Biomarkers in Multiple Sclerosis

– Monitoring disease activity and treatment efficacy

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Gothenburg, Sweden, 2018

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Cover illustration by Martina Nováková Lam

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ISBN 978-91-629-0438-8
http://hdl.handle.net/2077/54957

Printed in Gothenburg, Sweden 2018
BrandFactory AB Sverige

To patients with Multiple Sclerosis

Abstract

The pathophysiology of multiple sclerosis (MS) is complex with the presence of inflammation and neurodegeneration in all stages of the disease. The disease course, treatment response and outcome are highly variable in MS. There is a need for reliable biomarkers reflecting different parts of the pathophysiology of MS that may improve the decision-making between various treatment options. The aim of the thesis was to investigate the influence of different therapies on biomarker levels in cerebrospinal fluid (CSF) and blood, explore the relationships between inflammatory and degenerative biomarkers, their diagnostic value and the value of measuring brain atrophy, i.e. brain parenchymal fraction (BPF) and the thinning of retinal nerve fibre layer (RNFL) to detect signs of early degeneration.

In study I, treatment with natalizumab reduced 24S-hydroxycholesterol concentrations in CSF and serum and 27-hydroxycholesterol concentrations in CSF.

In study II, relapsing-remitting MS patients had higher levels of neurofilament light (NFL), CXCL13, chitinase-3-like-1 (CHI3L1), and chitotriosidase 1 (CHIT1) than controls. Subgroup analysis revealed higher levels of NFL, CXCL13 and CHIT1 in patients treated with first-line therapy compared to second-line therapy. NFL and CHIT1 levels correlated with relapse status, and NFL and CXCL13 levels correlated with the formation of new lesions on MRI.

In study III, the levels of NFL, CXCL13, and CHI3L1 decreased after treatment with fingolimod.

In study IV, high correlation between serum and CSF NFL was found. Serum concentrations of NFL were significantly higher in MS patients than in healthy controls and treatment reduced serum NFL levels. Patients with relapse or with radiologic activity had higher serum NFL levels than those in remission or those without new lesions on MRI.

In study V, all phenotypes of MS had increased NFL compared to HC. Increased glial fibrillary acidic protein (GFAP), lower BPF and RNFL were associated with progressive MS but not with other phenotypes of MS. Lower BPF and RNFL, indicating neurodegeneration, were associated with longer disease duration.

We showed that CSF biomarkers that represent different parts of the pathophysiology of MS were related to both clinical and radiological measures. The correlation between neurodegenerative and inflammatory biomarkers, and the lack of signs of neurodegeneration in the earliest

phases of relapsing-remitting MS, confirms the hypothesis regarding inflammatory-induced degeneration. The most important finding is that the blood-based biomarker NFL can reflect the disease activity and treatment efficacy. This finding is based on a large set of paired serum and CSF samples from a real-life cohort of patients across a wide clinical and therapeutic spectrum. Therefore, repeated serum NFL measurements may represent new possibilities for the monitoring of MS.

Popular scientific summary

Multiple sclerosis (MS) is a chronic autoimmune disorder that damages the central nervous system. It is the leading non-traumatic cause of neurological disability affecting young adults in the Western World. Approximately 2,500,000 people in the world have MS and it occurs in all parts of the world including Sweden where approximately 20.000 people suffer from it.

The disease is complex with inflammation and neurodegeneration present in all its stages. Its course, treatment response and outcome are highly variable. Therefore, there is a need for reliable measures that reflect its activity, disability progression and for the prediction of its severity. These measures are called *biomarkers* and they were the main focus of this thesis.

Over recent years there is accumulating evidence that disease modifying therapies not only reduce inflammation but also influence neurodegeneration in MS patients. Thus, the use of biomarkers, which reflect different parts of the disease, may improve the selection of the appropriate treatment option. Today, we use magnetic resonance imaging and a clinical neurologic investigation performed annually by a neurologist to assess the disease activity and evaluate the treatment response. However, MS is a very dynamic disease and therefore much of what occurs between examinations may be missed. Furthermore, the exact mechanism of action of certain disease modifying therapies is not clear.

The aim of the thesis was to find relationships between different biomarkers from cerebrospinal fluid, blood and imaging biomarkers found through magnetic resonance imaging. Further, we investigated the influence of different therapies on biomarkers and explored if we could use them to follow up the disease activity and the treatment response.

Several biomarkers from cerebrospinal fluid were measured. These substances were assessed before and after the treatment so the changes could be examined. We also investigated if the treatment response could be measured and what occurs during relapse and remission. We determined the thickness of the innermost layer of the eye using optical coherence tomography. This layer can also act as a biomarker and reflects what occurs in the brain. We measured brain volume with a new type of magnetic resonance imaging called Synthetic MRI. The changes in the eyes and brain volume are typical for MS patients. Thus, they also can be affected by the disease activity and the treatment.

We have found that biomarkers from cerebrospinal fluid were related to both clinical (i.e. relapse) and MRI measures and they have the ability to reflect the treatment efficacy of different disease modifying treatments. We found an association between neurodegenerative and inflammatory biomarkers and no evidence of neurodegeneration in the earliest phases of the disease. This confirms the hypothesis regarding inflammatory-induced degeneration. The most clinically important finding is that biomarker from blood called Neurofilament Light can reflect the disease activity and treatment efficacy. This biomarker is a protein that can be found in the neurons and is increased when these cells are destroyed. This finding is based on a large set of paired blood and cerebrospinal fluid samples from a real-life cohort of patients across a wide clinical and therapeutic spectrum and therefore, repeated Neurofilament measurements in peripheral blood may represent new possibilities for the monitoring of MS and can now be used even in regular MS care.

Populärvetenskaplig sammanfattning

Multipel skleros (MS) är en kronisk autoimmun sjukdom som skadar det centrala nervsystemet (CNS). Det är den främsta icke-traumatiska orsaken till neurologisk funktionsnedsättning bland unga vuxna i västvärlden. Sjukdomen förekommer i alla delar av världen med en ökande prevalens med avståndet från ekvatorn. Ungefär 2,5 miljoner människor har MS varav cirka 20.000 i Sverige. Sjukdomen är komplex och karakteriseras av både inflammation och neurodegeneration som utan behandling leder oftast till omfattande funktionsnedsättningar. Hittills inriktas all behandling på att minska inflammationen i CNS. Olika läkemedel har olika verkningsmekanismer och effekten av behandling varierar stort mellan olika patienter. Därför är det viktigt att utveckla pålitliga metoder och mått som avspeglar olika sjukdomsprocesser och som kan användas för att objektivt mäta sjukdomsaktivitet, funktionsnedsättning och sjukdomens svårighetsgrad. Dessa mått kallas för *biomarkörer* och de var huvudämnet för denna avhandling.

Under de senaste åren har det blivit allt tydligare att MS läkemedel inte enbart minskar inflammationen utan även neurodegenerationen vid MS. Idag används huvudsakligen klinisk neurologisk värdering och magnetkameraundersökning i valet av behandling och för monitorering av denna. Problemet är dock att MS är en väldigt dynamisk sjukdom och risken att missa sjukdomsprocesser och aktivitet är stor med dagens utvärderingsinstrument.

Målet med denna avhandling var att vid MS förbättra utvärderingsmetoderna av den sjukdomsmodifierande behandlingen och för att undersöka vilka sjukdomsprocesser som påverkas vid olika farmakologiska interventioner. Vi undersökte nivåer och relationer mellan olika biomarkörer i ryggvätska och blod samt hur dessa relaterades till kliniska sjukdomsmått och bildbiomarkörer från magnetkamera vid undersökning av hjärnan. Neurodegenerationen utvärderades dessutom med optisk koherenstomografi (mätning av näthinnans tjocklek) och syntetisk magnetkamera (automatiserad hjärnvolymbestämning). Både biomarkörer som avspeglar inflammation och neurodegeneration analyserades i ryggvätskan från patienter med MS och jämfördes med nivåerna hos friska kontrollpersoner. Biomarkörer mättes före och efter insatt MS läkemedel.

Vi har funnit att nivån av biomarkörer från ryggvätska var relaterade till klinisk sjukdomsaktivitet (skovfrekvens) och aktivitet bestämd med magnetkamera (nya eller tillväxande MS lesioner) och att de har förmåga att avspegla behandlingseffekten av olika MS läkemedel. Vi påvisade att inflammationshämmande behandling också minskade nivåerna av degenerativa biomarkörer i ryggvätskan. Emellertid fann vi inga tecken på neurodegeneration i de tidigaste faserna av sjukdomen. Detta bekräftar hypotesen om inflammatorisk inducerad neurodegeneration vid MS. Avhandlingens starkaste kliniska bidrag var att mätning av neurofilament light (NFL) i blod kan ersätta mätning av NFL i ryggvätska. Denna biomarkör är ett protein som finns i nervfibrerna. Vid skada, t.ex. orsakad av inflammation eller degeneration, läcker NFL ut i ryggvätska men också i blod i små mängder. Med en ultrakänslig metod kan NFL detekteras i blod. Genom upprepad blodprovstagning med bestämning av NFL kan behandlingseffekten monitoreras. Detta kan sannolikt bli ett viktigt komplement till dagens kliniska och neuroradiologiska utvärderingsmetoder.

Populárne vedecké zhrnutie

Skleróza multiplex (SM) je chronické autoimunitné ochorenie, ktoré poškodzuje centrálny nervový systém. Ide o jednu z hlavných netraumatických príčin neurologického postihnutia u mladých ľudí vo vyspelých krajinách sveta. Približne 2 500 000 ľudí vo svete má SM a táto choroba sa vyskytuje vo všetkých častiach sveta vrátane Švédska, kde SM trpí takmer 20 000 ľudí.

SM je zložité ochorenie s charakteristickým zápalom a neurodegeneráciou vo všetkých štádiách. Jeho priebeh a reakcia na liečbu sú veľmi rôznorodé, preto sú potrebné spoľahlivé vyšetrovacie metódy, ktoré odzrkadľujú jej aktivitu, zhoršenie stavu choroby a jej závažnosť. Tieto parametre sa nazývajú *biomarkery* a sú hlavným cieľom tejto práce. Ich meranie poukazuje na špecifický chorobný stav a jeho vývoj.

V posledných rokoch boli zhromaždené dôkazy, že terapie modifikujúce ochorenie nielen znižujú zápal, ale tiež ovplyvňujú neurodegeneráciu u pacientov so SM. Využívanie biomarkerov, ktoré odrážajú rôzne štádiá ochorenia, môže zlepšiť výber vhodnej liečby. Dnes sa používa vyšetrenie mozgu magnetickou rezonanciou a klinické neurologické vyšetrenie jedenkrát ročne, na základe ktorých vyhodnocujeme aktivitu ochorenia a reakciu na liečbu. SM je však veľmi dynamická choroba, a preto častokrát nezachytíme všetko, čo sa deje medzi vyšetreniami. Navyše nie je jasný presný mechanizmus účinku niektorých terapií.

Cieľom tejto práce bolo nájsť vzťahy medzi rôznymi biomarkermi z mozgovomiechového moku, z krvi a vyšetreniami magnetickou rezonanciou. Ďalej sme skúmali vplyv rôznych terapií na biomarkery a to, či ich môžeme použiť na sledovanie aktivity ochorenia a účinnosť liečby.

Merali sme niekoľko biomarkerov z mozgovomiechového moku pred a po liečbe, aby sme preskúmali, či by zmeny ich koncentrácie mohli odzrkadľovať odpoveď na liečbu a zistiť, čo sa deje v rôznych štádiách ochorenia.

Ďalej sme určovali hrúbku najvnútornejšej vrstvy sietnice oka pomocou optickej koherentnej tomografie. Táto vrstva môže byť tiež biomarkerom, nakoľko odráža to, čo sa deje v mozgu. Objem mozgu sme merali novým typom magnetickej rezonancie, ktorá sa nazýva syntetická magnetická rezonancia. Zmeny pozorované na sietnici oka a zmeny objemu mozgu sú typické pre pacientov s SM, ktoré môžu byť tiež ovplyvnené aktivitou ochorenia a liečbou.

Zistili sme tiež, že biomarkery z mozgovomiechového moku odrážali klinickú aktivitu ochorenia a aj aktivitu, ktorú sme namerali na magnetickej rezonancii mozgu a majú schopnosť odrážať aj účinnosť liečby. Odhalili sme vzťah medzi neurodegeneratívnymi a zápalovými biomarkermi a zároveň sme nenašli žiaden dôkaz neurodegenerácie v rannom štádiu ochorenia. Toto potvrdzuje hypotézu, že degenerácia nervového systémy pri SM je vyvolaná zápalom.

Najdôležitejším výsledok tejto práce je zistenie, že biomarker z krvi nazývaný Neurofilament Light môže odrážať aktivitu ochorenia a účinnosť liečby. Tento biomarker je proteín, ktorý sa nachádza v neurónoch a jeho koncentrácia je zvýšená pri deštrukcii týchto buniek. Výsledky štúdie sú založené na hodnotení početných párových vzoriek z krvi a mozgovomiechového moku pacientov, ktorí sú v rôznych štádiách ochorenia a liečia sa rôznymi liekmi v jednej zo štyroch univerzitných polikliník vo Švédsku.

Opakované meranie Neurofilamentu v krvi teda prináša nové možnosti monitorovania SM a tento biomarker v súčasnosti môže byť používaný aj v bežnej starostlivosti o pacientov s SM.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Novakova L**, Axelsson M, Malmeström C, Zetterberg H, Björkhem I, Karrenbauer VD, Lycke J

Reduced cerebrospinal fluid concentrations of oxysterols in response to natalizumab treatment of relapsing remitting multiple sclerosis.

Journal of Neurological Sciences 2015; 358(1-2): 201-206

II. **Novakova L**, Axelsson M, Khademi M, Zetterberg H, Blennow K, Malmeström C, Piehl F, Olsson T, Lycke J

Cerebrospinal fluid biomarkers as a measure of disease activity and treatment efficacy in relapsing-remitting multiple sclerosis

Journal of Neurochemistry 2017; 141(2): 296-304

III. **Novakova L**, Axelsson M, Khademi M, Zetterberg H, Blennow K, Malmeström C, Piehl F, Olsson T, Lycke J

Cerebrospinal fluid biomarkers of inflammation and degeneration as measures of fingolimod efficacy in multiple sclerosis

Multiple Sclerosis Journal 2017; 23(1): 62-71

IV. **Novakova L**, Zetterberg H, Sundström P, Axelsson M, Khademi M, Gunnarsson M, Malmeström C, Svenningsson A, Olsson T, Piehl F, Blennow K, Lycke J

Monitoring disease activity in multiple sclerosis using serum neurofilament light protein

Neurology 2017; 89(22): 2230-2237

V. **Novakova L**, Axelsson M, Malmeström C, Imberg H, Elias O, Zetterberg H, Nerman O, Lycke J

Searching for neurodegeneration in multiple sclerosis at clinical onset: diagnostic value of biomarkers

Manuscript - Submitted

Content

Abbreviations

AD	Alzheimers disease
AHSCT	Autologous hematopoietic stem cell transplantation
APC	Antigen presenting cell
AUC	Area under curve
BAFF	B-cell activating factor
BBB	Blood-brain barrier
BCB	Blood-CSF barrier
BioMS-EU	European union network for CSF biomarker research in multiple sclerosis
BPF	Brain parenchymal fraction
CD	Cluster of differentiation
CCL2	C-C motif chemokine ligand 2
CHIT1	Chitotriosidase
CHI3L1	Chitinase-3-like protein 1
CHI3L2	Chitinase-3-like protein 2
CI	Confidence interval
CIS	Clinically isolated syndrome
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	Computed tomography
CV	Coefficient of variation
CXCL10	C-X-C motif chemokine 10
CXCL13	C-X-C motif chemokine 13
DIS	Dissemination in space
DIT	Dissemination in time
DMT	Disease modifying therapy
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein Barr virus
EDSS	Expanded Disability Status Scale
ELISA	Enzyme-linked immunosorbent assay
FD-OCT	Fourier-Domain or Frequency-Domain Optical Coherence Tomography
FLAIR	Fluid attenuation inversion recovery
GA	Glatiramer acetate
GCIPL	Ganglion cell-inner plexiform layer
Gd	Gadolinium
GFAP	Glial fibrillary acidic protein

HC	Healthy controls
HD-OCT	High-Definition Optical Coherence Tomography
HLA	Human leucocyte antigen
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IFN	Interferon
IQR	Interquartile range
INDC	Control with inflammatory neurologic disease
JC	John Cunningham
kDa	Kilo Dalton
LLoQ	Lower limit of quantification
LP	Lumbar puncture
MBP	Myelin basic protein
MCI	Mild cognitive impairment
MCP-1	Monocyte chemoattractant protein 1
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging
MS	Multiple Sclerosis
MSFC	Multiple Sclerosis Functional Composite
MSSS	Multiple Sclerosis Severity Score
MV	Macula volume
NA	Not applicable
NEDA	No Evidence of Disease Activity
NFH	Neurofilament heavy
NFL	Neurofilament light
NGRN	Neurogranin
NINDC	Control with non-inflammatory disease
NZ	Natalizumab
OCB	Oligoclonal bands
OCT	Optical Coherence Tomography
OD	Patients diagnosed with other diseases
ON	Optic neuritis
OND	Other neurologic disorder or symptom
OR	Odds ratio
PASAT	Paced Auditory Serial Addition Test
PML	Progressive multifocal leukoencephalopathy
PPMS	Primary progressive multiple sclerosis
PrMS	Progressive multiple sclerosis
RIS	Radiologically isolated syndrome
RNFL	Retinal nerve fiber layer
ROC	Receiver operating characteristic
RRMS	Relapsing remitting multiple sclerosis
RCT	Randomized controlled trial
S	Serum

SC	Symptomatic controls
SD	Standard deviation
SDMT	Symbol digit modalities test
SNPs	Single nucleotide polymorphism
SPMS	Secondary progressive multiple sclerosis
SIMOA	Single-molecule array
SyMRI	Synthetic magnetic resonance imaging
TD-OCT	Time Domain Optical Coherence Tomography
TREM-2	Triggering receptor expressed in myeloid cells 2
T25-FW	Timed 25-Foot Walk
VCAM-1	Vascular cell adhesion molecule 1
VLA-4	Very late antigen 4
24OHC	24S-hydroxycholesterol
27OHC	27-hydroxycholesterol
9-HPT	9-Hole Peg Test

Introduction

Multiple sclerosis is a chronic disease of the CNS that leads to substantial disability in most patients. The early phase is characterized by relapses and the later phase by progressive disability. The pathophysiology of MS is heterogeneous, with multiple mechanisms involved at every clinical stage. Disease outcome is highly variable and there is a need of better prognostic markers for individual prediction of disease severity, rate of progression and treatment response.

Increased understanding of MS pathophysiology can facilitate identification of novel biomarkers. For example, the discovery of axonal damage in MS lesions implicated neurofilaments as a disease activity marker. However, due to the heterogeneity of the disease, the use of panels of multiple biomarkers may better reflect the different disease mechanisms involved in the pathogenesis of MS.

In this chapter an overview of MS and biomarkers is presented.

Multiple Sclerosis

MS is a chronic inflammatory autoimmune disorder that damages the CNS and is the leading non-traumatic cause of neurological disability affecting young adults in the Western World [1].

Epidemiology

Approximately 2,500,000 people in the world have MS and although the disease occurs in most parts of the world, the distribution is markedly uneven. The global median prevalence is 30 per 100 000 and MS prevalence increases with latitude, i.e. with the distance from the equator, but there are places with disproportionately high or low frequencies [2]. MS is common in regions populated by northern Europeans. However, this distribution is modified by migration. Migrations involving large numbers of people affect the distribution of MS and the risk of MS correlates with place of residence in childhood [2]. Migration from high-risk to low-risk regions in childhood is associated with a reduced risk, and from low to high prevalence parts of the world with an increased risk of developing MS by comparison with the population of origin [3, 4]. The incidence of MS is increasing over time [5, 6],

although these data can be confounded by heightened awareness of the disease and new diagnostic techniques and criteria. The incidence of MS peaks between 20 and 40 years [7] and it differs between the countries. In Sweden, the prevalence is 189/100 000 [8] and incidence is 10.2/100 000 [9]. MS is more common in women than in men at a ratio 2.3 : 1 in Sweden [8, 9]. In recent surveys, sex-ratio in different parts of the world was 3:1 [10, 11], potentially influenced by environmental factors [12]. The study based on data from the Swedish MS registry indicates that MS has increased in women during the 20th century [13].

Clinical course

In 80-90%, the clinical onset of MS is with a relapse, that is a transient period of neurological symptoms lasting from a few days to several weeks with most often complete or partial clinical recovery. The origin of the onset relapse in the CNS is most commonly the optic nerve, the spinal cord or the brainstem. New relapses usually occur with a rate that seldom exceeds 1.5 per year. This clinical course is designated **relapsing-remitting MS** (RRMS). With time, the recovery from each relapse is not complete and persistent symptoms accumulate. Without treatment, most RRMS patients will turn into **secondary progressive MS** (SPMS) after a mean disease duration of 15-20 years. Secondary progression is defined as a clinical condition with continuous progression that lasts for at least a year with no distinct remission. However, patients with SPMS may also have relapses that are superimposed on the progressive course. In 10-15% of patients, the course is progressive from the clinical onset and these patients are designated **primary progressive MS** (PPMS). The symptoms and rate of progression is similar in PPMS and SPMS.

Clinically isolated syndrome (CIS) is characterized as the first clinical presentation that is compatible with MS but does not fulfill the criteria for dissemination in time [14]. Patients with CIS may convert to RRMS if a repeated MRI shows one or more new MS lesions, or if a new relapse occurrs and thereby fulfills the diagnostic criteria [15]. Recently the diagnostic criteria were revised, reducing the number of patients who will be categorized as CIS [16].

Radiologically isolated syndrome (RIS) is characterized as an incidental imaging finding suggesting inflammatory demyelination without the clinical signs or symptoms [17]. Patients with RIS have increased risk of converting to MS if the diagnostic investigation reveals asymptomatic spinal cord lesions, gadolinium-enhancing lesions, or shows accompanying CSF findings indicating increased selective intrathecal IgG synthesis. Nowadays, RIS is not considered as a distinct MS phenotype [18].

Over recent years the increased understanding of MS and its pathophysiology prompted a revision of the clinical phenotypes (Figure 1). In addition to them, the occurrence of new disease activity (relapses or new lesion formation on MRI) and progression should be included in the characterization of patients [19]. Thus, patients are also categorized as active or not active and with or without progression to identify eligible patients for therapy.

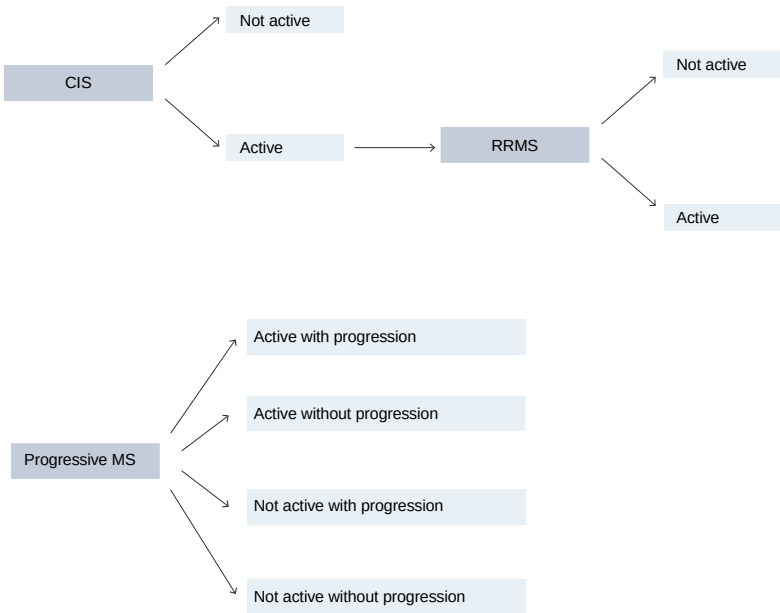


Figure 1. MS Phenotypes
The currently used classification of MS phenotypes is presented. Activity is characterized by a clinical relapse or MRI activity. Progression is evaluated by clinical examination.
CIS=clinically isolated syndrome, MS=multiple sclerosis, RRMS=relapsing-remitting multiple sclerosis-Redrawn from "New multiple sclerosis phenotypic classification." Lublin, F. D., Eur Neurol 2014, 72 Suppl 1:1-5.

Active disease is defined either clinically or radiologically. A clinical relapse is defined as an episode of new or increasing neurological disturbance lasting for at least 24 h, in the absence of fever or infection [20]. If new symptoms occur within 30 days after the last relapse, they count as the same relapse of the disease. The radiological activity seen on MRI is defined as the occurrence of contrast-enhancing T1 lesions or new and/or enlarged T2 lesions [18].

Progressive disease is defined clinically as steadily increased neurological dysfunction without recovery. However, the fluctuation in the disability

and phases of stability may occur during the progressive disease course [18]. Progression is measured by clinical neurological examination. Currently, there are not any established imaging measures of progression. Measures such as brain volume loss, increasing number and volume of T1-hypointense lesion, or diffusion tensor imaging could be considered.

Diagnosis

The diagnosis is based on typical clinical symptoms and the evidence of **dissemination in space** (DIS) and **dissemination in time** (DIT). Previously, MS diagnosis was made after two attacks with typical symptoms at least one month apart and from at least two separate areas of the CNS [21]. The diagnostic criteria used in this thesis [15] were revised in 2010, and the MS diagnosis can now be made after one attack with support of typical CNS lesions on MRI. **DIS** is characterized by at least one T2 lesion in at least 2 out of 4 typical CNS regions for MS; periventricular, juxtacortical, infratentorial and spinal. **DIT** is characterized by the presence of contrast-enhancing and non-enhancing lesions on one MRI scan or a new T2 and/or contrast-enhancing lesions on a follow-up MRI scan.

To diagnose RRMS, at least one clinical attack with abnormal findings on neurological examination must be present, together with MRI lesions indicating MS, and excluding the alternative diagnoses when applying the criteria. To support MS diagnosis, additional assessment with lumbar puncture and/or blood tests might be useful.

The SPMS can only be diagnosed in a person who has previously experienced RRMS. The transition from RRMS to SPMS is a gradual process with few or no relapses and a gradual worsening of symptoms over time. Taking medical history, performing neurological examination and repeating MRI help in diagnosing SPMS.

To diagnose PPMS, there needs to be at least one year of disease progression and 2 out of 3 of the following criteria; evidence of DIS in the brain, evidence of DIS in the spinal cord, positive oligoclonal bands in cerebrospinal fluid.

The latest revision of MS criteria in 2017 [16] enables earlier diagnosis of MS. The main differences to previous criteria [15] are the inclusion of the contrast-enhancing lesion that is the origin of new symptoms/relapse in the evaluation and that CSF finding of oligoclonal IgG bands can substitute for new relapses and lesion formation on MRI to fulfill the criterium of DIT (Figure 2).

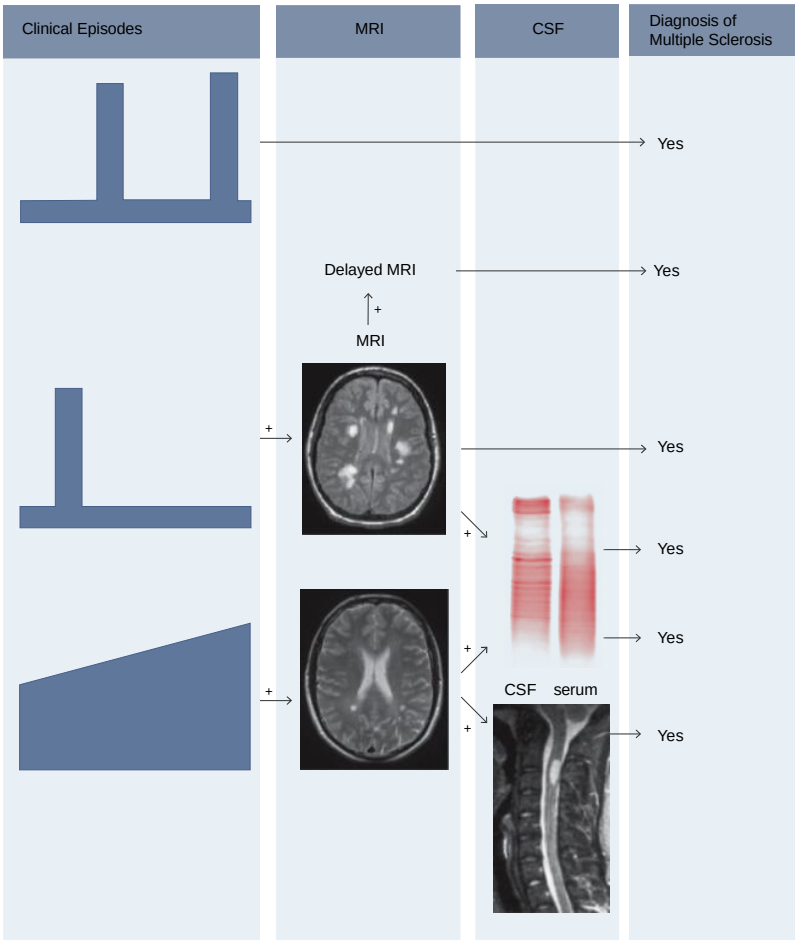


Figure 2. Criteria for the diagnosis of MS

The principle of MS diagnosis is to establish dissemination in time and space of lesions—ie, that episodes affecting separate sites within the CNS have occurred at least 30 days apart. MRI scan and CSF examination substitute for one of these clinical episodes. Dissemination in time on MRI requires the presence of a contrast-enhancing lesion and a lesion without contrast at any time. When a contrast-enhancing lesion is missing, dissemination in time on MRI requires a new lesion at any time compared with a reference scan. Dissemination in time on CSF requires the presence of oligoclonal bands. Dissemination in space on MRI requires the presence of at least one lesion in 2 of 4 regions (cortical/juxtacortical; periventricular; infratentorial; spinal). Primary progressive multiple sclerosis can be diagnosed after 1 year of a worsening neurological deficit and 2 of following 3 criteria: a positive brain MRI; a positive spinal cord MRI; positive oligoclonal bands.

CNS=central nervous system, CSF=cerebrospinal fluid, MRI=magnetic resonance imaging

Adapted from "Multiple sclerosis." Compston, A. and A. Coles, Lancet 2008, 372(9648):1502-1517.

Thus, the CSF analysis has previously not been necessary to diagnose RRMS. However, there is a number of differential diagnosis where CSF analysis might contribute to the diagnostic work-up. In CSF, the following findings are typical for MS: mild to moderate increase in the number of lymphocytes or monocytes indicating inflammation (>2 to usually <50 mononuclear cells/μL), moderately increased albumin ratio indicating interrupted blood-brain barrier, presence of oligoclonal IgG bands (2 or more) and/or elevated IgG index indicating immune activation. There is a number of other biomarkers that might contribute to the diagnosis of MS that have not yet been validated.

Etiology

The etiology of MS is still unknown, but the data indicates that the interaction between genes and environmental or lifestyle factors seems to be involved. MS is not a hereditary disease, but genetic factors contribute to the MS risk. MS risk in the general population is 0.2%. The MS risk within family [22] is 0.2% in a person married to an MS patient, 2.3% in siblings to MS patients, 1.7% in dizygotic twin to an MS patient, 1.2% in a child of an MS patient and 15% in a monozygotic twin to an MS patient.

Genetic susceptibility to MS is mostly associated with the human leukocyte antigen (HLA) region. The HLA class II alleles DRB1*1501, DRB1*0301 and DRB1*1303 expressed on cells of innate immune system are associated with increased risk of developing MS, whereas the HLA class I allele A2 is associated with decreased risk [23]. Additionally, genome-wide association studies have identified more than 100 common genetic variants (single nucleotide polymorphism, SNPs) associated with MS, mostly in genes related to the adaptive immune system [23-25]. A part of genes associated with MS are also associated with other autoimmune diseases, such as rheumatoid arthritis, psoriasis, and autoimmune thyroid disease [24]. The most important HLA risk allele (DRB1*1501) is not associated with other common autoimmune diseases. Thus, the DRB1*1501 allele might drive the CNS-specific autoimmunity, whereas the other SNPs associated with MS are probably more broadly connected with the regulation of the immune response. Many of the SNPs are associated with genes that are important for the function of the immune system [23]. On the other side, there is little overlap in genes between MS and primary neurodegenerative diseases [23].

Several infections have been suggested to be of importance in the etiology and pathogenesis of MS. However, none have repeatedly and convincingly been identified as a causative agent and MS is not a contagious disease. Nevertheless, there is evidence that human herpes- and retrovirus infection are involved in the development of MS [26]. Since MS is an autoimmune

disease, this process might be triggered by microorganisms in genetically susceptible individuals. Only the Epstein-Barr virus (EBV) is associated with increased MS risk [27]. Almost all MS patients (>99%) are EBV seropositive compared to general population (94%). MS risk is extremely low in seronegative individuals with OR 0.06 (CI 0.03-0.13). In addition, individuals with history of mononucleosis have increased MS risk with OR 2.3 (CI 1.7-3.0) [28].

Another factor that has been associated with increased risk for MS is lack of sun exposure and vitamin D. Low serum concentration of vitamin D is thought to modulate the differentiation of T lymphocytes and is related to an increased risk of MS [29, 30]. Higher latitude correlates with increased incidence and prevalence of MS [31]. This factor is related to sun exposure and vitamin D levels that might be protective. Sunlight leads to activation of vitamin D in the skin and it is difficult to know if it is the sunlight itself or if it is the vitamin D that exerts a protective effect. A recent study concluded that sun exposure and vitamin D status independently affect the risk of MS [32]. It could also be possible that lack of sunlight and vitamin D during childhood (or already during fetal life) may lead to later increased risk of MS. Although a reduction in lesion formation on MRI was noted during addition of vitamin D to INF beta [33], none of the randomized controlled trials demonstrated a significant reduction in relapse rate or EDSS in response to vitamin D supplementation and studies were not sufficiently powered to observe a clinical treatment effect. Therefore, we cannot conclude that vitamin D is a clinically effective treatment for MS patients. However, the effect of vitamin D supplementation in MS patients is still being investigated.

Several lifestyle factors relevant to MS risk that have been observed seem to have the greatest impact before the age of 20. It has been shown that both overweight [34] and night shift work [35] before the age of 20, but not when MS is already diagnosed, increase the risk of MS compared with population-based controls.

There is evidence that lifestyle and genetic risk factors both contribute to the disease risk. Both smoking and passive smoking are significant risk factors and the relative risk for MS development is approximately 1.5 for smokers compared to nonsmokers [36]. Interestingly, this risk is linked to the HLA type (DRB1 * 1501), and the disease risk is multiplied when they occur together [37-39]. A similar observation has been made in rheumatoid arthritis, but in connection with other HLA types. This shows that a common environmental factor (smoking) can contribute to the development of an autoimmune disease, but the genes (HLA) determine whether inflammation affects the joints or the nervous system. The mechanism for this is still unknown, but we can speculate that chemically reactive substances in tobacco smoke can modify our own proteins in the respiratory tract so that they become immunological reactive or otherwise contribute to the activation of immune cells. Continued smoking after the disease has broken out is

associated with a more serious disease course [36, 38]. In this context, it is important to note that non-smoking tobacco or nicotine replacement products were not associated with increased MS risk, which supports the fact that it is the smoke and not the nicotine itself that is disease-causing [40].

Pathology

The multiple focal areas of myelin loss accompanied by gliosis and axonal loss within the CNS, called plaques or lesions, appear as indurated areas, hence the term sclerosis. These plaques are the pathologic hallmark of MS [41-43]. The location of lesions in the CNS usually dictates the type of clinical deficit. The MS plaques consist of a variety of immunologic and pathologic features, including different degrees of inflammation, demyelination, remyelination and axonal injury [43, 44]. The immune system directly participates in the destruction of myelin and nervous cells [44]. The evolution of the individual lesion involves several stages: immune engagement; acute inflammatory injury of axons and glia; recovery of function and structural repair; post-inflammatory gliosis and neurodegeneration [45].

Inflammation is present in all lesion types and disease stages of MS, but its severity decreases with patient age and disease duration. Remyelination with the presence of newly formed myelin sheaths and oligodendrocyte precursor cells is frequently encountered within the active plaques of early MS. Axonal injury in MS is most pronounced during active inflammation and demyelination, and acute axonal injury occurring in early MS lesions contributes to the relapse-related disability observed predominantly during the inflammatory disease phases [43]. The presence of inflammatory cortical demyelination and meningeal inflammation is also common in the early disease stage. This contradicts a primary neurodegenerative process in the early stage of MS and suggests that neuronal and axonal injury in early cortical demyelination occurs as a result of inflammation. These lesions may drive the cortical demyelination and neurodegeneration in patients with both primary and secondary progressive MS [43]. The neurodegeneration in all demyelinated lesions is invariably associated with inflammation. In chronic inactive lesions from aging patients with long-standing progressive MS where the inflammatory process has died out, the neurodegeneration is also reduced to levels seen in control patients [43].

Pathologic characterization of MS lesions

The **acute MS plaque** inflammation is usually combined with demyelination and is typically characterized by myelin loss, infiltration of immune cells

and parenchymal edema [42]. The perivascular influx of immune cells includes lymphocytes (predominantly T cells), monocytes and macrophages (containing myelin debris). The degree of oligodendrocyte loss is variable, while the axonal injury is often extensive. Glial reactivity is present throughout the lesion but the glial scarring is not typical for acute plaque.

The **chronic plaque** is characterized by myelin loss and glial scarring. The temporal evolution progresses from chronic active plaque with the active destruction at the edge of the lesion to chronic silent plaque with absence of inflammation. The **chronic active plaque** is populated with activated microglia, macrophages and reactive astrocytes. The presence of antibodies and complement is more prominent in chronic active lesions. Areas of remyelination are present mostly at the edge of the lesion but can be present in the entire lesion. The **chronic silent plaque** is hypocellular, with little or no signs of inflammation, remyelination is uncommon and axonal density is low. The progression of the disease may be related to inflammation extending beyond focal lesions, including involvement of normal appearing white matter, as well as grey matter plaques [46]. **Grey matter plaques** are more common in progressive MS, but they can be developed early in the disease process. These lesions are characterized by infiltration with inflammatory cells and associated with neuronal loss and transected axons. These lesions are less inflammatory with fewer infiltrating T lymphocytes and microglia/macrophages and lacking the BBB breakdown. However, the meninges overlying the grey matter lesions contain B cell follicles [46, 47] and MS patients with ectopic B cells follicles had more rapid disease progression [47], supporting their role in the pathogenesis of these lesions.

Thus, MS lesions show pathologic heterogeneity with both inflammatory and neurodegenerative characteristics during all stages and there seems to be an association between inflammation and neuro-axonal injury [43, 48]. The degree of infiltration of T- and B cells correlated with lesion formation rate. Plasma cells infiltrates were more pronounced in patients with progressive MS but other inflammatory cells declined in older patients with long disease duration.

Pathophysiology

MS is considered as an autoimmune disorder and its exact cause is unknown. The perplexing issue of what allows the immune system to attack self tissues is a continuing focus of research. In MS, most of the CNS damage is a result of an abnormal immune-mediated response. This process includes innate immune system (macrophages, microglia, natural killer cells) and adaptive immune system (B- and T lymphocytes). Thus, both humoral

antibody-mediated and cell-mediated cytotoxic immunity are mounting the attack.

There are two major models that may explain the pathoetiology of MS [49]. The first model considers MS as a primary autoimmune disease with inflammation where autoreactive T cells cross the BBB and cause CNS damage. According to the second model, a primary axonal degeneration causes a secondary inflammation. However, there is a possibility that both processes take place simultaneously as we can see brain atrophy already in newly diagnosed patients, indicating early neurodegeneration. These early MS lesions are characterized by low-grade inflammation, including microglial activation. The grade of inflammation decreases while the degree of degeneration gradually increases as the disease evolves [50]. The causal relation between them is questionable. Although anti-inflammatory therapies have not been successful in progressive MS without signs of inflammatory activity, the early initiation of therapy might delay or even inhibit the onset of progressive disease. This indicates that the hypothesis regarding inflammatory-induced degeneration might be correct, at least in the relapsing-remitting phase of the disease [51, 52].

The role of the immune system in MS

Findings from animal models and immunological studies of patients with MS suggest a change in the involvement of the immune system during disease initiation and progression. Peripheral immune response targeting the CNS drives the disease process during the early phase, whereas immune reactions within the CNS dominate the progressive phase [53].

The role of the immune system and the major hypothesis in development of inflammatory and degenerative phases of MS disease are described below.

A) **The early inflammatory phase** (Figure 3)

Alternative 1 (Figure 3A):
CNS antigen-specific immune activation of autoreactive T cells occurs in the periphery (e.g. skin, intestines, lungs) and is transferred to the unaffected CNS. How T cells become abnormally activated towards CNS antigens remains unclear. Several infectious agents have been postulated to trigger the autoreactive T cells, mostly EBV [54, 55], together with other factors described in 'Etiology'. After migration to lymph nodes, a few of these antigen-specific T cells and B cells invade the CNS [56]. CD4+ T cells entering the perivascular space release cytokines locally and disturb oligodendroglial and astroglial homeostatsis [53]. Plasma cells accumulate in the brain and

release antibodies that target both the myelin sheath and glial cells. The release of inflammatory mediators will open the BBB and attract the influx of monocytes and additional lymphocytes, leading to formation of lesions. Most of the tissue damage during this phase is initiated by adaptive immune response and mediated by activated phagocytes (innate immunity).

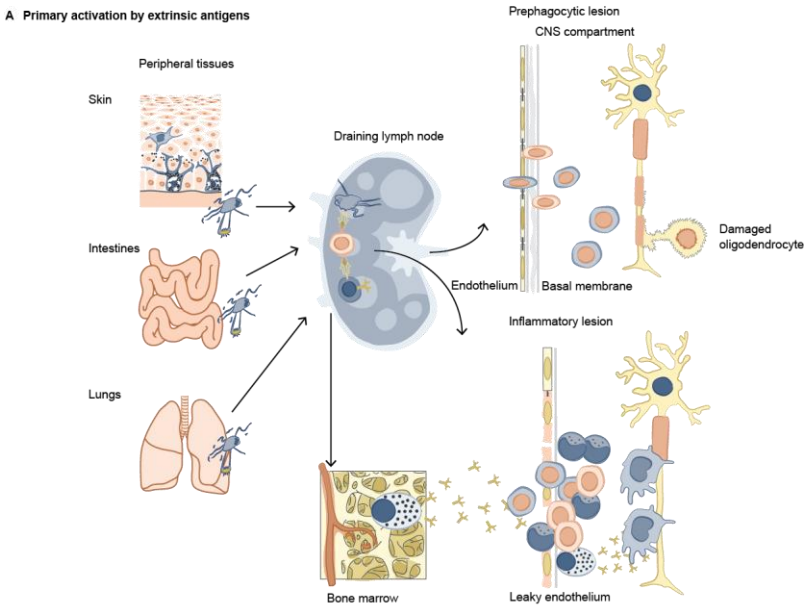
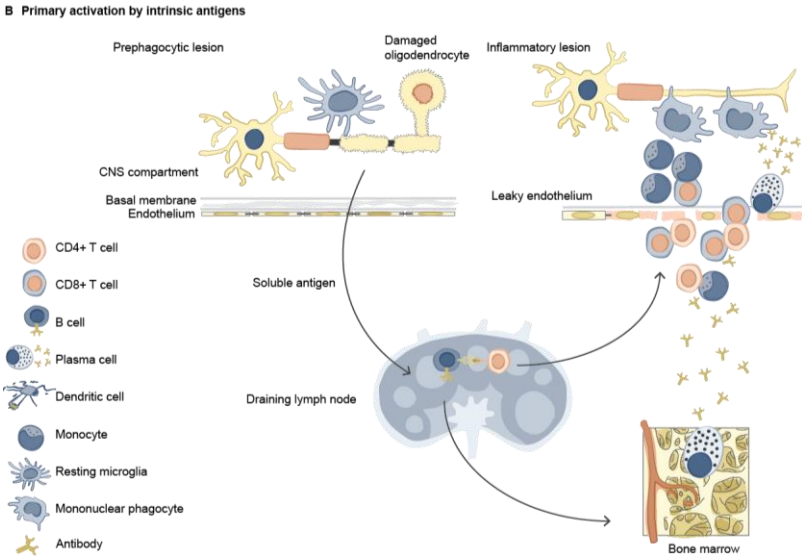


Figure 3. The primary neuroinflammation model

A: *Primary activation by extrinsic antigens.* Pathogens are processed in peripheral tissues (skin, intestines, and lungs) by dendritic cells. Dendritic cells migrate to draining lymph nodes and present these antigens to T cells. In the draining lymph nodes, B cells can also act as antigen-presenting cells after capture of soluble antigens by their B-cell receptor. As a result of interaction between B cells and T cells in a germinal centre reaction (an ordered process in which cells proliferate, undergo somatic hypermutation and class switch recombination), B cells proliferate and mature into antibody-secreting plasma cells that migrate to the bone marrow or inflamed tissue. Instructive cues for T-cell homing are produced by dendritic cells. In specific conditions, aberrant homing to the CNS can occur and a few activated T cells might invade the CNS compartment as pioneering cells (prephagocytic lesion). On reactivation with autoantigens, most likely in the perivascular space, T cells are able to invade the CNS parenchyma and create an inflammatory environment by secretion of cytokines. As a result, more immune cells (including monocytes and plasma cells that respond to chemoattractant factors produced in the developing lesion) are recruited and create a substantial inflammatory infiltrate (inflammatory lesion). Plasma cells that accumulate in the brain locally release antibodies that target the myelin sheath and glial cells, which might lead to dysfunction of these structures.



B: *Primary activation by intrinsic antigens.*Antigens are released from the CNS, in the absence of initial immune cell infiltrates (prephagocytic lesion), because of primary oligodendrocyte destruction. As a result of oligodendrocyte death, local microglial cells become activated. Although little evidence exists for active sampling of CNS antigens in the CNS parenchyma and export to draining lymph nodes by antigen-presenting cells, soluble CNS antigens can drain out of the CNS to deep cervical lymph nodes. Here, B cells might capture antigens via their receptor and present them to T cells. Whether B cells as antigen-presenting cells can prime naive T cells is still debated. However, several experimental scenarios suggest that T-cell priming by B cells is possible, in principle. Antigen-specific activation of T cells in draining lymph nodes results in an adaptive immune response that targets the CNS and is similar to that previously described (A).
In summary, in the first scenario (A), a pristine CNS would be targeted by an adaptive immune response. By contrast, in the second scenario (B), the homeostasis of the CNS would be intrinsically disturbed and thereby trigger an adaptive immune response that results in inflammatory demyelination.
Reprinted from The Lancet, Vol 14(4):411. Hemmer, B., M. Kerschensteiner and T. Korn, Role of the innate and adaptive immune responses in the course of multiple sclerosis. Copyright © 2015 Elsevier.

Alternative 2 (Figure 3B):
The initiation within the CNS causes activation of the resident microglia with secondary recruitment of adaptive and innate immune cells [56]. Thus, primary defect in oligodendrocytes (e.g. genetic) leads to their death and consecutive activation of microglial cells. How the initial oligodendrocytes damage is induced is unknown. The damage itself does not induce the autoimmunity against these cells. An alternative pathology of oligodendrocytes could result from infections that persist in them and evoke an adaptive immune response. However, no widespread infection of the CNS has been identified in MS patients [53]. Antigens migrate from the CNS into cervical lymph nodes to induce secondary adaptive immune response in the periph-

ery. The antigen presentation in the CNS and the transfer to lymph nodes is undertaken by dendritic cells. Primed T cells migrate to the CNS and produce cytokines there. However, this afferent pathway of the adaptive immune response is generally thought to be absent in the CNS. The glymphatic system might be an alternative antigen drainage way. The glymphatic system drains the CNS to the CSF and could serve as collector for waste antigens [57].

B) **The progressive phase of MS** (Figure 4)

Alternative 1:
According to the primary neurodegeneration model, damage to the axon-glial unit results in axonal degeneration and triggers a progressive neurodegenerative disease [50, 58]. Inflammation is a secondary response to tissue degeneration. These two arguments support this hypothesis: firstly, immunomodulatory and immunosuppressive intervention have mostly not changed the disease course of progressive MS [50]; secondly, the disability progress in progressive disease is independent of initial disease course and steady decline is reported in classic neurodegenerative disorders [59]. However, a primary axon-glial defect seems unlikely. No defect gene loci in MS patients essential in neuronal or glial function have been identified.

Alternative 2:
The compartmentalized inflammation drives the disease progress [48] and inflammation continues to damage the tissue in progressive MS, since markers of axonal injury correlate with immune cell infiltration in the lesions of patients with progressive MS as well. Furthermore, in older patients with progressive MS without active inflammation, the extend of axonal degeneration returns to the rate of normal aging [48]. Progressive MS might still be driven by inflammation, but is disconnected from systemic immune response. The inflammation would develop from focal accumulation of immune cells, T cells and monocytes influx, to more diffuse immune cell activation including microglia and B cells. Detection of B cells in inflammatory aggregates in the meninges of patients with progressive MS supports this hypothesis [60, 61]. The activation of microglial cells, with the presence of pro-inflammatory mediators and antibodies emerging from aggregates of meningeal cells would cause the axonal degeneneration.

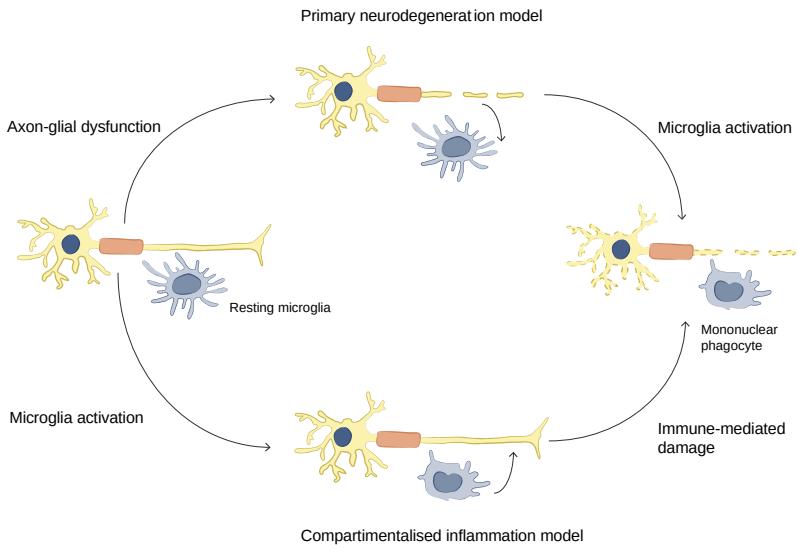


Figure 4. The primary neurodegeneration model
The acquired damage to the axon–glial unit results in axonal degeneration. This damage is caused by poor trophic support, metabolic disturbances, or a disturbed clearance of toxic (excitotoxic) mediators that result from pre-existing oligodendrocyte damage and myelin loss. Axonal degeneration would then cause a secondary activation of surrounding microglial cells. By contrast, the compartmentalised inflammation model suggests that the disease process is mainly driven by activation of microglial cells that could result from the continued presence of proinflammatory mediators and antibodies emerging from, for example, meningeal cell aggreates or from changes to the intrinsic state of microglia in response to prolonged inflammation. Activation of microglial cells would then cause axonal degeneration probably via the release of toxic mediators (eg, reactive species or glutamate)—the effects of which would be enhanced by pre-existing tissue damage that might restrict glutamate uptake and release iron. In both disease models, axonal degeneration would, in time, probably be followed by neuronal atrophy and possibly cell loss. These models are not mutually exclusive and might act synergistically to cause progressive axonal and ultimately neuronal degeneration.

The mechanism of inflammation and neurodegeneration in MS

Adaptive immune response by T lymphocytes is thought to mediate injury to myelin and neurons. How T cells become abnormally activated towards CNS antigens remains unclear. CD4+ T cells differentiate to several cell populations, including Th1, Th2, Th17 and T regulatory cells. In MS, there is a shift towards Th1 and Th17 cells and T regulatory cells with dysfunction that allow the inflammation to continue.

The BBB restricts the transport between blood and the CNS tissue. One of the earliest steps in lesion formation is the breakdown of the BBB. Enhanced expression of adhesion molecules on the surface of lymphocytes and macrophages seems to underlie the ability of these inflammatory cells to penetrate the BBB. Initially, leukocytes are rolling in the endothelium of the BBB. This is facilitated by upregulation of adhesion molecules located on endothelial cells, including VCAM-1. Out of a panel of leukocyte adhesion receptors, the α4 subunit of VLA-4 was identified as a crucial factor for T cell binding to CNS endothelium. Clinical trials of a humanized monoclonal antibody targeting the α4 subunit of VLA- 4, called natalizumab, also demonstrated efficacy in the treatment of MS [62]. The antibody blocks the α4 subunit of VLA-4 and interrupts the adhesion to its binding partner, VCAM-1. Hence, selective inhibition of specific adhesion molecules are effective at reducing leukocyte entry into the CNS. A multitude of adhesion molecules participate in effective leukocyte trafficking to and within the CNS and serve as potential targets for therapies in MS.

The trigger for the vascular changes in MS is unclear. Chemokines, a broad class of cytokines, mediating chemotaxis, also contribute to leukocyte migration to the CNS. The cytokines disrupt the BBB and allow the migration of immune cells between the endothelial cells into the CNS and eventually the process of inflammatory destruction of white matter takes place in MS [63]. In the CNS, other inflammatory cells are recruited, including CD8+ T cells, migcrolia and macrophages. The mechanism involved in axonal damage could be caused by CD8+ T cells via the release of cytotoxic granules and induction of apoptosis or direct transection of axons. Mononuclear phagocytes, such as microglia and macrophages, are the dominant immune cells located in the lesions in both RRMS and PrMS. These cells interact with cells of the adaptive immune system, but can also directly cause neuroinflammatory tissue damage. The phagocytes are mainly responsible for the myelin damage and removal of debris. The level of their activity in MS lesions can be staged by the presence of myelin degradation products in them [56].

In perivascular spaces bordering to active MS lesions, dendritic cells serve as APC at the BBB and contribute to the early inflammatory processes in MS [56]. Microglia found in active MS lesions serve as APC within the CNS. When activated microglia express greater amount of MHC II and other co-stimulatory molecules, thus promoting the pro-inflammatory response of T cells within the CNS [64]. Another APC involved in driving myelin-reactive CD4+ T cells in MS are B cells [65, 66]. However, not all interaction between APCs and T cells promote inflammation. For example, suppressor myeloid cells are capable of suppressing T cell function. In MS, the myeloid suppression is regulated by TREM-2, a trans-membrane signaling protein expressed by microglial cells, macrophages, monocytes and dendritic cells

[67]. This mechanism could be dysregulated by secretion of soluble TREM-2 which could prevent inhibitory function of transmembrane TREM-2 [67].

Except for the role as APC, B cell produces cytokines and B cell derived cytokines/chemokines were isolated from peripheral blood lymphocytes in MS patients [68]. Several chemokines and their receptors have been shown to influence B cell trafficking. Among them, CXCL13 play a central role and it is the most important determinant for B cell recruitment into the CNS. CXCL13 is increased in actively demyelinating MS lesions, secreted by macrophages in the perivascular cuffs but is not present in chronic inactive lesions.

The intrathecal production of immunoglobulins, which can be demonstrated by an OCB pattern on electrophoresis, detected in over 90% of MS patients [65], suggests an important role of humoral immune response (i.e. B-cell activation). The OCB are thought to be a product of clonally expanded B cells within the CNS and they target ubiquitous intracellular antigens released in cellular debris [69]. The OCB are characteristic for MS patients and they may persist over years [70]. The persistence of OCB indicates that the immunoglobulin forming B cells can survive in the brain over extended periods of time. The B cell survival is supported by astrocytes producing B cell growth factors including BAFF, CXCL10 and CXCL13, as well as by inflammatory activation enhancing their production. The role of CXCL13 in MS is supported by biomarker studies showing that the CSF concentration of CXCL13 is elevated in MS patients, correlates with conversion from CIS to definite MS, and is increased during relapse [71]. The presence of B cell follicles within the meninges, where CXLC13 is also found, confirms their roll in MS. There might be an association between the EBV infections and secondary lymphoid follicles in MS patients where the EBV-infected B cells are localized [72]. The role of B cells in MS pathogenesis is supported by the fact that anti-CD20 monoclonal antibody treatment, which deplete B cells, is effective in MS. B cells may promote inflammation in MS via direct and indirect effects on T cells, as B-cells are APC for T cells and this will modifiy secretion of pro-inflammatory cytokines.

These immune mediated responses leading to inflammation, with secretion of cytokines and antibodies, oxidative stress, mitochondrial dysfunction, ion channel dysfunction and inadequate regulatory function, cause damage to myelin, oligodendrocytes and axons (Figure 5) [73, 74]. The deterioration occurs early and continues throughout the entire course of MS and leads to brain and spinal cord atrophy and permanent disability. The inflammatory and degenerative responses appear to be closely intertwined, and might act synergistically [51]. As the biomarkers research indicates, the axonal injury in MS is related to inflammation-triggered neurodegeneration [75, 76]. Neurodegeneration contributes greatly to neurological disability in MS and is probably the dominant process in progressive MS. Whether neurodegeneration is an independent process in patients with MS or if its oc-

currence is secondary to inflammation remains unknown [77]. Although anti-inflammatory therapies are generally less effective in progressive MS than in RRMS, the early initiation of such therapies delay disability development [78] and might delay or blunt the conversion to progressive disease. Therefore, understanding neurodegeneration is fundamental in developing new therapeutic strategies, especially for progressive MS. Also, there is a need for sensitive biomarkers of neurodegeneration for measuring outcomes in clinical trials.

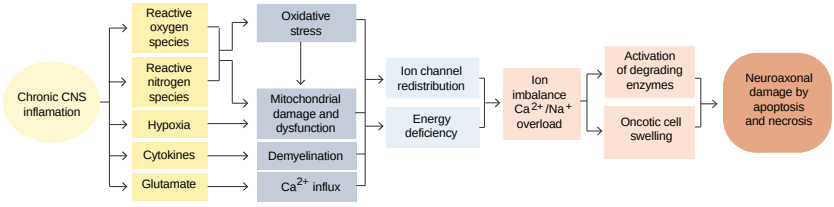


Figure 5. Cascades leading to inflammation-induced neuroaxonal injury
The scheme illustrates the prevailing hypothetical sequence of events eventually leading to neuroaxonal degeneration in multiple sclerosis. Chronic CNS inflammation lies at the root of deregulation of neuronal and axonal metabolism. The cascade culminates in the hallmarks of inflammation-induced neurodegeneration.
Adapted from Nature Reviews Neurology, Vol 10(4):225-238. Friese, M. A., B. Schattling and L. Fugger. Mechanisms of neurodegeneration and axonal dysfunction in multiple sclerosis. Copyright © 2014, Springer Nature

Treatment of MS

The pharmacological treatment of MS can be divided into relapse treatment, disease-modifying treatment and symptomatic treatment.

The **relapse treatment** ususally consist of methylprednisolone 1 g per day during 3 days. In cases of severe relapses that seem corticosteroid refractory, plasmapheresis may be considered. The shortterm effect of high dose methylprednisolone treatment improves symptoms and short term disability after an acute exacerbation [79]. However, there is no evidence that shows an effect of corticosteroid treatment on long term disability in patients with MS [79, 80]. Various symptoms of MS patients such as pain, spasticity, sleep disorders, sexual dysfunction or bowel and bladder dysfunction are treated with drugs against these disorders. With few exceptions the **symptomatic treatment** is not specifically approved for MS but is frequently used in several other conditions.

Disease modifying treatment

DMT can ameliorate the disease course and improve prognosis. In general, earlier initiation of DMT results in better outcomes (Figure 6). The first DMT for MS was approved more than 20 years ago. Over the years new therapies have evolved with different modes of action, efficacy, safety and adverse effects, administration and monitoring requirements. Their common feature is that they intervene with the immune system and reduce CNS inflammation in MS. The effect on progression of disability and atrophy rate of the brain and spinal cord are probably secondary to their anti-inflammatory effect. However, there are some therapies that show neuroprotective effects and improve regeneration in experimental animal models. Until recently, all approved DMTs are indicated for CIS or RRMS and the randomized controlled treatment trials in progressive MS have essentially been negative. However, recently ocrelizumab, an anti-CD20 antibody, has been approved for early and inflammatory active PPMS.

The DMTs can be divided into first-line and second-line therapies due to their efficacy and safety. The most common treatment strategy usually starts with a first-line DMT and the patient is monitored clinically and radiologically. In the case of disease activity, the therapy is switched to a second-line DMT with better efficacy. This **escalating treatment strategy** has the advantage of being safe but due to adverse events, inadequate tolerability and breakthrough disease activity many of the patients switch to other DMTs, usually the second-line DMTs. Thus, there is a risk of delaying effective treatment. However, in MS patients with high disease activity from onset, the treatment with second-line therapy could be initiated directly. The other major treatment strategy is **induction therapy**, where the treatment is

given once (AHSCT) or in short courses (alemtuzumab and cladribine). The immune system is extensively impaired for a limited time, allowing for a reconstitution of the immune sytem thereafter. Today, this strategy is mostly offered patients with aggressive RRMS. Over recent years, more effective DMTs have been used earlier in the course of MS and often also as the initial therapy.

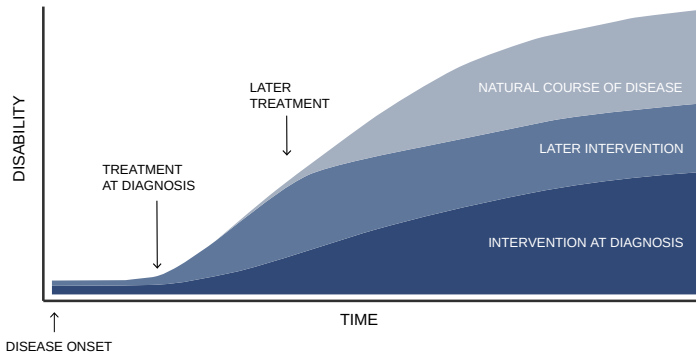


Figure 6. The window of therapeutic opportunity for disease modifying therapies
The disability progression with time depends on the time of the treatment initiation. The brightest colour represents the natural course of the disease. The darker colour represents delayed treatment intervention after the diagnosis. The darkest colour with the lowest rate of disability progression represents the treatment intervention at the disease onset.
Adapted from "Axonal pathology in multiple sclerosis: relationship to neurologic disability." Trapp et al., Curr Opin Neurol. 1999;12(3):295-302. "Neurodegeneration in Multiple Sclerosis: Relationship to Neurological Disability." Trapp et al., Neuroscientist. 1999;5:48-57. "Axonal transection in the lesions of multiple sclerosis." Trapp et al., N Engl J Med. 1998;338(5):278-85. "Early intervention with immunomodulatory agents in the treatment of multiple sclerosis." Jefferry et al., J Neurol Sci. 2002;197(1-2):1-8. "Therapy of relapsing multiple sclerosis. Treatment approaches for nonresponders." Cohen et al., J Neuroimmunol. 1999;98;29–36.

Currently used DMTs in Sweden and their modes of action are presented in Figure 7.

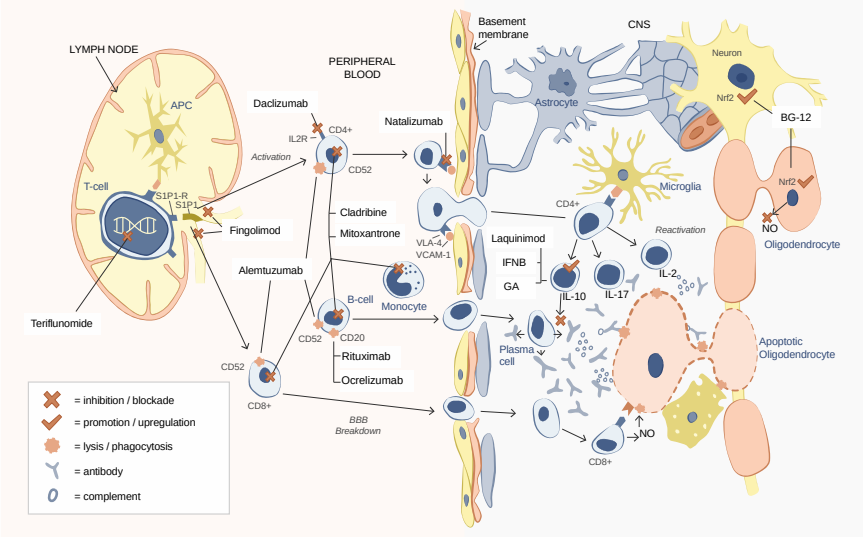


Figure 7. Modes of action of disease modifying drugs
Schematic representation of multiple sclerosis pathophysiology indicating points of treatment intervention. APC=antigen presenting cell, BBB=blood brain barrier, CNS=central nervous system, GA=glatiramer acetate, IFNB=interferon beta, IL2R=interleukin 2 receptor, NO=nitric oxide, Nrf2=nuclear factor (erythroid-derived 2)-like 2, S1P1=sphingosine-1-phosphate 1, S1P1-R=sphingosine-1-phosphate 1 receptor, VCAM-1=vascular cell adhesion molecule 1, VLA-4=very late antigen 4.
Adapted from "What Do Effective Treatments for Multiple Sclerosis Tell Us about the Molecular Mechanisms Involved in Pathogenesis? Buzzard at al., Int. J. Mol. Sci. 2012, 13(10), 12665-12709.

First-line DMTs and their modes of action

Interferon beta induces synthesis of immunomodulatory substances, production of anti-inflammatory cytokines, shift towards Th2 response.

Glatiramer acetate is a combination of four amino acids randomly polymerized into peptides causing shift to anti-inflammatory Th2 response.

Teriflunomide reduces the activity of the mitochondrial enzyme dihydroorotate dehydrogenase, which is crucial in pyrimidine synthesis and proliferation of T cells.

Dimethyl fumarate or **BG-12** activates the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) antioxidant response pathway. Its anti-inflammatory properties protect against oxidative stress-related neuronal death and myelin damage.

Second-line DMTs and their modes of action

Natalizumab is a human anti-α4-intergrin monoclonal antibody that inhibits leukocytes from entering into CNS through the BBB.

Fingolimod binds to sphingosine-1-phosphate receptors and blocks the exit of B cells and T cells from the lymph node.

Mitoxantrone is a synthetic anthracenedione derivative. It is an antineoplastic, immunomodulatory agent that affects B cells, T helper and cytotoxic cells, suppress and modulates the immune system.

Rituximab is a chimeric human/mouse anti-CD20 antibody that binds to the CD20 antigen on B cells and recruits the immune system to mediate cell death.

Ocrelizumab is a humanized anti-CD20 monoclonal antibody that binds to the same epitope as rituximab, the CD20 antigen in B cells, and mediate cell death of B cells with CD20 antigen.

Alemtuzumab is a humanized anti-CD52 monoclonal antibody that leads to depletion of circulating T cells and B cells.

Daclizumab is a humanized anti-CD25 monoclonal antibody that binds to the α subunit of the IL-2 receptor on T cells that are high-affinity receptors. The medium-affinity (consists of two β subunits CD122) receptors are not affected. The effect is a reduction of T cell response and expansion of CD56 bright natural killer cells.

Cladribine (2-chlorodeoxyadenosine) [81] is a synthetic chlorinated deoxyadenosine analog that is biologically active mostly in lymphocytes and provides targeted and sustained reduction of circulating T and B lymphocytes.

Although the primary effect of these DMTs is inhibition of inflammation, they seem to have some secondary neuroprotective effects, as they can restrict the clinical progression and the brain atrophy.

Neuroprotection aims at protecting the nervous tissue, with the ultimate goal of preventing neuro–axonal injury and loss. With the limited understanding of MS pathophysiology including the complex interactions between glia, immune cells and neurons, the key targets for neuroprotection remain elusive. The putative pathways for neuroprotection in MS include protection of neurons from direct injuries (e.g. excitotoxicity and oxidative stress), protection of oligodendrocytes that provide growth factors and important survival signals, regulation of other glial cells including microglia and oligodendrocytes, repair of the BBB, or boosting endogenous repair mechanisms [82]. The following groups of drugs have the potential to be directly neuroprotective in MS: sodium channel blockers, calcium channel blockers, glutamate antagonist, growth factors, nitric oxide blockers, sex hormones, phenols, statins, immunophilin ligands, peroxisome proliferator-activated receptor γ agonist, agents with pleiotropic effects.

Evaluation of MS

There are various ways to measure the disease. In this thesis we explored new biomarkers in relation to the conventional measures described below.

The disease activity can be measured clinically as a relapse, which consists of new or worsening signs and symptoms caused by inflammation in the CNS. These episodes develop quickly, last at least 24 hours and can continue for several days to weeks. Relapses can be followed by a full recovery or by a partial recovery with some lasting disability. The average number of relapses during one year in a group of patients in a clinical study, is defined as annual relapse rate.

The radiological disease activity is measured by MRI, as the number of contrast-enhancing T1 lesions or as a number of new and/or enlarging T2 lesions. The contrast-enhancing T1 lesions, that appear bright on an MRI scan after administration of an intravenous imaging contrast agent (gadolinium), are a sign of active inflammation since the enhancement in average remains for 3 weeks. The hyperintense T2 lesions appear bright and can be tracked over time to measure MS progression. T2 lesions are both active and chronic and their age cannot be determined in these images. Further, hypointense T1 lesions show sites of significant tissue injury and nerve cell death, referred to as "black holes" due to their appearance on an MRI scan, and they are usually a sign of irreversible damage.

The disability can be defined as the loss of abilities that results from damage to the CNS and may be irreversible. Expanded Disability Status Scale (EDSS) [83] and Multiple Sclerosis Severity Score (MSSS) [84] are the most common scoring instruments to evaluate the disability in MS.

EDSS is a clinician-administered assessment scale scoring the neurological function, based on the evaluation of seven functional systems of the CNS (visual, brainstem, pyramidal, cerebellar, sensory, bladder and bowel, cognitive and walking ability). It consists of an ordinal rating system from 0 (normal neurological status) to 10 (death due to MS) in 0.5 increment intervals (when reaching EDSS 1). The lower scale values of the EDSS (0-4.0) measure impairments in the functional systems based on the neurological examination, while the values above EDSS 4.0 are mainly based on the walking distance, and the values above EDSS 6 are based on the need of assistance (unilateral, bilateral or wheelchair). The EDSS measurements have limited inter-rater and intra-rater reliability, and show greater variability for lower EDSS scores (1.0-3.5) than for higher score values [85]. The increase of EDSS is not linear, thus a difference between the values 1.0 and 2.0 is caused by a much smaller neurological deficit than an increase from 6.0 to 7.0.

The EDSS and its predecessor DSS have been used in nearly every MS clinical trial in the last 40 years. The changes in EDSS can be defined as confirmed disability progression or confirmed disability improvement and

these two measures are commonly used surrogate endpoints in MS trials. **Confirmed disability progression** is defined as an increase in EDSS score (by 1.0, if EDSS score was 0-5.5 at baseline, or by 0.5 if EDSS score was ≥6.0 at baseline), that is sustained over a pre-determined time period of usually 3 or 6 months. Participants with SPMS are more likely to worsen than to improve. Participants with RRMS, however, were equally likely to have sustained improvement as sustained worsening. Even if worsening was defined as a 1.0 point increase in the EDSS sustained for 6 months, there was no significant difference in the frequency of worsening vs improvement. The definitions of disability progression used in clinical trials were suggested to be inadequate [86]. Therefore, an additional measure in clinical trials was defined. **Confirmed disability improvement** is defined as a decrease in EDSS score of at least 1 point for all patients, regardless of baseline EDSS score, with the decrease in disability sustained for ≥12 weeks and the absence of a relapse. This measure is sensitive to treatment effects in clinical trials over 2 years [87].

MSSS determines the severity of the disease by combining the grade of neurological disability expressed by EDSS and disease duration [84]. In clinical trials, the use of other measurements as the MSFC is recommended to provide information on dimensions not covered in the EDSS, such as upper limb function or cognitive skills.

Multiple sclerosis functional composite (**MSFC)** [88] is a combined measure of three separate disability assessments that was first introduced in the late 1990s. The MSFC is calculated based on leg, hand and cognitive function. **Timed 25-Foot Walk (T25-FW)** determines walking speed by measuring how fast a patient can walk 25 feet. **Nine-Hole Peg Test (9-HPT)** measures arm, wrist and hand function by timing the speed in which a patient can move nine pegs into nine holes and then remove them, using one hand at a time. **Paced Auditory Serial Addition Test (PASAT)** measures cognitive function by testing thinking speed and calculation ability.

To briefly examine the cognition in MS, **SDMT** together with PASAT are suggested as part of the Brief Cognitive Assessment in MS (BICAMS) [89]. SDMT is considered a robust cognitive test for visuo-spatial ability and processing speed not affected by age, education, gender and socioeconomic status [90] and proposed as a single screening cognitive assessment tool in patients with MS [89]. However, a drawback with SDMT is that repeated testing with SDMT has a practice effect that may affect the test result [91].

The goal of treatment with DMT is No Evidence of Disease Activity (**NEDA**) [92]. NEDA is defined as absence of new and/or enlarging T2 lesions, absence of contrast-enhancing lesions, absence of confirmed disability progression, and absence of relapses [93]. Even if fulfilling NEDA, a proportion of patients still may have a disease activity that may not be revealed. Thus, additional measures have been proposed, e.g. brain atrophy or

biochemical biomarkers that may contribute to NEDA for accessing disease-free status and might improve the assessment of disease activity in MS [94].

There are many aspects of the disease that are not captured and cannot be evaluated by currently used clinical and MRI measures. Therefore, there is an unmet need for new biomarkers to measure different aspects of the disease.

Biomarkers

A biomarker is defined as a 'characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention' [95]. The ideal biomarker is strongly associated with the disease and absent in healthy individuals; it is specific for one disease and is absent in other diseases; it captures a biological important aspect of the disease; the concentration of the biomarker reflects the severity of the disease and can predict the prognosis; the effect of the therapy reflects the change in the concentration of the biomarker [96, 97].

In heterogenous diseases as MS, biomarkers may contribute to characterizing patients, the pathophysiological pattern of the disease, and for evaluating therapeutic response [98, 99]. Various biomarkers in blood and CSF have been explored, but few of them are validated for use in clinical practice [96]. Additionally, biomarkers can be used as surrogate endpoints in clinical trials, i.e. biomarkers substitute for the clinical endpoint. The complete process of biomarker development usually takes more than 20 years (Figure 8) [100].

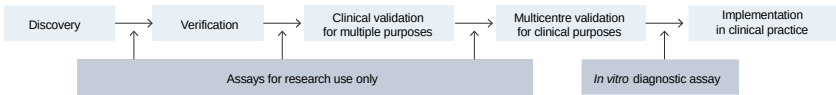


Figure 8. Schematic representation of the process of biomarker development
The light blue boxes indicate the steps to be followed, dark blue boxes indicate the type (and quality) of assays that are typically used. The first three steps can be performed rapidly (in a couple of years), but the complete process to clinical implementation usually takes >20 years.

Categorization

The biomarkers used in MS can be categorized according to their function in clinical use (I) or according to their function reflecting the pathophysiology of the disease (II).

I) **Predictive biomarkers** are used to identify individuals who are at risk of developing the disease, e.g. relatives to MS patients or asymptomatic individuals with signs of MS on MRI. They may also predict severity of the disease and conversion between phenotypes such as CIS to RRMS or RRMS to SPMS.
Diagnostic biomarkers are used to distinguish healthy individuals from individuals with a disease and they are used to set a diagnosis e.g. in patients seeking for clinical symptoms indicative of a demyelinating disorder. They can improve the sensitivity and specificity of the diagnostics by identifying individuals with MS and certainly distinguish them from patients with other neurological diseases [96].
Disease activity biomarkers can reflect various pathophysiological processes of the disease and they can help to distinguish patients with mild from more aggressive disease. This may facilitate treatment decision-making. Also, they can help to identify the different stages of the disease, e.g. identify patients with transition to the progressive disease phase or to distinguish which disease processes are most prominent (e.g. inflammatory or neurodegenerative) at different timepoints [96].
Treatment response biomarkers can reflect the efficacy of a treatment or identify patients who are at risk of treatment failure or at risk of adverse drug reaction. They can also be used to guide the treatment decision [96].

II) **Disease related pathophysiological mechanisms:**
Immunological activation/dysfunction;
Disruption of BBB;
Demyelination;
Glial activation/dysfuction;
Neuroaxonal damage;
Oxidative stress;
Remyelination and repair.

In this thesis, diagnostic biomarkers, the biomarkers of disease activity and the therapeutic efficacy biomarkers are explored. Further, their relationship to various pathophysiological mechanisms is described.

Body fluid biomarkers

It is advantageous to study the biomarkers that reflect pathological processes of MS in CSF rather than in blood due to its proximity to the CNS. The CSF is produced by the choroid plexus, brain interstitium, and meninges. It circulates in a craniocaudal direction and is exchanged 3-5 times daily. The vascular system is separated from the CSF by the blood-CSF barrier (BCB), and from the brain by the BBB. The function of the barriers is to protect the brain from toxic and pathogenic agents, and their disruption plays a key role in a number of CNS disorders, including MS. Recently, the glymphatic system was decribed [57], facilitating the clearance of interstitial solutes from brain parenchyma. Both aging and neuroinflammation are associated with enduring reactive gliosis, which may impair the glymphatic pathway function, slowing the clearance of toxic metabolites from the brain and facilitating the development of neurodegeneration [57].

The CSF consists of mainly (80%) blood-derived molecules, the rest consists of brain-derived and intrathecally produced molecules. The transport of the molecules across the BBB and BCB occur by passive diffusion (e.g. albumin, immunoglobulins – thus the barriers are permeable for both small molecules and macromolecules) or is facilitated by active transport (e.g. glucose). The transport is mainly regulated by aquaporin-4 channels, which are abundantly located at the blood-brain and brain-CSF interfaces. There is a specific CSF/blood ratio of blood-derived molecules that could be changed during pathological processes.

The composition of the CSF is not constant and the levels of various proteins differ due to several factors. These factors are the BCB and BBB function, site of sampling (ventricular vs lumbar), the diurnal fluctuations, molecular size of blood-derived molecules (e.g. IgM vs. albumin) and circadian rhythm (e.g. glucose). In MS, the CSF is collected via LP and alterations of lumbar CSF are mainly influenced by processes bordering to the ventricular and spinal CSF space and less by processes in cortical areas remote from the ventricles [101].

To minimize the preanalytical factors, to be able to replicate the studies and exchange the samples, a consensus protocol for the standardization of CSF collection and biobanking was etablished [102]. It recommends to withdraw at least 12 mL of CSF by performing the LP between the vertebra body L3-L5, to discard the samples with blood-contamination, to use the polypropylene tubes with screw cap, and to note the time and date of withdrawal. Further, the other body fluids should be collected simultaneously, the samples should be stored at room temperature before, during and after spinning, and the samples (CSF together with blood) should ideally be frozen within 1 hour in at least 2 aliquots with minimum 0.1 mL volume and stored at -80∘C. Afterwards, the controls have been defined to make them comparable [103] and the guidelines for uniform reporting of body fluid

biomarker studies in neurologic disorders were established to improve the quality of biomarker research [104].

Caution should be made when measuring the treatment effect of DMTs in MS. Biomarkers that correlate with treatment effect can provide misleading information about the efficacy, if these biomarkers are not involved in pathogenic pathways of the disease [105]. Likewise, analyzing biomarkers in blood when examining CNS diseases can be inaccurate. The blood biomarker concentration may reflect its release from peripheral tissues if the studied biomarker is not CNS-specific. Hence, the inflammatory biomarkers relevant for MS may also be affected by inflammatory process in the periphery. Therefore, when exploring biomarkers for MS, it is more appropriate to choose a CNS-specific substance to be sure that the analysis reflects the mechanism of the disease. Otherwise, we might measure the impact on the systemic immune system caused by DMTs, but not their real effect on the pathological processes in the CNS.

Biomarkers investigated in this thesis

Previous investigations of CSF biomarkers revealed altered profiles of the proteins that reflect different aspects of the pathophysiology of MS. In this thesis, we explored the significance of the following protein biomarkers for MS presented in Figure 9.

The studied biomakers are divided into two groups: biomarkers of inflammation and biomarkers of neurodegeneration. Their function, predictive value, association to clinical course, relation to disease activity, response to treatment and correlation to other biomarkers are described separately for each biomarker.

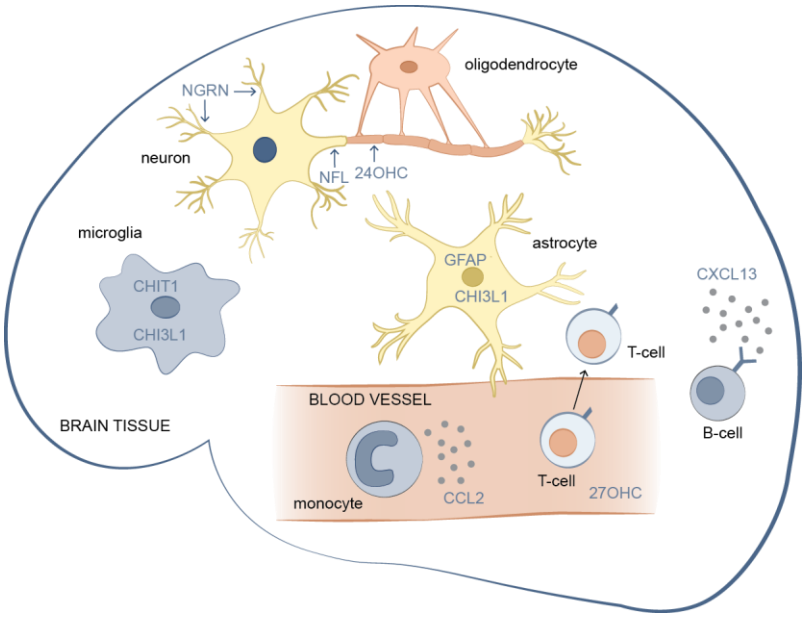


Figure 9. Cells producing the studied biochemical biomarkers
The figure shows the brain and the cells producing various biomarkers. Neuronal axons produce neurofilament; neuronal dendrites produce neurogranin; oligodendrocytes and cell membranes of neurons produce 24S-hydroxycholesterol; astrocytes produce glial fibrillary acidic protein and chitinase-3-like-1; microglia cells produce chitinase-3-like-1 and chitotriosidase; B cells produce C-X-C motif chemokine 13. CCL2 is produced by many cell types, including endothelial, fibroblasts, epithelial, smooth muscle, mesangial, astrocytic, monocytic, and microglial cells. Almost all cells of the body can synthesize 27OHC and its CSF levels correlate with the corresponding levels in the circulation.
CCL2=C-C motif chemokine ligand 2, CHIT1=chitotriosidase, CHIT3L1=chitinase-3-like-1, CXCL13=C-X-C motif chemokine 13, GFAP=glial fibrillary acidic protein, NFL=neurofilament light, NGRN=neurogranin, 24OHC=24S-hydroxycholesterol, 27OHC=27-hydroxycholesterol

Biomarkers of inflammation

The biomarkers of inflammation include biomarkers reflecting immunological activation or dysfuction and biomarkers reflecting disruption of the BBB.

Chitinase-3-like protein 1 (CHI3L1, also known as YKL-40) and **chitinase-3-like protein 2 (CHI3L2)** are expressed by microglia, macrophages, epithelial cells, and astrocytes and are involved in the pathogenesis of chronic autoimmune disorders. Both are most abundantly associated with astrocytes and microglial cells in inflammatory MS plaques [106].

CHI3L1 is a prognostic marker of conversion from CIS to MS and of disability progression in MS. High CSF levels of CHI3L1, together with NFL, have

been associated with shorter time to conversion from CIS to MS [107-109]. In patients with ON, increased CSF levels of CHI3L1 predicted cognitive disability, and combined with age, CHI3L1 predicted conversion to MS [110].The CSF CHI3L1/CHI3L2 ratio discriminated PrMS from RRMS, with lower CHI3L2 levels in PrMS [109]. CSF CHI3L1 was increased in PrMS and RRMS with active disease [111-113], and correlated with the number of Gd-enhancing lesions [111]. However, the levels in RRMS in remission were similar to HC [111] or increased compared to HC [112]. Plasma CHI3L1 was increased in PrMS but there was no difference was found between RRMS in relapse and remission [114]. The CSF levels of CHI3L1 were decreased by treatment with mitoxantrone or natalizumab [112], and with daclizumab [115]. Serum CHI3L1 was suggested to have a role as response biomarker to IFN beta in RRMS [116]. However, serum CHI3L1 levels were increased in GA but unchanged in INF beta treated patients and further analysis showed that CHI3L1 was increased in the INF beta non-responder group, and mainly unchanged in the responder group. CSF CHI3L1 is associated with other biomarkers of leukocyte infiltration (e.g. CXCL13) and with neurodegenerative biomarkers (NFL) [117]. CSF CHI3L1 correlated with NFL in CIS patients and with GFAP in RRMS patients [118].

Chitotriosidase (also known as chitinase 1, CHIT1), is a member of the chitinase family, secreted by activated macrophages. In MS, it is a marker of microglial activation, and thus represent a responder of innate immunity [119, 120]. Elevated CHIT1 levels were associated with conversion to MS in patients with CIS [121]. CSF CHIT1 was elevated in MS patients compared to HC and other neurological patients. CHIT1 activity in blood significantly associated with the MS clinical course (higher in SPMS compared to RRMS, $p=0.01$) and EDDS ($p<0.001$). CSF/plasma CHIT1 ratio predicted clinical deterioration ($r=0.91$; $p<0.001$) [122]. The CHIT1 index corrected for BBB function was elevated in MS patients with increased mononuclear cells and OCB in CSF compared to those with normal CSF [120]. In another study, no association between plasma CHIT1 activity and MS clinical course or clinical response to IFN beta treatment was found [123]. Treatment with natalizumab reduced CHIT1 levels [119]. Thus, CHIT1 may be useful in clinical trials where microglia is targeted or as a safety biomarker in trials where the brain serves as a bystander organ [119]. In addition, CHIT1 levels were found to correlate with MBP levels in CSF and can be a biomarker of demyelination or phagocytic activity [124].

Chemokines are members of the chemoattractant cytokine family. They play key roles in the trafficking of leukocytes.

C-X-C motif chemokine 13 (CXCL13) is a B-cell chemokine. The concentration of CXCL13 in CSF is increased in patients with active RRMS and in patients converting from CIS to MS [125]. CSF CXCL13 levels $\geq$15.4 pg/ml showed a good positive predictive value and specificity for a MS diagnosis and for a clinical relapse within one year from onset [126]. The CSF CXCL13

levels were increased in RRMS and PPMS compared to controls [127]. Natalizumab treatment of RRMS reduced the CSF CXCL13 levels [71] and a reduction of the CXCL13 levels was also found in PrMS after treatment with mitoxantrone or rituximab [128]. CSF CXCL13 levels correlated with CSF cell count, total protein levels, IgG Index and the presence of CSF IgG OCB and IgM OCB.

C-C motif chemokine ligand 2 (CCL2), also known as **monocyte chemoattractant protein-1 (MCP-1**), is an important chemokine for the recruitment of monocytes/macrophages and T cells to the CNS. CCL2 and its receptor CCR2 have been shown to be involved in various neurodegenerative disorders including Alzheimer's disease, MS, and ischemic brain injury [129], and to have a pathogenic role in experimental autoimmune encephalomyelitis [130]. CCL2 levels are consistently decreased in the CSF of patients with chronic neuroinflammatory conditions, despite abundant expression within MS lesions. CCL2 is consumed by migrating inflammatory cells, which down regulate CCR2, as they cross the BBB [130]. CCL2 levels were decreased in CSF and serum of all MS patients [131] with the lowest CSF levels during acute relapses in RRMS [132-134]. The CCL2 response to DMT showed contrary results. The CSF concentration of CCL2 increased after relapse or acute ON treated with methylprednisolone [135, 136]. Serum CCL2 levels were higher in MS patients treated with IFN beta than in HC, particularly among those patients with a stable form of the disease [137]. However, in another study CSF CCL2 levels were not modified by methylprednisolone or IFN beta therapy [133]. CSF levels of CCL2 correlated with intrathecal IgG production [132, 135] and the CSF level of NFL [132].

Biomarkers of neurodegeneration

Glial fibrillary acidic protein (GFAP) is expressed by astrocytes. The concentration of GFAP in the CSF is thought to reflect astrogliosis or glial activation/dysfunction as it was isolated from MS lesions with severe astrocytosis [138]. Increased concentrations of GFAP have been associated with the progression of disability in MS with the highest concentration in patients with SPMS [75, 108, 139, 140]. GFAP showed no relation to relapse [140]. The treatment with natalizumab in RRMS [141] and the treatment with mitoxantrone or rituximab in PrMS [128] did not influenced the CSF GFAP levels.

Neurofilaments are structural components of myelinated axons that are composed of three main subunits, known as light (NFL), medium, and heavy (NFH), depending on their migration on polyacrylamide gel electrophoresis. There are more proteins in the neurofilament family, e.g. alpha-internexin and peripherin. Neurofilaments are released into the CSF after axonal injury [142] in diverse neurological disorders, including MS [143]. A number of

important studies were conducted with the pioneering work led from Sweden [76, 142, 144] and research groups from around the world have verified the main results.

The neurofilament light (NFL) protein is one of the most studied biomarkers of disease activity and treatment response in patients with MS. The CSF NFL concentration at disease onset may predict disease severity and conversion from CIS to MS [108, 110, 145-148]. NFL levels are increased in the CSF during all stages of MS [75, 149], with the highest levels following acute relapses [75, 76, 149], during contrast enhancing lesions [128, 141] or new T2 lesions [147] on MRI. The NFL concentration is decreased by effective treatment with DMTs. CSF NFL was decreased after treatment with fingolimod [150] and natalizumab [141] in RRMS and after treatment with mitoxantrone or rituximab in PrMS [128]. The NFL was suggested to be a better biomarker for monitoring treatment effects than NFH [151].

Serum NFL correlated with CSF NFL [152-155]. Increased levels were found during the relapse and correlated with EDSS, brain atrophy, lesion volume and the occurrence of contrast enhancing lesions [153, 155-157]. The NFL levels in blood were decreased after treatment with IFN-beta 1a [157] and with fingolimod [155].

Neurogranin (NGRN) is a postsynaptic protein enriched in dendritic spines. Elevated levels of NGRN have been found in subjects with mild cognitive impairment and Alzheimer's disease [158, 159]. In CSF, NGRN is a marker of synaptic integrity and its levels are influenced by neurodegeneration [160]. This biomarker has not been studied in MS.

Tau is a protein that binds to microtubules, stabilize them and take part in regulation of the axonal transport [161]. Tau is concentrated predominantly in neuronal axons [162], with a gradient of tau along the axon with the highest level around the synapse [163]. Tau function depends on its phosphorylation state that changes its conformation. This can cause decreased binding to microtubules and may contribute to the collapse of cytoskeleton, synaptic transmission and trophic support in neurons. These processes are important in tau-mediated neurodegeneration [161, 164]. The CSF tau concentrations were increased in MS compared to HC [165-169], but no significant difference among different subtypes of MS could be detected, although highest levels were found in very early disease stages [166]. Increased tau levels predicted conversion from CIS to MS [167, 170]. Tau levels correlated with EDSS, the number of T2 lesions [167] and were increased in MS patients with Gd-enhancing lesions and in those with intrathecal IgG synthesis, supporting the notion that axonal damage is influenced by inflammatory activity [166]. Combination of MRI results and CSF tau concentration improved predicting conversion from CIS to MS [166]. In patients with RRMS, CSF tau was a marker of poor outcome in patients with RRMS; increased CSF tau levels were correlated to the disability, shorter time to next relapse and increase in EDSS [171]. Other studies have not

been able to confirm tau as a biomarker in MS [108, 172-174]. Tau concentrations were not a useful marker of mitoxantrone treatment response [169] and tau has not been studied in other DMTs.

Oxysterols

24S-hydroxycholesterol (24OHC, cerebrosterol) and **27-hydroxycholesterol (27OHC)** are the most abundant cholesterol oxidation products. **24OHC** is a brain-specific cholesterol hydroxylation product that passes freely over the BBB [175]. Almost all 24OHC in blood circulation originates from the brain [175-177] and has been suggested as a potential biomarker for neurodegeneration [178, 179]. In contrast, almost all cells of the body can synthesize **27OHC**. Its CSF levels correlate with the corresponding levels in the circulation [180] and the flux into the brain is to some extent dependent upon the integrity of the BBB [180].

Imaging biomarkers

The most commonly used imaging biomarkers of disease activity are the number of new and/or enlarged T2 lesions and T1 lesions with contrast enhancement. The disease activity measured by MRI is much higher than the relapse rate. For every relapse the MRI will show approximately 10 new lesions [181, 182]. The magnitude of MS disease is often measured by T1- and T2 lesion volumes but degeneration is reflected better by measuring the brain and spinal cord atrophy. Atrophy measurements such as brain parenchymal fraction (BPF) on T1 weighted images better correlate with disability progression and treatment effects on disability [183]. However, so far volume measurements that are clinically relevant and could offer an in vivo measure of neurodegeneration have not been accessible in the clinical practice of MS. In this thesis, we explored brain atrophy and retinal nerve fibre layer (RNFL) as imaging biomarkers.

Brain atrophy

Generally, brain volume decreases by 0.7-1.0% per year in MS patients, i.e. three times faster compared to healthy individuals [184, 185]. Significant reduction of brain volume is demonstrable already early in the disease and even at the clinical onset of MS and is influenced by DMTs [185, 186]. Brain atrophy had emerged as a measure of disease progression in MS [187], and was shown to be present during all stages of MS and in all types of disease course [188, 189] with the similar rate of brain volume loss [190]. Especially, grey matter loss occurred within the first year after CIS onset and brain volume loss predicted conversion to MS [191-193]. Brain atrophy was asso-

ciated with disability in MS independent of the amount of inflammatory lesions [194] and was also associated with cognitive impairment and fatigue. It showed a prognostic value, as the degree of brain atrophy was associated with subsequent increase of disability [194]. Patients with early RRMS had significantly lower subcortical deep grey matter but not cortical grey matter volumes compared to patients with CIS. This subcortical atrophy developed during the first 4 years of the RRMS. Thus, grey matter atrophy is relevant for disease progression from the earliest clinical stages [195].

The presence and degree of neurodegeneration in early MS is inconsistent in various studies due to heterogeneity existing between different methods for the detection of brain atrophy and inclusion of different MS populations. Brain atrophy rates were greater in CIS and RRMS subjects than in controls and greater atrophy rates were observed in CIS patients who developed clinically definite MS compared to subjects who did not [196]. In another study, there was no significant whole brain atrophy in CIS at clinical presentation compared to controls and no significant difference in the whole normalized grey matter volume between CIS and controls. Only specific areas showed significant atrophy, including areas of bilateral thalamic, hypothalamic, putamen and caudate atrophy [197]. In previous studies, the patients with CIS had higher number of lesions on MRI [193, 195] than in subsequent studies, which might be an effect of the revised diagnostic criteria of MS [15]. With the new diagnostic criteria, MS is diagnosed at the earliest stages and this could affect the degree of neurodegeneration present at the diagnosis.

The retinal nerve fibre layer

RNFL is the innermost layer of the retina and consists of unmyelinated axons of the retinal ganglion cells that acquire myelin sheath after leaving the eye (Figure 10). Over recent years, it has become clear that RNFL correlates with several clinical and MRI outcomes in MS [198-200], e.g. with brain atrohy/BPF [201-203]. Peripapillary RNFL thinning was associated with an increased risk of clinical deterioration [204]. However, one study did not show any change in peripapillary RNFL between newly diagnosed MS and HC [205]. Thinning of RNFL correlated with disability progression in newly diagnosed patients and in patients with early RRMS [206]. Thus, determining the thickness of RNFL may reflect the general evolution of neurodegeneration in MS and could be useful for assessing neurodegeneration [201].

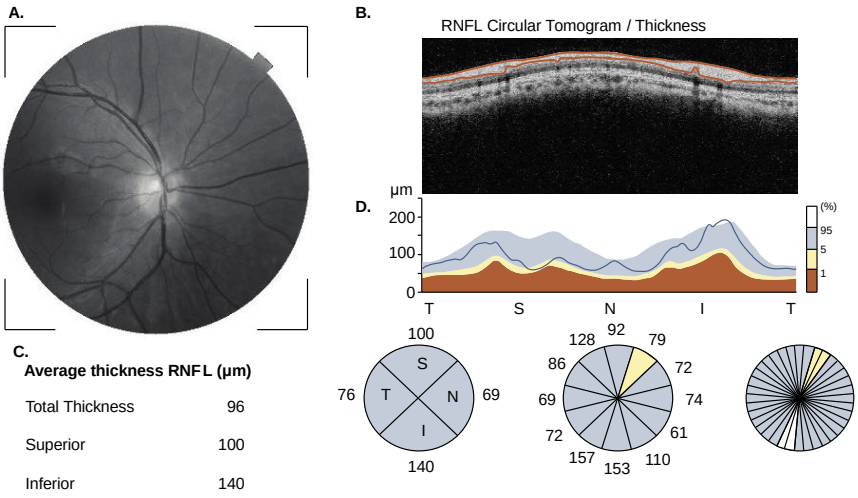


Figure 10. OCT report showing the RNFL measurements
A.Retina with the papilla in the center. B. The optical tomography image with the retinal layers, RNFL thickness is marked with brown colour. C. Average RNFL thickness in different region of the retina. D. The graph representing absolute RNLF thickness in µm and relative thickness compared to reference population in %. The blue line represents the absolute RNFL thickness of the examinated subject, light blue colour represents the thickness within the normal range between 5th and 95th percentile of reference population, yellow colour represents the thickness in the range between 1st and 5th percentile, brown colour represents the thickness below 1st percentile, white colour represents the thickness above 95th percentile.
RNFL=retinal nerve fibre layer, S=superior, I=inferior, T=temporal, N=nasal

NEDA was associated with steady RNFL thickness and patients with clinical evidence of disease activity had increased rate of axonal retinal atrophy independently from brain MRI lesions [207]. The neurodegenerative biomarkers measure an important part of the disease and have been proposed as an addition to the definition of NEDA [208] and to the measurement of disease-free status [94].

Acute optic neuritis (ON) is a common early onset symptom of MS occurring in approximately 20-25% of cases, and optic nerve disease affects the majority of patients with MS at some point during the course of the disease. ON causes reduced RNFL thickness [201] as a consequence of axonal transection with subsequent retrograde degeneration of axons [198]. Even the unaffected eye showed significant thinning of the RNFL compared with HC. The loss of visual function in MS patients without ON is an example of neurodegeneration in the absence of local inflammation [209]. The estimated annual decrease in RNFL thickness in MS patients with no history of ON is

approximately 0.5-2 μm compared with 0.1 μm in healthy controls [210, 211]. Interestingly, retinal loss was found in classical neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease.

Methods for measuring biomarkers

Enzyme-linked immunosorbent assay (ELISA)

ELISA is a method that uses antibodies and color change to identify a substance. Seven of the studied biomarkers in the CSF were analysed by a type of ELISA called sandwich ELISA, either in-house or commercial ELISA.

A sandwich immunoassay is a method using two antibodies, which bind to different sites on the antigen. In this thesis, the antigen was the studied biomarker (Figure 11). The antibody coated well is a solid surface containing the capture antibody, which is highly specific for the antigen. The analyte (CSF or blood) containing the antigen (biomarker) is added, followed by addition of a second antibody referred to as the detection antibody. The detection antibody binds the antigen at a different epitope than the capture antibody. As a result, the antigen (biomarker) is captured between the capture and the detection antibody as in a sandwich. The immunoassay sensitivity is determined by the binding affinity of the antigen to the antibody. As the antigen concentration increases, the amount of detection antibody increases, leading to a higher measured response.

To quantify the amount of bound antibodies, different reporters can be used. These reporters (i.e. enzyme, fluorophore, or biotin) can be directly attached to the detection antibody or to a secondary detection antibody which binds the detection antibody. In the case of using a secondary antibody, the capture antibody and the detection antibody must be from different species (i.e. if the capture antibody is a rabbit antibody, the detection antibody would be from goat, chicken, etc., but not rabbit). If the detection antibody is directly labeled with the reporter, then the capture and detection antibodies can be from the same species.

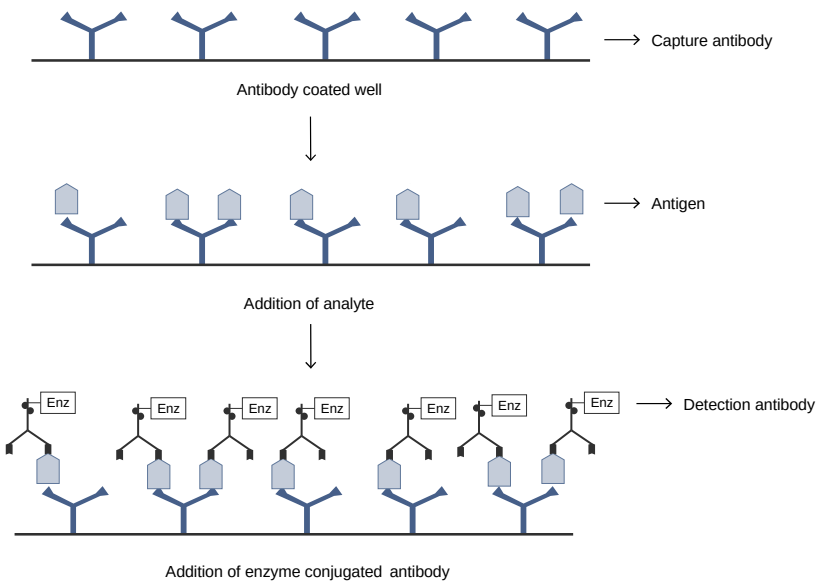


Figure 11. Diagram of a sandwich ELISA
The capture antibody is attached to a solid surface creating an antibody coated well. The analyte containing the antigen is added and binds to the antibody, then the second antibody with conjugated enzyme, called detection antibody, is added. The capture and detection antibody bind to different sites in the antigen. As a result, the antigen is located between the antibodies as in a sandwich, therefore it is called "sandwich ELISA". The addition of the enzyme's substrate leads to color development.The amount of colour (absorbance) is directly proportional to the analyte concentration.
Reprinted from Assay Guidance Manual (E-book). Chapter Immunoassay Methods; Karen L. Cox, BS, Viswanath Devanarayan, PhD, Aidas Kriauciunas, Joseph Manetta, BS, Chahrzad Montrose, PhD, and Sitta Sittampalam, PhD.; Published May 1, 2012; Last Update: December 24, 2014. https://www.ncbi.nlm.nih.gov/books/NBK92434/

Polyclonal antibodies often contain multiple epitopes and the same affinity purified polyclonal antibody can be used as the capture and labeled detection antibody. The substrate for the enzyme is added to the reaction forming a colourimetric signal that can be detected. The amount of signal is proportional to the amount of the detected antigen in the sample.

The reporter used to measure the amount of attached detection antibody determines the detection mode. If the reporter is a substrate of an enzyme, the detection is colourimetric and a spectrophotometric plate reader is used. Several types of enzyme reporters have been developed in order to increase sensitivity in an immunoassay. If the reporter is a fluorophore tagged antibody, the amount of antibody is measured using a fluorescent plate reader. If the detection antibody is labeled with biotin, there is the

flexibility to use a number of different types of streptavidin conjugated reporters.

Single molecule array (SIMOA) assay

Simoa has the ability to detect biomarkers at ultra-low levels, which have traditionally only been detectable in CSF. The principle of SIMOA is explained in Figure 12.

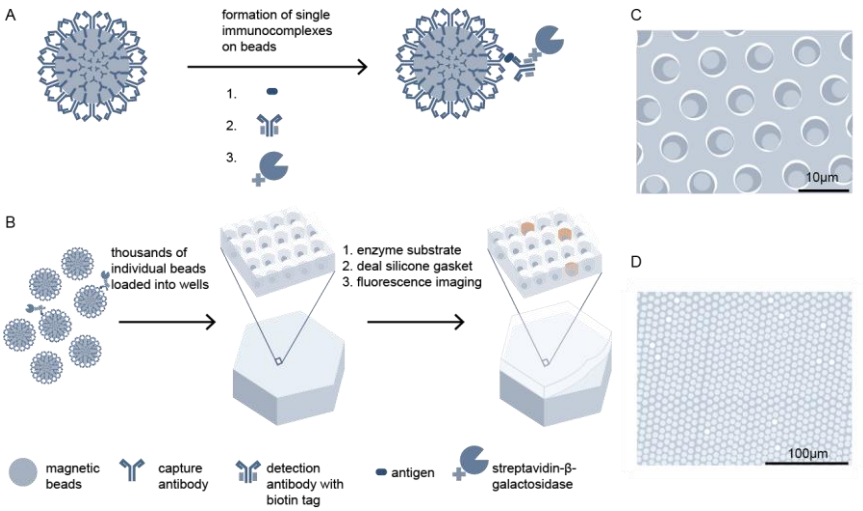


Figure 12. Single molecule array assay
(a,b) Single protein molecules are captured and labeled on beads using standard ELISA reagents (a), and beads with or without a labeled immunoconjugate are loaded into femtoliter-volume well arrays for isolation and detection of single molecules by fluorescence imaging (b). (c) Scanning electron micrograph of a small section of a femtoliter-volume well array after bead loading. Beads (2.7 µm diameter) were loaded into an array of wells with diameters of 4.5 µm and depths of 3.25 µm. (d) Fluorescence image of a small section of the femtoliter-volume well array after signals from single enzymes are generated. Whereas the majority of femtoliter-volume chambers contain a bead from the assay, only a fraction of those beads possess catalytic enzyme activity, indicating a single, bound protein molecule. The concentration of protein in bulk solution is correlated to the percentage of beads that carry a protein molecule.

Fluorescence Spectrometry/Spectroscopy (or Fluorometry)

Fluorescence spectrometry is a method to determine the concentration of an analyte in solution based on its fluorescent properties (Figure 13). A beam with a wavelength varying between 180 and ~800 nm passes through a solution in a cuvette. The light that is emitted by the sample is measured from an angle. In fluorescence spectrometry both an excitation spectrum (the light absorbed by the sample) and an emission spectrum (the light emitted by the sample) can be measured. The concentration of the analyte is directly proportional with the intensity of the emission.

There are several parameters influencing the intensity and shape of the spectra. When recording an emission spectrum, the intensity is dependent on the excitation wavelength, concentration of the analyte solvent, path length of the cuvette and self-absorption of the sample.

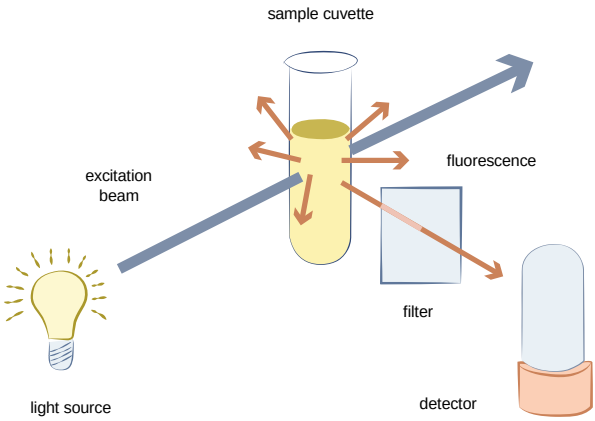


Figure 13. Fluorimeter scheme
The figure shows a fluorescence schematic. The light source is in a 90 degrees angle with the detector. The sample is located at the intersection of the two beam paths.

Mass Spectrometry

Mass spectrometry measures the mass-to-charge ratio of ions to identify and quantify molecules in simple and complex mixtures. A solid, liquid or gas sample is ionized with electrons to break the molecules in the sample into charged fragments (ions). These ions are then accelerated in an electromagnetic field, where they are deflected and separated according to their mass-to-charge ratio. After detection results are displayed as spectra of the relative abundance of detected ions. The atoms or molecules in the sample

can be identified by correlating known masses to the identified masses or through a characteristic fragmentation pattern (Figure 14).

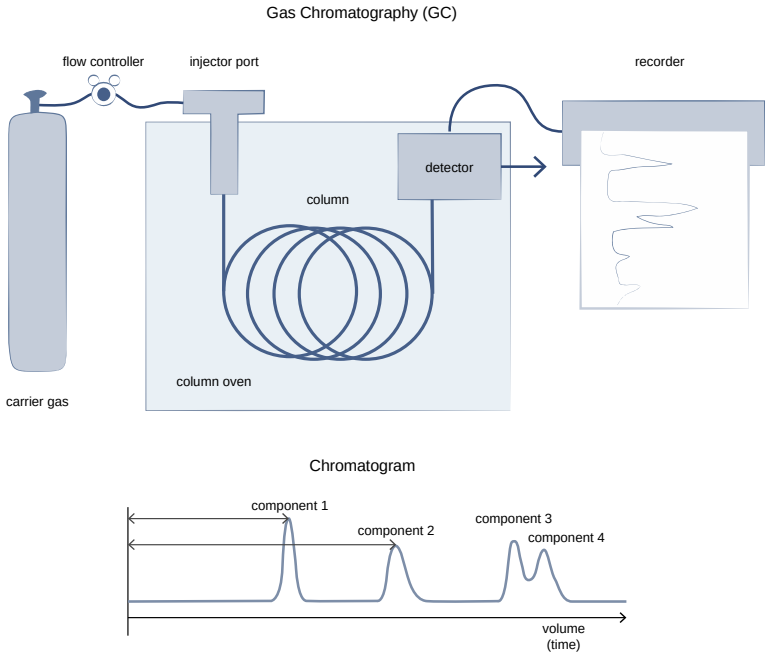


Figure 14. Gas Chromatography Mass spectrometry
A simple gas chromatography is shown. It includes a tank of gas, pressure and flow regulators to control the gas flow, an oven, an injector to allow injection of a small volume of the sample mixture under pressure. A sample is ionized with the electrons from the gas to break the molecules in the sample into charged fragments (ions). These ions are then accelerated in an electromagnetic field (in the column), where they are deflected and separated according to their mass-to-charge ratio. They are detected (in the detector) and results are displayed as spectra of the relative abundance of detected ions (in the chromatogram). The atoms or molecules in the sample (components in the chromatogram) can be identified by correlating known masses to the identified masses or through a characteristic fragmentation pattern.

Isotope dilution mass spectrometry is a reference technique for quantitative analysis. It combines the sensitivity and selectivity of mass spectrometry instruments with the precision and accuracy associated with the use of internal standards (=isotopically-enriched form of analyte). Isotope-labeled proteins are the optimal internal standards for quantitative proteomics as they closely mimic the behavior of their natural counterparts during the analytical process. Known amounts of isotopically-enriched substance are added to the analyzed sample. Mixing of the isotopic standard

with the sample effectively "dilutes" the isotopically-enriched standard and the amount of analyte can be calculated (Figure 15).

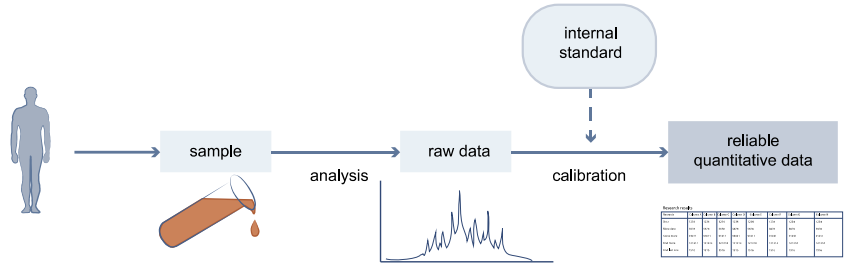


Figure 15. Isotope dilution mass spectrometry
A sample is analyzed and the results are presented as raw data. The isotope-labeled proteins are used as internal standards for quantification of the data. This technique combines the sensitivity and selectivity of mass spectrometry with the precision and accuracy associated with the use of internal standards.

Nephelometry

The albumin in CSF and serum were analyzed by nephelometry. The light passing through the sample solution and the amount of scattered radiation is measured generally at 90° (Figure 16). The measurement of intensity of scattered light as a function of concentration of dispersed phase is the basis of analysis of nephelometry.

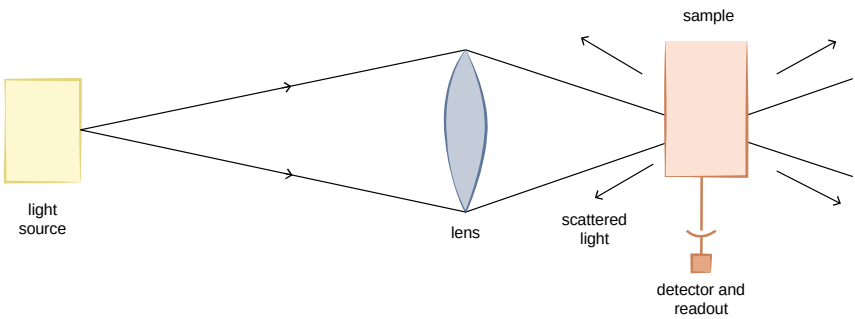


Figure 16. Nephelometry
The light passes through the sample solution and the amount of scattered light is measured at 90° (detector and readout). The intensity of scattered light is measured and compared to the amount from known solutions.

It is important to note that in nephelometry, incident and scattered light are of the same wavelength, whereas in fluorometry, scattered light is of a different wavelength than incident light.

The albumin ratio = CSF albumin / S albumin, is a better measure of BBB deficiency than CSF albumin, because albumin is not produced in the CNS but diffuses into CSF via the BBB from the bloodstream.

Isoelectric focusing (IEF)

Proteins are separated according to their isoelectric point by IEF followed by immunoblotting (Figure 17). OCB that are detected in CSF by IEF are often seen in CNS inflammatory disease, and have high specificity and sensitivity for MS diagnosis [212]. Detection of two or more IgG positive bands, indicates an intrathecal oligoclonal IgG production. More than 95% of patients with MS have OCB of IgG class in CSF that are not detectable in serum, thereby providing powerful evidence for the diagnosis of MS. At the present time, this is the only diagnostic biochemical biomarker available to support the MS diagnosis.

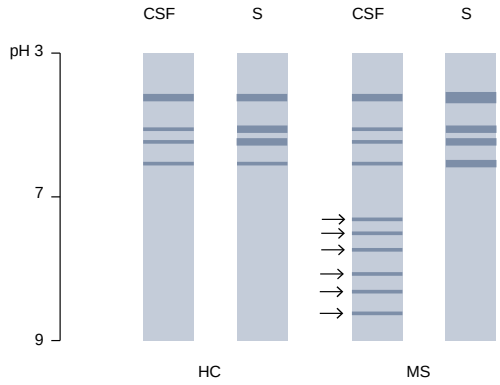


Figure 17. Isoelectric focusing
An ampholyte solution is incorporated into a gel. A stable pH gradient is established in the gel after application of an electric field. CSF and serum are added and electric field is reapplied. After staining, proteins are shown to be distributed along pH gradient according to their pH values. This qualitative method is used to detect OCB in CSF. OCB can be detected in the alkaline region of the gel (pH>7). The number of OCB is counted with the naked eye. The CSF proteins are compared with serum protein to detect if they exist in both fluids or selectively in CSF. The results of isoelectric focusing are presented for a healthy control without OCB and for a MS patient with OCB selectively in CSF (arrows).
CSF=cerebrospinal fluid, HC=healthy control, MS=multiple sclerosis
OCB=oligoclonal bands, pH=potential hydrogen

The IgG concentration is usually increased at a high level of oligoclonal IgG bands, and is measured as IgG index.

IgG index = (CSF IgG / S IgG) / (CSF albumin / S albumin) is elevated in about 70% of MS patients, but rarely in OCB-negative MS patients. Normally, IgG index is <0.63.

Because of lower diagnostic sensitivity, IgG index cannot replace the analysis of OCB in CSF in the diagnosis of MS. However, when elevated, it is additional evidence for an augmented B cell response within the CNS which is compatible with MS [212].

Magnetic resonance imaging

Over recent years conventional MRI has broadened its use from essentially a diagnostic tool to assessing disease activity, rate of neurodegeneration and monitoring efficacy of therapeutic intervention. The MRI protocol for MS usually consists of **T1-weighted image with gadolinium enhancement**, **T2-weighted and fluid-attenuated inversion recovery (FLAIR) images**. On T1-weighted images MS lesions appear hypointense, meaning that they display as dark areas, either as "black holes" that are areas of permanent axonal damage or as areas of edema, which are not permanent and disappear on subsequent scans. On T2-weighted images MS lesions appear hyperintense, meaning that they appear as bright spots on the MRI image. The accumulation of MRI activity may be determined by measuring the volume of brain lesions and this measure is designated as **lesion load**.

However, there are still several shortcomings with conventional MRI. MRI scanning in routine investigations is time consuming with relatively long scan times, and the difference of signal intensity of the images between specific MR scanner settings makes comparisons unreliable. This is especially evident when measuring atrophy development and lesion volumes on the basis of conventional MRI images.

Synthetic MRI is a method that generates conventional images based on MRI quantification (Figure 18). It potentially decreases both examination time and enables quantitative measurements [213-215]. By adding this software to conventional MRI, automated quantification is possible with only slightly increased examination time compared to conventional MRI. On healthy individuals the method has the ability to differentiate between white matter, grey matter and CSF [216]. In MS, this method has been used to quantify BPF [217]. The diagnostic accuracy and volumetric measurements of synthetic MRI were compared to conventional MRI in patients with MS and in healthy controls in a validation study which concluded synthetic MRI can be a good alternative suitable for MS studies [218].

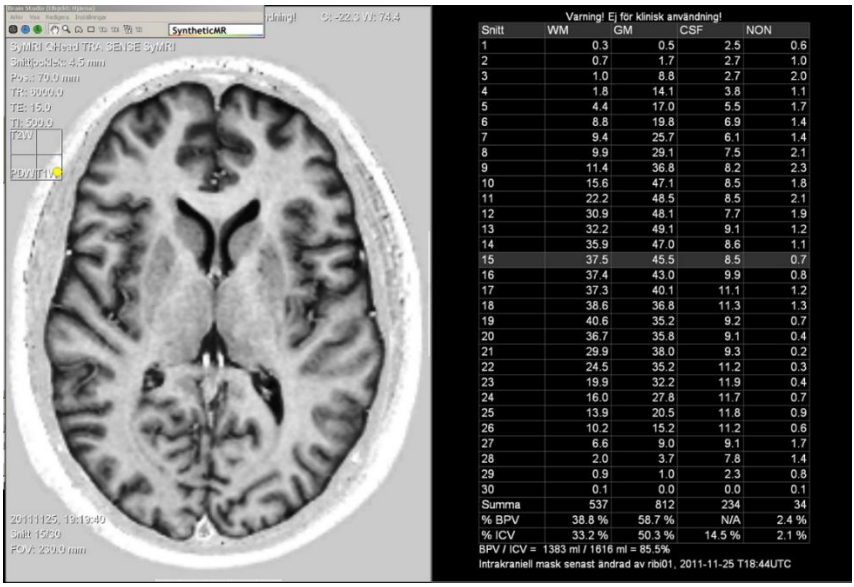


Figure 18. Example of a report from examination performed by Synthetic MRI
The picture on the left shows one slice of the brain, T1 weighted image performed by Synthetic MRI. The table on the right shows the volumetric data provided for every slice of the brain, including white matter (WM), grey matter (GM), cerebrospinal fluid (CSF) and remaining tissue (NON). On the bottom, the sum of the slice volumes and percentages of the brain volumes are shown. The highlighted slice number 15 is the presented slice of the brain.
BPF [%] = BPV [ml] / ICV [ml]
BPF=brain parenchymal fraction, BPV=brain parenchymal volume, CSF=cerebrospinal fluid, GM=grey matter, ICV=intracranial volume, WM=white matter

The automated segmentation methods displayed varying degrees of similarity to the manual reference, with SyMRI being the most similar [219]. SyMRI showed good characteristics for calculating whole brain volume in cross-sectional studies [217] and was used in our study. This method showed shortest processing time, lowest repeatability error of brain volume, BPF, intracranial volume and grey matter fraction when compared to other methods [218].

Optical coherence tomography

OCT is a non-invasive technique that generates a cross-sectional image of the RNFL by scanning the retina with a laser beam. OCT can measure the RNFL thickness, including ganglion cell layer thickness, macula volume and macula thickness (Figure 19).

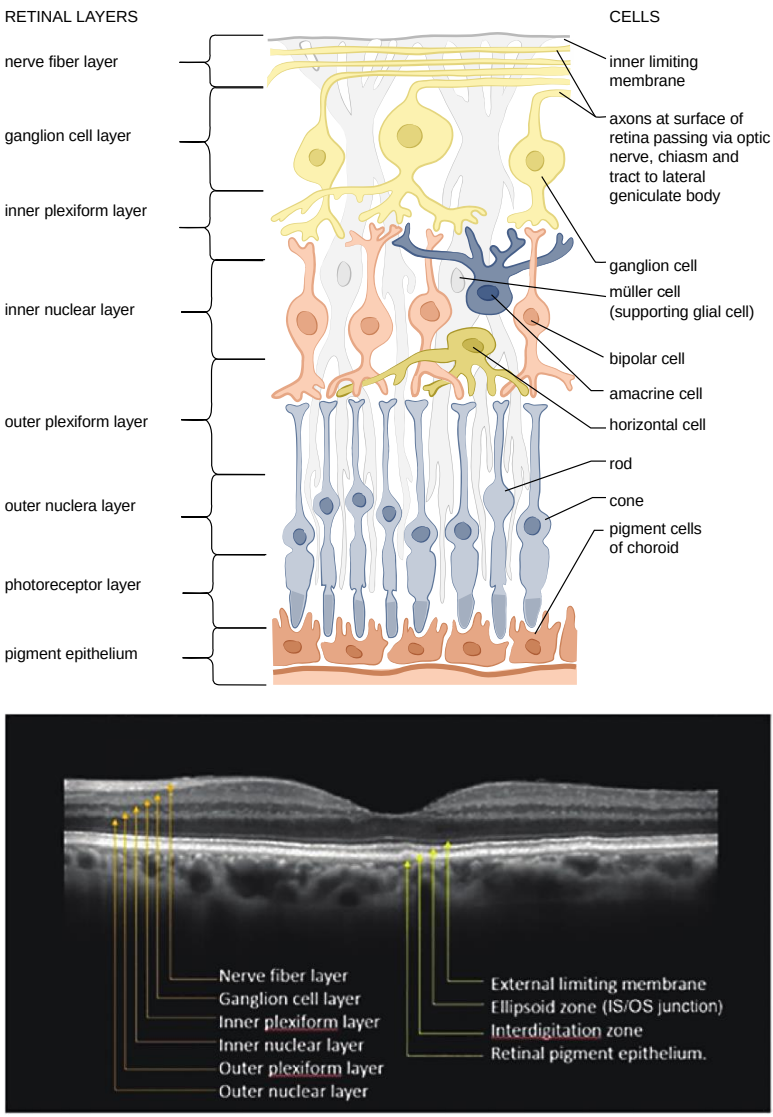


Figure 19. Retinal layers
The retinal layers and cells creating these layers. OCT image of the retina with the layers demonstrated. Note that the nerve fiber layer is the innermost layer of the retina.

OCT is an imaging technique which works in a similar way to ultrasound, but using light waves instead of sound waves. By using the time-delay in-

formation of light waves, which have been reflected from different depths inside a sample (retina), an OCT system can reconstruct a depth-profile of the sample structure (retinal layers). The classic OCT technology first described in 1991 is now called time-domain (TD) OCT because the depth is sampled one point at a time by moving reference mirror to produce an axial scan (Figure 20) [220]. This limits the speed and sensitivity of imaging. Therefore, faster technique called Fourier-domain (FD) OCT detecting the entire depth simultaneously was developed. Spectral domain (SD) OCT and frequency domain (FD) OCT refer to the same technology. In FD-OCT, the reference mirror is stationary and the interference between the sample and the reference beams is detected as a spectrum. The spectrum is spread out with a grating and captured by a high-speed line camera. Fourier tranform of the spectral interferogram produces axial scan. Fourier tranform transforms a function of time (a signal) to a function of frequency, in a similar way to how musical chords can be expressed as the frequencies (or pitches) of its constituent notes (Figure 21).

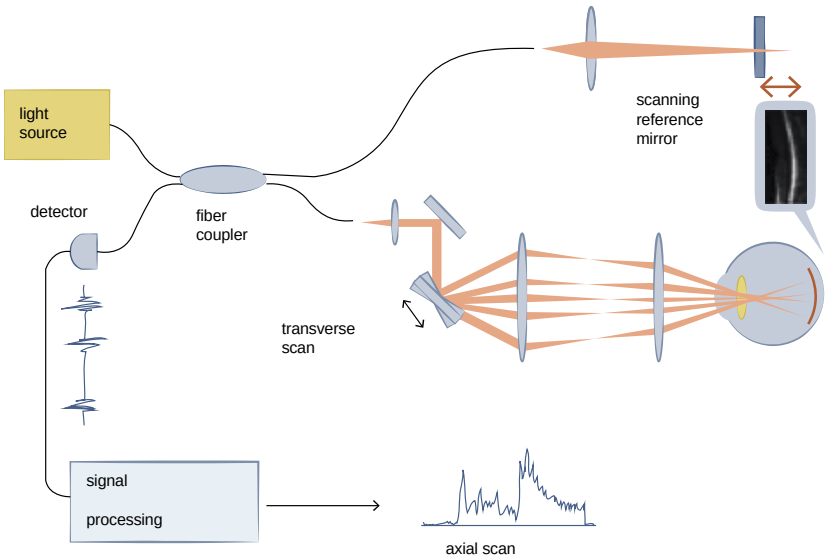


Figure 20. Schematics of time-domain OCT

The reference mirror (upper right) moves back and forth over a distance that corresponds to the axial (depth) range of interest in the sample. The data acquisition is synchronized with the scan cycle of the reference mirror. Each cycle provides one axial scan that contains information on the reflected signal strength versus depth in the sample. A transverse scan mirror steers the probe beam over a transverse dimension. The OCT image is a representation of reflected signal strength over axial and transverse dimensions.

Reprinted from Huang, D. OCT Terminology — Demystified! A pioneer of the technology provides a translation of the latest jargon [Internet] Ophthalmology management 2009 [cited 31 Jan 2018]. Available from: https://www.ophthalmologymanagement.com/issues/2009/april-2009/oct-terminology-demystified!

Besides the technical terms, there are even more terms introduced for marketing purposes. E.g. Topcon uses the name "3D OCT" to emphasize the fact that FD-OCT is able to acquire a 3D data and those can be viewed in 3D viewing software. Zeiss calls the Cirrus system "HD-OCT" to emphasize the high-definition aspect of FD-OCT. With speeds ranging from 17,000 to 40,000 axial scans per second, the current generation of FD-OCT retinal scanners can all acquire a 1,000-line high-definition OCT image in a largely motion-free time frame of 25 to 59 milliseconds.

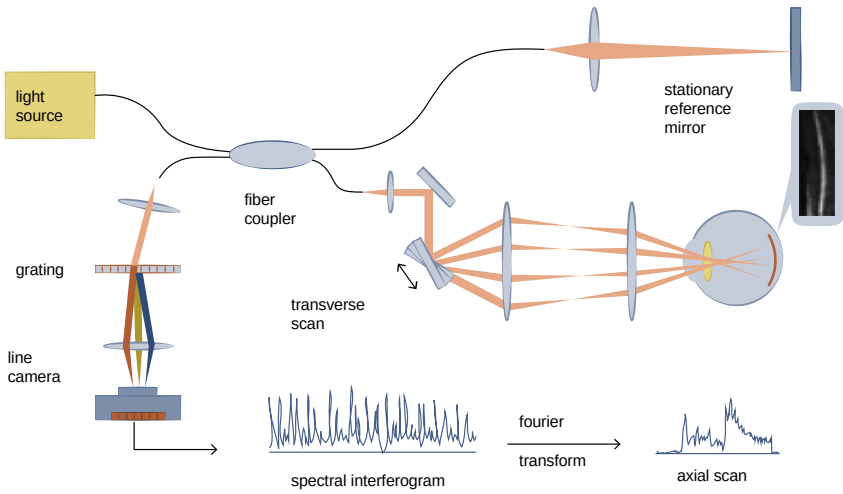


Figure 21. Schematics of a spectrometer-based Fourier-domain OCT
The reference mirror is stationary. The spectral interferogram is the spectrum of the combined reference and sample reflections. Reflections from different depths of the sample produce spectral modulations of different periodicities. The Fourier Transform converts the spectral modulations into depth information (axial scan).
Reprinted from Huang, D. OCT Terminology — Demystified! A pioneer of the technology provides a translation of the latest jargon [Internet] Ophthalmology management 2009 [cited 31 Jan 2018]. Available from: https://www.ophthalmologymanagement.com/issues/2009/april-2009/oct-terminology-demystified!

OCT's imaging depth (Figure 22) is typically limited to a few millimetres, less than ultrasound, MRI, or X-ray computed tomography (CT), but its resolution is greater. This comparison is reversed with respect to confocal microscopy. Like ultrasound, the acquisition time of OCT is short enough to support tomographic imaging at video rates, making it much more tolerant to subject motion than either CT or MRI. It does not require physical contact with the sample, and may be used in air-filled hollow organs (unlike ultrasound). OCT uses non-ionising radiation at biologically safe levels, allowing

for long exposure times, and its level of complexity is closer to ultrasound than to CT or MRI, allowing for the low-cost portable scanners.

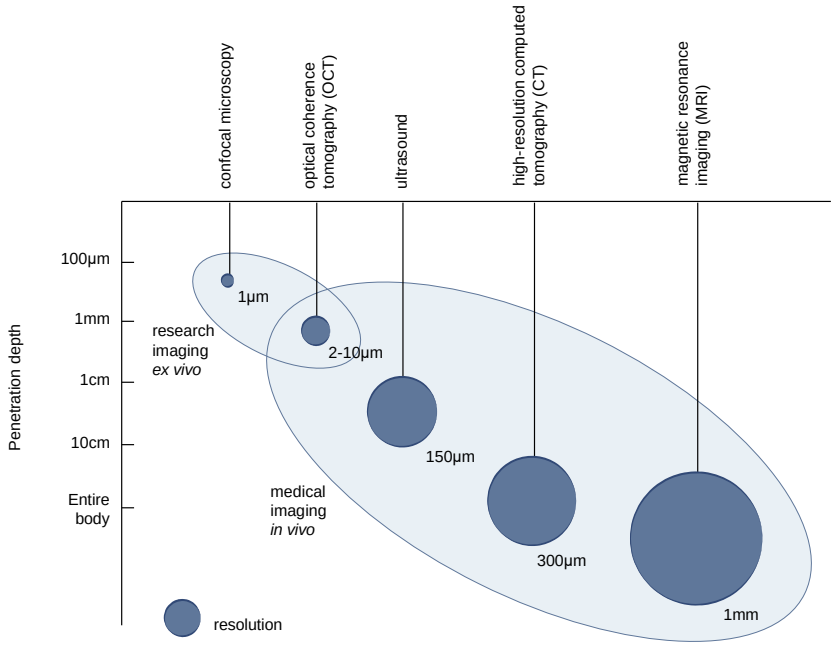


Figure 22. OCT resolution
Comparison of OCT resolution and imaging depths to those of alternative techniques. The “pendulum” length represents imaging depth, and the “sphere” size represents resolution.
Reprinted from OBEL. Introduction to OCT [Internet] The Optical+Biomedical Engineering Laboratory. Available at: http://obel.ee.uwa.edu.au/research/fundamentals/introduction-oct/ [Accessed 31 Jan 2018]

Aims

The pathophysiology of MS is complex with the presence of inflammation and neurodegeneration in all stages of the disease. The disease course, treatment response and outcome are highly variable in MS. There is a need for reliable biomarkers that reflect disease activity, disability progression and predict the disease severity. Over recent years there is accumulating evidence that DMTs not only reduce inflammation but also influence neurodegeneration in MS. Thus, the use of biomarkers that reflect different parts of the pathophysiology of MS may improve the decision-making between the various treatment options and may also improve the monitoring of treatment response.

The aim of the thesis was to:

- Find relationships between clinical, MRI measures and biomarker levels in CSF (Paper I-III, V).
- Explore associations between inflammatory and degenerative biomarkers in CSF (Paper I-III, V).
- Investigate the influence of different therapies on biomarker levels in CSF and blood (Paper I-IV).
- Determine the correlation between NFL levels in CSF and serum, and the influence of DMTs with different efficacy on NFL levels in CSF and serum (Paper IV).
- Explore the diagnostic value of different biomarkers from SyMRI, OCT and CSF (Paper V).
- Find evidence of early neurodegeneration in MS by the use of OCT, SyMRI and levels of CSF biomarkers (Paper V).

Patients and Methods

Patients selection

All patients in our studies were consecutively enrolled and were diagnosed with MS according to the McDonald criteria 2010 [15].

Paper I and IV

The study cohort consisted of patients with RRMS (n=31) recruited at the MS Center, Department of Neurology, Sahlgrenska University Hospital, Gothenburg, Sweden during 2006-2008 and constituted a sub-group of a larger population that had been described previously [141]. All patients included in the study started the treatment with natalizumab and were examinated before and after 12 months of natalizumab treatment.

Paper II and IV

Patients with RRMS who had an indication to switch therapies because of breakthrough disease activity (n = 40), tolerability/adverse events (n=2), or risk of progressive multifocal leukoencephalopathy because of John Cunningham virus antibody positivity during natalizumab therapy (n=17) were enrolled in the study at the MS centers of Sahlgrenska University Hospital, Gothenburg (n=17), and Karolinska University Hospital, Stockholm, Sweden (n = 42) during 2012-2013. A total of 59 patients were enrolled, 7 were treatment naive or without DMT for ≥ 1 year, 33 were on first-line (IFN beta) and 19 were on second-line treatment (natalizumab) for at least 1 year before the study. All patients on different MS treatments were examined once.

Paper III and IV

Patients with RRMS (n=43) were enrolled in the study at the MS centers of Sahlgrenska University Hospital, Gothenburg (n=17) and Karolinska University Hospital, Stockholm, Sweden (n=26) during 2012-2013. All patients were prospectively followed 4-12 months after the treatment start with

fingolimod. According to their previous treatment, these patients were divided into first-line (n=26; IFN beta, glatiramer acetate and teriflunomide) and second-line treatment group (n=14; natalizumab).

Paper IV

Patients with MS (n=286), patients with other neurologic disorders or symptoms (n=45), and HC (n=42) [103] were enrolled in the study at the neurology departments of 4 Swedish hospitals: University Hospital of Umeå, Umeå; Sahlgrenska University Hospital, Gothenburg; Karolinska University Hospital, Stockholm; and Örebro University Hospital, Örebro. The patients in this study were previously included in other studies on biomarkers performed by the MS research group at Sahlgrenska University Hospital. The 286 patients with MS consisted of 204 with RRMS and 82 with PrMS. The latter group included 19 with PPMS and 63 with SPMS. A subgroup of these patients (n=148), including 98 with RRMS and 50 with PrMS, were followed up prospectively and examined before and after a median of 12 months (range 0–46 months). A small proportion remained untreated (n=10), and the remaining (n=138) were treated with the following DMTs: glatiramer acetate (n=2), glatiramer acetate plus mitoxantrone (n=1), IFN beta (n=3), oral weekly methotrexate (n=7), mitoxantrone (n=15), fingolimod (n=21), rituximab (n=23), natalizumab (n=63), alemtuzumab (n=2), and cyclophosphamide (n=1).

In conjunction with baseline sampling, most patients escalated their DMT (n=68) from less effective DMTs (IFN beta, glatiramer acetate, high doses of IV immunoglobulin, or oral weekly methotrexate) to more effective DMTs (alemtuzumab, cyclophosphamide, fingolimod, glatiramer acetate plus mitoxantrone, mitoxantrone, natalizumab, or rituximab). A second group of patients were not treated at the time of sampling and either were treatment naive (n=36) or had a prior treatment terminated >3 months previously (n=14). A third group (n=20) had changed to another DMT with similar efficacy because of adverse effects or unsatisfactory adherence. Participants with OND served as controls and were divided according to established definitions [103] into those with inflammatory neurologic diseases (n=15) and those with non-inflammatory neurologic diseases (n=16).

Paper V

All patients with clinical MS symptoms (n=271) were enrolled at the MS Center of Sahlgrenska University Hospital between April 2014 and June 2016. After a diagnostic investigation, these patients were classified accord-

ing to the final diagnosis, as follows: patients with CIS (n=4) or patients with RRMS with disease duration <2 years (early RRMS; n=93), patients with RRMS with disease duration ≥2 years (established RRMS; n=39), symptomatic controls (SC; n=89) and patients diagnosed with other diseases (OD; n=46). Further, patients with progressive MS (PrMS; n=23) were selected from the MS registry (www.msreg.net). Each group was examined once. None of the patients were treated with immunomodulatory or immunosuppressive treatment at the inclusion in the study. We retrospectively searched the local diagnostic registry in May 2017 and found 7 patients with MS that had been missed. Thus, the study population was considered an unselected incidence cohort of individuals with suspected MS that were referred to a Swedish Multiple Sclerosis Center.

Paper I-V

Blood donors and university students served as HC. None of the controls had any neurological signs or history of neurological disease. The HC in all five studies were examined once.

Analytical methods

All analyses, except oxysterols, were performed by board-certified laboratory technicians at the Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, according to protocols approved by the Swedish Board for Accreditation and Conformity Assessment. Oxysterols were analyzed at the Biochemistry Laboratory, Karolinska University Hospital, Huddinge, Stockholm.

In this thesis, following analytical methods were used:

The **CSF NFL** protein was measured with a sensitive sandwich ELISA method (NF-light® ELISA kit, UmanDiagnostics AB, Umeå, Sweden). Intra- and inter-assay coefficients of variation were below 10%. The lower limit of quantification (LLoQ) of the assay was 31 pg/mL (=31 ng/L).

This method was established by Karlsson and Rosengren at the Sahlgrenska University Hospital at the end of the 1980s and resulted in a first ELISA 1996 [144]. Their method was based on rabbit polyclonal anti-NFL IgG that was used as the secondary antibody. Bound secondary antibody was detected using peroxidase-conjugated donkey anti-rabbit IgG. The LLoQ was 125 ng/L. This method was further developed by Niklas Norgren and Torgny Stigbrand in Umeå together with Rosengren and Karlsson, using monoclonal anti-NFL antibodies [221]. Using this method, CSF samples were incubated in pre-coated anti-NFL plates. Two monoclonal antibodies

highly specific for NFL were used as secondary antibodies. Streptavidin horse radish peroxidase was used for detection.This method was more sensitive with a lower LLoQ and most importantly, it gave an unlimited access to antibody [142]. This is the method used for all the analysis in this thesis. Under optimal conditions an inter-laboratory CV of 14% should be achievable for this NfL ELISA [222]. However, all analysis in this thesis were performed in a single laboratory.

CSF GFAP was measured with in-house ELISA [223]. The plates were coated with hen anti-GFAP IgG followed by incubation with CSF samples for 2 h, then rabbit anti-GFAP IgG was added as a secondary antibody and incubated for 1 h, both at room temperature. Detection was performed with peroxidase-conjugated donkey anti-rabbit IgG and 3,3',5,5'-tetramethylbenzidine substrate. The absorbance was read at λ 490 nm, and the LLoQ was 16 pg/mL.

CSF tau was measured with an ELISA (INNOTEST hTAU Ag, Fujirebio, Ghent, Belgium). The LLoQ of the assay was 75 ng/L, and intra- and interassay coefficients of variation were below 10%.

CSF CXCL13 was measured with ELISA (Human CXCL13/BLC/BCA-1 Immunoassay, R&D Systems Inc., Abingdon, United Kingdom), according to the manufacturer's instructions. The average intra- and inter-assay coefficients of variation were below 10% and the LLoQ was 7.8 pg/mL.

CSF CHI3L1 was analyzed with solid phase sandwich ELISA (Human Chitinase 3-like 1 Quantikine ELISA Kit, R&D Systems Inc., Minneapolis, MN). The intra-assay coefficient of variation was below 7% and the LLoQ was 8.15 pg/mL.

CSF chitotriosidase activity was measured with an in-house method in which 100 μL of a fluorescence-linked substrate, 4-MU-β-D-N,N'N''-triacetylchitotrioside (Sigma M5639, MO, USA) was added to 100 μL CSF and incubated for 15 min at 37◦C. In the presence of chitotriosidase, the β-chain cleavage leads to release of the fluorescence element into the samples, which is quantified by spectrofluorometry (Jasco, Cremella, Italy). A serial dilution of pooled reference plasma was used as a standard from which activity was calculated. The intra- and inter-assay coefficients of variations were below 10% and the LLoQ was 0.2 nkat/L.

CSF CCL2 was analyzed with solid phase sandwich ELISA (Human CCL2/MCP-1 Quantikine ELISA Kit, R&D Systems Inc., Minneapolis, MN). Intra- and inter-assay coefficients of variation were below 8% and the LLoQ of the assay was 10 pg/mL.

CSF NGRN was measured using an in-house ELISA with the monoclonal antibody Ng7 (BioLegend, CA, USA) as the capture antibody and the rabbit anti-NGRN antibody (Upstate, MA, USA) as the detecting antibody. The mean intra-assay coefficient of variation was 17% and the LLoQ was 125 pg/mL.

24OHC and 27OHC in serum and CSF were analyzed with isotope-dilution mass spectrometry using deuterium-labeled internal standards, as described elsewhere [224]. In brief, serum and CSF samples were subjected to saponification to hydrolyze oxysterol esters. The hydrolysis was performed at 22∘C for 2 hours with concentration 0.35 M KOH. In order to prevent cholesterol autoxidation during sample preparation and handling, cholesterol was separated from oxysterols by solid-phase extraction using silica columns. A deuterium-labeled internal standard was added for both oxysterols analyzed.

Serum NFL levels were determined using the NF-Light kit from UmanDiagnostics (NF-light® ELISA kit, UmanDiagnostics AB, Umeå, Sweden), transferred onto the SIMOA platform using a homebrew kit (Quanterix Corp, Boston, MA, USA), as previously described [225]. Intra- and inter-assay coefficients of variation were below 10%. The LLoQ was 1.95 pg/mL. Comparison of the NFL analysis by ELISA, electrochemiluminiscence immunoassay, and SIMOA revealed that SIMOA is more sensitive than the two other methods. The levels of NFL in serum analyzed by SIMOA, and NFL in CSF analyzed by ELISA, were highly correlated in patients with various neurological disorders [152]. NFL in blood can be measured with SIMOA even at low NFL concentration in CSF, as present in healthy subjects [225].

Ethics

All patients and controls participated voluntarily in the studies and informed consent was obtained from all subjects after oral and written information was provided. The Regional Ethical Review Boards in Uppsala (Dnr 2005:253), Stockholm (Dnr 2009:2107) and Gothenburg (Dnr 895-13) approved the studies.

Statistics

Statistical analysis was performed using Microsoft Office Excel, IBM SPSS Statistics software (Paper I-V) and R statistics software (Paper V). Only complete cases were included in all statistical analysis (Paper I-V).

Visual inspection of the data and Shapiro–Wilk test of normality was used to evaluate the distribution of the biomarkers. Nonparametric tests were used to analyze the non normally distributed data (Paper I-IV). The Mann–Whitney U-test was used for unpaired data to investigate group differences, Wilcoxon signed rank sum test was used for analysis of matched pair data, e.g. before and after NZ treatment, and Kruskal–Wallis H test was used to analyze several independent samples.

Multiple regression analysis was performed to test the influence of gender, age, EDSS, and MSSS on the biomarkers (Paper I-III).

Multiple correlations were adjusted by Hochberg (Paper II-III).

Correlations between biomarkers were analyzed by the Spearman rank correlation test (Paper I-V). The receiver operating characteristic (ROC) curve estimation was performed with the assumption of nonparametric distribution. The Youden index, expressed as "Sensitivity + Specificity – 1", was used to calculate optimal cutoffs that maximize both sensitivity and specificity (Paper IV).

Group comparisons were performed with the T-test for continuous variables (Paper V). Correlations were visualized in correlation matrix plots, for all observations and for subgroups of individuals. The biomarkers were clustered and reordered with hierarchical clustering. The equation "1 – the correlation" was used as a distance metric and the average link. Anotherwords, we joined the particular pair of clusters with the smallest average distance between pairs of biomarkers (Paper V).

The principal component analysis was used to visualize high-dimensional data and identify patient clusters. Standardized data were decomposed into uncorrelated directions and sorted according to how well they explained the variance in the data (Paper V).

Prediction analyses were performed to determine whether biomarkers could discriminate between SC, RRMS, and OD groups. All the biomarkers identified with the CSF, OCT, and SyMRI analyses were tested separately. For this purpose, a classification tree model with gini impurity as splitting criterion was used (Paper V). Algorithms for constructing classification tree model usually work top-down, by choosing a variable (i.e. biomarker) at each step that best splits the groups of patients. Different algorithms use different metrics for measuring the best split, generally by measuring the homogeneity of the target biomarker within the subgroups. Gini impurity is a measure of how often a randomly chosen subject from the group would be incorrectly diagnosed if it was randomly diagnosed according to the distribution of diagnosis in the subgroup. The size of the tree was selected based on a 10-fold cross validation to minimize the cross-validated misclassification error.

To remove the effect of age on the biomarkers in group comparisons and in prediction analyses, age adjustments were performed for variables that were significantly related to age (at 10% level) in the HC group. These variables included white and grey matter volumes, BPF, NFL, GFAP, tau, and the albumin ratio. Age adjustments were performed by subtracting the age trend observed with the HC data, which was estimated with linear regression, from the observed values in the variables of interest. Moreover, NFL, GFAP, tau, the albumin ratio, the number of lymphocytes, and the IgG index were log-transformed prior to analysis (Paper V).

All statistical tests were performed at a 5% level of significance, without corrections for multiple testing. This was justified by the exploratory nature of this study and the limited group sizes (Paper V).

Results

The contribution of CSF biomarkers in the diagnostic work-up of MS (Paper V)

Our study showed that OCB are the most important CSF biomarker in MS diagnostics. Furthermore, increased NFL levels in CSF showed diagnostic value in MS, above all in separating healthy subjects seaking for neurological symptoms, from those who had MS or other neurological diseases. All subjects free from neurological disease had normal levels of NFL, except persons with ON that showed minor signs of axonal damage. Thus, it is highly improbable to suffer from a neurological disease, when having neurological symptoms but normal NFL levels in CSF.

In the unselected cohort of individuals with suspected MS, biomarkers for discriminating between SC, OD, and RRMS were measured. We found that the most important biomarkers were intrathecal IgG production, the IgG index, the presence of OCB, the NFL and the number of lymphocytes. Using only the intrathecal IgG production, 93% of the RRMS patients and 96% of the SC were correctly predicted. Adding NFL, the sensitivity increased to 97% for diagnosing MS. On top of intrathecal IgG production and NFL, no additional biomarkers improved the MS diagnostics. The OCT and SyMRI biomarkers did not contribute to the discrimination between the patient groups or correct diagnosis, neither when they were studied separately, nor when all biomarkers were included in the analysis.

Using the principal component analysis, two clusters of biomarkers were observed, where CSF biomarkers mainly contributed to the first principal component and SyMRI biomarkers mainly to the second principal component. Furthermore, the CIS/RRMS and PrMS groups were clustered together and separated from the HC and SC groups along the first principal component, due to differences in the inflammatory biomarkers detected in the CSF.

Searching for early degeneration in MS (Paper V)

Degenerative biomarkers in CSF

All phenotypes of MS exhibited significantly elevated NFL levels (CIS/early RRMS, 63.2%; established RRMS, 63.9%; PrMS, 38.1%) compared to HC (4%) and SC (0%) ($p<0.001$). NFL levels were higher in the newly diagnosed RRMS groups compared to the PrMS group, but they were within the normal range in the HC and SC groups. The PrMS group had significantly higher GFAP levels compared to the HC, SC, CIS/early RRMS ($p<0.001$) and established RRMS ($p=0.0013$) groups. There was no significant difference in the levels of GFAP between the CIS/early RRMS and established RRMS groups. However, the both RRMS groups had higher GFAP levels than the HC and SC groups. Tau levels were lower in the HC and SC groups compared to the CIS/early RRMS group ($p=0.006$ and $p=0.014$, respectively), the established RRMS group ($p<0.001$ and $p=0.002$, respectively) and the PrMS group ($p<0.001$ and $p=0.001$, respectively). No significant differences in tau levels were found between patients with different multiple sclerosis phenotypes, although tau levels tended to be lower in the CIS/early RRMS group than in the PrMS group ($p=0.05$).

Degenerative imaging biomarkers

The SyMRI data showed that the BPF was reduced in the PrMS group compared to the HC, SC and newly diagnosed RRMS groups ($p<0.001$). There was no significant difference in BPF between the HC, SC and newly diagnosed RRMS groups, although patients with CIS/early RRMS tended to have higher BPF values than those in the established RRMS group ($p=0.063$). White matter and grey matter volumes were not significantly different between the different multiple sclerosis phenotypes.

The PrMS group had thinner RNFL and lower MV compared to the HC, SC and RRMS groups ($p<0.001$). No significant differences in the RNFL or MV were found between the HC, SC, and newly diagnosed RRMS groups. None of the biomarkers for degeneration were increased in the SC or HC groups, and we did not find any signs of pathology in the SC group that could distinguish it from the HC group.

Relationships between different biomarkers (Paper II, V)

Correlations between biomarkers and hierarchical clustering of biomarkers were performed for all untreated MS patients and controls, and also separately for patients with CIS/RRMS, and the HC group (Paper V). The biomarkers creating clusters had higher correlation and thereby closer relationship to each other. When all individuals were included in the analysis, two distinct clusters were observed. One cluster consisted of inflammatory biomarkers in the CSF (number of lymphocytes, IgG index, intrathecal IgG production, and OCB), the NFL and the GFAP in the CSF. The other cluster consisted of neurodegenerative biomarkers (brain volume measures, RNFL, and MV). When the CIS and RRMS groups were analyzed separately, a similar cluster pattern was observed. However, the HC group showed a different cluster pattern. The cluster with inflammatory biomarkers in the CSF, the CSF NFL and the CSF GFAP previously observed, now appeared as two separate clusters. The cluster with brain volume measures, RNFL and MV was non-existent in HC (Paper V).

We found correlations between biomarkers of inflammation and degeneration in MS patients. GFAP correlated with CHI3L1 ($r = 0.352$, $p = 0.007$) and NFL correlated with CHI3L1 ($r = 0.330$, $p = 0.011$) and CHIT1 ($r = 0.298$, $p = 0.023$). CHIT1 correlated with two other inflammatory biomarkers, CHI3L1 and CXCL13 ($r = 0.499$, $p < 0.001$ and $r = 0.298$, $p = 0.023$, respectively). The CSF cell count (mononuclear cells) highly correlated with the inflammatory biomarker CXCL13 ($r = 0.670$, $p < 0.001$), whereas the correlations between CSF cell count and NFL ($r = 0.294$, $p = 0.024$) and CCL2 ($r = 0.341$, $p = 0.008$) were lower (Paper II).

CSF biomarkers for measuring disease activity and progression (Paper II, V)

Our studies showed the ability to measure the disease activity with several biochemical biomarkers, as their levels in CSF were increased in MS patients with clinical and radiological activity (Paper II).

The CSF levels of NFL, CHI3L1 and CHIT1 were higher in patients with relapse than in patients with clinical remission ($p = 0.002$, $p = 0.006$ and $p = 0.006$, respectively; Paper II). NFL and CXCL13 were higher in patients with new T2 lesions than in patients without new T2 lesions ($p = 0.007$ and $p = 0.014$, respectively; Paper II; Figure 23). Concentrations of CXCL13 and CCL2 correlated with occurrence of Gd-enhancing lesions ($r = 0.614$, $p = 0.001$ and $r = -0.445$ and $p = 0.026$, respectively) and with the number of

contrast enhancing lesions (r = 0.622, p = 0.001 and r = -0.505 and p = 0.010, respectively; Paper II; Figure 23).

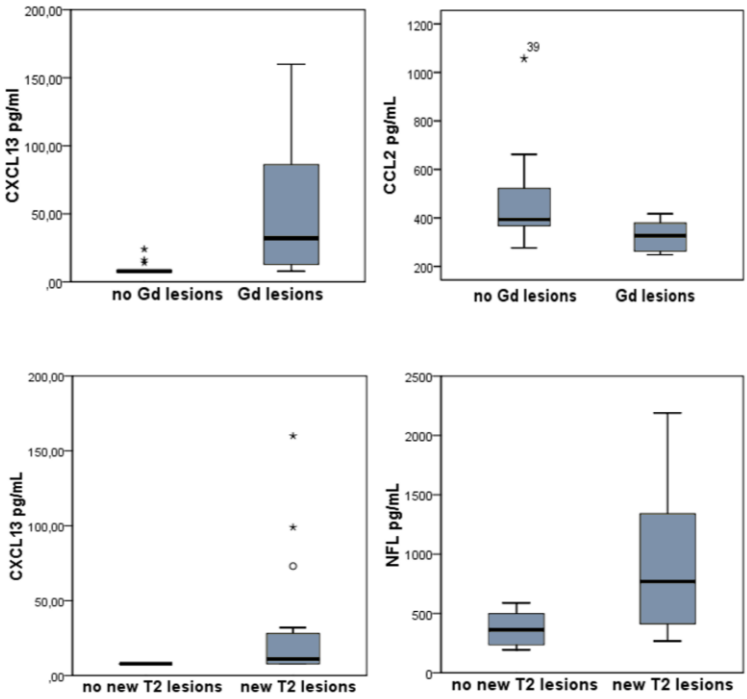


Figure 23. The significant relationships between the biomarker concentrations and MRI measures
Levels of CXCL13, NFL and CCL2 in cerebrospinal fluid in multiple sclerosis patients with or without one or more gadolinium enhanced lesions or T2 lesions. The box indicates the IQR, bar indicates the median, and whiskers indicate the 95% CI. Extreme values are marked with open dots (± 1.5 × IQR) or with asterisks (± 3 × IQR).
CCL2=C-C motif chemokine ligand 2, CI=confidence interval, CXCL13=C-X-C motif chemokine 13, Gd=gadolinium, IQR=interquartile range, NFL=neurofilament light

The group of patients without clinical or radiological activity had increased CHIT1 and CHI3L1 levels in CSF indicating a residual disease activity where the currently used tools are not sufficient to detect it (Paper II).

Degenerative biomarkers were associated with disability and disease progression. The disease duration and the EDSS were negatively correlated with the RNFL ($r=-0.24$, $p=0.0030$ and $r=-0.267$, $p<0.001$, respectively) and the BPF ($r=-0.358$, $p<0.001$ and $r=-0.366$, $p<0.001$, respectively). The disease duration was positively correlated with the EDSS ($r=0.283$, $p<0.001$) and the GFAP ($r=0.311$, $p<0.001$; Paper V).

CSF biomarkers for monitoring therapeutic efficacy (Paper I, II, III)

The treatment with natalizumab and fingolimod reduced the CSF levels of NFL (Paper I, III), and the treatment with natalizumab also reduced CHI3L1, CHIT1, and CXCL13 (Paper II, III). Patients with MS had higher concentrations of NFL, CXCL13, CHI3L1 and CHIT1 than controls ($p <0.001$), but the concentration of GFAP, CCL2 and NRGN did not differ significantly between patients and controls. Patients receiving first-line treatment had significantly higher concentrations of NFL, CXCL13 and CHIT1 than those receiving second-line treatment ($p=0.008$, $p=0.001$ and $p=0.026$, respectively). The levels of NFL, CXCL13, CHI3L1 and CHIT1 in CSF were significantly higher in untreated patients and in patients receiving first-line treatment compared to controls ($p <0.001$). In patients receiving second-line treatment, only CHI3L1 and CHIT1 were significantly elevated compared to controls ($p = 0.001$ and $p = 0.009$ respectively; Paper II; Figure 24).

The levels of NFL, CXCL13, and CHI3L1 decreased ($p < 0.05$) after fingolimod treatment. A further subgroup analysis revealed reduction in NFL ($p < 0.001$), CXCL13 ($p = 0.001$), CHI3L1 ($p < 0.001$), and CHIT1 ($p = 0.002$) in patients previously treated with first-line therapies. In contrast, the levels of all analyzed biomarkers were essentially unchanged in patients switching from natalizumab to fingolimod (Paper III; Figure 25).

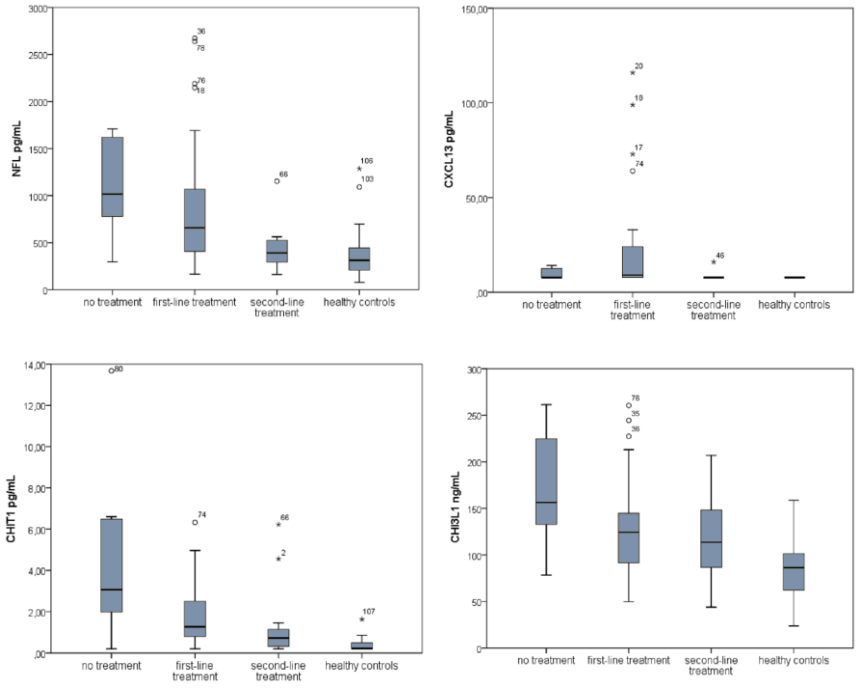


Figure 24. Levels of NFL, CXCL13, CHIT1 and CHI3L1 in CSF in MS patients and HC

Levels of NFL, CXCL13, CHIT1 and CHI3L1 in cerebrospinal fluid in multiple sclerosis patients compared with levels obtained in healthy controls. The box indicates the IQR, bar indicates the median, and whiskers indicate the 95% CI. Extreme values are marked with open dots (± 1.5 × IQR) or with asterisks (± 3 × IQR).

CHIT1=chitotrisidase, CHI3L1=chitinase-3-like protein 1, CI=confidence interval, CSF=cerebrospinal fluid, CXCL13=C-X-C motif chemokine 13, HC=healthy controls, IQR=interquartile range, MS=multiple sclerosis, NFL=neurofilament light

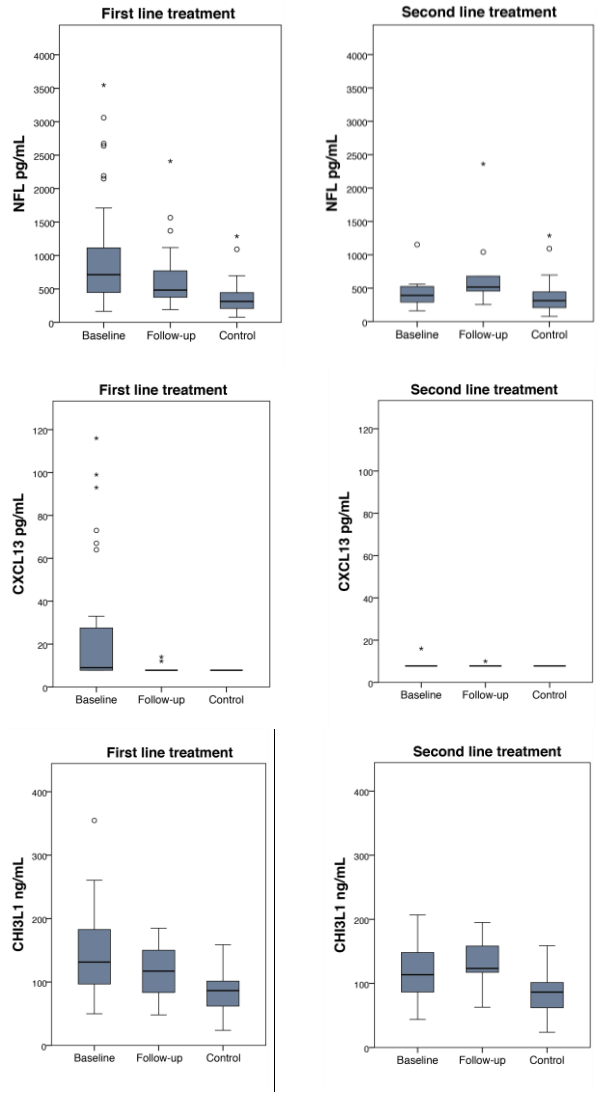


Figure 25. The levels of NFL, CXCL13 and CHI3L1 in MS patients before and after fingolimod treatment compared to HC

The levels of biomarkers in cerebrospinal fluid before and after 4–12 months of fingolimod treatment (n = 43). The levels were compared with levels obtained in age-matched healthy controls (n = 39). The box indicates the IQR, bar indicates the median, and whiskers indicate the 95% CI. Extreme values are marked with open dots (± 1.5 × IQR) or with asterisks (± 3 × IQR).

CHI3L1=chitinase-3-like protein 1, CI=confidence interval, CXCL13=C-X-C motif chemokine 13, HC=healthy control, IQR=interquartile range, MS=multiple sclerosis, NFL=neurofilament light

Adapted from Multiple Sclerosis Journal, Vol 23(1):62-71. Novakova et al, Cerebrospinal fluid biomarkers of inflammation and degeneration as measures of fingolimod efficacy in multiple sclerosis.

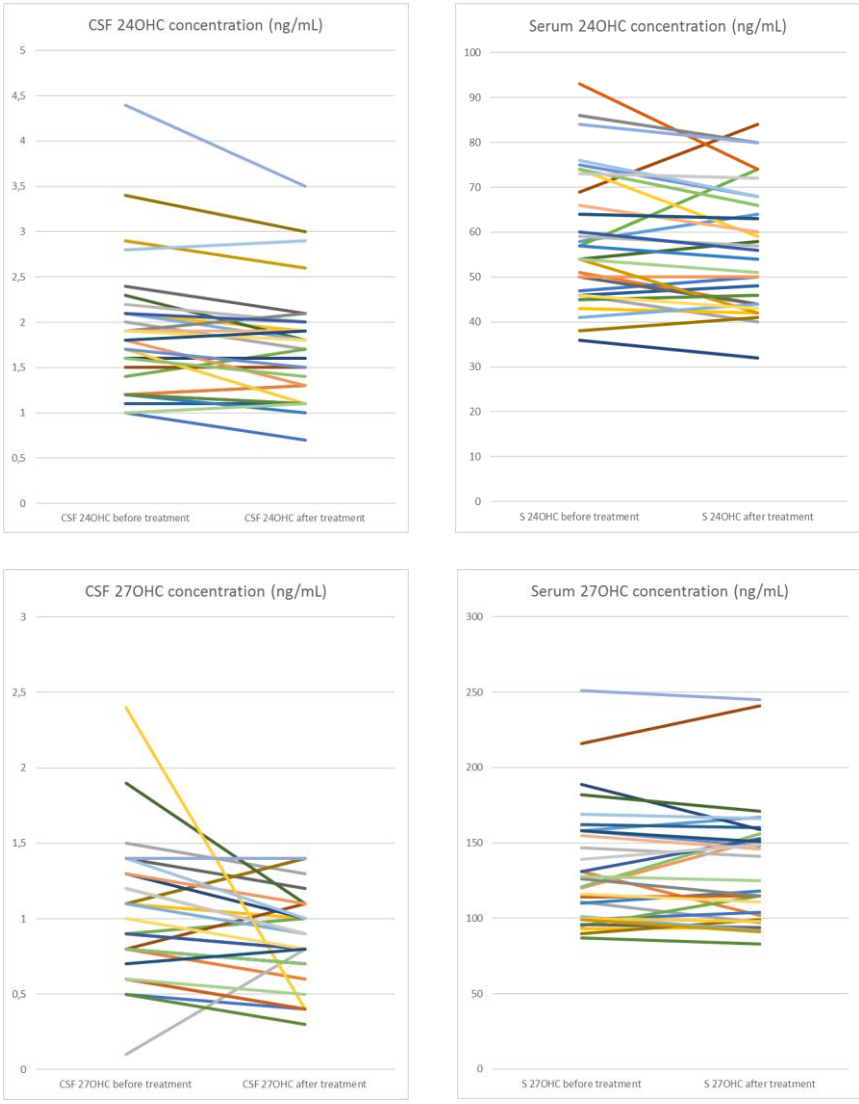


Figure 26. The levels of oxysterols in MS patients before and after natalizumab treatment
The concentrations of 24OHC and 27OHC oxysterols in CSF and serum before and after 12 months of natalizumab treatment in MS patients (n=31).
CSF=cerebrospinal fluid, MS=multiple sclerosis, S=serum, 24OHC=24S-hydroxycholesterol, 27OHC=27-hydroxycholesterol
Reprinted from J Neurol Sci, Vol 358(1-2):201-206. Novakova et al, Reduced cerebrospinal fluid concentrations of oxysterols in response to natalizumab treatment of relapsing remitting multiple sclerosis.

24OHC and 27OHC in CSF were decreased after treatment with natalizumab (Paper I). Natalizumab treatment reduced CSF 24OHC concentrations (p=0.002), CSF 27OHC concentrations (p=0.01) and serum 24OHC concentrations (p=0.029). There was no significant effect of the treatment on serum 27OHC concentrations (Figure 26).

Furthermore, serum concentrations of 24OHC correlated with Symbol Digit Modalities Test scores before (r=0.5, p=0.007) and after (r=0.403, p=0.033) natalizumab treatment. Thus, serum 24OHC correlated with visuo-spatial ability and processing speed in RRMS, therefore it could be a potential biomarker of cognition (Paper I; Figure 27).

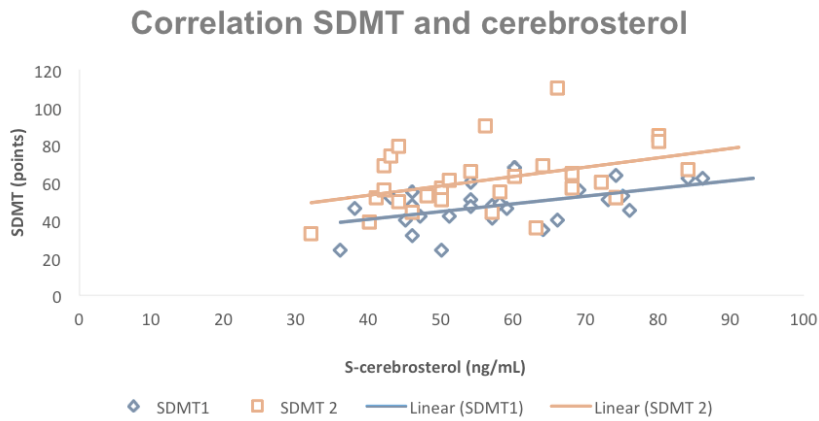


Figure 27. Correlation between SDMT and 24OHC in MS patients before and after natalizumab treatment

Correlation between SDMT and 24S-hydroxycholesterol in serum. Before treatment (SDMT1) r = 0.5, p = 0.007. After treatment (SDMT2) r = 0.403, p = 0.033.

S=serum, SDMT=Symbol Digit Modalities Test, MS=multiple sclerosis, 24OHC=24S-hydroxycholesterol/cerebrosterol

GFAP, NGRN, MCP-1 could not measure the response to the DMTs (Paper II, III).

Moving from CSF to blood: serum NFL for monitoring disease activity and treatment response (Paper IV)

An important limitation of the usefulness of CSF biomarkers is the requirement for repeated LPs. Therefore, the serum NFL was explored. Our study showed a high correlation between NFL levels in serum and CSF in MS patients ($r=0.629$, $p<0.001$), HC ($r=0.385$, $p<0.001$) and patients with OND ($r=0.740$, $p<0.001$; Figure 28).

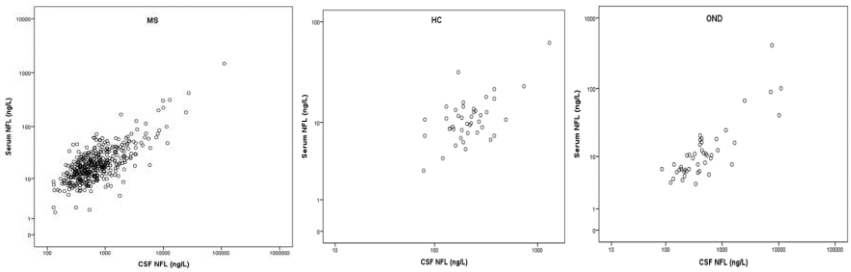


Figure 28. Correlations between serum and CSF NFL in MS patients, in HC and in patients with OND
Correlation between NFL concentrations in serum and CSF (A) in patients with MS was r=0.620 (95% CI 0.558–0.675, p<0.001), (B) in HCs was r=0.385 (95% CI 0.092–0.616, p<0.001), and (C) in patients with OND was r=0.740 (95% CI 0.571–0.849, p<0.001).
CI=confidence interval, HC=healthy control, MS=multiple sclerosis, NFL=neurofilament light, OND=other neurological disorder or symptom.

We showed that measuring disease activity is possible in serum, as the NFL levels in serum were increased during relapse and during the presence of Gd-enhancing lesions (Figure 29).

The NFL levels in serum decreased after a year of treatment in both patients who initiated first-line or second-line therapy, and in those who escalated treatment from first-line to second-line therapy (Figure 30).

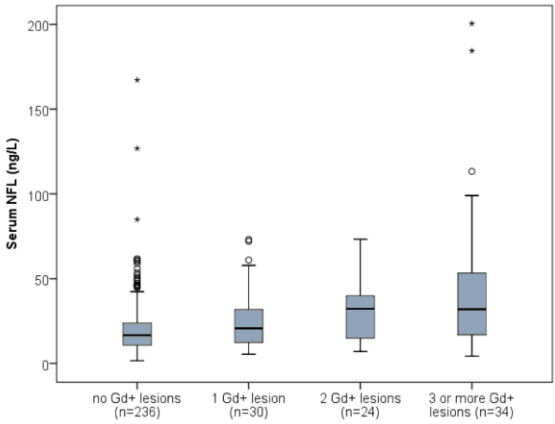


Figure 29. The levels of NFL in serum and the number of Gd-enhancing lesions

Serum NFL concentration in patients with no Gd-enhancing lesions (n=236) was 16.8 (IQR 10.5–24.6) ng/L, with 1 Gd-enhancing lesion (n=30) was 21.3 (IQR 12.8–36.5) ng/L, with 2 Gd-enhancing lesions (n=24) was 31.9 (IQR 14.5–39.9) ng/L in serum, and with >3Gd-enhancing lesions (n=34) was 31.9 (IQR 17.4–55.6) ng/L. Box indicates IQR; bar indicates median, and whiskers indicate 95% confidence interval. Extreme values are marked with open dots (±1.5 IQR) or with asterisks (±3 IQR).

IQR=interquartile range, MS=multiple sclerosis, NFL=neurofilament light.

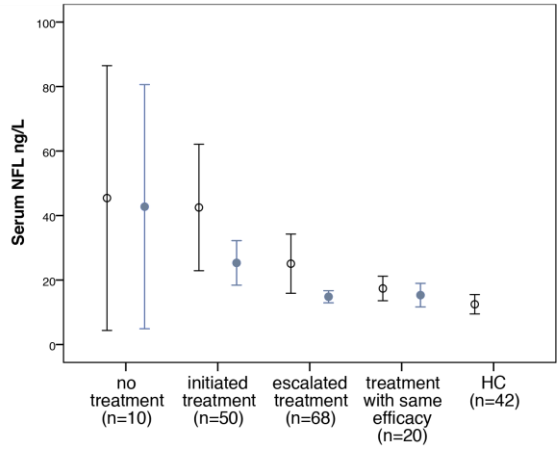


Figure 30. The levels of NFL in serum in MS patients at baseline and follow-up and in HC

Serum NFL concentrations at baseline and follow-up in patients with MS who remained untreated, in patients with MS who initiated treatment with DMTs, in patients with MS who escalated DMT to more effective therapy, in patients with MS who changed treatment between DMTs of similar efficacy, and in HCs.

The figure shows median (dots) and 95% confidence interval (lines) of serum NFL concentrations. Black color represents serum NFL at baseline. Blue color represents serum NFL at follow-up.

DMT=disease-modifying therapy, HC=healthy control, MS=multiple sclerosis, NFL=neurofilament light.

Discussion

In this thesis we showed that DMTs for MS influence biomarker concentrations in CSF. The therapeutic effect on clinical as well as on MRI outcomes correlated with CSF biomarker levels. We also found that the effect was not attributed to inflammatory biomarkers but also involved degenerative biomarkers. In fact, we could to some extent demonstrate the impact from DMTs on different parts of the pathophysiology of MS. The study populations were consecutively included from different MS centers in Sweden and represent "real life" subjects. Thus, we showed that it is possible to monitor treatment effects with several of the investigated biomarkers. The most promising out of the studied CSF biomarkers was NFL, a marker of axonal damage. Although high level of NFL is a marker of neuro-axonal degeneration in several neurological degenerative disorders, NFL seems to better reflect inflammatory activity than progression in MS. This characteristic of NFL was confirmed in this thesis. The usefulness of NFL has been limited due to the need of repeated lumbar punctures. This obstacle has been removed with the development of ultrasensitive immunoassay, allowing determination of NFL in blood. We showed that NFL levels in blood correlated strongly with the levels in CSF and that similar results were observed in blood and CSF after the treatment with different DMTs. Thus, it seems plausible that axonal damage, the culprit of disability in MS, can be monitored with NFL levels in blood.

Monitoring treatment efficacy with biomarkers in CSF

In recent years, a number of different DMTs have emerged and the need for objective biomarkers is growing in order to facilitate the decision-making process when choosing the most appropriate therapy. In this process, the development of reliable biomarkers for prediction, disease activity and progression is obvious. The presented studies explore the value of biochemical biomarkers as a complement to clinical and MRI measures. The reduction or increase in biomarker levels could be used to determine the treatment efficacy.

The effect of DMTs on the biomarkers levels were shown in the studies included in this thesis and in other studies during recent years. The DMTs decreased CSF levels of NFL [II, III, 128, 141, 150] and serum levels of NFL [IV, 153, 155]. They reduced CSF levels of CHI3L1 [II, III, 112], CHIT1 [II, III,

119] and CXCL13 [II, III, 71]. In addition, higher NFL and CXCL13 levels were found in patients treated with first-line therapies compared to second-line therapy, reflecting the rate of the treatment efficacy [II]. Thus, there is accumulating evidence suggesting that these biomarkers may be useful in the evaluation of DMT intervention. Interestingly, even though the currently used DMTs are all essentially immunomodulatory, the neurodegenerative biomarkers NFL [III, 150], 24 OHC [I] and beta amyloid [226] were reduced by DMT. These findings support the use of multiple biomarkers, which may better reflect the interplay between pathological processes in MS and the complexity of the disease.

Our studies confirmed the association between disease activity, disease progression, and the levels of CSF biomarkers in MS. The increased CSF levels of NFL, CXCL13, CHI3L1 and CHIT1 were associated with relapse rate and formation of new lesions on MRI [II, III, IV] and higher levels of CSF GFAP were associated with disease progression [V]. The correlation between biomarkers and disease activity [75, 76, 107, 120, 125, 128, 129, 133, 141, 227, 228] and disease progression [75, 107, 139] have been reported previously and our studies showed similar results.

We showed that patients with clinical and radiological stability may still show signs of residual inflammatory activity. In paper III, some patients displayed increased CSF levels of CHI3L1, CHIT3 and CXCL13 without relapse or MRI activity. This means that there may be inflammation that escapes detection of current methods. However, one drawback of measuring inflammatory biomarkers is that they are not CNS specific and therefore inflammatory processes outside the CNS may influence CSF levels. Inflammatory activity captured from biomarkers in blood does not reflect the rate of inflammation in MS. Nevertheless, the interplay between the peripheral and CNS immunity is higher than previously thought [53] and migration of active immune cells and chemokines across the BBB is extensive [44, 53]. Although the effect of DMTs is essentially on the systemic or peripheral immune system, the impact on the CNS inflammation is profound. Hence, the effect of DMTs on inflammatory biomarkers in blood or CSF may be difficult to interpret. Increased levels of these biomarkers in blood or even CSF may not reflect the ongoing pathological processes in MS.

Several studies on CSF biomarkers have shown their usefulness in determination of prognosis, in assessing the disease activity and therapeutic efficacy. An important limitation of their use is the requirement for repeated lumbar puncture. With the ultrasensitive SIMOA technology [229], the measurement of NFL levels in serum/plasma is now possible. We observed increased levels of NFL in serum in MS patients during relapse or in the presence of Gd-enhancing lesions, and serum NFL levels reflected the efficacy of DMTs [IV]. We also showed high correlation between NFL determined in serum and CSF in MS patients, in HC and in patients with OND [IV]. In line with our previous results in CSF [75, 76, 128], similar relationships

between serum NFL levels and disease activity were found [153]. For the first time, we could reflect the efficacy of various DMTs by determining the serum NFL concentration in a real-life cohort of patients. This finding implies that monitoring of disease activity in MS could be simplified by taking repeated blood samples instead of performing lumbar punctures. Previous studies on serum/plasma NFL showed increased concentrations also in other conditions damaging CNS, e.g. in HIV-related encephalopathy [225], in traumatic brain injury [230], or in American football players after an intensive playing season [231]. Thus, determination of serum NFL may be a useful biomarker in several neurological conditions.

Current monitoring of disease activity, progression and therapeutic efficacy in MS rely on clinical and MRI assessments, often performed only once or twice a year. Diffuse or low intensive activity may not be captured and these methods do not determine the pathophysiological effects of intervention with DMTs. In this thesis [I-IV] we have shown that the CSF biomarkers NFL, CXCL13, CHI3L1, CHIT1, 24S-OH are influenced by DMTs indicating that several pathological processes are inhibited. In addition, serum NFL may add important information on activity and degeneration in MS that is currently missed with clinical and MRI measures. Serum NFL could improve the monitoring of MS patients to maintain a disease-free status as it is highly unlikely to have disease activity while having normal levels of serum NFL [IV]. The regular determination of serum NFL could be added to the current NEDA definition [92, 94].

Exploring the interplay between inflammation and neurodegeneration

Studying specific biochemical biomarkers contributes to our understanding of MS pathophysiology and how inflammation and degeneration intertwine.

In paper V, correlations between biomarkers showed two distinct clusters, one consisting of inflammatory biomarkers in the CSF (number of lymphocytes, IgG index, intrathecal IgG production, and OCB), the NFL and the GFAP in CSF. The other cluster consisted of neurodegenerative biomarkers (brain volume measures, RNFL, and MV). These two clusters could separate MS patients from HC. The degenerative biomarkers were associated with disability and longer disease duration.

Although the investigated biomarkers could be separated into degenerative and inflammatory biomarkers, we found that they were also correlated in MS patients [II]. However, there was no evidence of early neurodegeneration in newly diagnosed RRMS patients [V]. The GFAP level (representing the degree of astrogliosis), the thickness of RNFL and the BPF (representing

the degree of degeneration) were unaffected in CIS and early RRMS and similar to the levels found in HC.

The lack of signs of degeneration in early MS and the correlation between inflammatory and degenerative biomarkers are both in line with the hypothesis that inflammation is the primary event and responsible for neurodegeneration, at least in the early stages of MS [51, 52]. However, there was no correlation between two degenerative biomarkers, NFL and 24OHC [I]. This could probably be explained by differences in their origin, metabolism and elimination. Even if both 24OHC and NFL are classified as degenerative biomarkers, they represent different aspects of the pathology of MS and both their turnover and elimination differ. NFL in CSF is a marker of axonal damage with the peak concentration shortly after relapse but 24OHC is affected by a plethora of pathological processes. Besides neuronal damage, the CSF 24OHC concentration is influenced by the number of metabolically active neurons [178], hydroxylation [232, 233] and the rate of the 24OHC clearance from the CSF [176]. However, concentrations of CSF 24OHC are fairly constant in adults, and less than 1% of the total excretion of 24OHC is via the CSF [233]. Therefore, the reduction of 24OHC by DMT is probably more influenced by reduced neuronal damage and degeneration than changes in neuronal metabolism. Almost all of the other studied oxysterol 27OHC observed in CSF originates from peripheral blood and the concentration is to some extent depending on the functional state of the BBB [233]. Thus, the biomarkers reflect various aspects of the MS pathophysiology and the influence of DMTs on various pathological processes in MS.

Improving MS diagnostics with biomarkers

Until recently, the diagnostic criteria of MS [15] were based purely on clinical and typical MRI findings. The value of CSF findings in the diagnosis of MS has been limited after the introduction of MRI in the assessment of MS. In the 2010 revision of the McDonald critera, the CSF assessment was not included in the diagnostic critera of RRMS [15]. However, in many countries determination of increased intrathecal IgG synthesis has remained a valuable analysis in the diagnostic work-up of MS, and the usefulness in the differential diagnosis of MS has never disappeared. Recently, a new revision of the McDonald criteria of MS was published [16]. One of the major changes was the re-introduction of OCB in CSF, indicating selective intrathecal IgG synthesis in the CSF/CNS compartment. In patients with clinical and MRI findings supporting dissemination in space, it is not necessary to find evidence of contrast enhancement in at least one MS lesion or at least one new lesion on a subsequent MRI examination to show DIT. The presence of CSF OCB can substitute the DIT requirement [16], so the CSF examination has

regained its importance. This is in line with the results in paper V, where we confirmed that except for conventional MRI, the CSF biomarkers (cell count, albumin ratio, IgG index and OCB) are the most reliable and robust diagnostic biomarkers in MS. In case of negative CSF biomarkers, CSF NFL could increase the diagnostic accuracy [V]. Thus, NFL is a potential diagnostic biomarker, which has previously also shown predictive value and correlated to disease severity [145, 146, 156]. NFL also showed high sensitivity when separating healthy subjects, presenting with neurological symptoms, from those who had MS or other neurological disease [V]. Normal NFL levels were found in persons essentially free from neurological disease [V]. Hence, persons with symptoms indicative of a demyelinating event but with normal CSF NFL levels are highly unlikely to suffer from neurological CNS disease.

Strengths and limitations

The strength of our studies is the consecutive inclusion of patients, creating an unselected material [I-V]. Inclusion of untreated patients with different disease duration at the time of diagnosis [V] enabled measurement of neurodegenerative biomarkers in untreated MS patients at different stages of the disease and in non-MS patients (=symptomatic controls without multiple sclerosis, but showing symptoms indicative of MS). This allowed us to evaluate neurodegeneration and to differentiate the groups of patients. All patients seeking for suspected MS symptoms were included in the study and assessed with the extensive diagnostic battery of different biomarkers to evaluate them and to explore the presence of early degeneration in MS. The methods used for measuring neurodegeneration are widely available and easy to use in daily MS care. Another strength of the study is the statistical calculation of how these methods can contribute to diagnostics and the comprehensive matrix of the relationships between all biomarkers.

The limitation is the cross-sectional design in paper II and V lacking the evaluation of predictive measures and treatment effects. However, the purpose of these two studies was to focus on the diagnostic work-up and the attempt to refine the diagnostics, stratification of the MS patients and the evaluation of relationships between the biomarkers.

In order to correctly interpret CSF biomarker data in relation to disease activity or treatment efficacy, certain aspects must be considered. Firstly, the treatment duration may influence biomarker levels. Both first- and second-line MS therapies usually achieve an effect on MRI and clinical measures after several months of treatment. This was taken into consideration when including patients in our studies. Patients classified as treated with DMTs were treated more than 1 year in order to be sure that the

treatment has reached its effect. Untreated patients were without DMTs for at least 1 year. Also, the length of wash-out periods may have influenced biomarker levels at baseline. This was taken into account when discussing the results [III]. Secondly, some of the biomarkers are age-dependent e.g. NFL [75, 76, 117], GFAP [75, 223], CHI3L1 [117], and CCL2 [129]. In our studies, all correlations were adjusted for age or the groups were age-matched. Thirdly, repeated lumbar puncture may not be feasible in some patients because of side effects or fear of the sampling procedure. Therefore, the possibility of clinical monitoring of therapeutic interventions using CSF biomarkers is limited. Results from analysis of CSF biomarkers may essentially contribute to the evaluation of new DMTs in phase II trials or in making difficult therapeutic decisions easier for selected patients.

CSF biomarkers are related to different pathological processes involved in MS and could provide an additional dimension in the assessment of treatment efficacy even in clinical practice. However, the CSF concentrations of CXCL13 and CHIT1 were frequently below the detection levels of the immunoassay, their usefulness for monitoring therapeutic efficacy seemed therefore limited [II, III].

Our study on serum NFL [IV] was based on a large set of paired serum and CSF samples from a real-life cohort of patients across a wide clinical and therapeutic spectrum. They support serum NFL as a biomarker for monitoring disease activity and treatment intervention in MS in clinical settings. We found that serum and CSF NFL concentrations were highly correlated and reacted similarly during the different stages of MS and in response to treatment with DMTs. This paired serum and CSF material is a strength of our study.

Owing to the non-randomized design of our studies, we cannot rule out that reduced biomarker levels could be due to regression to the mean. However, this explanation seems less plausible because of the magnitude of the biomarkers reduction. It should also be noted that treatment was not selected on the basis of biomarker levels, which therefore should not have contributed to a selection bias regarding those parameters. This is one disadvantage of our observational study compared to the RCT on fingolimod in RRMS [156] where the placebo group is available. The treatment effect on biomarker levels could be assessed in relation to the biomarker levels determined in the placebo treated patients. On the contrary, the advantage of our study is the unselected real-life population showing that practical use of serum NFL in daily MS care is possible.

Conclusion and Future Perspective

In this thesis, we showed the impact of treatment on the serum and CSF biomarkers and correlation of these biomarkers with clinical and radiological measures. Furthermore, we showed that serum NFL is a potential biomarker for monitoring disease activity and treatment efficacy in clinical settings. Finally, we showed that CSF NFL increased the diagnostic accuracy in MS and that OCT and SyMRI measures did not contribute in the diagnostic work-up.

Out of the studied biomarkers, NFL showed the most clinically relevant results being able to help in diagnosing MS, monitoring the disease activity and the response to the DMTs. Furthermore, it is the one of the few known neurospecific biomarkers available in peripheral blood. NFL in CSF plays an important role in MS diagnostics and might help distinguish patients seeking for neurological symptoms who are healthy from those with MS or other neurological disorders.

The pattern of NFL changes was almost identical in plasma and CSF. Thus, plasma NFL may be a valuable tool to evaluate CNS injury in MS but also in other neurological conditions. Plasma NFL may replace CSF analysis despite the differences of NFL concentrations in the two fluids [225].

Further investigations on serum/plasma NFL in large and longitudinal patient cohorts are needed before it can be established as a biomarker to measure disease activity, to determine prognosis, and to measure treatment response in patients with MS and other neurological diseases in clinical practice. The assessment of temporal changes in serum NFL concentration in relation to clinical disease activity and new MRI lesions needs to be explored. Also, a study to determine sensitivity and specificity of CSF and serum NFL in a larger population is planned. The biomarkers have the ability to describe various pathophysiological mechanisms in MS, and they could be used to measure specific effects of an intervention.

The imaging biomarkers of neurodegeneration, RNFL and BPF, were correlated to the disease duration, and MS patients with longer disease duration showed a higher level of neurodegeneration. Thus, these imaging biomarkers have potential to measure neurodegeneration prospectively. It is questionable if all available DMTs can reduce neurodegeneration in MS, and their usefulness will be the objective of longitudinal prospective studies. BPF and RNFL need to be further evaluated in order to establish their place in measuring treatment efficacy and to determine their value as prognostic biomarkers. The long-term follow-up of patients is planned to im-

prove the counselling of patients regarding diagnosis, prognosis and in the decision-making between various MS therapies.

Acknowledgements

This thesis is a result of approximately 5 years of work and many persons have been involved. Firstly, thanks to my main supervisor **Professor Jan Lycke** for giving me the chance to discover what research is about, for all the patience and support from the beginning, and for reading and improving all the work I have done. To my co-supervisor **Professor Henrik Zetterberg**, thank you for your enthusiasm, you are an inspiration.

A very special thanks goes out to my colleagues and co-authors **Markus Axelsson** and **Clas Malmeström** for helping me along the way, collecting the data and for keeping me company at lunch and coffee breaks.

Co-authors **Kaj Blennow, Mohsen Khademi, Fredrik Piehl, Tomas Olsson, Martin Gunnarsson, Anders Svenningsson, Peter Sundström, Ingemar Björkhem, Virginija Danylaité Karrenbauer, Henrik Imberg, Olle Elias och Olle Nerman** for their valuable contributions to our papers included in this thesis.

Professor emeritus **Oluf Andersen** who first introduced me to the MS research.

I am also grateful to the whole MS team at Sahlgrenska University Hospital for taking care of our patients and for helping me whenever I needed it, with a special mention to **Birgitta Heimer**, **Snezana Josifovska**, **Pia Levander**, **Karin Ängbacke**, **Gisela Andblad** and **Hedvig Myhrberg**. To the research nurses **Anne Brandt**, **Cecilie Scheiene**, **Steffi Unger**, **Viktoria Sjöstedt** and **Lena Silberberg** for the hard work you do every day in our studies, for assessing the patients and for spreading a good mood at the department, with a special mention to Anne for all your support.

I am also grateful to the following MS-ologists: **Björn Runmaker**, **Ólöf Elíasdóttir, Tatiani Fainekou**, **Sofia Sandgren** and **Peter Vaghfeldt** for your cooperation.

All my colleagues at the Neurology Department for their assistance with the inclusion of patients in our studies. You are all fantastic colleagues!

My boss **Mikael Edsbagge** for giving me the time to work on this thesis. To the university staff at the Institute of Neuroscience and Physiology at Sahlgrenska Academy, in particular to those working at the Department of Clinical Neuroscience for making this thesis possible.

My research would not be possible without funding. I am grateful to Swedish State Support for Clinical Research (ALFGBG), NEURO Sweden, the Research Foundation of the Multiple Sclerosis Society of Gothenburg, the Edith Jacobsons Foundation, Helena Ahlin Foundation and Odd Fellow

Karlstad, pharmaceutical companies Novartis, Biogen and TEVA (unconditional grants) for their financial support.

To my life-coach and the best man in the world, **Carl-Johan**, I cannot thank you enough for your love, wisdom, support, and incredible friendship. My super ambitious sisters, **Martina** and **Kristína**, for helping me keep up the pace. A special thanks to Martina for sharing her experience, and for the artwork and graphical design of this thesis. My parents, **Anna** and **Marko**, and my parents-in-law, **Ann-Charlotte** and **Michael**, because you are always there and help us unconditionally. I am also grateful to my other family members and friends who have supported me along the way.

And finally, last but by no means least, thanks to all the patients and healthy participants for taking part in our studies, for your time and interest in participating and contributing to new knowledge and scientific progress.

Thanks!

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