during the past decades, starting in 1993 when interferon beta was approved for treatment of RRMS and escalating in 2004 with the appearance of the first high efficacy drug, natalizumab. There is now an increasing number of immunomodulatory disease modifying drugs (DMD) available for patients with RRMS. These drugs have different mechanisms of action, efficacy and side effect profiles [20-22], as briefly presented in Table 2.

Natural disease course and response to treatment vary a lot in the MS population, making personalized treatment decisions challenging. Lack of good prognostic biomarkers in clinical routine also pose an obstacle to personalized treatment. It is currently debated whether escalation therapy (starting with a DMD with moderate effect and switching to a DMD with higher efficacy if signs of breakthrough disease activity are detected) or induction therapy (starting with a high efficacy DMD) is the best approach in newly diagnosed RRMS. In highly active disease, immune reconstitution therapy may be the best choice. Autologous hematopoietic stem cell transplantation (AH SCT) results in profound and long lasting immune reconstitution and is probably the most effective treatment for MS when the right patients are selected for this procedure [23]. However, the risks and patient discomfort related to AH SCT can be deterring. Some view alemtuzumab and cladribine as pulsed immune reconstitution therapies, since they have profound effects on lymphocyte subpopulations and can have longstanding effects on disease activity after the two initial years of pulsed treatment administration [24]. Anti-CD20 therapy results in B cell depletion and is highly effective in RRMS [25]. During the last years anti-CD20 therapy with rituximab has become the most widely prescribed DMD for RRMS in Sweden [26]. Several of the currently available DMDs were introduced in clinical treatment practice during or after the time period when the cohort study underlying this thesis was conducted. After the termination of the study the treatment profile of the cohort has changed dramatically. Most patients are now treated with a DMD and almost all patients that were on interferon beta have switched to oral treatments or rituximab. Had the study started today, several patients would have been started on treatment earlier and DMDs with higher efficacy than interferon beta would have been used.

When initiating, monitoring and evaluating treatment with a disease modifying drug, the patient's overall satisfaction with the current treatment, compliance, side effects, other health conditions and sometimes pregnancy related issues and economy must be considered in addition to treatment efficacy. This can sometimes be challenging in clinical practice. Considering treatment efficacy alone, it can be discussed how this should be measured in RRMS and it can also be discussed when treatment efficacy is good enough. It has been proposed that RRMS should be treated until no signs of disease activity or minimal signs of disease activity remain. However, there is no consensus regarding what constitutes signs of disease activity and how to monitor disease activity optimally, to be further discussed later.

Table 2. Overview of the current treatment options for relapsing remitting multiple sclerosis and their mechanisms of action [20, 22, 23] (not all drugs listed)

Generic name	Trade name in Sweden	Mechanism of action (MOA)
Interferon beta	Avonex, Extavia, Plegridy, Rebif	MOA not completely known. Interferons are cytokines with antiviral, antiproliferative and immunomodulatory effects. May promote Th2 deviation in the immune system and affect Treg function.
Glatiramer acetate	Copaxone	MOA not completely known. Glatiramer acetate is a mixture of four amino acids that form proteins similar to myelin basic protein. May promote Th2 deviation and affect Treg function.
Natalizumab	Tysabri	Monoclonal antibody against VLA-4 on lymphocytes. Prohibits activated lymphocytes to enter the CNS from blood.
Fingolimod	Gilenya	Sphingosine-1-receptor modulator. Prohibits activated lymphocytes from leaving secondary lymphoid organs and thereby reduces the entry of inflammatory cells into the CNS.
Dimethyl fumarate	Tecfidera	MOA not completely known. Activates transcription factor Nrf2, leading to increased expression of proteins that may protect against oxidative stress. Also reduces lymphocyte counts in blood.
Teriflunomide	Aubagio	MOA not completely known. Affects mitochondria and causes inhibition of T cell and B cell proliferation by reducing the pyrimidine synthesis in these cells.
Rituximab, Ocrelizumab	Mabthera, Ocrevus	Monoclonal antibodies against CD20. Results in B cell depletion through antibody- and complement-mediated cytotoxicity. B cell depletion, in turn, affects other immune cells like T cells.
Alemtuzumab	Lemtrada	Monoclonal antibody against CD52 on T cells and B cells. Results in profound immunosuppression and shift towards regulatory cells and memory cells.
Cladribine	Mavenclad	Deoxyadenosine analogue resistant to adenosine deaminase. Induces profound lymphopenia.
Autologous hematopoietic stem cell transplantation (AHSCT)*	-	High-dose chemotherapy with autologous stem cell support. Involves immunoablative treatment followed by reconstitution of the immune system from CD34+ autologous stem cells. Potentially eradicates the autoimmune cells and processes that cause and maintain MS.

**AHSCT is not a single pharmacological agent, it is instead a complex procedure involving stem cell harvesting, immunoablative chemotherapy and re-infusion of stem cells, as well as administration of bone marrow stimulating agents and infection prophylaxis.*

Disease activity in multiple sclerosis

A relapse is a classic sign of disease activity, but absence of relapses does not mean that there is no disease activity. New hyperintense T2 lesions, Gadolinium-enhancing lesions and/or hypointense T1 lesions in follow-up MRIs are also well-established signs of disease activity, with or without clinical symptoms. Sustained Expanded Disability Status Scale (EDSS) progression may also be a sign of disease activity. The no evidence of disease activity (NEDA) concept can be useful when discussing disease activity. NEDA-3 denotes a situation where a patient has (1) no relapses, (2) no sustained EDSS progression and (3) no MRI activity. Of note, relapse, sustained EDSS progression **or** MRI activity means that a patient does not fulfil NEDA-3. NEDA-3 has been criticized for not necessarily picking up diffuse low grade disease activity and neurodegeneration. Increased brain volume loss and abnormal levels of some body fluid biomarkers are considered by many to be signs of disease activity as well, and including such parameters in the NEDA concept would expand it to NEDA-4 or NEDA-5 and make it a more comprehensive disease activity evaluation tool (Figure 3) [27, 28]. However, methodological problems regarding brain volume loss measurements have prevented this parameter from being widely used. As for body fluid biomarkers there is an ongoing debate on which biomarkers to use. Until recently, the most promising biomarkers have been analyzed in CSF. The need for CSF is a big obstacle for widespread use of a biomarker in clinical practice. Since EDSS does not reflect cognition well at all, it has also been suggested to include a parameter reflecting cognition in the NEDA concept [29], although there is no consensus regarding what would constitute such a parameter.

Figure 3. Schematic representation of the “no evidence of disease activity” (NEDA) concept, here illustrated by a possible version of NEDA-5, where no increased brain atrophy and no elevation of NFL have been added to the original NEDA-3.

Cytokines and chemokines

Cytokines are secreted proteins with diverse functions, mediating and regulating many activities in the immune system, including proliferation, differentiation, activation and migration of innate immune cells (*e.g.* macrophages and natural killer cells) as well as adaptive immune cells (*e.g.* T cells and B cells) [30]. Cytokines are of major importance in cell signaling in the immune system. They are produced by classical immune cells as well as by *e.g.* endothelial cells and fibroblasts. In the CNS, cytokines are not only produced by immune cells, including microglia, but also by astrocytes and oligodendrocytes. After secretion, cytokines can act in an endocrine, paracrine or autocrine way. They bind to cell-surface cytokine receptors, initiate intracellular signaling and eventually alter cell functions. Cytokine redundancy refers to the fact that more than one cytokine can often mediate the same effect, *e.g.* by binding to the same receptor on a specific cell type and thereby initiating the same intracellular events. Cytokine pleiotrophism refers to the ability of a cytokine to induce different biological actions in different cells, *e.g.* by binding to different receptors or by initiating different intracellular events in different cells although binding the same cell-surface receptor.

Chemokines are small chemotactic cytokines that stimulate leucocyte movement and regulate the migration of leucocytes from the blood to tissues and within the lymphoid tissues. The nomenclature of chemokines is based on whether two conserved N-terminal cysteine residues have a non-conserved amino acid between them (CXC) or not (CC). Chemokines are attractive biomarkers because they recruit specific inflammatory cells that reflect the ongoing type of inflammation. Also, chemokines are generally present at higher concentrations than cytokines, which facilitates reliable measurements especially in CSF. In general, CXCL13 can be considered as a B cell chemoattractant, whereas other chemokines measured in this thesis were chosen because they recruit different settings of T helper cell subsets. CXCL10 can be used as a marker for inflammation mediated by a Th1 response and CCL22 for a Th2 response, while CXCL1 and CCL20 are markers for a Th17 response. CXCL8 (formerly called IL-8) is also induced in Th17 responses, and a key recruiter of neutrophils typical for Th17. However, CXCL8 can also be induced by other, general inflammatory pathways.

The complement system in multiple sclerosis

The complement system is a very old part of the innate immune system, with important functions in the defence against infections. It also has many other immunoregulatory functions and it is involved in tissue modelling and organ development, including the CNS. The complement system encompasses approximately 50 proteins that are either circulating or membrane bound. The complement system provides protection against infection by acting as opsonins (C3b, C4b) and anaphylatoxins (C3a, C5a), and by mediating cell lysis (terminal complement complex sC5b-9). In autoimmune antibody mediated diseases, complement activation plays an important role by its strong ability to enhance inflammation and cause tissue damage. The complement system can be activated through three pathways; the classical pathway is initiated by antigen-antibody complexes, the alternative pathway by foreign or damaged cell surfaces and the mannose-

binding lectin pathway by foreign carbohydrates. All three pathways converge at the cleavage of C3 to C3a and C3b. These cleavage products are highly bioactive proteins involved in phagocytosis and inflammation processes. Further downstream activation of the terminal complement pathway results in the formation of sC5b-9 complexes (sometimes called membrane attack complexes) that are potentially cytolytic. Massive and unmotivated complement activation would be very harmful to the host and complement activation is therefore tightly regulated by complement control proteins. Hepatocytes are the main producers of complement proteins in the periphery. In the CNS, complement proteins are produced by both neurons and glial cells [31]. Complement activation has been demonstrated in both acute and chronic MS lesions [14, 17, 32]. C1q staining is present in MS lesions [17], suggesting that the classical pathway is important for complement activation in MS. Antibody- and complement-mediated myelin phagocytosis as well as sC5b-9 mediated lysis are involved in demyelination processes in MS [31]. However, sublytic sC5b-9 concentrations may also protect oligodendrocytes from apoptotic cell death, indicating a dual role for complement in demyelination [31].

Body fluid biomarkers in multiple sclerosis

Given the heterogeneity of MS, with a big variation in disease activity and prognosis, and with an increasing number of drugs with different mechanisms of action, it would be a great advantage to have biomarkers that could facilitate disease activity assessments and individualized treatment.

A biomarker, or biological marker, can be 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 [33]. Biomarkers can serve to separate health and disease states, to differentiate between disease states, to predict outcome/prognosis and to evaluate treatment response. In MS, routine MRI parameters, like T2 lesions and Gadolinium enhancing lesions, and routine CSF parameters, like IgG index and oligoclonal bands, can be called biomarkers. IgG index is a measure of intrathecal immunoglobulin synthesis that takes IgG levels in blood and blood-brain-barrier integrity into account. Oligoclonal bands in the CSF, without corresponding bands in plasma, result from intrathecal immunoglobulin synthesis. Increased IgG index and presence of oligoclonal bands in CSF are found in about 95% of MS patients. These abnormalities can also be found in inflammatory and infectious conditions affecting the CNS and are hence not specific for MS. The mononuclear cell count in CSF can be normal or moderately elevated in MS. If elevated, the mononuclear and total leucocyte cell counts are usually lower than in CNS infections. Optical coherence tomography (OCT) can measure the retinal nerve fiber layer thickness, which is a biomarker for neurodegeneration in MS [34, 35].

As for body fluid biomarkers other than the abovementioned routine CSF parameters, candidates have classically been identified at protein level, but increased levels of nitric oxide metabolites have also been proposed as disease activity biomarkers [36, 37] and the lipid mediators 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadeca-

dienoic acid (13-HODE) were elevated compared with controls in the patient cohort discussed in this theses [38]. Potential biomarkers at microRNA level have attracted increasing interest in recent years [39]. MicroRNAs are small non-coding RNA fragments that exert post-transcriptional regulation of gene expression by effects on mRNA. On mRNA level (gene expression data from microarrays) and gene level (genome-wide association studies) it may often be more appropriate to talk about risk factors, risk profiles and disease susceptibility, but some may be called candidate biomarkers as well [40, 41]. In this thesis, a panel of established or potential neurodegenerative and neuroinflammatory protein biomarkers were analyzed, some only in CSF, others in both CSF and serum/plasma. Here follows a brief overview of these proteins.

Neurofilament light chain (NFL) and neurofilament heavy chain (NFH)

Neurofilament proteins are part of the neuronal cytoskeleton, *e.g.* providing structural support for axons. They are highly specific neuronal proteins that are very stable in vitro [42]. Three major neurofilament subunits exist and based on their molecular mass they are named neurofilament heavy chain (200-220 kDa), neurofilament intermediate chain (145-160 kDa) and neurofilament light chain (68-70 kDa). NFL and NFH can be measured with enzyme-linked immunosorbent assays (ELISA) in CSF. Increased levels reflect axonal damage and many studies have reported elevated NFL levels in CSF in MS, with still higher levels during relapses than in remission [40, 43]. Of note, increased levels of NFL are not specific for MS. As can be expected, considering that neurofilaments are neuronal cytoskeleton proteins, NFL can be elevated in stroke, traumatic brain injury, neurodegenerative disease and non-MS neuroinflammatory conditions like CNS infections and autoimmune encephalitis. This means that NFL cannot be used to differentiate between MS and other conditions that cause axonal damage. Instead, the value of NFL in the diagnostic phase is to aid in separating health and disease. When a diagnosis of CIS or RRMS had been determined, the value of NFL is in indicating disease activity. NFL in CSF has been shown to be associated with visual outcome and development of MS after optic neuritis [44], to be a risk factor for conversion from CIS to MS [45] and to decrease on various treatments for MS [46-48]. Until recently, measurements of NFL levels have only been performed in CSF, which has limited its clinical use. Since CSF analysis is routinely performed in Sweden in the diagnostic work-up when there is a suspicion of MS, NFL could easily be included in the analysis panel at this point in time. To exploit the full potential of NFL in CSF in the MS context, monitoring over time would be desirable. This would require repeated lumbar punctures though, which would be time consuming as well as inconvenient for patients. Recently high sensitivity detection of NFL in blood using single molecule array (Simoa) methodology has enabled measurement of NFL in serum or plasma, with these levels correlating relatively strong with CSF levels in several studies [49-52]. S-NFL has also been shown to decrease in parallel with CSF-NFL on treatment [52, 53].

Glial fibrillary acidic protein (GFAP)

GFAP is a protein that is expressed in astrocytes in the CNS. Its precise functions are unknown. Elevated levels of GFAP in CSF can be considered as a marker of glial scarring. In MS, elevated levels of GFAP in CSF has been reported to correlate with disease progression and EDSS [54, 55].

Chitinase-3-like-1 (CHI3L1)

CHI3L1, also known as YKL-40, is a protein that binds chitin but lacks chitinase activity. Its functions are unknown. Its secretion from activated astrocytes, microglia/macrophages and neutrophils is induced by proinflammatory cytokines. Increased levels have been demonstrated in neuroinflammation, including MS [56, 57]. Increased CHI3L1 is an independent risk factor for conversion from CIS to MS and is associated with shorter time to conversion [44, 58]. Also, CHI3L1 levels in CSF are higher in relapses and in patients with Gadolinium enhancing lesions [59]. Levels decrease after treatment with natalizumab and fingolimod, respectively [48, 57].

Matrix metalloproteinase-9 (MMP-9)

MMP-9 is a proteolytic enzyme involved in the breakdown of extracellular matrix in normal physiological processes (*e.g.* embryological processes and wound healing) as well as in pathological processes (*e.g.* cancer, infection and inflammation). MMP-9 levels in CSF have been reported to be increased in MS [60-64] and to decrease on natalizumab treatment [65].

Osteopontin (OPN)

OPN is an extracellular matrix protein involved in bone remodelling, wound healing, cancer biology and inflammatory diseases. It is expressed in T cells, dendritic cells, macrophages and NK cells and can act pro-inflammatory by increasing IL-12 and IFN-gamma production, while suppressing IL-10 production. OPN has been reported to be elevated in CSF from MS patients [64], to be higher during relapses and correlate with EDSS [66] and to decrease on natalizumab treatment [65]. A metaanalysis conclude that both peripheral blood and CSF levels of OPN are increased among MS patients [67].

Interleukin 6 (IL-6)

IL-6 is one of the major proinflammatory cytokines in the acute inflammatory response. IL-6 is produced by a large number of cells including monocytes, endothelial cells, T cells and other cells, in response to pathogen-associated molecular patterns, IL-1 and TNF α . IL-6 increases the synthesis of acute phase proteins in the liver, stimulates B cell differentiation towards antibody producing plasma cells and stimulates Th17 differentiation in T cells. IL-6 is also involved in chronic inflammation and autoimmunity and anti-IL-6 therapy is sometimes used in the treatment of rheumatoid arthritis. In MS, elevated IL-6 levels have been reported in CSF [68, 69].

CXCL1

CXCL1, induced by Th17 cytokines, is a major neutrophil chemoattractant expressed by *e.g.* astrocytes and microglia. CXCL1 binds to the chemokine receptor CXCR2 on neutrophils. Oligodendrocytes also express CXCR2, and in MS, both CXCL1⁺ astrocytes and CXCR2⁺ oligodendrocytes have been detected in active lesions [70], indicating that CXCL1 may help to recruit oligodendrocyte progenitor cells to sites of inflammation. Elevated CXCL1 levels in CSF from MS patients in comparison to controls have been reported [71, 72].

CXCL8

The IL-17 family cytokines (IL-17A and IL-17F) produced by Th17 cells target macrophages and epithelial cells, among others, to produce CXCL8. CXCL8 is a major neutrophil chemoattractant that also stimulates neutrophil degranulation as well as macrophage phagocytosis. It is one of the central cytokines involved in early inflammation.

Elevated CXCL8 levels in CSF from MS patients in comparison to controls [73] and decrease on treatment with rituximab [73] and natalizumab [74] have been reported.

CXCL10

CXCL10 is produced by *e.g.* monocytes, T cells, astrocytes and endothelial cells in response to INF- γ , a typical Th1 cytokine. CXCL10 is a chemoattractant for monocytes/macrophages, dendritic cells, NK cells, activated B cells and Th1 cells. CXCL10 binds to the CXCR3 receptor. In MS, CXCL10 is thought to recruit activated T cells and macrophages to the sites of inflammation in the CNS. Elevated CXCL10 levels in CSF from MS patients in comparison to controls [71-73, 75] and decrease on treatment with rituximab [73] and natalizumab [74] have been reported.

CXCL13

CXCL13 is a strong B cell chemoattractant that is important for B cell migration and also for development of B cell follicles and secondary lymphoid structures. CXCL13 levels have been shown to be associated with risk of conversion from CIS to MS [76]. In MS, CXCL13 has been reported to be elevated in CSF [75, 77, 78] and to be associated with disease exacerbations and unfavorable prognosis [76]. CXCL13 levels decrease after treatment with natalizumab and rituximab, respectively [47, 79].

CCL20

CCL20 binds to CCR6 and is a strong chemoattractant for lymphocytes, mainly Th17 cells, in MS. Elevated levels of CCL20 in peripheral blood from MS patients has been reported [80, 81].

CCL22

CCL22 is produced by dendritic cells and monocytes/macrophages. It is a Th2 attracting chemokine that is also involved in NK cell recruitment. In MS, elevated levels of CCL22 in CSF [71, 74] and decrease after treatment with natalizumab [74] has been reported.

Fatigue in multiple sclerosis

In addition to focal neurological symptoms and signs, patients with MS often experience more general symptoms like fatigue, cognitive impairment and depression. Fatigue can be described as “a subjective lack of physical and/or mental energy that is perceived by the individual or the caregiver to interfere with usual or desired activity”, although there is no universally accepted definition of the phenomenon [82]. Fatigue is reported as one of the worst symptoms by many MS patients and it can affect daily life [83] and work productivity [84] negatively. The pathophysiology of fatigue is incompletely understood [85]. MRI and EDSS assessments do not well reflect fatigue experience in MS patients [84]. Possible links between inflammation and fatigue in MS have been suggested [86] but although IL-6 [87-89] has been implicated, its association with fatigue is not firmly established and published data on NFL, CXCL1, CXCL10 and CXCL13 levels in CSF in relation to fatigue scores are lacking. Treatment of fatigue mainly involves non-pharmacological interventions, *e.g.* energy management strategies, exercise, life style changes and mental coping strategies. Symptomatic pharmacological treatment with amantadine, modafinil or methylphenidate is sometimes prescribed, although scientific evidence supporting this is largely lacking. Reduced fatigue experience in MS patients has been reported for some DMDs, *e.g.* natalizumab [90, 91].

Theoretically, primary fatigue is related to the actual pathophysiology of the MS disease, *i.e.* demyelination, axonal loss and neuroimmunological circumstances in the CNS, while secondary fatigue is related to other circumstances accompanying the MS disease, *e.g.* depression, sleep disturbance, reduced performance in daily life activities and socio-economic consequences of the MS disease [92]. Side effects from medication may also be the cause of fatigue. However, in clinical practice it is often difficult to discern what is primary fatigue and what is secondary fatigue. Regarding depression and fatigue, a bidirectional cause-effect relationship is conceivable and there are difficulties with the classification of fatigue as primary or secondary when it is associated with depression. Also, fatigue experience is not restricted to MS patients and, furthermore, some MS patients have non-MS related fatigue. The total prevalence of fatigue varies depending on how it is defined and measured, as well as depending on patient sample [85]. It has been reported to be 47% in patients with clinically isolated syndrome (CIS) [93] and 83% and 95%, respectively, in patients with MS [84, 94].

Due to the subjectivity of fatigue, interviews and self-report instruments are commonly used to assess it, *e.g.* the modified fatigue impact scale (MFIS) [95]. Fatigability, on the other hand, refers to an objective decline in performance during continuous performance of a prolonged task or comparing performance on a probe task before and immediately after prolonged performance of a separate fatigue-inducing task [96]. Motor fatigability as well as cognitive fatigability can be estimated but there is a lack of consensus regarding optimal methods for this [97]. Perceptions of fatigue and fatigability are distinct phenomena and not necessarily associated [98, 99].

AIMS

Overall aims

The overall aim of this thesis was to evaluate neuroinflammatory and neurodegenerative parameters in CSF and blood for their potential as biomarkers in patients with newly diagnosed CIS and RRMS. The hypothesis was that body fluid biomarkers could be identified for prediction of disease activity defined by clinical and imaging parameters and that they could be used for assessment of ongoing disease activity as well. The clinical significance of this would be to facilitate individualized disease modifying treatment in RRMS. We also wanted to increase our understanding of the inflammatory and degenerative nature of newly diagnosed CIS and MS, including exploration of complement activation and possible associations between CSF biomarkers and fatigue.

Specific aims

Paper I: To assess whether concentrations of a panel of neurodegenerative and neuroinflammatory markers at baseline were able to predict disease activity during two years of follow-up in patients with CIS and RRMS.

Paper II: To correlate neurofilament light chain protein in serum and cerebrospinal fluid, to evaluate if they were associated with other disease activity parameters and to identify parameters associated with number of new T2 lesions and brain volume loss during four years of follow-up in patients with CIS and RRMS.

Paper III: To assess correlations between fatigue scores and other self-assessment scores reflecting anxiety, depression and quality of life, as well as correlations between fatigue scores and neuropsychological test results, clinical data, MRI data and inflammatory and neurodegenerative parameters in CSF and serum/plasma, in a cohort of patients CIS and RRMS.

Paper IV: To assess if the complement system was activated in CSF and plasma from patients with CIS and RRMS, to assess if markers of complement activation at baseline were associated with disease activity during follow-up and to assess if complement levels were associated with biomarkers of neuroinflammation and axonal damage.

METHODS

Study subjects and study design

The basis for this thesis is a prospective longitudinal cohort study where 44 patients with CIS or newly diagnosed MS were consecutively enrolled and followed-up by the same neurologist (Irene Håkansson) at the Department of Neurology, University Hospital of Linköping, Sweden. All patients fulfilled the revised McDonald criteria from 2010 for CIS or MS. At baseline all patients were untreated. Patients then received immunomodulatory treatment according to current Swedish clinical practice. The first patient was included in September 2009 and the last four year follow-up was completed in May 2016. The study was approved by The Regional Ethics Committee in Linköping and written informed consent was obtained from all participants.

At baseline and at one year, two years and four years of follow-up, patients underwent clinical neurological examination, sampling of saliva, peripheral blood and CSF and MRI of the brain. MRI of the spinal cord was only included if clinically indicated. The MRI study protocol included quantitative MRI (qMRI) at all four occasions, while magnetic resonance spectroscopy (MRS) was performed at baseline, at one year and at four years of follow-up. A neuropsychological evaluation was performed at baseline and at one year, two years and four years of follow-up. At these occasions, patients also completed questionnaires regarding life quality, fatigue, anxiety and depression. Three patients did not participate in the neuropsychological evaluation in the study protocol, in all three cases based on requests from the patients themselves. The study design is graphically presented in Figure 4. The PPMS subgroup in the cohort study turned out to be very small, consisting of only three patients. This was considered too few to allow meaningful statistical analyses and conclusions and therefore the PPMS patients were not included in the papers.

Two patients dropped out of the study, one after one year and another one after two years of follow-up. The reason for leaving the study was the same for both patients, they moved away from Östergötland and did not want to travel to Linköping for study visits. Patient characteristics of the 41 patients with CIS or RRMS at baseline are presented in Table 3 and Table 4.

Two patients were initially accepted for inclusion in the cohort study but were excluded before completion of baseline evaluation and these two are not included in the 44 patients described above. One of these two patients was excluded due to confirmation of another diagnosis (initial MRI revealed a trigeminal neurinoma that explained her hemifacial sensory symptoms) and the other one was excluded due to early withdrawal of consent (he did not complete baseline evaluation).

For peripheral blood and CSF, 23 age- and sex-matched healthy controls (HC) were recruited from healthy blood donors. Healthy controls were free from past and current neurological and autoimmune disease and their clinical neurological examinations were normal, as were routine findings in CSF. No medication was allowed in healthy controls,

except oral contraceptives. Since the three PPMS patients were not included in the papers underlying this thesis, one of the healthy controls was excluded, in order to better maintain a 2:1 ratio of patients to controls and also to better maintain optimal age- and sex-matching. Characteristics of the 22 healthy controls involved in the papers are presented in Table 3.

Table 3. Patient and healthy control characteristics at baseline.

Clinical and laboratory data	Patients n=41	Healthy controls n=22	p-value
Women/men (% women)	32/9 (78 %)	17/5 (77 %)	0.9
Age ^a (years)	31 (24-36)	32 (26-41)	0.3
Diagnosis (CIS/RRMS)	19/22	N/A	
Relapse within last 2 months (yes/no)	23/18	N/A	
Mean disease duration ^b (months)	11.8	N/A	
Median disease duration ^b (months)	3.5	N/A	
Disease duration ^b (number of subjects)		N/A	
0-1 months	10		
1.25-3 months	10		
3.25-12 months	13		
13-36 months	5		
37-120 months	3		
Median EDSS	2.0	N/A	
EDSS (number of subjects)			
0	6	22	
1.0	12		
1.5-2.0	14		
2.5-3.0	4		
3.5-4.5	5		
CSF mononuclear cell count ^a (x10 ⁶ /L)	4.6 (1.8-9.2)	2.1 (0.9-2.7)	0.001
Albumin ratio ^a	4.8 (3.4-6.0)	4.7 (3.6-5.3)	0.5
IgG index ^a	0.7 (0.5-1.1)	0.5 (0.5-0.5)	<0.001
IgG synthesis index ^a	1.3 (1.0-2.1)	0.9 (0.9-1.0)	<0.001
Oligoclonal CSF IgG bands (pos/neg)	33/8	0/22	<0.001

P-values from Chi-square test for sex distribution and oligoclonal bands and from Mann-Whitney U-test for age and CSF data. ^a: Median and within brackets interquartile range. ^b: Disease duration refers to time from first symptom suggestive of demyelinating disease. N/A: not applicable

Table 4. Patient diagnoses, relapse status and treatment status over time

Clinical and laboratory data	Baseline	1 year	2 years	4 years
Number of subjects	41	41	40	39
Diagnosis (CIS/RRMS)	19/22	12/29	9/31	7/32
Relapse within last 2 months (yes/no)	23/18	4/37	2/38	2/37
Treatment (number of subjects)				
No DMT	41	17	18	18
Interferon- β 1b	0	18	12	5
Interferon- β 1a	0	1	1	1
Dimethylfumarate	0	0	0	2
Fingolimod	0	1	2	3
Natalizumab	0	4	7	10

DMT: disease modifying treatment

Figure 4. Study design: A prospective longitudinal cohort study of patients with clinically isolated syndrome or newly diagnosed relapsing-remitting multiple sclerosis.

Clinical evaluation

Clinical evaluation included history and physical examination. A general medical history was obtained, with special emphasis on neurology. Close care was taken to recognize relapses and disease progression. Treatment evaluation was also thorough in patients on disease modifying drugs or pharmacological treatment aiming to reduce symptoms. If not spontaneously reported, all patients were specifically asked if they had experienced pain, fatigue, cognitive dysfunction, anxiety or depression. This was systematically conducted in order to reduce the risk of not recognizing these symptoms, which can otherwise sometimes escape attention. The physical evaluation included assessment of general condition, heart and lung auscultation, blood pressure measurement and neurological examination. EDSS and timed 25-foot walk was documented at all study visits.

EDSS is a widely used disability assessment tool in MS. The EDSS version used in this cohort study is the one defined by Kurtzke in 1983 [100] and the clinical meaning of the various EDSS scores are presented in Table 5. EDSS is based on eight functional systems (pyramidal function, cerebellar function, brainstem function, sensory function, bowel and bladder function, visual function, higher cerebral function and “other”). Based on a clinical neurological examination the patient is assigned a functional system score for each functional system. The functional system scores are weighed together in a resulting EDSS score, where the scale goes from zero (no deficits) to ten (death due to MS). Low EDSS scores are more sensitive to details and changes in the neurological examination, whereas high EDSS scores depend on impairments of walking ability and activities of daily living. EDSS is an ordinal rating scale, which should be remembered in statistical analyses of EDSS scores.

CSF collection, handling of CSF and routine CSF analyses

A lumbar puncture is a procedure where a needle is inserted between two lumbar vertebrae, in this cohort study L2/L3 or L3/L4, into the subarachnoid space. In neurology, a lumbar puncture is usually performed to collect CSF for analyses. In this cohort study, almost all lumbar punctures were performed with the patient lying on her or his left side and all of them were performed between 09 and 12 a.m. The first 1-2 mL of CSF were sent for neuroborreliosis diagnostics, the second 1-2 mL were sent for routine CSF analyses and the following 12 mL were fetched by personnel from the research laboratory. These 12 mL were centrifuged (300 x g for 10 minutes) and the supernatant was aliquoted and immediately frozen and stored at -70°C. Plasma samples were collected directly after CSF collection. Routine CSF analyses were performed at the Department of Clinical Chemistry, University Hospital in Linköping, and included total leucocyte count, mononuclear cell count, erythrocyte count, albumin ratio, IgG index and determination of oligoclonal bands by isoelectric focusing of CSF and serum.

Table 5. The clinical correlates of the various EDSS scores

EDSS	Clinical meaning
0.0	Normal neurological exam
1.0	No disability, minimal signs in 1 FS
1.5	No disability, minimal signs in more than 1 FS
2.0	Minimal disability in 1 FS
2.5	Mild disability in 1 or minimal disability in 2 FS
3.0	Moderate disability in 1 FS or mild disability in 3 - 4 FS, though fully ambulatory
3.5	Fully ambulatory but with moderate disability in 1 FS and mild disability in 1 or 2 FS; or moderate disability in 2 FS; or mild disability in 5 FS
4.0	Fully ambulatory without aid, up and about 12 hours a day despite relatively severe disability, able to walk without aid 500 meters
4.5	Fully ambulatory without aid, up and about much of day, able to work a full day, may otherwise have some limitations of full activity or require minimal assistance; relatively severe disability; able to walk without aid 300 meters
5.0	Ambulatory without aid for about 200 meters; disability impairs full daily activities
5.5	Ambulatory for 100 meters, disability precludes full daily activities
6.0	Intermittent or unilateral constant assistance (cane, crutch or brace) required to walk 100 meters with or without resting
6.5	Constant bilateral support (cane, crutch or braces) required to walk 20 meters without resting
7.0	Unable to walk beyond 5 meters even with aid, essentially restricted to wheelchair, wheels self, transfers alone; active in wheelchair about 12 hours a day
7.5	Unable to take more than a few steps, restricted to wheelchair, may need aid to transfer; wheels self, but may require motorized chair for full day's activities
8.0	Essentially restricted to bed, chair, or wheelchair, but may be out of bed much of day; retains self care functions, generally effective use of arms
8.5	Essentially restricted to bed or wheelchair much of the day, some effective use of arms, retains some self care functions
9.0	Restricted to bed or wheelchair, can communicate and eat
9.5	Unable to communicate effectively or eat/swallow
10.0	Death due to MS

FS: functional system

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) is a well-established technique for detection of antigens or antibodies. The principal steps of a so-called sandwich ELISA are as follows: Wells on a plate are coated with capture antibodies specific for the antigen in question. Samples are added to the wells and if the antigen in question is present, it binds the capture antibody. Antibodies specific for another epitope of the antigen in question are added and bind the antigen. These detection antibodies can be enzyme conjugated (direct sandwich ELISA), or, if the primary detection antibody used is unlabeled, a secondary enzyme conjugated detection antibody is added (indirect sandwich ELISA). Unbound antibody-enzyme conjugates are removed by repeated washings. A

substrate is then added and converted by the enzyme to a color that can be measured to determine the presence and quantity of the antigen in question in the samples.

ELISA methodology was used to determine levels of NFL, NFH, GFAP, CHI3L1, MMP-9 and OPN in CSF from patients and controls, as detailed in paper I and II. It was also used to determine levels of C3 in CSF as well as C3a and sC5b-9 in both plasma and CSF, as detailed in paper IV.

Multiplex bead assay

With this method, multiple analytes can be simultaneously detected in one sample. Beads internally dyed with fluorescent dyes, where each type of bead is coated with antibodies specific for one of the antigens of interest (capture antibodies), are added to the samples and bind to their antigens (if present). Detection antibodies are then added and also bind their antigen, creating an “antibody-antigen sandwich”. A phycoerythrin complex is then added and bind the detection antibodies. Finally, a dual laser detection instrument based on flow cytometry principles is used. The bead complexes are scanned one at a time by one laser that determines the bead type, thereby identifying the specific analyte, and another laser that determines the magnitude of the phycoerythrin signal, thereby quantifying the amount of the specific analyte.

Multiplex bead assay methodology was used to determine levels of CXCL1, CXCL8, CXCL10, CXCL13, CCL20 and CCL22, as detailed in paper I and II. It was also used to determine IL-6 levels in CSF and plasma in paper III and to determine C1q levels in plasma in paper IV.

Single molecule array (Simoa)

This technique enables detection of very low concentrations of antigen in a sample, improving sensitivity from the pg/mL range of conventional ELISA to the fg/mL range. Capture-antibody covered magnetic beads are added to the samples and bind antigen if present. Enzyme-linked detection antibodies are then added similarly to a sandwich ELISA. In the presence of substrate, beads are then loaded into extremely small wells holding only a single bead per well. Wells containing beads that have captured the target antigen will be detected due to their fluorescence and the technology allows detection of beads capturing only a single molecule. The ratio of the wells containing beads that have captured antigen to the total number of wells containing a bead corresponds to the concentration of the analyte in the sample.

Simoa methodology was used to determine levels of NFL in serum in paper II.

Nephelometry and immunoturbidimetry

Nephelometry and immunoturbidimetry both measure the turbidity (cloudiness) of a sample to determine the level of an analyte. Antibodies to the antigen of interest (the protein that is to be measured) are added to samples. Immune complexes form and precipitate, increasing the turbidity of the sample. When light is passed through the reaction solution, some light is scattered by the sample, some light is absorbed by the sample and the rest passes through the sample. Nephelometry is based on the principle that small particles in a diluted sample suspension scatter laser light that is passed through the suspension. The amount of scattered light is measured and compared to the amount of scatter from known concentrations. The concentration of the antigen can thereby be determined from a standard curve. While nephelometry measures the amount of light scattered, immunoturbidimetry instead measures the amount of light absorbed by the sample.

In paper IV, C3 levels in plasma were determined using nephelometry methodology and high-sensitivity C-reactive protein (hsCRP) levels in plasma were determined using immunoturbidimetry.

Magnetic resonance imaging

Magnetic resonance imaging can provide excellent images of the human brain and spinal cord and is used both in the diagnostic process and in the follow-up of CIS and RRMS. As indicated in Figure 1, there is usually more MRI activity than relapse activity and evaluation of the presence or absence of new T2 lesions is of major importance in the follow-up of MS patients.

When placed in a very strong magnetic field in an MRI camera, the randomly oriented magnetic fields of hydrogen atoms (protons) in the body align their axes with the external magnetic field. Radio waves are sent into the body, tipping the hydrogen atoms a bit. When reorienting themselves to the external magnetic field again, the hydrogen atoms emit radio waves that can be measured and processed to yield interpretable images of *e.g.* the brain. Hydrogen atoms have different relaxation properties depending on what tissue they are located in. By measuring *e.g.* relaxation times and applying advanced mathematics on the data, contrasts between tissues can be achieved and presented as images. T2 hyperintense lesions, or T2 lesions, are bright areas seen in so called T2 weighted MRI images. They represent the total number of focal MS lesions. T1 hypointense lesions appear dark in so called T1 weighted MRI images. If very dark, hypointense lesions are called “black holes” and represent permanent tissue loss. If not very dark, they may represent edema in an area with active inflammation. Gadolinium enhancing lesions, or Gd+ lesions, enhance Gadolinium contrast that has been injected intravenously. Gadolinium normally can't pass from the blood to the CNS. However, when the blood-brain barrier is disrupted during either a clinical relapse or clinically silent inflammation, Gadolinium can enter the areas of active inflammation and these areas are thereby highlighted in MRI images. MS lesions that don't enhance Gadolinium

are considered to be older than two or three months. Focal MS lesions affect gray matter as well as white matter in the CNS. White matter lesions are much easier to detect in standard MRI and for that reason a lot of focus has been on white matter lesions. The T2 lesion count in standard MRI represents the number of white matter lesions in MS and T2 lesions are routinely monitored in MS patients. Selected magnetic resonance images from one of the cohort patients are shown in Figure 5.

All MRI examinations of cohort patients were performed on a 1.5 T Achieva MRI system (Philips Healthcare, Best, The Netherlands), as detailed in Supplementary material in the papers. All MRI examinations were reviewed by the same neuroradiologist and the main parameters evaluated in the papers were the total number of T2 lesions and the number of new T2 lesions during follow-up.

Figure 5. Magnetic resonance images of the brain from one of the cohort patients. This patient received natalizumab early, due to highly active disease. Lesions in the corpus callosum are present in the sagittal T2 FLAIR image to the left, periventricular white matter lesions are present in the axial T2 FLAIR image in the middle and a Gadolinium enhancing lesion is present in the axial T1 image, acquired after intravenous Gadolinium injection, to the right.

Brain volume measurements

Brain volume and brain volume loss are clinically interesting parameters in MS as well as in some other neurological diseases. Abnormally low regional or whole brain volume and abnormally high brain volume loss over time signals brain atrophy. Assessing brain volume is more complicated than detecting and counting T2 lesions. Developing methods for brain volume measurements is an ongoing process and there is no golden standard among existing methods. Some of the existing methods are labor intense and time consuming. Also, conventional magnetic resonance imaging lacks an absolute scaling. Quantitative MRI is instead based on the quantification of the longitudinal R1 relaxation rate ($1/T_1$ relaxation time), the transverse R2 relaxation rate ($1/T_2$ relaxation time) and the proton density. These parameters can be used to create synthetic MR images. They can also be used to calculate volumes of gray matter, white matter and CSF, respectively (Figure 6), as well as the volume not classified as gray matter, white matter or CSF (a volume including *e.g.* blood vessels), based on physical properties rather than relative image characteristics. In this cohort study we used a fast and highly automated method for whole brain volume measurements. Based on quantitative MRI data, SyMRI® version 8.0 (SyntheticMR, Linköping, Sweden) was used to calculate total brain volume expressed as brain parenchymal fraction (BPF), the ratio of total brain parenchyma volume to total intracranial volume.

Figure 6. Gray matter (green in left image), white matter (pink in middle image) and CSF (blue in right image) segmentation in synthetic magnetic resonance images from one of the cohort patients, based on quantitative MRI data.

Questionnaires

Several questionnaires were included in the study. Fatigue was assessed with the Modified Fatigue Impact Scale (MFIS) [95]. Anxiety and depression was assessed with the Hospital Anxiety and Depression scale (HAD) [101]. Disease impact on daily life and life quality was assessed with the Multiple Sclerosis Impact Scale (MSIS-29) [102] and the Short Form 36 (SF-36) [103].

Neuropsychological tests

Several neuropsychological tests [104] were included in this cohort study and they were administered and interpreted by neuropsychologists. Data from four of these tests were included in paper III: (1) The Delis–Kaplan Executive Function System (D-KEFS) initial letter Verbal Fluency Test (VFT), where the total score from three trials with different letters was recorded, (2) the D-KEFS Trail Making Test (TMT) with number-letter switching, (3) the Paced Auditory Serial Addition Test (PASAT) with numbers presented every 2.4 seconds and (4) the Auditory Consonant Trigram Test (ACTT), where the total number of correct letters was recorded.

Verbal Fluency Test

In the VFT, study subjects were instructed to verbally produce as many words as possible starting with the letter F, A and S, respectively, for each letter during 60 seconds. Total number of words produced were recorded. The VFT reflect mainly executive function and requires language function and ability to speak.

Trail Making Test

In the TMT with number-letter switching, study subjects were instructed to connect dots on the test form in sequential order, alternating between numbers and letters, as fast as possible. The correct sequence starts with 1-A-2-B-3-C and ends at 16-P. The time needed to complete this task was recorded. The TMT reflect mainly executive function and cognitive processing speed. The test requires vision and upper extremity motor function that allow the patient to see the numbers and letters and to efficiently draw lines between them.

Paced Auditory Serial Addition Test

In the PASAT version used in this cohort study, single digits were presented auditory to study subjects every 2.4 seconds and study subjects were instructed to add each new digit to the one immediately prior to it and give the sum as an answer. The PASAT score is the total number of correct answers (maximum 60). The PASAT is a challenging test that assesses auditory information processing speed and flexibility, working memory, attention and arithmetic capability. Basic requirements are ability to hear and ability to speak.

Auditory Consonant Trigram Test

In ACTT, an auditory trigram (three random letters, *e.g.* FTB) was presented verbally to the study subject, who was asked to recall the trigram after a time delay during which a distracting task (counting backwards) was performed. 15 trigrams were presented to study subjects and the ACTT score was the total number of correct letters. The ACTT assesses working memory, cognitive processing speed and divided attention. Like for the PASAT, basic requirements for ACTT are ability to hear and ability to speak.

Statistical methods

Statistical analyses were performed using SPSS for Windows, version 23, and GraphPad Prism 6. Many data sets showed a non-parametric distribution and therefore, in all four papers, the Mann-Whitney U-test was usually used to compare two independent groups and non-parametric bivariate correlation analysis (Spearman) was usually used for correlation analyses. However, in paper II, the relationship between NFL in CSF and serum was examined using both Spearman correlation analysis and Pearson correlation analysis when sample size was >50, and repeated measures analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons was used to compare repeated BPF measurements in patients over time. Analysis of covariance (ANCOVA) was used to compare complement levels in patients and controls, controlling for high-sensitivity C-reactive protein. Wilcoxon matched-pairs signed rank test was used to compare levels of immunological markers before and after treatment and to compare baseline and follow-up levels in patients. Bivariate linear regression and multiple regression were used to evaluate how well certain continuous predictor variables, *e.g.* NFL, explained certain continuous outcome variables, *e.g.* brain volume loss over time and number of new T2 lesions over time. In paper I and II, logistic regression was used when investigating if continuous variables, *e.g.* NFL, could predict dichotomous outcome variables, *e.g.* NEDA-3. Receiver operating characteristic (ROC) curves were derived from logistic regression to investigate the discriminatory power of *e.g.* NFL for separation of patients and controls as well as separation of patients with and without disease activity during follow-up.

Ethics statement and patient consent

All studies included in this thesis were approved by The Regional Ethics Committee in Linköping. Verbal and written informed consent was obtained from all participants.

RESULTS AND DISCUSSION

Neurodegenerative and neuroinflammatory markers in patients and in healthy controls (paper I, II)

At baseline, when no patient had started DMD treatment, CSF levels of NFL, NFH, CHI3L1, MMP-9, CXCL1, CXCL8, CXCL10, CXCL13 and CCL22 were significantly higher in patients than in controls, whereas GFAP and OPN levels did not differ (Table 6). Patients and controls had similar levels of chemokines in plasma. Patients also had significantly higher levels of NFL in serum than controls at baseline (Table 7). NFL levels decreased in the patient group during follow-up (Table 7), probably related to treatment effect and lower proportions of patients in relapse at follow-ups.

Table 6. Cerebrospinal fluid levels of neurodegenerative and neuroinflammatory markers in patients at baseline and in healthy controls

	Patients n=41	Healthy controls n=22	p
NFL	895 (300-2060)	212 (151-289)	<0.001
NFH	35 (16-67)	16 (16-16)	≤0.01
GFAP	464 (348-636)	438 (342-474)	0.26
CHI3L1	96362 (75542-177092)	71080 (60078-98891)	≤0.01
MMP-9	221 (61-624)	61 (61-61)	<0.001
OPN	83 (52-125)	65 (43-109)	0.27
CXCL1	17 (8-23)	8 (8-8)	≤0.01
CXCL8	25 (22-30)	17 (14-22)	<0.001
CXCL10	1091 (814-2093)	539 (440-816)	<0.001
CXCL13	4 (2-17)	UD	<0.001
CCL22	21 (8-57)	UD	<0.001

Concentrations are given in pg/ml and presented as median and within brackets inter-quartile range. UD = Under detection limit. P-values from Mann-Whitney U-tests, patients compared to healthy controls, significant differences in bold.

Table 7. Neurofilament levels in patients over time and in healthy controls

	Baseline n=41	One year n=41	Two years n=40	Four years n=39	Healthy controls n=22
S-NFL	17 (12-22)	14 (8-20)†	13 (9-16)†	11 (8-16)†	11 (8-14)*
CSF-NFL	895 (300-2061)	381 (248-682)†	327 (202-513)†	374 (242-515)†	212 (151-289)**

Concentrations are given in pg/mL and presented as median and within brackets inter-quartile range. NFL was measurable in all subjects. †: Significantly lower level at follow-up than at baseline, p -values from Friedman test followed by Dunn's test to correct for multiple comparisons ≤ 0.01 . *: Significantly higher levels in patients at baseline than in healthy controls, $p=0.002$ in Mann-Whitney U-test. **: Significantly higher levels in patients at baseline than in healthy controls, $p\leq 0.001$ in Mann-Whitney U-test.

Correlation between NFL in CSF and serum (paper II)

The correlation between S-NFL and CSF-NFL in samples from controls and from patients at baseline is presented in Figure 7. Spearman's rho was 0.65 ($p<0.001$) and the correlation coefficients were similar when the control group and the patient group were analyzed separately (Spearman's rho 0.59 and 0.63, respectively).

Figure 7. Correlation between S-NFL and CSF-NFL, all samples from patients at baseline and healthy controls ($n=63$). Logarithmic scales are used on graph axes for improved visibility (increased separation of data points), whereas statistical analyses were performed on non-transformed linear data. Spearman's rho 0.65 ($p<0.001$) and Pearson's r 0.74 ($p<0.001$).

Correlations between biomarkers in CSF (paper I)

As can be seen in Table 8, there were many positive correlations among the markers in our panel. Correlations between NFL and several of the chemokines as well as with CHI3L1 and MMP-9 were at or above Spearman's rho 0.50. In a wider perspective, the results in the table below are in line with MS pathophysiology involving changes in many immunological parameters. The coexisting increase and correlations with neurodegenerative markers indicate that inflammation and degeneration are parallel processes, although further conclusions can not be drawn from this study. Many inflammation related parameters increase in active MS, and decrease on effective treatment. From a clinical perspective, all parameters that can be measured are not necessarily useful. It is important to try to identify the best parameter or combination of parameters to use in various situations in clinical practise.

Table 8. Map of correlation analyses between neurodegenerative and neuroinflammatory markers in CSF at baseline

	NFH	GFAP	CHI3L1	MMP-9	OPN	CXCL1	CXCL8	CXCL10	CXCL13	CCL22
NFL	0.64*	0.47*	0.60*	0.50*	0.30*	0.35*	0.41*	0.54*	0.54*	0.50*
NFH	1.00	0.45*	0.33*	0.23	0.28*	0.12	0.24	0.31*	0.30*	0.20
GFAP		1.00	0.51*	0.02	0.38*	0.10	0.18	0.18	0.17	0.02
CHI3L1			1.00	0.38*	0.45*	0.36*	0.35*	0.50*	0.40*	0.35*
MMP-9				1.00	0.11	0.38*	0.41*	0.63*	0.80*	0.80*
OPN					1.00	0.22	0.15	0.18	0.21	0.11
CXCL1						1.00	0.69*	0.61*	0.35*	0.42*
CXCL8							1.00	0.68*	0.34*	0.43*
CXCL10								1.00	0.61*	0.61*
CXCL13									1.00	0.75*
CCL22										1.00

Magnitude of correlation coefficients (Spearman's rho) are shown, p-values according to bivariate non-parametric correlation analyses (Spearman). Correlation coefficients ≥ 0.50 in bold. * $p \leq 0.001$

Neurofilament levels for separation of patients and healthy controls (paper I)

The ability of CSF-NFL alone to discriminate between patients and controls was assessed. With logistic regression analysis based on NFL, 71% of patients (29 out of 41) and 82% of HC (18 out of 22) could be correctly classified, with an overall correctly classified proportion of 75% (47 out of 63 individuals). Sensitivity and specificity for different NFL values were determined using ROC curve analysis (Figure 8A). An NFL cut-off at 200 ng/L captured 85% of patients but had a specificity of only 50%. An NFL cut off at 450 ng/L captured 71% of patients and had a specificity of 86%. An NFL cut off at 850 ng/L captured 49% of patients and had a specificity of 100% in our cohort.

Neurodegenerative and neuroinflammatory markers at baseline in relation to conversion from CIS to RRMS during follow-up (paper I, II)

Paper I: Within the CIS group, NFL levels in CSF at baseline were significantly higher in patients converting to RRMS during two years of follow-up than in non-converters, and in logistic regression analysis NFL correctly classified 78% of converters (7 out of 9) and 90% of non-converters (9 out of 10). The overall correctly classified proportion of patients was 84% (16 out of 19). An ROC curve for NFL at baseline in relation to conversion from CIS to RRMS during two years of follow-up is presented in Figure 8C. With a cut off value of ≤ 500 ng/L for NFL, 89% of CIS patients not converting to RRMS were identified, and the specificity was 70%.

Paper II: Within the CIS group at baseline ($n = 19$), CXCL10, NFL, and MMP-9 in CSF were significantly higher ($p \leq 0.05$ in Mann-Whitney U tests) in patients that converted to RRMS during 4 years of follow-up ($n = 12$) than in non-converters ($n = 7$). For the 12 CIS patients that converted to RRMS during the study, multiple regression modelling of time to conversion resulted in a highly significant model (adjusted R^2 0.81, $p=0.006$) when including number of T2 lesions at baseline and baseline CSF levels of CXCL1, CXCL10, CXCL13 and NFL.

Neurodegenerative and neuroinflammatory markers at baseline in relation to disease activity during follow-up (paper I, II)

An ROC curve for CSF-NFL at baseline in relation to disease activity (NEDA-3) during two years of follow-up is presented in Figure 1 B. With a cut off value of ≥ 450 ng/L for NFL, 93% of patients showing disease activity were identified, and the specificity was 67%. For comparison, a cutoff value of ≥ 770 ng/L had a sensitivity of 74% and a specificity of 83% and a cut off value of ≥ 200 ng/L had a sensitivity of 96% and a specificity of 33%.

Several neurodegenerative and neuroinflammatory markers in CSF at baseline were significantly higher in patients that showed signs of disease activity during two years of follow-up than in patients that did not, see Figure 9.

Figure 8. Receiver–operating characteristics curves for neurofilament light chain (NFL). (A) Presence of disease. NFL in cerebrospinal fluid (CSF) at baseline and separation of patients ($n=41$) and healthy controls ($n=22$). Area under curve (AUC) is 85%. (B) Prediction of disease activity. NFL in CSF at baseline and separation of patients with ($n=27$) and without ($n=12$) evidence of disease activity during 2 years of follow-up. AUC is 85%. (C) Prediction of conversion from clinically isolated syndrome (CIS) to relapsing remitting multiple sclerosis (RRMS). NFL in CSF at baseline in patients with CIS ($n=19$) and separation of patients not converting to RRMS during 2 years of follow-up ($n=10$) and patients converting to RRMS during 2 years of follow-up ($n=9$). AUC is 92%.

Legend to Figure 9 (figure on next page). Neurodegenerative and neuroinflammatory markers at baseline in healthy controls and in patients subgrouped by disease activity during 2 years of follow-up. Concentrations are given in pg/mL. Lines show median and interquartile range. ** $P \leq 0.01$ (Mann–Whitney U-test), *** $P < 0.001$ (Mann–Whitney U-test), ns $P > 0.01$ (Mann–Whitney U-test). Healthy controls (HCs) ($n=22$). No evidence of disease activity (NEDA) ($n=12$): patients with NEDA during 2 years of follow-up (no relapses, no brain magnetic resonance imaging (MRI) activity (new or enlarging T2 lesions or gadolinium (Gd)-enhancing lesions) and no disability worsening). Evidence of disease activity (EDA) ($n=27$): patients with evidence of disease activity during 2 years of follow-up (relapses, brain MRI activity (new or enlarging T2 lesions or Gd-enhancing lesions) or disability worsening). CHI3L1, chitinase-3-like-1 protein; GFAP, glial fibrillary acidic protein; MMP-9, matrix metalloproteinase-9; NFH, neurofilament heavy chain; NFL, neurofilament light chain; OPN, osteopontin

Figure 9. Neurodegenerative and neuroinflammatory markers at baseline in healthy controls and in patients subgrouped by evidence of disease activity during 2 years of follow-up. Detailed legend on previous page.

Neurodegenerative and neuroinflammatory markers in relation to brain volume loss and T2 lesions during follow-up (paper II)

Mean BPF decrease from baseline to four year follow-up was significantly higher in patients with EDA-3 than in patients with NEDA-3 during one year (-1.6 vs -0.7, $p=0.001$), but not two (-1.4 vs -0.7, $p=0.02$) and four (-1.3 vs -0.6, $p=0.06$) years of follow-up, if applying a significance level of 0.01. BPF decrease during four years of follow-up correlated significantly with the number of T2 lesions in baseline brain MRI but not with the number of new T2 lesions during one, two and four years of follow-up. It also correlated significantly with S-NFL and CSF-NFL at one year follow-up and with baseline CHI3L1 as well as CHI3L1 at follow-ups. BPF over time is illustrated in Figure 10.

Interestingly, CSF-NFL correlated significantly with both number of new T2 lesions and BPF decrease during follow-up, whereas MMP-9 and inflammatory chemokines correlated mainly with new T2 lesions and CHI3L1 levels correlated mainly with BPF decrease (Table 9).

Table 9. Selected correlations between neuroinflammatory and neurodegenerative markers, T2 lesions and BPF change during follow-up

	T2, BL	New T2, 1 y	New T2, 2 y	New T2, 4 y	BPF change, 4y
S-NFL, BL	0.33	0.55*	0.43	0.44	-0.24
S-NFL, 1y	0.45	0.43	0.33	0.34	-0.56*
S-NFL, 4y mean	0.41	0.43	0.32	0.35	-0.42
CSF-NFL, BL	0.29	0.60*	0.64*	0.51*	-0.20
CSF-NFL, 1y	0.59*	0.59*	0.58*	0.50*	-0.54*
CSF-NFL, 4y mean	0.41	0.55*	0.60*	0.53*	-0.35
CSF-MMP-9, BL	0.46	0.53*	0.47	0.42	-0.16
CSF-MMP-9, 2y mean	0.38	0.51*	0.51*	0.46	-0.26
CSF-CHI3L1, BL	0.49	0.42	0.36	0.26	-0.55*
CSF-CHI3L1, 2y mean	0.42	0.30	0.24	0.20	-0.54*
CSF-CXCL1, BL	0.51*	0.56*	0.42	0.23	-0.34
CSF-CXCL10, BL	0.50*	0.58*	0.48	0.41	-0.26
CSF-CXCL10, 2y mean	0.47	0.51*	0.42	0.34	-0.37
CSF-CXCL13, BL	0.37	0.51*	0.52*	0.46	-0.26
CSF-CXCL13, 2y mean	0.40	0.49	0.52*	0.45	-0.33

Magnitude of correlation coefficients (Spearman's rho) from bivariate non-parametric correlation analyses (Spearman) are shown. *: Spearman's rho ≥ 0.50 and $p \leq 0.001$

S: serum, CSF: cerebrospinal fluid, BL: baseline, y: year

T2, BL: Number of T2 lesions in baseline brain MRI.

New T2, 1y, 2y and 4y: Number of new T2 lesions in brain MRI during one, two and four years of follow-up, respectively, compared to baseline MRI.

BPF change, 4y: Brain parenchymal fraction decrease during four years of follow-up.

2y mean: The mean of levels at baseline, one year and two year follow-up.

4y mean: The mean of levels at baseline, one year, two year and four year follow-up.

Figure 10. Brain parenchymal fraction (BPF) in patients at baseline and during follow-up. BPF at baseline (BL) and at 1 year (1y), 2 years (2y), and 4 years (4y) of follow-up, mean with standard deviation. ** $p \leq 0.01$ in repeated measures ANOVA with Bonferroni correction for multiple comparisons. ns: not significant

Clinical relevance of paper I and paper II

Body fluid biomarkers

In general, biomarkers can serve to separate health and disease states, to differentiate between disease states, to quantify disease activity, to predict outcome/prognosis and to evaluate and predict treatment response. The first two papers in this thesis focus a lot on NFL, a neuron specific biomarker of axonal damage. NFL in CSF was considered a new and promising biomarker in MS when the planning of this cohort study took place in 2009, to a large extent based on publications from Swedish researchers [43, 55, 105]. Patients in our cohort were included from 2009 to 2012 and followed up for four years, ending in 2016. During those years published data supporting NFL as a biomarker in MS accumulated.

In paper I, we show that NFL in CSF performs well for separation of patients and healthy controls and also as a prognostic biomarker of disease activity during follow-up in CIS and RRMS. The use of NFL in clinical practice can aid in differentiating between early neuroinflammatory/neurodegenerative disease and “health” as in *e.g.* mild and transient unspecific sensory symptoms. NFL is not specific for MS though, and therefore this biomarker can not be used to differentiate between MS and other diseases that cause axonal damage. NFL levels may also help us individualize treatment in CIS/RRMS. Considering the heterogeneity in MS, body fluid biomarkers like NFL can aid in prediction of disease activity and in evaluation of ongoing disease activity/treatment response, thereby impacting decisions on treatment strategy.

An important treatment goal in modern MS treatment regimens is to suppress disease activity as much as possible, sometimes expressed as “treating to NEDA”. Defining and evaluating disease activity is of major importance for decisions on therapy initiation, therapy continuation and therapy change. In clinical practice, the initial NEDA-3 concept (no relapses, no sustained EDSS progression and no focal MRI activity) is still dominating. In agreement with many other researchers in the MS field, I believe that an expanded NEDA concept would better reflect disease activity in MS. Incorporating parameters reflecting accelerated brain volume loss and axonal damage could allow detection of aspects of disease activity that are not picked up with the NEDA-3 concept. Brain volume loss and NFL could perhaps better reflect diffuse and ongoing pathological processes in normal appearing gray and white matter (tissue that looks normal in conventional MRI sequences), changes that we know are present from histopathology studies [106, 107] as well as from neuroimaging studies [108, 109]. Our initial findings strengthened our opinion that NFL in CSF is a clinically useful biomarker in CIS and RRMS and that NFL could be included in the expanding NEDA concept. To monitor NFL levels in CSF would however require repeated lumbar punctures for CSF sampling. This would cause patients more discomfort and be more time consuming for neurologists than using a biomarker that could be monitored by repeated blood sampling. An MS disease activity biomarker in blood is therefore desired. The first biomarker in serum/plasma to show consistent promise in MS is NFL. Unlike cytokines and chemokines and some other protein biomarkers, the only source of NFL is neurons. Whereas NFL in serum/plasma is by no means specific to axonal damage caused by MS, it is at least specific for axonal damage, as opposed to *e.g.* inflammatory cytokines or chemokines that can be elevated due to chronic inflammatory conditions outside the CNS or due to temporary inflammation during infections.

In paper II analysis of NFL in serum was included. Major aims of that study were to evaluate the correlation between NFL in CSF and serum and to compare them as biomarkers. A strong correlation would support that NFL in serum is a reliable biomarker, although sampled in a body fluid traditionally considered to be “further away” from the CNS than CSF. In paper II, we report a correlation between NFL in serum and CSF that was in the same range as reported by others [50, 52]. Evidence supporting that S-NFL reflect disease activity in MS is accumulating [50, 51, 110, 111] and we agree with that. However, we note that CSF-NFL correlated stronger with NEDA-3, new T2 lesions and brain volume loss during follow-up than S-NFL did. In paper II we also found that baseline NFL was associated with new T2 lesions as well as brain volume loss during follow-up, whereas CHI3L1 in CSF was mainly associated with brain volume loss and CXCL1, CXCL10, CXCL13, CCL22 and MMP-9 in CSF were instead mainly associated with new T2 lesions. This implies that NFL may reflect focal as well as diffuse CNS-pathology in MS, whereas the chemokines mainly reflect focal inflammatory lesions. In conclusion, we argue that NFL could be included in the NEDA concept, although cut-off levels need to be settled. We believe that CSF-NFL is a better biomarker than S-NFL but we recognize the obvious practical inconveniences of repeated lumbar punctures. We suggest that both CSF-NFL and S-NFL can be included in the diagnostic work-up of suspected CIS/RRMS. In follow-up it may in most cases be more suitable to monitor S-NFL, a notion supported by the fairly strong correlation between CSF and serum levels in our study and by other larger studies. In one recent study, involving 259 patients, high S-NFL was associated not only

with contrast enhancing lesions and new T2 lesions but also with future EDSS worsening as well as brain and cervical spinal cord volume loss [111]. In another recent study [53], S-NFL in 589 patients with RRMS and 35 controls were analyzed. Mean S-NFL level was approximately 30 pg/mL in the patient group and 17 pg/mL in the control group (for comparison, medians were 17 pg/mL in our patient cohort and 11 pg/mL in our controls). S-NFL at baseline correlated with T2 lesion load and Gadolinium enhancing lesions at baseline [53]. Prognostically, S-NFL was associated with new T2 lesions, relapses, brain volume loss and EDSS progression [53]. Also, S-NFL was increased in patients without Gadolinium enhancing lesions compared with controls [53]. This supports that S-NFL reflects ongoing neuronal damage that is not detectable with conventional MRI, caused by diffuse neuroinflammation/neurodegeneration in normal appearing white and gray matter. Conventional MRI without Gadolinium enhancing lesions can be viewed as showing some of the things that have happened in the brain in the past, while NFL can be said to measure, in real time, the ongoing axonal damage status. This has inspired a simile of peripheral blood NFL levels being “the neurologist’s CRP” [112]. NFL in serum/plasma may become the first body fluid biomarker to be used clinically to monitor disease activity/treatment response in MS and as such it could conveniently be evaluated even several times per year, with one treatment goal being to normalize elevated NFL levels. In a subgroup of patients from a DMT trial [113], where S-NFL was analyzed in samples obtained every twelfth week during 96 weeks, NEDA-3 patients had consistently low and stable S-NFL levels, whereas EDA patients had significantly higher and more variable S-NFL levels (data presented by Peter Calabresi at theECTRIMS congress 2017). NFL in serum/plasma can be informative in clinical trials on disease modifying drugs, on group level, but before introducing this biomarker on a broad front in clinical practice, at individual level, some issues need to be resolved. These include standardization of measurements, establishing accepted and age adjusted cut off levels and establishing reference ranges for normal values based on data from large numbers of healthy controls. How to handle the impact of comorbidities possibly affecting NFL levels also has to be considered.

In our cohort, CXCL1, CXCL10, CXCL13 and CCL22 levels in CSF were associated with new T2 lesions during follow-up. The chemokines CXCL10 and CXCL13 have been reported to be elevated and to correlate with disease activity when measured in the CSF, but not in plasma, by others as well [75, 79, 114]. Elevated levels of CCL22 in CSF [71, 74] and decrease after treatment with natalizumab [74] has been reported by others too, as has elevated CXCL1 levels in CSF from MS patients in comparison to controls [71]. These chemokines could perhaps be of some value in the diagnostic process, as well as give some prognostic information, in addition to NFL. However, the need for analysis in CSF limit their use during follow-up. It remains an open question if these or other chemokines add enough extra information to motivate their use in clinical practise.

We found higher CSF levels of CHI3L1 and MMP-9 in patients than controls at baseline, with no significant differences between relapse and remission. Increased levels of CHI3L1 and MMP-9 have been demonstrated by others as well [57, 60]. Our data indicate that MMP-9 levels at baseline predict new T2 lesions during follow-up, whereas this was less clear for CHI3L1. Results from other studies on MRI activity in relation to MMP-9 and CHI3L1 in CSF are diverging and difficult to compare due to different study designs

and parameters evaluated [58, 78, 115], whereas data indicating that DMDs decrease MMP-9 and CHI3L1 levels are more convincing [48, 57, 116]. Elevated CHI3L1 levels have been linked to a higher risk of conversion from CIS to MS [44, 58]. In our material, MMP-9 at baseline was higher in converters than non-converters, whereas CHI3L1 was not, and multiple regression analysis of time to conversion suggested that NFL in combination with some of the chemokines may be better biomarkers in this area. In conclusion, CHI3L1 and MMP-9 could be of value as biomarkers in MS, but data from our studies as well as from others are not as uniform and convincing as data on NFL.

Brain volume

Focal MS lesions affect gray matter as well as white matter in the CNS, but white matter lesions are more easily visualized with conventional MRI. White matter lesions are central both in the diagnostic phase and during follow-up. The T2 lesion count in standard MRI represent the number of white matter lesions. However, it is well known from pathological studies that cortical and deep gray matter lesions also exist in MS [117-119] and both demyelination and neuronal damage, including synapse loss, has been demonstrated in gray matter lesions as well as in so called normal appearing gray matter [120]. With the deployment of new MRI protocols gray matter lesions can be visualized [121]. The number of T2 lesions and T2 lesion volume in conventional MRI correlate poorly to clinical disease manifestations and disease progression in MS [122], although lesion volume has been reported to predict long term disability [123]. Increased whole brain volume loss is present already at the CIS stage [124, 125] and is thought to represent total tissue loss in CIS and MS, *i.e.* the sum of demyelination, neuroaxonal loss and glia cell loss. Increased whole brain volume loss has been reported to be associated with increased cognitive dysfunction [126, 127] and predict EDSS progression [128]. A meta-analysis of MRI data from over 10 000 patients that had participated in various clinical trials concluded that the combination of brain volume loss and new T2 lesions predicted disability progression in RRMS better than either of these two parameters on their own [129]. It has been suggested that brain volume loss could be included in the expanding NEDA concept.

Measuring brain volume as BPF, like we did in the patient cohort, is non-invasive and logistically easy, only marginally extending the time in the MRI-scanner. However, the use of BPF measurements, as well as other brain volume quantification methods, are hampered by technical imperfections in the acquisition and processing of MRI data, variability of estimates using different imaging methods, physiological variation in brain volume and difficulties in identifying optimal cut off levels for annual brain volume loss [27, 130, 131]. Brain volume can be affected by alcohol, hydration status, time of the day, medications (the so called pseudoatrophy that can occur during the first year after starting an effective DMD for MS as well as effects of other drugs like *e.g.* lamotrigine and diuretics) and concomitant pathophysiological conditions (*e.g.* diabetes) [130]. A cut off for normal versus increased brain volume loss set at an annual brain volume loss of -0.4% has been suggested by some researchers [132, 133], a cut off level that had a sensitivity of 65% and a specificity of 80% for separation of MS patients and healthy controls

in one of these studies [133]. This suggested cut off level has been questioned or disputed by others [134, 135]. Based on the various problems mentioned above, it has been argued that although suitable for scientific studies, BPF measurements are not suitable for assessing changes in individual patients over months or a few years [130]. Consensus guidelines from the magnetic resonance imaging in multiple sclerosis (MAGNIMS) group, a European collaborative research network, state that the use of longitudinal brain volume assessment as a marker of disease progression in individual patients cannot be considered to be reliable at present [136].

Mean BPF decrease in our cohort was 0.49/year from baseline to one year, 0.50/year from baseline to two years and 0.36/year from baseline to four years. This is in line with some other reports but rather low compared to yet other studies [137-139]. Normal brain volume loss in adults is considered to be 0.1-0.3% per year [133], with higher total brain volume loss than 0.4% per year reported for older individuals [140]. We unfortunately had no data on brain volume from our healthy controls. To assess the test-retest reliability of our BPF measurement method, one patient with RRMS was scanned six times in repetition with only ten minutes between start of each scan. Between each scan the patient exited the scanner. Mean BPF was 92.2, the range was 91.7-93.1 and the coefficient of variation (the standard deviation divided by the mean value expressed as per cent) was 0.56%. Although observed in only one individual, this amount of variation in measurements on one single individual in one single day is very problematic, since it is so big in relation to expected annual brain volume loss. It implies that although brain volume loss can be demonstrated on group level with the method we used, very long follow-up or a better way of determining brain volume would be needed for clinically useful brain volume data on individual level. Including brain volume loss in the expanding NEDA concept, for use in the evaluation of treatment efficacy in individual patients on an annual basis, is probably unwarranted at present, regardless of methodology used. On the other hand, assessments of brain volume loss on a group level can be valuable as an outcome parameter in clinical trials. Technical development, standardization of measurements and more frequent brain volume measurements over time could increase the clinical usefulness brain volume measurements on individual level in the future. Also, importantly, focal or partial volumes may be better parameters to monitor than total brain volume. Thalamic volume has been reported to be associated with cognitive dysfunction [141, 142] and, in addition, deep gray matter volume has been linked to EDSS progression as well [143]. Cortical lesions and cortical volume have been shown to correlate with cognitive performance, disability progression and conversion to progressive MS [144]. Corpus callosum measurements have been shown to correlate with both physical and cognitive dysfunction [145, 146]. Upper cervical cord area has been linked to EDSS already at early stages of MS [147].

To conclude on brain volume measurements in our cohort, we do not think that brain volume measurements based on the BPF methodology that we used would be advisable to include in an expanding NEDA concept for routine clinical follow-up of MS patients.

Fatigue scores in relation to other parameters (paper III)

MFIS scores were significantly higher in patients who either spontaneously reported tiredness as a symptom, or answered affirmative when asked about the presence of this symptom, than in patients who did not ($p \leq 0.001$ at baseline as well as at one year follow-up). MFIS did not correlate with age, time from first symptom or EDSS, at baseline or at one year follow-up (all Spearman's ρ s < 0.4 and $p > 0.01$). Also, MFIS did not differ significantly with regard to, diagnosis (CIS/RRMS) or relapse status (in relapse/not in relapse), at baseline ($p = 0.68$ and 0.32 , respectively) or at one year follow-up ($p = 0.86$ and 0.92 , respectively). Women had somewhat higher MFIS scores at baseline ($p = 0.04$) and at follow-up ($p = 0.05$). At one year follow-up, interferon beta treated patients ($n = 18$) and untreated patients ($n = 15$) had similar MFIS, HAD-A, HAD-D, MSIS-29 physical, MSIS-29 psychological, SF-36 physical and SF-36 mental scores (all $p > 0.5$ in Mann-Whitney U-tests). There were no significant differences between baseline scores and one year follow-up questionnaire scores, except for HAD-A, where baseline scores were significantly higher ($p = 0.001$).

MFIS scores correlated significantly with other self-reported questionnaire data; HAD-A, HAD-D, MSIS-29 physical, MSIS-29 psychological, SF-36 physical, SF-36 mental, as well as with ACTT results at baseline and at one year follow-up, see Table 10.

Table 10. Correlations between MFIS and HAD-A, HAD-D, MSIS-29 physical, MSIS-29 psychological, SF-36 physical, SF-36 mental and ACTT, at baseline and at one year follow-up

	HAD-A	HAD-D	MSIS-29 physical	MSIS-29 psycho- logical	SF-36 physical	SF-36 mental	ACTT
MFIS, BL	0.45*	0.63**	0.74**	0.77**	-0.64**	-0.70**	-0.49*
MFIS, 1y	0.48*	0.55**	0.78**	0.78**	-0.54**	-0.57**	-0.58**

*Correlation coefficients (Spearman's ρ) from bivariate non-parametric correlation analyses (Spearman) are shown. *: $p \leq 0.01$, **: $p \leq 0.001$*

MFIS: Modified Fatigue Impact Scale, HAD-A: Hospital Anxiety and Depression scale anxiety score, HAD-D: Hospital Anxiety and Depression scale depression score, MSIS-29 physical: Multiple Sclerosis Impact Scale physical score, MSIS-29 psychological: Multiple Sclerosis Impact Scale psychological score, SF-36 physical: Short Form 36 physical component summary score, SF-36 mental: Short Form 36 mental component summary score, ACTT: Auditory Consonant Trigram Test, BL: baseline, 1y: one year follow-up.

There were no significant correlations between MFIS scores and neuroinflammatory/neurodegenerative markers (IL-1 β , IL-6, CXCL1, CXCL10, CXCL13 and CCL-22 in plasma, NFL in serum and IL-6, CXCL1, CXCL10, CXCL13, CCL22, NFL, CHI3L1 and GFAP in CSF), or between MFIS scores and BPF, total number of T2 lesions in the brain, BPF decrease from baseline to one year and number of new T2 lesions during one year follow-up. There were also no significant differences in MFIS, HAD-A, HAD-D, MSIS-29 physical,

MSIS-29 psychological, SF-36 physical and SF-36 mental scores between patients fulfilling NEDA-3 (n=18) and patients not fulfilling NEDA-3 (n=20) during one year of follow-up (all $p>0.01$), indicating that the NEDA-3 concept fails to properly reflect important aspects of newly diagnosed CIS and RRMS, like fatigue, mood and cognition, in the short time (one year) follow-up perspective.

In paper III we also report that IL-6 in CSF from patients was significantly higher than in healthy controls at both baseline and one year follow-up (both $p\leq 0.01$), whereas plasma levels did not differ (both $p>0.01$).

To summarize, in paper III we report significant and consistent correlations between MFIS scores and other self-reported questionnaire data as well as ACTT performance at both baseline and one year follow-up in the cohort, whereas MFIS scores showed no significant correlations with conventional disease state/activity parameters, including clinical, neuroimaging, and neuroinflammatory/neurodegenerative markers. Does this suggest that no associations exist between fatigue and conventional disease state/activity parameters, or did we for some reason not detect them? It is important to understand that fatigue is a subjective, complex and multifactorial phenomenon. Whereas neuroimaging parameters and neurofilament levels in an MS patient usually reflect impact related to the MS disease alone, a patient's fatigue experience can be influenced by many other parameters than strict MS pathophysiology. Psychosocial secondary effects of the MS disease as well as non-MS related circumstances in life can affect MFIS scores. We had no means of controlling for these factors, which may have influenced our results, and we do not claim to have studied either primary fatigue or fatigability in MS. The MFIS in itself also carries limitations. It equals an ordinal scale and its questions and answer options can be interpreted and handled in different ways by different persons, restricting *e.g.* possibilities to compare different individuals. The limitations of the MFIS as well as potentially unevenly distributed non-MFIS related response biases could have affected our results. There is also the aspect of what MFIS actually measures, whether variation in the low score range like in our study is clinically relevant and whether it is meaningful to use a cut off score to dichotomize into fatigue and non-fatigue groups in the way that has been done in some other studies. In general, fatigue research projects could benefit from combining quantitative and qualitative research methodology and from approaches taking socioeconomic and cultural contexts into account. Implications for clinical practise from our study and other studies are that patient perceived fatigue, while being both common and "invisible", cannot easily be quantified (neither in itself, nor by a surrogate marker) or linked with commonly used objective disease state/activity parameters. This makes fatigue especially important to recognize and put into context during consultations. If more objective assessments of fatigue experience in patients are desired, other parameters than EDSS, total brain volume, T2 lesions or markers of neuroinflammation and neurodegeneration need to be evaluated. Motor fatigability as well as cognitive fatigability can be estimated, although consensus regarding optimal methods is lacking [97] and bearing in mind that patients' perceptions of fatigue and fatigability test results are not necessarily associated [98, 99].

A final remark on this subject is that using questionnaires may aid in discussions about fatigue or quality of life, but that questionnaires cannot, in my opinion, substitute the

patient-physician dialogue. Many other factors than the MS disease affect the individual patient's subjective experience of life quality and fatigue. Also, the consequences of the actual MS disease vary a lot between individual patients. As neurologists we interact with whole individuals, in varying life contexts, when we meet our MS patients. Their MS may be our primary focus and of course we need to consider objective parameters like *e.g.* new T2 lesions during follow-up of our patients. However, subjective symptoms, as well as patients' questions, views, fears, preferences, needs for non-pharmacological interventions/aid *etc.*, must be noted and addressed in parallel. In addition to fundamental requirements like a well-functioning health care system in general, this requires clinical skills and well-working physician-patient relationships that cannot be replaced by any biomarker measurements or questionnaires.

Complement levels and complement activation in CIS and RRMS (paper IV)

Levels of C1q, C3, C3a and sC5b-9 were determined in both plasma and CSF in healthy controls and in patients. In unadjusted comparisons, CSF-C1q and P-C3 were significantly higher in the patient group as a whole, as well as in relapse (defined as onset of relapse within two months previous to CSF sampling) and remission subgroups, than in controls. Relapse patients also had significantly higher levels of CSF-C3 and CSF-C3a than controls.

Complement levels in CSF can be affected by leakage over a damaged blood brain barrier and therefore CSF complement levels were evaluated in relation to albumin in CSF. CSF-C1q and CSF-C3a did not correlate with the albumin level in CSF.

Complement levels in plasma can also be affected by systemic inflammation and therefore we evaluated complement levels in relation to hsCRP. Levels of hsCRP were significantly higher in patients than in controls, with similar levels in relapse and remission. Of note, both patients and controls had a low median hsCRP (<2 mg/L). Nevertheless, the difference between the groups may be of importance. We cannot determine whether higher hsCRP levels in patients are related to the MS disease itself or represent a higher prevalence in the patient group of conditions unrelated to MS that raise hsCRP. Increased levels of hsCRP in RRMS patients compared with healthy controls have been reported previously [148-150], whereas others have reported hsCRP levels to be similar in MS patients and healthy controls [151, 152], although higher in relapses than in remission [151]. In patients, hsCRP correlated significantly with P-C3 (Spearman's rho 0.59, $p \leq 0.001$), but not with the other complement components.

Comparison of complement levels in patients and controls using ANCOVA with hsCRP as a covariate showed that a significant difference remained for CSF-C1q ($p=0.01$) but not for P-C3 ($p=0.14$) on whole group level. For the relapse subgroup compared with controls, a significant difference remained for CSF-C1q ($p=0.006$) and CSF-C3a ($p=0.05$), but not for P-C3 ($p=0.14$) and CSF-C3 ($p=0.06$). For the non-relapse subgroup compared with controls, no significant differences for CSF-C1q ($p=0.16$) or P-C3 ($p=0.35$) remained. Taken together, differences in plasma between patients and controls did not remain

significant when adjusted for hsCRP. In contrast, some differences in CSF-C1q and CSF-C3a levels remained when adjusted for hsCRP (Figure 11).

Figure 11. Levels of C1q (A) and C3a (B) in cerebrospinal fluid in patients at baseline and in healthy controls. CSF: cerebrospinal fluid, All pat: All patients (n=41), Relapse: patients in relapse (n=23), HC: healthy controls (n=22). *: $p \leq 0.05$, **: $p \leq 0.01$. P-values from Mann-Whitney U-tests as well as from ANCOVA controlling for hsCRP.

P-C3 has previously been reported to be elevated in progressive MS, but not in RRMS [153], while others have not found significant differences between MS patients and controls [154]. We found higher absolute levels of P-C3 in our cohort than in controls, but after adjustment for hsCRP, this difference disappeared. Elevated levels of CSF-C3 in patients and a weak correlation between CSF-C3 and EDSS have been reported [155], which we did not see in our cohort. The conflicting reports on P-C3 and CSF-C3 levels could be related to differences in methodology and patient populations. C3 levels can also be difficult to interpret since C3 is an acute-phase reactant and increased consumption caused by complement activation can be masked by increased synthesis of C3 in response to inflammation. Another aspect to keep in mind when interpreting levels of complement components in CSF is that these proteins exist in about 1000-fold higher levels in blood and that leakage over the blood-brain-barrier and contamination of blood at lumbar puncture may have an impact on the results. CSF-C3 was found to correlate with the CSF albumin level in our study and we cannot exclude that the CSF levels are affected by blood-brain barrier damage allowing C3 to pass from blood to CSF. As for contamination of blood at lumbar puncture, the vast majority (all but three CSF samples) had erythrocyte counts below $10 \times 10^6/L$.

Interestingly, baseline CSF-C3a was associated with the numbers of T2 lesions and NEDA-3 status during follow-up (Figure 12), meaning that baseline CSF-C3a carried some prognostic information. However, viewed in a biomarker perspective, the differences between patients and controls were small and the prognostic value of CSF-C3a was clearly surpassed by other markers in this cohort, *e.g.* NFL [114, 156, 157]. Thus, our results do not support the use of CSF-C3a as a single biomarker in early MS, although its association with T2 lesions could possibly add value to combined panels.

Figure 12. A: Baseline levels of C3a in cerebrospinal fluid (CSF-C3a) in patients with (n=16) and without (n=25) new T2 lesions in brain MRI at one year follow-up, compared to baseline. P-value from Mann-Whitney U-test. B: Baseline levels of CSF-C3a in patients not fulfilling NEDA-3 (EDA, n=21) and in patients fulfilling NEDA-3 (NEDA-3, n=20) during one year of follow-up. P-value from Mann-Whitney U-test.

In CSF at baseline, C1q correlated significantly with CXCL1, CXCL8, CXCL10, MMP-9, CHI3L1, NFL and GFAP, and C3a correlated significantly with CXCL1, CXCL8, CXCL10, CXCL13, CCL22, MMP-9, CHI3L1 and NFL. In general, C1q and C3a in relapse patients showed stronger associations with the neurodegenerative/neuroinflammatory markers than patients that were not in relapse at the time of CSF sampling. Selected correlations are shown in Table 11. Our results on CSF-NFL are somewhat in line with a previous report of CSF-C3 levels correlating with NFL in MS [155]. However, the patient group in that study included progressive MS in addition to RRMS [155].

Table 11. Selected correlations between complement factors (C1q and C3a) and neurodegenerative/neuroinflammatory markers in cerebrospinal fluid, in patients at baseline

	CXCL1	CXCL8	CXCL10	CXCL13	CCL22	MMP-9	CHI3L1	NFL	GFAP
C1q all	0.41**	0.55**	0.59**	0.35	0.35	0.45**	0.47**	0.41**	0.44**
C1q rel	0.55**	0.49*	0.69**	0.47*	0.50*	0.64**	0.31	0.47*	0.29
C1q rem	0.31	0.64**	0.57**	0.30	0.30	0.28	0.73**	0.41	0.54*
C3a all	0.50**	0.49**	0.49**	0.58**	0.53**	0.50**	0.64**	0.56**	0.38
C3a rel	0.47*	0.38	0.54**	0.68**	0.55**	0.56**	0.73**	0.38	0.41*
C3a rem	0.36	0.46*	0.20	0.30	0.37	0.27	0.46*	0.56*	0.23

Magnitude of correlation coefficients (Spearman's rho) are shown.

**: Spearman's rho ≥ 0.40 and p 0.02-0.05, **: Spearman's rho ≥ 0.40 and p ≤ 0.01*

All: all patients (n=41), rel: patients in relapse (n=23), rem: patients in remission (n=18)

We have found no previous reports of CSF-C3a being elevated during relapse, correlating with neuroinflammatory or axonal damage markers in CSF, or correlating with number of T2 lesions in brain MRI, neither in a cross sectional setting nor in a prospective setting. C1q levels have been studied previously, and increased levels of CSF-C1q in MS have been reported before [158]. We confirm this finding and show that CSF-C1q is associated with established CSF markers of neuroinflammation and neurodegeneration. Since C1q and C3a levels were not correlated with albumin levels or systemic inflammation, the increased CSF levels indicate complement activation and inflammation within the CNS.

Treatment effects

It would have been interesting to be able to address the question of how much different disease modifying drugs lower NFL levels. Our cohort was unfortunately too small to address this kind of research question. Out of curiosity, as well as to get some practice in relevant statistical analyses in this area, I compared interferon-beta and natalizumab treated patients with regard to NFL levels before and during treatment. Although the number of patients were small in the two treatment groups, statistical analysis indicated differences in treatment effects, see Figure 13. In CSF, NFL was significantly lower in natalizumab treated patients than in interferon-beta treated patients at both two and four years (p-values from Mann-Whitney U-test ≤ 0.05), although baseline levels did not differ significantly. CSF-NFL was also significantly lower at both two and four years than at baseline in natalizumab treated patients and at two, but not four, years in interferon-beta treated patients (p-values from Wilcoxon matched pairs signed rank test ≤ 0.05). In serum, NFL was significantly lower in natalizumab treated patients than in interferon-beta treated patients at four (but not at two) years, although baseline levels did not differ significantly. S-NFL was also significantly lower at both two and four years than at baseline in natalizumab treated patients, whereas it was not lower at follow-ups in interferon-beta treated patients. This is in line with data on treatment effects on NFL levels in CSF that has been reported by others [46, 78]. Treatment effects on S-NFL levels have also been reported by several groups, with more pronounced effects seen in patients treated with high efficacy DMDs [52, 53].

Figure 13. NFL and treatment effects. Baseline (BL), two (2y) and four (4y) year levels of NFL in patients treated with interferon-beta (IFN) ($n=12$ at two years and $n=5$ at four years) and natalizumab (NAT) ($n=7$ at two years and $n=10$ at four years) in CSF at (A) two and (B) four years of follow-up, respectively, and in serum at (C) two and (D) four years of follow-up, respectively. All patients were untreated at baseline.

Strengths, limitations and statistical considerations

General strengths of this cohort study are the broadly characterized patient group, with clinical data, imaging data and immunological data available, the prospective longitudinal design and that no patient had started treatment with a DMD at baseline. Patients were followed-up by the same neurologist in a standardized way. The presence of blood and CSF from sex- and age-matched healthy controls is also a strength, although controls were not prospectively followed-up. Of note, the controls were symptom free individuals, not “other neurological diseases” or patients investigated due to some kind of symptoms and where no disease was found. General limitations are sample size, the confounding introduced by varying treatment regimens among the patients, the lack of MRI data from healthy controls and the high number of statistical comparisons/correlations performed.

A sufficiently large and representative sample of the MS population is an essential requirement for every clinical MS study. Larger sample sizes give more reliable results with greater precision and power. Separate validation samples/cohorts are also desirable for verification of initial findings. As for power, *i.e.* the probability of finding a statistically significant difference given that there really is a difference between groups, it depends on *e.g.* the variability of the parameter in question, the precision of the measurements of the parameter in question, how big the difference actually is and what significance level that is chosen. Samples of the total population should also be representative, *i.e.* accurately reflect the total population’s characteristics, to allow study results to be generalized to the total population. Standard representativeness parameters in medical research are age and sex. In MS research it could also be *e.g.* the proportion of smokers or HLA DRB1*15:01 positive patients. The characteristics just mentioned can be evaluated, whereas suboptimal representativeness in the form of for instance the presence of three individuals that are extreme “immunological outliers” in a sample could be harder to detect and could have more profound effects on results in a small sample than in a large sample. Regarding sample size in my patient cohort, it is rather low, due to various practical reasons. Regarding representativeness, the median age (31 years) is in line with the total MS population, whereas the proportion of men (22%) is lower than in the total MS population.

Because of multiple testing, a stringent p-value of ≤ 0.01 was usually considered to be significant in Mann-Whitney U-tests, Wilcoxon matched-pairs signed rank tests, Spearman correlations and linear regression analyses. For some comparisons, where patient numbers were small and comparisons few, a p-value of ≤ 0.05 was considered significant in Mann-Whitney U-tests and Wilcoxon matched-pairs signed rank tests. In correlation analyses, stringent significance levels of Spearman’s $\rho \geq 0.5$ or ≥ 0.4 were most often used, usually in combination with the requirement of a p-value of ≤ 0.01 . Nevertheless, the number of group comparisons and exploratory correlation analyses performed is potentially problematic due to the risk of false positive findings. We tried to reduce this risk by using the strict significance levels mentioned above and also by looking for consistency in overall patterns of correlations and making use of the longitudinal design of the study. Reviewers’ opinions on significance levels also had an impact on final cut-offs, in part explaining some inconsistencies between the papers. Interestingly, Reviewers

occasionally expressed opposite opinions on our significance levels, with one regarding them too strict while the other one thought they were too generous.

There is always a risk of false positive and false negative findings in research. These can be the result of non-representative samples, laboratory errors *etc.*, but they can also be caused by pure chance as well as statistical decisions regarding cut off levels. With stringent significance levels, positive findings are more likely to really be true but the false negative rate is higher than with a less stringent significance level. The opposite applies to liberal significance levels. The number of comparisons/correlations that are evaluated is also important. Generally speaking, with a high enough number of analyses there will be false positive findings in spite of an ever so stringent significance level. With a significance level of $p=0.05$ in group comparisons, on average one in 20 will be a false positive. With $p=0.01$ it will be one in 100 and with $p=0.001$ it will be one in 1000. The commonly used strategy to dichotomize in significant versus non-significant is sometimes problematic in itself. It can be difficult to define what a meaningful difference between groups really is and of particular note, a significant difference is not necessarily clinically meaningful. Also, it may be difficult to motivate why a correlation with a Spearman's rho of 0.51 is reported as significant while correlation with a Spearman's rho of 0.49 is not. Regarding correlations it is also important to realize that they don't say anything at all about causality. If the variables A and B correlate, it could be that A causes B or the other way around, or it could be that another factor causes both A and B, or that A and B have independent causes that are either associated or by pure chance happen to affect A and B in a way that make them correlate.

Despite the statistical limitations discussed above, we would like to emphasize that the consistent findings of NFL repeatedly performing well as a biomarker in our small cohort strongly support the clinical usefulness of NFL as a biomarker in MS.

CONCLUSIONS

Paper I and II

In paper I, CSF levels of NFL at baseline performed well at separating patients from healthy controls as well as at predicting disease activity during two years of follow-up in recently diagnosed CIS and RRMS. In paper II, a fairly strong correlation between NFL levels in CSF and serum was demonstrated (Spearman's ρ 0.65 and Pearson's r 0.74, both $p < 0.001$). However, CSF-NFL levels showed an overall stronger correlation profile than serum levels with regard to number of new T2 lesions and therefore seemed to reflect disease activity better. CSF-NFL was associated with both new T2 lesions and BPF decrease during four years of follow-up, whereas CHI3L1 was associated mainly with brain volume loss and CXCL1, CXCL10, CXCL13 and MMP-9 were associated mainly with new T2 lesions. Paper II also confirmed CSF-NFL as a prognostic biomarker for conversion from CIS to MS. Paper I and II support CXCL1, CXCL10, CXCL13, CHI3L1 and MMP-9 in CSF as biomarkers in CIS and RRMS and further add to the accumulating evidence that CSF-NFL and S-NFL are clinically useful biomarkers that could be included in the expanding NEDA concept.

Paper III

Fatigue scores in patients with newly diagnosed CIS and RRMS were associated with other self-assessment questionnaire data and to some extent with neuropsychological test performances, whereas associations with EDSS, total brain volume, number of T2 lesions or protein neurodegenerative/neuroinflammatory markers, including NFL levels in CSF, could not be demonstrated. Study results indicate that subjective fatigue scores are not well reflected by some commonly used and objectively measurable disease parameters.

Paper IV

C1q and C3a levels were elevated in CSF from patients with newly diagnosed CIS and RRMS compared with healthy controls. Thus, the complement system is activated in CSF, close to the ongoing inflammation in CNS, in newly diagnosed CIS and RRMS. CSF-C1q and CSF-C3a in patients were associated with several other markers, indicating that they are integrated in the neuroinflammatory and neurodegenerative processes. Study results also indicate that baseline CSF-C3a levels in patients may carry prognostic information in relation to new T2 lesions, NEDA-3 status and conversion from CIS to RRMS during follow-up. However, our results do not support the use of CSF-C3a as a single biomarker in RRMS.

Taken together, this thesis:

- Confirms and extends the knowledge of NFL as a useful biomarker in CIS and RRMS, for prediction of disease activity.
- Suggests that NFL, rather than total brain volume loss, could be included in an expanded NEDA concept.
- Shows that different biomarkers seem to have different profiles in the prediction of disease outcome:
 - NFL was associated with NEDA-3 as well as brain volume loss
 - Chemokines CXCL1, CXCL10 and CXCL13, matrix metalloproteinase MMP-9 and complement component C3a were associated mainly with new T2 lesions
- Shows that the currently investigated biomarkers must be measured in CSF; no correlations were found between plasma biomarkers and disease outcome, with the important exception of NFL.
- Supports the use of NFL in serum as a biomarker in CIS and RRMS, considering its fairly strong correlation with NFL in CSF, its associations with disease activity and the practical advantages of blood sampling over CSF sampling in a long term monitoring perspective.
- Illustrates that MFIS scores can be associated with other self-report questionnaire data, including quality of life, while not with EDSS, total brain volume, T2 lesions or markers of neuroinflammation and neurodegeneration.
- Shows that a moderately sized cohort study can reveal important findings, in part due to adherence to a strict protocol and careful follow-up over several years.

FUTURE PERSPECTIVES

Regarding MS research in general, I believe that applying bioinformatics approaches on genomics, epigenomics, transcriptomics, proteomics and metabolomics data from large cohorts will be rewarding for further elucidation of MS pathophysiology as well as further development of clinical biomarkers and disease modifying drugs. MS immunology is complex and focusing on one or a few proteins only will never give us the complete picture. Multicenter and multiprofessional collaboration could enable the collection of large and representative patient population samples, validation cohorts and age- and sex-matched healthy controls. It could also facilitate the creation of larger biobanks, standardized clinical and MRI evaluations, standardized protocols for collecting, handling and storing samples, assay development and standardization, as well as make bioinformatics expertise available to more researchers. However, there will still be a need for smaller clinical cohorts to *e.g.* verify the results and usefulness of biomarkers in various clinical settings. Although large sample sizes have many advantages, for a biomarker to be clinically useful, it needs to perform well in small patient groups as well.

Another important future area is the increasing sensitivity and precision in measuring low concentrations of biomarkers. Proteins in body fluids are the ideal biomarkers, and here new techniques have made it possible to reliably measure low concentrations of proteins. In this thesis, a Simoa was used for detection of NFL in serum. Simoa, and other high-sensitivity platforms, are able to measure also other brain-specific molecules in serum, which opens up new possibilities to explore and monitor degenerative processes in the brain. For exploration, and maybe also for clinical use, multiplexing would be a great advantage, allowing measurements of several (hundreds) of proteins at the same time. Here, the proximity extension assay (PEA, Olink proteomics) poses several advantages since it combines high sensitivity (PCR-based amplification) with high specificity (two epitopes detected), and is multiplexed. The extensive data output from these assays require advanced statistics to be optimally and correctly handled and interpreted.

Regarding the cohort study underlying this thesis, magnetic resonance spectroscopy data as well as data on cortisol in saliva from patients are available for analyses. Flow cytometry data on cell populations in blood from both patients and healthy controls are also available. Moreover, remaining stored samples could be used in new research projects, potentially pooled with other samples to increase sample size. In addition to serum, plasma and cell free CSF, there are lysed cells from CSF and blood in PAXgene tubes that could be used in RNA studies, blood cells in EDTA tubes that could be used in DNA studies and peripheral blood mononuclear cells that could be used in lymphocyte functional studies.

In Linköping, a colleague neurologist is currently collaborating with several other hospitals to recruit patients to the SouthEast Sweden NeuroInflammation Cohort (SESNIC) study. The SESNIC study is a prospective longitudinal study that aims to validate and extend findings in the current CIS cohort regarding early biomarkers (baseline samples in untreated patients) for prediction of prognosis and disease activity. The SESNIC study

will have the advantage of a larger sample size than my study cohort. Also, for exploration, as mentioned above, several cohorts can be combined to achieve larger sample sizes. Of particular interest is the possibility to identify responders and non-responders to treatment, which in fact could be done for natalizumab using a network- and omics-based approach [156]. Another exciting Linköping-based project is a multi-center study with the aim to use pregnancy as a model to understand mechanisms of improvement and worsening of MS during pregnancy.

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