Investigation of the pathophysiology of progression in multiple sclerosis

Studies on cerebrospinal fluid biomarkers

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We work in the dark – we do what we can – we give what we have.
Our doubt is our passion and our passion is our task.
The rest is the madness of art.

Henry James (1843-1916)

Abstract

Multiple Sclerosis (MS) is considered an autoimmune disease of the central nervous system (CNS). It usually starts with a relapsing remitting (RR) course that eventually transforms into progressive (P)MS, showing neurodegenerative features. The pathogenesis behind the transition from RRMS to PMS is essentially unknown.

The aim of this thesis was to investigate if biomarkers in the cerebrospinal fluid (CSF) of MS patients could provide new insights into the pathophysiology of MS progression, and if biomarker levels could reflect disease activity, disability progression, or therapeutic efficacy.

Three study designs were established. The first was cross sectional and comprised MS patients, healthy controls (HC) and control subjects with another inflammatory disease. The second used a long-term follow-up setting in which RRMS, PMS and HC were assessed twice 8-10 years apart. The third used immunomodulatory or immunosuppressive intervention (natalizumab, mitoxantrone or rituximab) and assessed MS patients pre- and 12-24 months post-treatment. CSF biomarkers were analyzed for i) axonal damage (neurofilament light, NFL), ii) astrogliosis (glial fibrillary acidic protein, GFAP), iii) amyloid precursor protein metabolism (BACE1 activity, and sAPP/Aβ metabolites) iv) B-cell regulation (CXCL13) and v) intrathecal IgG synthesis (IgG index, oligoclonal IgG bands (OCB)).

Increased mean GFAP levels were found in all courses of MS with the highest levels in PMS, whereas the mean NFL level of this MS population was not different from that of HC (Paper I). At long-term follow-up GFAP levels correlated with disability and had prognostic value. In contrast, increased NFL levels were found in another MS population compared to HC (Paper IV). This discrepancy might be explained by differences in disease activities between the investigated populations and due to improved sensitivity of the NFL immunoassay. We found signs of downregulation of BACE1 activity (Paper II) and sAPP/Aβ metabolism (Paper III) in MS. The levels of sAPP/Aβ in MS were generally decreased compared to HC suggestive of impaired neuronal function in MS. Mass spectrometry studies indicated that the sAPP/Aβ metabolism was changed in PMS compared to HC by formation of other decomposition products.

We demonstrated, in opposite to the general view, changed number and pattern of OCB in CSF over time, which correlated to CXCL13 levels (Paper V). Natalizumab treatment increased sAPP Aβ metabolites towards HC levels. Immunosuppressive treatment (mitoxantrone, rituximab) reduced NFL and CXCL13 in PMS. Interestingly, significantly lower NFL levels were found prior to immunosuppression in PMS patients previously treated with interferon beta or glatiramer acetate, suggest-

ing an impact on axonal damage also with first line MS therapies. Immunosuppressive treatment did not influence the number or pattern of OCB (Paper V).

In conclusion, our studies present evidence that increased immune activity plays a critical role in PMS for axonal damage and seemed to influence sAPP/Aβ metabolism. In PMS, the reduced NFL level following immunosuppressive treatment clearly supports a relationship between CNS inflammation and neurodegeneration. Biomarkers in CSF provide unique information about the pathophysiology in PMS, and may serve as complement to clinical and MRI measures for assessment of disease activity, progression, severity and therapeutic efficacy.

Key words: multiple sclerosis, cerebrospinal fluid, biomarker, disease progression, NFL, GFAP, CXCL13, BACE1, sAPP/Aβ, IgG, oligoclonal IgG bands, IgG index

Populärvetenskaplig sammanfattning

Multipel Skleros (MS) är en av de vanligaste orsakerna till neurologiskt handikapp bland unga vuxna i västvärlden. I Sverige lever 17 500 personer med sjukdomen. Minst två tredjedelar av de drabbade är kvinnor. MS är en autoimmun sjukdom som drabbar det centrala nervsystemet (CNS). Vanligtvis debuterar sjukdomen med relapsing remitting (RR) MS där återkommande försämringsepisoder (skov) följs av hel eller delvis återhämtning (remission). Efter i genomsnitt 15-20 år övergår sjukdomen i sekundär progressiv (SP) MS där en gradvis försämring inträder. Vid MS föreligger inflammatoriska cellinfiltrat i avgränsade områden (plaque) i hjärna och ryggmärg. Där skadas nervfibrernas isolering (myelin), myelinbildande celler (oligodendrocyter), stödjeceller (astrocyter) och nervcellernas utskott (axon). I SPMS sjunker eller upphör skovfrekvensen och den neuro-axonala förstörelsen är utbredd med förtvining (atrofi) av både vit och grå substans i CNS. Man vet idag att det är förstörelsen av axon som framförallt orsakar sjukdomssymptomen och den progressiva neurologiska funktionsförlusten.

Sista decenniernas genombrott vad gäller behandling av MS har ingivit hopp om att kunna förbättra prognosen för många MS patienter. Framgångarna har dock i princip gällt RRMS och förståelsen för vilka mekanismer som är speciella för progressiv (P) MS är fortfarande bristfällig.

Tanken bakom avhandlingens studier var att med hjälp av biomarkörer i ryggmärgsvätska (CSF), hitta samband för att öka förståelsen av dessa mekanismer. Vi ville också karaktärisera biomarkörerna för att bestämma deras förmåga att avspegla sjukdomsaktivitet, progression och terapeutiska effekter vid PMS.

I CSF undersöktes nivåer av i) neurofilament (NFL), en axonskademarkör, ii) gliafibrillärt surt protein (GFAP), en markör för astrocytaktivering, iii) BACE1 aktivitet och amyloid precursor protein/betamyloid (sAPP/Aβ) metabolismen och iv) tecken på B-cells reglering av inflammation (CXCL13) och en ökad produktion av immunoglobuliner.

Studierna har byggts upp kring tre studiedesigner med tillhörande patient- och kontroll populationer. I den första användes en tvärsnittsanalys, i den andra gjordes en långtidsuppföljning, och i den tredje studerades effekten på biomarkörer under immunomodulerande eller immunosuppressiv terapi (natalizumab, mitoxantrone eller rituximab). Patienterna som långtidsuppföljdes och de som behandlades undersöktes vid två tillfällen med intervall på 8-10 år respektive 1-2 år.

CSF biomarkörerna visar att inflammation utgör en betydande del av sjukdomsmekanismen också vid PMS och att den går att påverka med läkemedel som dämpar immunaktiviteten. NFL nivåerna var stegrade vid PMS. Behandling med cellgiftet

mitoxantrone och det immundämpande medlet rituximab sänkte NFL nivåerna. Vi såg en tydlig koppling mellan immunrespons (CXCL13 nivåer och inflammation synlig på magnetkamera(MRI)) och axonskada (NFL).

Under hela MS förloppet sågs tecken på aktivering av hjärnans stödjeceller med ökat läckage av GFAP till CSF. Detta var mest uttalat vid hög sjukdomsaktivitet som ett tecken på ett akut astrocytsvar men sågs också vid långvarig sjukdom som ett troligt mått på den sammanlagda spridda plaque bildningen i CNS. GFAP nivåerna visade ett samband med klinisk funktionsnedsättning och visade prognostiskt värde men påverkades inte av terapi.

BACE1 aktivitet och sAPP/Aβ metabolismen har tidigare framförallt studerats vid Alzheimer's sjukdom, men har sista åren också studeras vid inflammatoriska sjukdomar. I våra studier fann vi att BACE1 aktivitet och sAPP/Aβ nivåerna sjönk vid MS generellt, sannolikt som tecken på pågående inflammation och möjligen störd nerv funktion. Vid RRMS ökade nivåerna mot de normala efter natalizumab behandling vilket inte skedde efter mitoxantronebehandling av SPMS. Genom utvidgade studier med mass spectrometri sågs tecken på att metabolismen inte bara är sänkt utan också ändrad vid PMS genom att andra nedbrytningsprodukter bildas.

En generell uppfattning är att immunglobolinproduktionen av oligoklonala IgG band i CSF är oförändrade över tid. Våra observationer motsäger detta och visar en koppling till att OCB bildningen är relaterad till B-cells aktiviteten. Antalet band och mönstret av OCB ändrades över tid men inte av behandling med mitoxantrone.

Våra studier av biomarkörer i CSF talar för att inflammatorisk aktivitet har betydelse för degenerativa processer såsom axonal skada och ändrad sAPP/Aβ metabolism. Vi visar att detta samband även finns vid PMS och att immunhämmande behandling kan påverka dessa processer. Några av dessa biomarkörer kan komma att få betydelse för värdering av sjukdomsaktivitet (NFL, CXCL13), progression och svårighetsgrad (GFAP) och monitorering av terapi (NFL, CXCL13, sAPP/Aβ metaboliter) vid progressiv MS.

List of original articles

Paper I

M. Axelsson, C. Malmeström, S. Nilsson, S. Haghighi, L. Rosengren, J. Lycke
Glial fibrillary acidic protein: a potential biomarker for progression in multiple sclerosis.
J Neurol 2011; 258: 882-888

Paper II

N. Mattsson, **M. Axelsson**, C Malmeström, G Wu, R Anckarsäter, S Sankaranarayan, U Andreasson, S Fredrikson, A. Gundersen, L Johnsen, T Fladby, A Tarkowski, E Trysberg, A Wallin, H Anckarsäter, J. Lycke, O Andersen, AJ. Simon, K Blennow, H Zetterberg
Reduced cerebrospinal fluid BACE1 activity in multiple sclerosis
Mult Scler 2009 ; 15: 448-454

Paper III

K. Augutis®, **M. Axelsson**®, E. Portelius, G. Brinkmalm, U. Andreasson, M. K Gustavsson, C. Malmeström, J. Lycke, K. Blennow, H. Zetterberg and N. Mattsson
®contributed equally
Cerebrospinal fluid biomarkers of ß-amyloid metabolism in multiple sclerosis
Mult Scler published online 15 October 2012

Paper IV

M. Axelsson, C. Malmeström, M. Gunnarsson, H. Zetterberg, P. Sundström, J. Lycke®, A. Svenningsson®
®contributed equally
Immunosuppressive therapy reduces axonal damage in progressive multiple sclerosis
Manuscript-Submitted

Paper V

M. Axelsson, N. Mattsson, C. Malmeström, H. Zetterberg, J. Lycke
The influence from disease duration, clinical course, and immunosuppressive therapy on the synthesis of intrathecal oligoclonal IgG bands in MS
Manuscript-Submitted

Contents

List of abbreviations

Aβ	Beta amyloid
APP	Amyloid precursor protein
BACE1	β-site APP cleaving enzyme
BBB	Blood-brain barrier
CD	Cluster of differentiation
CIS	Clinically isolated syndrome
CNS	Central nervous system
CSF	Cerebrospinal fluid
CXCL 13	Cys-X-Cys motif ligand 13
CV	Coefficient of variation
DIS	Dissemination in space
DIT	Dissemination in time
DMT	Disease modifying treatment
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein Barr virus
EDSS	Expanded disability status scale
ELISA	Enzyme linked immunosorbent assay
FLAIR	Fluid attenuated inversion recovery
FS	Functional system
GA	Glatiramer acetate
Gd^{+}	Gadolinium enhancement
GFAP	Glial fibrillary acidic protein
HC	Healthy controls
HLA	Human leukocyte antigen
HR	Hazard ratio
IFNB	Interferon beta
IL	Interleukin
Ig G	Immunoglobulin G
IP	Immunoprecipitation

kDa	kilo Dalton
LC-MS/MS	liquid chromatography
MAG	Myelin-associated glycoprotein
MALDI-TOFMS	matrix assisted laser desorption/ionization time-of-flight mass spectrometry
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MSFC	Multiple sclerosis functional composite scale
MS-MS	Tandem mass spectrometry
MSSS	Multiple sclerosis severity score
Mx	Mitoxantrone
NAB	Neutralizing antibodies
NFH	Neurofilament-heavy chain
NFL	Neurofilament-light chain
NFM	Neurofilament-medium chain
NRG1	Neuregulin 1
Nz	Natalizumab
OCB	Oligoclonal IgG bands
OR	Odds ratio
PML	Progressive multifocal leukoencephalopathy
PMS	Progressive multiple sclerosis
PPMS	Primary progressive multiple sclerosis
RRMS	Relapsing remitting multiple sclerosis
sAPP	soluble amyloid precursor protein
SAS	Spinal anaesthesia subjects
SD	Standard deviation
SLE	Systemic lupus erythematosus
SPMS	Secondar progressive multiple sclerosis
Th 2 cells	T helper 2 cells
TNFα	Tumor necrosis factor α
VCAM-1	Vascular cell adhesion molecule 1

1. Introduction

1.1 General background

Multiple Sclerosis (MS) is an organ-specific autoimmune disease of the central nervous system (CNS). The aetiology and pathogenesis of MS remain largely unknown. There is evidence that it may begin as a primarily inflammatory disorder that subsequently takes on degenerative features; however the inflammation activity seems to vary widely between patients (1).

MS is initially dominated by focal white matter inflammatory infiltrates, with demyelinating lesions also appearing in the cortex and deep grey matter of the brain. Additionally, the CNS tissue shows diffuse and global changes, including signs of widespread inflammation (2), microglial activation (3), neuron and axon damage (4), oligodendrocyte depletion (5), and astrogliosis (6). Irreversible degeneration appears early in the disease process (7), and brain atrophy may already be observable at the clinical onset of MS (clinically isolated syndrome; CIS).

The initial clinical course is usually relapsing-remitting (RR) with transient episodes of neurological symptoms. In most cases, this eventually changes into a secondary progressive (SP) course characterized by continuous accumulation of neurological disability with or without superimposed relapses. Over time, the inflammatory activity decreases and CNS degeneration becomes more prominent. The role of inflammation in neurodegeneration and the pathophysiology behind secondary progression are essentially unknown. Further investigations are required to determine whether they are independent from each other, or if inflammation is responsible for secondary degeneration.

Magnetic resonance imaging (MRI) has become the dominant method for diagnosis, monitoring disease activity, and evaluating treatment effects. Although, MRI may differentiate between inflammatory activity and degenerative processes to some extent, this technique cannot be used to identify different pathophysiological processes of MS. Moreover, during the progressive phase of MS, the appearance of new lesions may be undetectable due to confluent lesion formation, and the methods for determining lesion volume and atrophy are laborious and commonly unavailable.

Biochemical biomarkers of body fluids, especially cerebrospinal fluid (CSF), have increasingly gained attention in studies of MS. For decades, selective detection of oligoclonal IgG bands in CSF has been used for diagnostic purposes (8). More recently, it has been found that several inflammatory and CNS parenchymal biomarkers seem to reflect important pathological processes in MS, with some being related to disease activity, disease severity, and clinical course (9).

As patients change from RRMS to SPMS, they exhibit several features that resemble those of known neurodegenerative diseases. They develop irreversible disability, are relatively unresponsive to immune modulatory or immunosuppressive treatment, and show brain and spinal cord atrophy. In the present thesis, we explored the pathophysiology behind this transformation by measuring the levels of different biomarkers in CSF. Our major objectives were to find biomarkers that are associated with clinical course, predict disease severity, and reflect the effects of therapeutic intervention.

1.2 MS epidemiology

MS is the main non-traumatic cause of neurological disability among young adults in Sweden. The Swedish MS prevalence is among the highest in the world (189/100 000), with approximately 17 500 diagnosed cases as of the end of 2008 (10). Previous calculations show a Swedish yearly MS incidence of between 3.9-5.2/100 000 (11, 12), but a recent nationwide study estimates this incidence to be twice as high (Ahlgren et al., unpublished data). MS distribution varies widely in different parts of the world; temperate climate zones are considered high-risk areas (13), and the risk seems to increase with the distance from the equator (14). There are exceptions to this latitude gradient, with prevalence being lower in northern Norway (73/100 000) (15) and higher on Sardinia (152/100 000) (16) than in surrounding areas at the same latitude. In Sweden, the MS prevalence increases by 1% for women and 1.5% for men per degree of latitude increase (10). Studies of populations with mixed genetic background at the same latitude have suggested that the risk of MS development depends on ethnicity (16). In the 1970s, it was found that people with African and Asian backgrounds, respectively, had 50% and 20% lower MS risks compared to Caucasians. However, a recent investigation showed reversed numbers, with an increased MS risk among African Americans compared to other groups (17).

MS with relapse onset affects women twice as often as men, while this female preponderance is less obvious in primary progressive MS (PPMS). The female/male ratio in Sweden is 2.35 (10). Several studies have found that RRMS occurs with an increased female/male ratio (18). The increased MS risk for women seems to be dependent of the year of birth and geographic location (19), with the highest female/male ratio (4.55) observed among patients born in 1980-1989 in northern Europe. Socioeconomic factors (20) or migration from low- to high-risk areas (21) might affect the gender ratio. These observations appear to suggest on-going changes in MS incidence, but this has not been confirmed (10).

1.3 Aetiology of MS

Although the aetiology of MS is essentially unknown, there is accumulating evidence that both genetic and environmental factors influence MS risk.

1.3.1 Genes and MS

An increased risk is found in genetic relatives of MS patients, but the heredity of MS is complex. The background risk of MS is about 0.2% in Sweden (10). For a person with an affected relative, the risk of MS development is estimated to be 25-30% for a monozygotic twin, 3-5% for a dizygotic twin or sibling, 2% for a parent or child, and 1% for other second and third degree relatives (22, 23).

The search for MS risk genes was initially focused on genes regulating the immune system. Among all genes, the strongest association has been found with the HLA class II genotype DRB1*15:03 (on the short arm of chromosome 6); this genotype is carried by 28-33% of northern Caucasian MS patients compared to 9-15% of healthy controls, having an OR of 3.08 (24). Protective genes have also been isolated among MS patients. HLA A*02 is the most potent independent risk reducer, with an OR of 0.73 (25). HLA-C*05 and HLA B*44 have also been found to reduce MS risk, both independently and in combination with each other (26, 27).

Genome-wide association studies in vast multinational MS and matching control populations have detected at least 57 risk loci, the majority with OR in the range of 1.1-1.3 (28, 29). Notably, the majority of identified non-HLA genes have also been located in or near immune system-regulating genes. One-third of the MS-associated genes are also associated with other autoimmune diseases (30, 31).

1.3.2 Environmental and lifestyle factors and MS risk

Among the environmental and lifestyle factors that have been suspected to influence MS risk and prognosis, few have been convincingly associated with MS in repeated studies.

1.3.2.1 Infections and MS

Many infectious agents have been suspected to either trigger MS or maintain the disease as a chronic CNS infection. The proposal that infections are involved in MS aetiology and pathogenesis is based on the observation that people who migrate during adulthood from low to high MS prevalence areas or vice versa retain their original risk, but their children are at the risk determined by their new location (32, 33). It has been suggested that MS incidence rose after increased migration from high MS prevalence areas to isolated environments (Faroe, Sardinia, etc.), leading to “MS epidemics” (34-36).

1.3.2.2 Epstein-Barr virus

Epstein-Barr virus (EBV) is one of several human herpesviridae that, after an initial infection, hibernate in humans; EBV predominantly hibernates in B lymphocytes. Among adult humans, 90-95% are seropositive, compared to almost 100% of MS patients (37, 38). Individuals who are seronegative for EBV have an OR of only 0.06 for developing MS compared to EBV-seropositive persons (39). Furthermore, previous clinical mononucleosis increases the risk of developing MS by 2-3 times (40). An intriguing but unconfirmed finding is the detection of EBV-infected B cells in germ-like follicle structures of the meninges in SPMS patients (41).

1.3.2.3 Tobacco smoke

Tobacco smoke seems to influence the risk and prognosis of MS. The relative risk of MS development is higher among current smokers compared to individuals who have never smoked (OR, 1.6) (42). MS risk is also increased among children of smokers (OR, 2.12) (43) and for non-smoking adults who are exposed to tobacco smoke (OR, 1.3) (44). Compared to non-smokers, smokers have a higher risk of developing MS from CIS (HR, 1.8) (45), and have a higher lesion load and increased atrophy development seen on MRI (46).

1.3.2.4 Vitamin D and sun exposure

It is well documented that MS prevalence increases with higher latitude (14), and this association has been connected to low sunlight exposure and less endogenous vitamin D production (47). It has been suggested that UVB radiation has an independent protective effect on MS risk (48, 49). High serum level of 25-hydroxy vitamin D is a risk reducer for MS (OR, 0.59) (50), and vitamin D seems to suppress disease activity. It has been demonstrated that peroral vitamin D treatment leads to a lower relapse rate compared to in a placebo-treated group (51, 52), and supplemental vitamin D therapy has been suggested as a potential MS treatment.

1.3.3 Combination of genes and environmental or lifestyle factors

Like many other diseases, MS seems to emerge due to a combination of genetic vulnerability and harmful environment. Recent studies have shown that >80% of the isolated MS risk genes are regulated by vitamin D (53, 54), including HLA DRB1*15:01. Among smokers, presence of the HLA DRB1* 15:01 genotype and absence of the protective HLA A*02 genotype gives an OR of 13.5 for MS development (55), compared to an OR of 4.9 in non-smokers with the same gene set. The current knowledge of risk genes is an important resource to promote better understanding and identification of pathological processes. This information may also be useful in establishing risk profiles for individual environmental factors and predicting treatment effects.

1.4 Clinical course of MS

In 80-90% of cases, the clinical onset of MS is transient and followed by complete or partial recovery from symptoms (23) *(Figure 1)*. The symptoms may have mono- or multifocal origin in the CNS, most commonly involving the optic nerve, the spinal cord, or the brain stem. New relapses usually occur with an annual rate of 0.5-1. Over time, the recovery from each relapse becomes less complete, and persistent symptoms accumulate. In an untreated MS population, a majority of RRMS cases turn into SPMS after a median time of approximately 15-20 years from disease onset (23, 56). SPMS patients may initially have relapses that are superimposed over the on-going clinically progressive process. In most cases, clinical progression consists of spastic para- or tetraparesis, cerebellar ataxia, or spastic hemiparesis, and the symptoms gradually become more complex and severe with increasing decline of neurological functions. In 10-15% of patients, the course is progressive from onset; these patients are designated PPMS.

After clinical onset and before there is evidence for the chronic and disseminated disease of MS, the term CIS is used (57). A majority of patients with CIS will eventually be diagnosed with definitive MS once dissemination in space and time is fulfilled *(Figure 2)*.

The term benign MS is often defined as a case with an EDSS of ≤2.0-3.0 after a disease duration of 10-15 years (58). Studies of benign MS show the frequent presence of non-motor symptoms, like fatigue, pain, and cognitive impairment (59). The term is retrospective and it is not possible to predict the individual clinical course in MS; it has been proposed that a better definition is needed (60).

It is debated whether different MS disease courses reflect different pathogenic mechanisms. RRMS and SPMS are per definition parts of the same disease, and the majority of untreated RRMS patients will proceed to develop SPMS. On the other hand, PPMS has a different clinical presentation and gender distribution; however, little evidence supports different mechanisms behind this process. PPMS and SPMS involve the same age of onset and similar disability development (61). The set of risk genes (HLA and non-HLA genes) also seems to be the same for both subtypes (28).

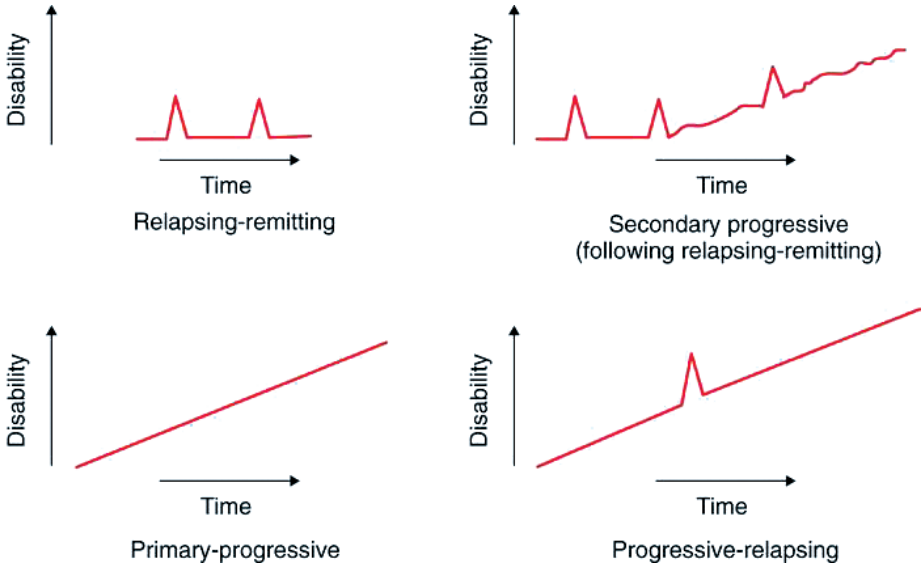

Figure 1
Clinical subtypes of MS. The figure shows the two main types of onset, relapsing or progressive, and totally four subgroups depending on the further course of disease. (Adapted from (64) reprinted with permission from with permission from Wolters Kluwer Health.)

1.5 Diagnostic criteria in MS

The diagnostic workup of MS is currently based on MRI results rather than clinical measurements; however, MS diagnosis still requires evidence of CNS white matter lesions that are disseminated in time (DIT) and space (DIS) and not better explained by any other diagnosis. In parallel with improved radiological and biochemical methods and the unification of clinical evaluations among neurologists, the MS diagnostic criteria have been repeatedly modified and revised. The progression from the criteria of Schumacher (8) and Poser (62) to the current revised criteria of McDonald (63) represents an on-going process of simplifying the diagnostic workup to allow early diagnosis without losing diagnostic sensitivity and specificity. This evolution has included almost complete elimination of the influence of other paraclinical methods, such as visual evoked potentials or detection of oligoclonal IgG bands in CSF. Evidence of DIT and DIS can be obtained by a single or repeated MRI, or by observation of new clinical attacks.

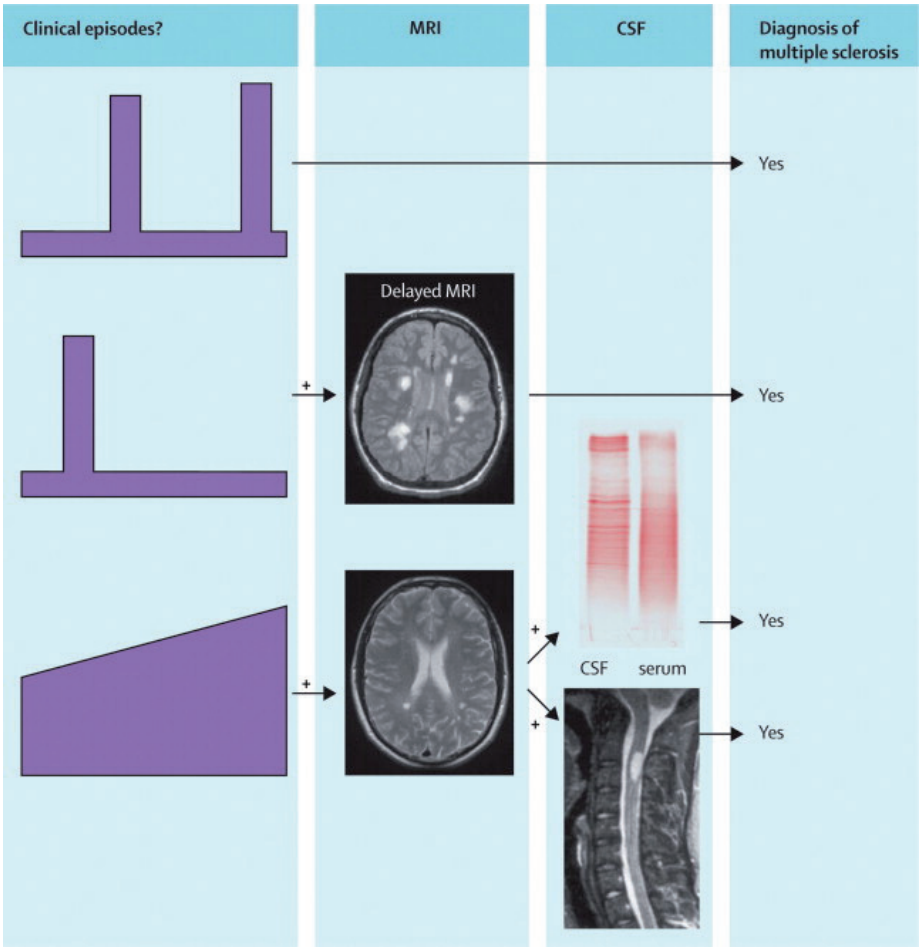

Figure 2
Localization of lesions and demonstration of dissemination in space (different locations in CNS) and time have been core concepts in diagnosing MS. Clinical and paraclinical investigations are usually applied. (From (23), reprinted with permission from Elsevier).

In contrast with previous MS diagnostic criteria, the criteria of McDonald include PPMS. The diagnosis of PPMS requires continuous worsening of CNS symptoms over at least one year combined with paraclinical signs on MRI, or signs of inflammation in CSF, such as increased IgG index and/or selective formation of oligoclonal IgG bands (OCB). SPMS diagnosis is based on evidence of continued worsening for at least six months, without being associated with relapses (64), although some SPMS patients have relapses superimposed on their progression. The transition

from RRMS to SPMS can be difficult to assess, especially in RRMS patients with remaining disability following severe relapses and in SPMS patients who continue to have relapses.

1.6 Disease-modifying MS treatment

1.6.1 Interferon beta

In the mid-1990s, interferon beta-1b (IFNB-1b) and interferon beta 1a (INFB-1a) were approved as the first disease-modifying therapies for MS. INFB-1a is identical to endogenous synthesized interferon beta, while INFB-1b lacks glycosylation and differs by one amino acid substitution and one amino acid deletion. These IFNBs are now available as different products that are administered either subcutaneously or as intramuscular injections, with a frequency ranging from every other day to once weekly.

IFNBs belong to a group of cytokines that acts on target cells through the IFN-alpha/beta receptor (65). They activate a cascade of genes that induce the synthesis of proteins with immunomodulatory properties, but the exact function remains obscure. IFNBs agonize the production of anti-inflammatory cytokines (e.g. IL-4 and 10), promoting a shift toward a Th2 response (66); they also seem to inhibit production of pro-inflammatory cytokines (e.g. IL-12, IL-17, IL-23, osteopontin, IFN-gamma, and TNF-alpha) (67, 68). IFNB also appears to stimulate T-cell apoptosis by down-regulating anti-apoptotic proteins (69). The mechanisms are complex and far from completely understood.

IFNB treatment of RRMS reduces the relapse rate by 30%, and reduces MRI lesions and disability development. In CIS, trials have demonstrated benefits of early treatment, although the long-term effect is uncertain (70). IFNB treatment of progressive MS, with few exceptions, has failed to reduce neurological disability (71, 72); however, it significantly affects relapse rate and lesion development (71, 73, 74).

The most common adverse effects include flu-like symptoms and skin reactions (75), which are the major causes of treatment discontinuation. Neutralizing antibodies (NAB) against IFNB may develop in 5-35% of patients, depending on the INFB product (76), and a high NAB titre can reduce or completely eliminate the effects of the drug (77).

1.6.2 Glatiramer acetate

Glatiramer acetate (GA) is a combination of four amino acids (L-alanine, L-glutamic acid, L-lysine, and L-tyrosine) that are randomly polymerized into peptides. These

peptides have immunomodulatory effects that are poorly understood. GA attaches to the MHC II complex and seems to disrupt antigen presentation, which might block MBP-reactive T cells causing a shift to an anti-inflammatory Th2 state (78, 79). Since GA does not pass the blood-brain barrier (BBB), this process occurs in the periphery and activated T cells migrate into the CNS and induce anti-inflammatory effects (80). GA has been shown to affect the antigen-presenting properties of B-cells. Although anti-GA antibodies are frequently seen, they do not seem to have an inhibitory effect (81, 82).

Treatment of RRMS patients with GA seems to have a clinical effect of the same magnitude as INFB treatment; head-to-head studies have not shown IFNB to be superior to GA (83-85). In progressive MS, GA treatment induced no significant clinical effects but on MRI lesion development (86, 87).

GA is generally well tolerated, but injection reactions occur in a majority of patients (75). One specific injection-related adverse effect is the immediate post-injection systemic reaction (IPISR) that includes dyspnoea, palpitation, flushing, and anxiety occurring for between 30 seconds and 30 minutes (88).

1.6.3 Natalizumab

Natalizumab (Nz) is a humanized monoclonal antibody directed against the α4β1-integrin molecule on mononuclear leukocytes. By blocking the interaction of α4β1-integrin with the endothelial vascular cell adhesion molecule-1 (VCAM-1) ligand, Nz inhibits leukocyte migration across the BBB. Pivotal clinical trials have shown that Nz treatment decreases relapse rates by approximately 70% versus placebo, and clinical and MRI measurements showed that 37% of Nz-treated patients were disease free, compared to 8% of placebo-treated patients (89). No randomized placebo-controlled clinical trial has yet been performed in progressive MS patients.

The major problem with Nz treatment in MS is the appearance of progressive multifocal leukoencephalopathy (PML), which is caused by an opportunistic polyoma virus (JC virus) infection colonizing the kidneys and bone marrow. In the absence of appropriate immune defence of the CNS, mutated JC virus has the ability to cause a massive CNS infection. Although the effect of Nz can be reversed, PML often causes lasting neurological disability and, in about 20% of cases, death (90, 91). Strategies to minimize the risks of PML include selection of patients without previous immune suppressive treatment and those that are negative for JC virus antibodies.

1.6.4 Mitoxantrone

Mitoxantrone (Mx) is a synthetic DNA-intercalating anthracenedione derivate that affects B cells, T helper cells, and cytotoxic T cells, and can both suppress and mo-

dulate the immune system (92). Mx does not cross an intact BBB. It is the only drug approved for use in SPMS (in the USA, but not Europe). Two randomized controlled studies have shown efficacy in all stages of MS, except PPMS, with effects on both relapse rate and EDSS progression (93, 94).

However, Mx exerts dose-dependent toxicity on many organs, leading to increased risk of acute myeloid leukaemia, cardiac dysfunction in about 1% (95), and increased risk of serious infections. The cumulative lifetime dose should not exceed 100 mg/m^2 for MS treatment (96).

1.6.5 Rituximab

Rituximab is a chimeric human/mouse anti-CD20 antibody. The Fab domain binds to the CD20 antigen on B lymphocytes, and the Fc domain recruits the immune system to mediate cell death (97). This antibody-dependent cytotoxicity is induced by either apoptosis or complement-dependent cytotoxicity (98-100). Because CD20 expression is unique to B cells, the beneficial effects of rituximab in MS support a role of B cells in MS pathogenesis. It is unclear how much of the effect of rituximab is generated outside the CNS, but determination of the BBB penetration shows a CSF/plasma ratio of 1/1000, which might be sufficient to also eliminate B cells from the CSF compartment (101).

Although rituximab is not registered for MS treatment, a number of open-label studies and case reports have shown clinical and radiological beneficial effects (102); it is used off-label and considered a potent agent for MS therapy. One phase II study of rituximab treatment in patients with RRMS showed significantly reduced relapse rate and lesion formation (103). In contrast, a phase III study in patients with PPMS showed non-significant differences; however, reduced disease progression was observed in a subgroup of patients younger than 51 years with Gd-enhancing (Gd^+) lesions (104).

2. Immunopathogenesis and pathology of MS

2.1 General considerations

MS is considered to be an autoimmune disease characterized by focal lesions disseminated throughout all parts of the CNS, and involving both grey and white matter. Widespread diffuse pathology is also seen in normal-appearing tissue. In the early phase of MS, inflammatory activity dominates, with a high rate of lesion formation; subsequently, this activity declines and neurodegeneration takes over (2). However, there is accumulating evidence that signs of neurodegeneration—including neuroaxonal loss, astrogliosis, and CNS atrophy—are already evident at disease onset (105). It remains to be determined whether neurodegeneration in MS is secondary to destructive immune activity or essentially a parallel and primary event.

2.1.1 MS lesion (plaque) formation

MS lesion or plaque formation typically involves focal inflammation with blood-brain barrier (BBB) breakdown and immune cell infiltration. Inflammation seems to be initiated in two steps. CD8+ T cells and activated microglia initially dominate, causing myelin destruction (106, 107). This destruction is followed by infiltration of activated macrophages, B cells, and T cells, and local secretion of pro- inflammatory cytokines and chemokines and their receptors (108, 109). Active lesions often give rise to BBB disruption, which can be detected by Gd^{+} enhancement on MRI (110, 111). As inflammation declines, MS lesions may either progress to a chronic inactive stage that is characterized by astrogliosis and insufficient remyelination, or show sufficiently remyelinated axons that appear as “shadow plaques” on MRI. Some lesions, designated as chronic active plaques, show preserved immune activity of lower intensity *(Figure 3)*; these lesions slowly expand at the border, while activity ceases at the centre (3). MS progression involves decreased active lesion formation, along with increased expanding chronic lesions. In progressive MS, inflammation seems to become more compartmentalized and the integrity of the BBB is maintained, allowing only low levels of protein exchange (111) *(Figure 4)*.

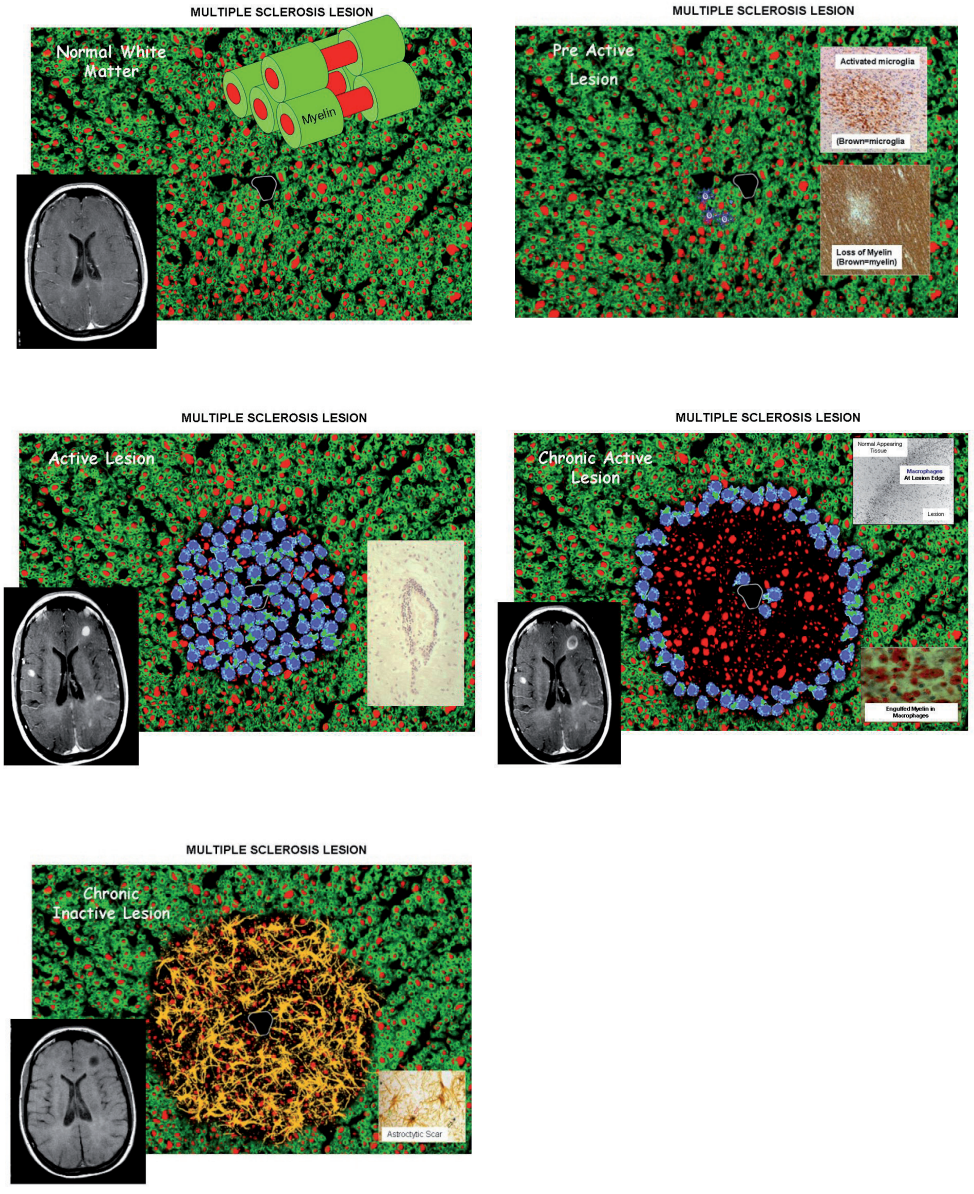

Figure 3
Schematic, MRI and microscopic images of MS lesions in different stages.
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2.1.2 Axonal degeneration

Axonal damage occurs in both early and late stages of MS, predominantly in active plaques (early and chronic), and correlates to the activity of lymphocytes and activated microglia (112). Ferguson et al. described accumulation of amyloid precursor protein (APP; a marker for axonal dysfunction or injury) all over active lesions and at the border of chronic active lesions (113). Axonal degeneration is also seen in non-lesion matter. Diffuse axonal injury and destruction are associated with widespread and diffuse low-grade inflammation, microglial activation, astrocytic gliosis, and mild demyelination (114), illustrating an active neurodegenerative process. The quantity of diffuse injury increases over time, and is more pronounced in progressive MS. Axonal loss outside of plaques could also be due to Wallerian degeneration, in which proximal axonal damage causes distal axonal degeneration. However, the extent of diffuse white matter injury does not correlate with the amount of focal white matter lesions, and only weakly correlates with cortical demyelination (2, 115).

2.1.3 Astrogliosis

Reactive gliosis with or without scar formation is a general feature of any kind of CNS damage and is a prominent feature in MS pathology (6). In areas outside of the plaques, the picture is diffuse with widespread areas of hypertrophic astrocytes with up regulated GFAP expression (116). In more severely affected areas, the astrocytes proliferate outside of their normal tissue architecture. In plaques, in addition to astroglial activation, dense and compact glial scars are formed (117); recent studies suggest that these scars act as neuroprotective barriers that stop inflammatory cells and predominantly form along the plaque borders (118). Glial scars interact with other cell types, and their extra cellular matrix contains substances that inhibit cellular migration (119).

2.1.4 Suggested patterns of MS pathology (120)

Investigation of the heterogeneity of MS pathology has led to the suggestion that there are four different and distinct immunopathogenetic patterns; these findings have been considered proof that there are different types of MS that may respond differently to treatments (121, 122). Pattern I shows T cells and macrophages around blood vessels, preserving oligodendrocytes but with no complement activation. Pattern II is like pattern I, but with complement activation. Pattern III shows diffuse inflammation, distal oligodendrogliopathy, microglial activation, and a loss of myelin-associated glycoprotein (MAG); contrary to patterns I and II, pattern III has no association with blood vessels. Pattern IV includes sharp bordered lesions, and oligodendrocyte degeneration with a rim of normal-appearing white matter; no

complement activation or MAG loss is detected. These observations are based on biopsy or autopsy materials from severely disabled patients, and the results have not been confirmed. It remains to be clarified whether these patterns represent different disease subgroups or different stages of the same disease.

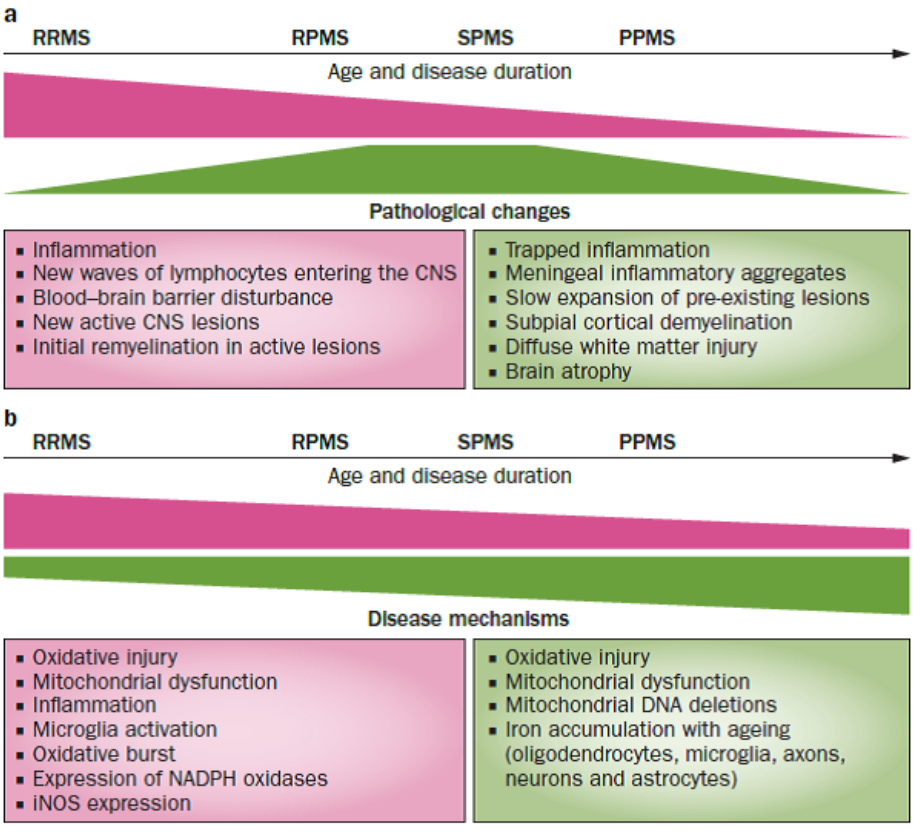


Figure 4
Schematic presentation of the evolution of structural pathology and disease mechanisms during the course of MS. a) Pathological features associated with conversion of RRMS (pink) to PMS (green). b) Changes in disease mechanisms associated with conversion of RRMS (pink) to PMS (green). The bars indicate the the extent of these differences in relation to increasing age and disease duration. Although no pathological or mechanistic feature is exclusive either for RRMS or PMS, major quantitative differences in their occurrence are evident between these stages. Abbreviations: iNOS, inducible nitric oxide synthase, RPMS relapsing progressive MS. From (131). Reprinted with permission from Nature Publishing Group.

2.2 The hallmarks of grey matter pathology

In MS, involvement of the deep grey matter and cortex is seen as either demyelination or retrograde degeneration from white matter lesions. Grey matter pathology and white matter pathology share many common features, but there are also fundamental differences. The cortex exhibits both focal and diffuse pathology, including atrophy (123), as is also observed in early stages of MS (124). Focal changes predominantly appear early on, and are often dominated by intense inflammation with perivascular infiltrates and large amounts of lymphocytes throughout the tissue, accompanied by activated macrophages and microglia (125). Losses of neurons, axons, and synapses are more pronounced in early-stage cortical plaques (2, 125). In general, cortical lesions are more commonly seen during the progressive phase of MS and in the subpial cortical layers (2), often in the vicinity of ectopic B-cell follicles of the meninges (126). The distance from the white matter seems to determine the content of inflammatory cells in grey matter lesions, with the subpial lesions dominated by activated microglia, apoptosis, and neuronal atrophy (127). Similar to white matter pathology in MS, global tissue loss is widespread in normal-appearing cortex, contributing to atrophy development (128).

2.3 Meningeal pathology

Topographically associated with subpial cortical lesions, meningeal B-cell follicle-like structures have been characterized in progressive MS (129, 130). Although it has not been confirmed, the B cells in these structures have been identified as immune reactive for Epstein-Barr virus (EBV) (41). These tertiary inflammatory germinal centres are predominantly found in SPMS (126), and are suggested to have a role in the pathogenesis of progressive MS. It is noteworthy that these findings indicate that MS can no longer be considered a disease affecting only nervous tissue.

3. Pathophysiology of progressive MS

3.1 General considerations

There are three main hypotheses explaining the pathophysiology of progressive MS and its relation to RRMS (131):

1. MS is a primary neurodegenerative disease. Dysfunctional and dying cells trigger an early immune response. Neurodegeneration may be modified or amplified by the reactive inflammation.

2. MS is a primary inflammatory disease that, over progressive stages, changes in anatomical location and intensity, making it untreatable using existing immune therapies.

3. MS develops through a combination of different pathological mechanisms, starting as an inflammatory disease and subsequently involving neurodegenerative processes. Delayed damage or neuronal death occurs when the reserve capacity is used and the anatomical structures are destroyed.

One general consideration is that axonal loss accumulates over the course of MS, eventually reaching a threshold at which the disease shifts to slow progression (132). It is likely that this process involves the imbalance between tissue injury and repair (133) and the consumption of compensatory mechanisms (132).

3.1.1 Microglial activation

Tissue injury in progressive MS is associated with chronically activated microglia (3), and activated microglia are found in normal-appearing white matter (134). Similar microglia involvement is seen in other neuroinflammatory and neurodegenerative diseases (135). Microglia are known to generate oxidative bursts and to induce demyelination and axonal damage; however, they also have neuroprotective functions (135).

3.1.2 Altered ion homeostasis

In normally myelinated axons, voltage-gated Na^+ channels are highly concentrated at the nodes of Ranvier. Loss of myelin is a major structural change during progressive MS. A number of studies have reported changes of ion homeostasis in demyelinated neurons, and redistribution of ion channels along the axon as a functional compensation. Altered expressions of voltage-gated Ca^{2+} channels (136), glutamate receptor (137), and Na^+ channels (138, 139) have been observed, which can lead to intra-axonal Ca^{2+} accumulation and eventually axonal death (131). This delayed

process can be induced by inflammation-mediated demyelination and axonal injury that occurred several years earlier.

3.1.3 Mitochondrial dysregulation

Mitochondrial injury is observed in the demyelinated axons of MS lesions (140, 141). Axons have excessive energy demands when they are not supported by myelin, and mitochondrial function is critical during axonal injury. Increased mitochondrial number and size are seen in axons in active plaques, which normalize after remyelination (142-144). Mitochondrial damage and loss in nerve cell bodies could accelerate axonal death and induce a state of "virtual hypoxia" (140). Redistribution of mitochondria is observed within damaged nerve cells, as well as increased numbers of defective mitochondria (145). One major cause of mitochondrial dysfunction is oxidative stress induced by inflammation (145). In active MS lesions, it is likely due to increased production of enzymes and oxygen free radicals (146, 147). Thin-calibre axons are more severely affected than thick ones because they have less mitochondria relative to their axonal surface area (140). The mitochondrial damage also affects oligodendrocytes and their ability to remyelinate injured axons (148, 149). Moreover, the release of high levels of extracellular Fe^{2+} during inflammation and tissue damage may lead to additional oxidative stress on mitochondria and axons (150, 151).

4. Measurements of disease activity and disability progression in MS

4.1 Relapse rate

Measuring the annual relapse frequency is a standard method for evaluating MS disease activity in clinical routine and clinical trials. A relapse is defined as a patient-reported or objectively observed event that is typical of an acute inflammatory demyelinating event in the CNS, current or historical, with duration of at least 24 hours, and in the absence of fever or infection (63). The average annual relapse rate is about 0.5-1 in an untreated population (89). A long observation time or large patient groups are needed to confirm altered activity.

4.2 Clinical scales of neurological disability, progression and severity

Clinical scales are used to score the progression of neurological deficit over time in clinical routine and clinical trials. A number of scales have been tested, with the aim of finding objective measurements of disability development in MS. Such measures should be reliable, MS specific, validated, and easy to use and interpret in clinical practice as well as in clinical research. The scales we used in this thesis are widely accepted and validated.

4.2.1 Expanded Disability Status Scale, EDSS

The currently dominant scale for clinical scoring is the Expanded Disability Status Score (EDSS), which gives patients a score from 0 to 10. Derived from the DSS (152), the EDSS is based on the evaluation of seven functional systems (FS) by targeted neurological examination. A synthesis of these evaluations gives an EDSS of up to 3.5 for which individual FS scores (with some exceptions) count equally. For scores in the range 4.0 to 6.5, walking ability is weighted as just as important as the FS scores. Scores of over 6.5 are given as an evaluation of the patient's independence and autonomy in ambulation. A score of 10 indicates death by MS. Obviously, this system leads to non-linear score development with a possibility of overlooking the deficit development, especially at higher scores. EDSS is an ordinal scale, which is only suitable for non-parametric statistics. Furthermore, the intra-rater repeatability is reportedly low (153), especially in mental and sensory FS where anamnestic information is necessary. Agreement between raters has been tested, and is only achieved with acceptance of differences ≤ 1.5 EDSS score (154). When using this scale, it should be noted that two EDSS steps are needed to confirm

clinical change (155), underlining the limitations of the EDSS in clinical studies or for therapy revisions. A major strength of the EDSS is its worldwide use and ease of use and interpretation.

4.2.2 Multiple Sclerosis Severity Score, MSSS

The Multiple Sclerosis Severity Score (MSSS) represents an attempt to determine the disability progression rate in MS; it is claimed that this scale measures MS severity and has predictive properties. Individual disease duration was combined with EDSS in 9892 patients from 11 countries (156) to establish the general EDSS development over the course of MS. By combining disease duration on the y-axis and the EDSS score on the x-axis, the MSSS score can be found in the matrix. Scores range from 0.01 to 9.99. Obviously the scores are non-linear and can only be calculated with non-parametric statistics. Patients with EDSS 10 (death) were not included in the investigation, underrepresenting severe cases of MS; MSSS scores were also not included for patients with disease duration exceeding 30 years. Thus, MSSS is based on a large—but in some aspects, historical—population. It could be questioned whether this population is still relevant for measuring MS severity.

4.2.3 Progression index

The progression index is calculated by EDSS/disease duration (157, 158). The main concept is the same as in MSSS, but the progression index has less predictive value. It has no limitations for use with patients with disease duration of above 30 years, but it has been scarcely used in previous studies.

4.2.4 Multiple Sclerosis Functional Composite, MSFC

The MSFC was established in 1999 as a complement to EDSS for overcoming the weaknesses of the widely used scale (159). The main idea was to use quantitative measurements and compare them with those derived from a large control population. The MSFC is based on three specific tests that examine walking speed in a short range (timed 25-foot walk; T25W), fine motor function and coordination in the arms (nine-hole peg test; 9HPT), and cognitive function (paced serial addition test, 3 sec; PASAT3). The test results are converted to a z-score and normalized to a control population. The z-score describes the number of standard deviations of a patient score in comparison with a reference population. This score has relatively high inter-rater agreement (160). Some studies claim that, compared to the EDSS, the T25W part of the MSFC is a better prognostic tool (161, 162), while other studies have reported that the T25W is equal or even inferior (163-165). The MSFC is time consuming and requires specific tools. It is mainly used in clinical treatment trials as a complement to the EDSS.

4.3 MRI

MRI has become the most important MS diagnostic tool (see MS diagnosis, page 20). The use of MRI in MS has increased along with the growing possibilities and demands for accurate diagnosis, assessment of disease activity and progression, and evaluation of therapeutic efficacy. Much of our current knowledge concerning disease activity, neurodegeneration, atrophy development, and grey matter involvement has been attained using MRI research. In phase II clinical trials, MRI measurements are used as surrogate markers for therapeutic outcome, and phase III clinical trials include several MRI-based secondary and tertiary objectives.

4.3.1 T1-weighted images

A T1 image is created by measuring the time that it takes protons to return to the magnetic field axis. MS lesions may appear as hypointense areas ("black holes"). These are preceded by Gd^+ lesions, reflecting destructive inflammation. The degree of hypointensity correlates with the degree of pathological severity (166) and persistent hypodense areas reflect irreversible tissue destruction with axonal loss (166). T1 lesions correlate more strongly with clinical deficits than pathology seen on other sequences (167).

4.3.2 Gadolinium-enhanced T1-weighted images

A T1 image with Gd^+ lesions reflects a damaged BBB (168). BBB disruption appears during acute inflammation and may be detected for up to five weeks. Contrast enhancement is often connected to clinical symptoms, i.e. relapses (169). This sequence is a fundamental part of the diagnostic criteria, as it provides evidence of dissemination in space and time (63).

4.3.3 T2-weighted images

A T2 image is created by measuring the proton dephasing following a transverse pulse, and is useful for describing the anatomy and composition of the central nervous system. Lesions detected on T2-weighted images can reflect a wide diversity of pathological processes, such as inflammation, demyelination, and glial scar formation. This is the sequence that visualizes the expansion of MS in the CNS. T2 lesions appear as focal hyperdense areas with typical location (periventricular, infratentorial, or juxtacortical) and appearance (ovoid and >3 mm in diameter). T2 lesions are the main diagnostic source for establishing dissemination in time and space (63).

4.3.4. Fluid-Attenuated Inversion Recovery (FLAIR)

Fluid-Attenuated Inversion Recovery (FLAIR) produces T2-weighted images.

Using an inversion-recovery technique, the inversion time (TI; the time between inversion and excitation pulses) is carefully chosen for the CSF signal (170). In MS, FLAIR imaging is more sensitive for detecting lesions close to the ventricles than T2-weighted lesions, since these may not be distinguishable from the signal of CSF (171). FLAIR can also detect juxtacortical and cortical lesions (172, 173), but the appearance of infratentorial lesions may be false positive (174).

4.3.5. MRI as a surrogate marker for MS activity and progression

In CIS, the appearance of one or more T2 lesions predicts conversion to definitive MS (175), and the number of T2 lesions at disease onset may predict disability development (176). The disease activity measured by MRI is much higher than that estimated clinically. Monthly MRI performance shows approximately 10-fold more new lesions compared with the number of new relapses (177, 178). However, increasing lesion load is not a common feature of PMS. Disability progression is better correlated with atrophy measurements, such as brain parenchymal fraction on T1-weighted images (179). The annual rate of whole brain atrophy is approximately 0.5-1% in MS patients, compared to 0.2-0.5% in healthy individuals (180-182). Significant brain volume reduction is also evident early in the disease, involving both grey and white matter (183). Atrophy of the grey matter and spinal cord seem to predict disability progression (184, 185). Brain atrophy measurements have been proposed for predicting outcome following neuroprotective therapies in MS trials (186).

4.4 Biochemical biomarkers in MS

4.4.1 General considerations

Biomarkers are physical, functional, or biochemical indicators of physiological or disease processes; they can have diagnostic properties, provide information about the risk of disease development, reflect disease activity or disease severity, and have predictive or prognostic properties. They are also used to investigate responses to therapies, or discern adverse events and drug interactions. Additionally, biomarkers may be used to explore important pathophysiological mechanisms in disease progression. In clinical trials, biomarkers can act as surrogate endpoints, i.e. substitutes for clinical endpoints. New biomarker development involves many challenges, and the use of biomarkers carries the risk that a biomarker may not measure what it is supposed to and instead reflect other processes.

In MS, MRI measurements (discussed above) are the most frequently used biomarkers. However, CSF biomarkers are the most widely studied among the biochemical

biomarkers in diseases affecting the CNS in general, and specifically in MS. MS is considered a CNS-specific disease and thus CSF is topographically near the disease process. However, the impact on the CSF differs depending on what CNS areas are affected. Frontal, parietal, or occipital regions of the cortex are considered CSF-distant, and pathological processes in these areas have less impact on CSF composition (187). The BBB creates an environment that is relatively isolated and partially protected from pathological processes affecting the rest of the body. However, about 80% of the protein content and all immune cells in the lumbar CSF are blood derived, and immune cells regularly migrate across the BBB in both directions. Under physiological conditions, blood-derived proteins enter the CSF compartment via passive diffusion and show a specific CSF-to-blood ratio (188), and these conditions change during some pathological processes.

Lumbar puncture side effects, including perceived discomfort of the patient, post-punctional headache, and minor risk for CNS infections or haematoma, makes CSF less attractive compared to blood. The development of blood-derived biomarkers for monitoring is therefore sought. Indeed, some serum and plasma biomarkers reflecting different immune mechanisms, show association with MS course or disease activity like osteopontin, LIGHT and metalloproteinases (189-192). However, structural biomarkers have been investigated in blood and the results to date have been inconclusive. For example, Eikelenboom et al. (2011) found no significant correlation between neurofilament heavy in blood and in CSF (193). Other studies have revealed diagnostic biomarkers for demyelinating diseases; aquaporin 4 antibodies have been included in the diagnostic criteria for neuromyelitis optica (194); and recently, KIR 4.1 antibodies were discovered in 50% of MS patients (195).

4.4.2 Structural biomarkers

4.4.2.1 Neurofilament protein

The neurofilament protein is the major component of the axonal cytoskeleton and is only found in nerve cells. Its function is to maintain the axonal structure, and it is essential in physiological processes like axonal transport (196). The neurofilament protein can be divided into three subunits: the 61-kDa neurofilament light (NFL) protein, the 103-kDa neurofilament medium (NFM) protein, and the 111-kDa neurofilament heavy (NFH) protein. The three subunits each have a common head and rod region, but differ in the tail region (196, 197) *(Figure 5)*.

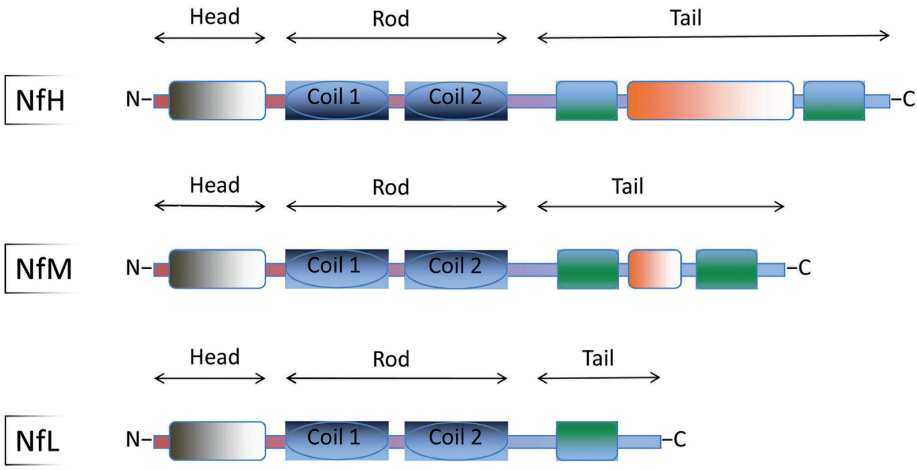


Figure 5
Schematic representation of the different neurofilament subunits. NFH: neurofilament heavy chain, NFM : neurofilament medium chain, NFL: neurofilament light chain, N: N-terminus, C: C-terminus. (199) Reprinted with permission from Sagepub.

These three subunits are assembled to interfilament structures of 8-10 nm. Neurofilaments are highly phosphorylated and the degree determines the three-dimensional structure and axonal diameter (198). During axonal damage, neurofilament leaks out into the surrounding tissue and further disperses into the CSF and blood. The pathological process of how the intermediate filaments are dissolved is not fully understood, and the further pathways of the three different subunits are uncertain but are clearly not identical (199, 200). While NFM remains poorly studied, the other two are increasingly valued as biomarkers of axonal damage in several neurological diseases. NFL levels in CSF are increased in many CNS diseases, such as herpes simplex virus encephalitis and tick borne encephalitis (201), cerebral vasculitis (202), atypical Parkinson disorders (PSP, MSA-C, MSA-P and CBD) (203), and ALS (204, 205). A number of studies have shown elevated neurofilament levels in MS. NFL levels are particularly elevated in active RRMS, early in the disease and during relapse but have been elevated in all courses of the disease (206-209). NFH is also elevated in patients with SPMS, and is related to disease progression and to brain atrophy on MRI (210, 211).

Neurofilament has also been experimentally measured in brain autopsy samples to verify axonal loss (112), and only been detected in blood (NFH) where massive axonal damage has occurred (212). The differing degrees of phosphorylation of the same protein in different situations and the similarities between the subunits in the

head region (213) create challenges in creating reliable assays that are free from cross reactions (214).

4.4.2.2 Glial fibrillary acidic protein (GFAP)

Glial fibrillary acidic protein (GFAP) is the major intermediate filament of the astrocyte cytoskeleton. It is thought to maintain the mechanical strength and outer shape of the astrocyte, and is essential for the processes of reactive astrogliosis and glial scar formation (215). Although GFAP has been isolated in various tissues, it is generally considered a CNS-specific protein (216, 217). GFAP consists of three domains: a head, rod, and tail. It can form homodimers as well as heterodimers with other proteins (218), it has a molecular weight of 43-49 kDa, and the proteins are arranged in fibre bundles of 8-9 nm (219). GFAP was originally isolated and characterized in MS lesions with severe astrogliosis (220). Elevated CSF GFAP levels are observed in pathological CNS conditions, such as dementia (221) and normal pressure hydrocephalus (222). In MS, augmented CSF GFAP levels are related to relapse (223) and a correlation has been observed between GFAP levels and disability in SPMS patients (208). Increased serum levels of GFAP are seen in patients with severe head injury (224) and are related to outcome in subarachnoid haemorrhage (225).

4.4.2.3 Albumin ratio

Albumin ratio (CSF/plasma albumin) is used as a measure of BBB function (226). Albumin is a liver-produced, 67-kDa heavy plasma protein that passively diffuses over the BBB. The ratio is positively correlated to age, and is also elevated in several pathological processes (227). Most MS patients have albumin ratio values below the upper reference level (228); thus, this measure is not used as an independent marker in MS. However, the BBB affects the measurements of other biochemical markers, such as intrathecal IgG production (229).

4.4.3 Inflammatory biomarkers

4.4.3.1 CXCL13

Cytokines are small proteins (~25 kDa) that function as mediators for intercellular communication. CXCL13 is 8-10 kDa, and is the 13th identified member of the cys-x-cys (CXC) motif ligand cytokine sub-family of the 17 members described in humans. This protein is also called ANGIE, ANGIE2, “B-cell chemoattractant”, BCA-1, BLC, and BLR1L.

CXCL13 is selectively chemotactic for B cells in the B1 and B2 subsets, and stimulates the chemokine receptor CXCR5 (230). CXCL13 is produced by a number of

cell types; it seems to control the organization of B cells within follicles in lymphoid tissue (231) and to indirectly influence immunoglobulin synthesis. From T-cell lymphomas, it is known that T cells can express CXCL13, reflecting their germinal centre origin (232). Elevated CXCL13 levels in CSF are seen in infectious diseases, such as Lyme borreliosis (233) and viral encephalitis (234). In MS, elevated levels of CSF CXCL13 reportedly predict conversion from CIS to definite MS (235). The levels are elevated during relapse, and correlate to IgG synthesis and the appearance of OCB (234, 236).

4.4.3.2 Oligoclonal IgG bands (OCB)

IgG in OCB produced in the CNS is characteristic of MS (237) and found in almost all patients (>95%) (228, 238, 239). In physiological processes, OCB are produced by clonally expanded plasma cells and plasma blasts (240, 241). Although target antigens for CSF antibodies in MS patients have been isolated, the congruence and pathological relevance of these antibodies remain obscure. Until recently, OCB were used as a diagnostic marker for all types of MS; however, the latest revision of the diagnostic criteria only uses OCB as a diagnostic marker for PPMS (63).

4.4.3.3 IgG Index

IgG produced in the CNS is the oldest identified biomarker in MS (242). To distinguish IgG produced outside the BBB, the IgG index is calculated as follows: (CSF IgG (mg/L)/serum IgG (mg/L))/(CSF albumin (mg/L)/serum albumin (mg/L)), thus compensating for the BBB function. An elevated IgG index is seen in 70-90% of MS patients, and is usually normal in OCB-negative patients (243-245). Use of the IgG index has been limited since its sensitivity is lower than that of OCB. In spite of its close connection to OCB, the IgG index is more closely related to immune activity (124, 234, 246) and treatment effects (247); therefore, the IgG index has been valued as a marker of immune suppression and treatment effect (247).

4.5.4 Biomarkers of the sAPP/Aβ metabolism

Amyloid precursor protein (APP), its degradation products, and the involved enzymes have been most extensively studied in Alzheimer´s disease, where the CSF levels provide diagnostic information (248). The *figure 6* below describes the three known APP processing pathways, which are dependent on the involvement of three secretases: α, β, and γ.

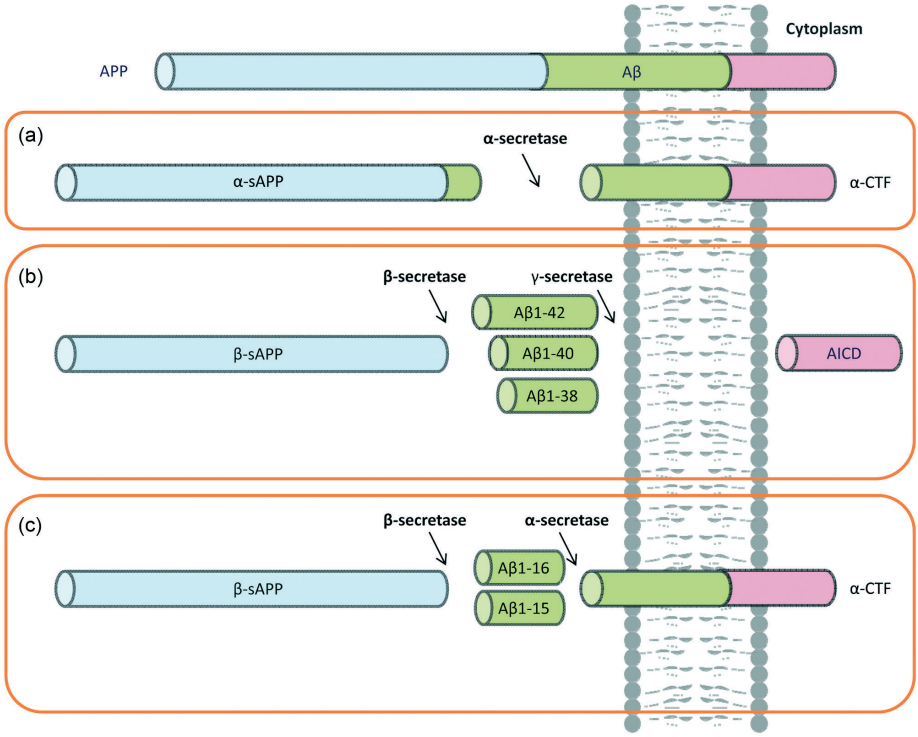


Figure 6
APP processing pathways.
(a) α-Secretase cuts APP within the Aβ domain, inhibiting Aβ formation and releasing α-sAPP. (b) APP may be sequentially processed by BACE1 at the N-terminal portion of Aβ and by γ-secretase at the C-terminal. In fact, γ-secretase may cut APP at different positions, producing Aβ peptides with different C-terminal amino acids. Other enzymes are involved in the production of Aβ peptides having other N-terminal amino acids (for example: AβX-42, AβX-40 and AβX-38). (c) APP may undergo combined α- and β-secretase processing, producing a range of short Aβ peptides. Aβ: amyloid beta; APP: amyloid precursor protein; BACE1: β-site APP cleaving enzyme 1; C-terminal: the acid end of an amino-acid molecule; CTF: C-terminal fragment; N-terminal: the amino end of an amino-acid molecule. Paper III Reprinted with permission from Sagepub.

4.5.4.1 β-site APP cleaving enzyme, BACE1 (β-secretase)

As shown in the above figure, BACE1 (β-secretase) cleaves APP, resulting in the release of N-terminal β-cleaved soluble APP (β-sAPP). The C-terminal fragment is further processed by γ-secretase to yield different Aβ peptides and the APP intracellular domain (AICD), or by α-secretase to form shorter fragments. APP can also undergo α-secretase-mediated cleavage, resulting in α-sAPP release (249). Studies in BACE1-knockout mice suggest that this enzyme has various functions besides

its role in APP metabolism, including cell differentiation, immunoregulation, and a physiological role in cleaving neuregulin 1 (NRG1) (250, 251). NRG1 is an axonal-expressed membrane-bound factor required for CNS myelination (252). BACE1-mediated release of the soluble ectodomain of NRG1 is required for the activation of myelination by oligodendrocytes (250).

Increased BACE1 levels have been reported in Alzheimer's disease and in persons with mild cognitive impairment (253, 254), as well as in traumatic brain injury and ischemic brain injury (255, 256). Little is known about BACE1 activity related to MS.

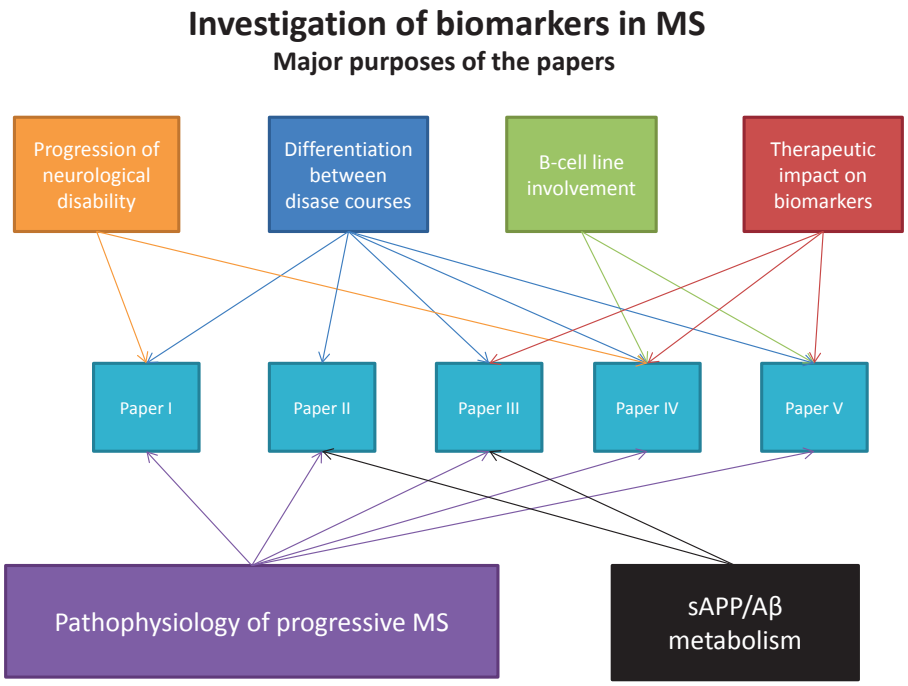
4.5.4.2 sAPP (α-sAPP and β-sAPP) and the Aβ peptides

In Alzheimer's disease, the Aβ 1-42 levels are reduced, while α-sAPP and β-sAPP are unaffected (257). Reduced CSF levels of Aβ peptides as well as α-sAPP and β-sAPP are observed in inflammatory conditions of the CNS, such as Lyme disease (258), opportunistic cerebral infections due to HIV (259), bacterial meningitis (260), and systemic lupus erythematosus (SLE) (261). Little is known about how APP metabolism in the CNS is affected in MS, and the CSF markers have not been systematically evaluated.

5. Aims of the study

The purpose of this project was to investigate if biomarkers in the CSF of MS patients

- can differentiate between different clinical courses of MS
- are associated with disease activity
- are associated with progression of neurological disability in MS
- are useful for monitoring therapeutic efficacy in different stages of MS
- may provide new insights into the pathophysiology of MS progression.



6. Subjects and methods

6.1 MS populations

This thesis includes three major cohorts of MS patients, of which the majority were consecutively recruited at the Department of Neurology, Sahlgrenska University Hospital, Gothenburg, Sweden. The first cohort consisted of patients included in a cross-sectional investigation of the sAPP/Aβ pathway in MS (Paper II); patients in this cohort were also recruited from two other MS centres: Akershus University Hospital, Oslo, Norway and Karolinska University Hospital, Stockholm, Sweden. The second cohort comprised a sub-group of patients from the first cohort, who were additionally examined in long-term follow-up setting (Papers I, II, III, and V). The third cohort consisted of patients who were assessed before and after 12-24 months of immunosuppressive or second-line immunomodulatory treatment (Papers III, IV, and V); patients in this cohort were also recruited from two other MS centres: Umeå University Hospital, Umeå and Örebro University Hospital, Örebro, both in Sweden.

All MS patients recruited before 2001 were diagnosed according to the Poser criteria (62). Patients recruited thereafter were diagnosed according to the criteria of McDonald (262) and later revisions (63, 263). Informed consent was obtained from all subjects, and studies were approved by the regional ethical boards of Gothenburg and Uppsala.

6.1.1 Patients included in the cross-sectional study of the sAPP/Aβ pathway (Paper II)

This cross-sectional study included 100 patients (33 men and 67 women) from three different populations (Gothenburg, Oslo, and Stockholm). The population from Gothenburg consisted of 66 patients (25 men and 41 women) and has been described previously (208). This group comprised 23 RRMS patients who were included at the time of an acute relapse, 18 RRMS patients in clinical remission, and 25 SPMS patients. The mean age was 39.5 years (range, 17-59 years), mean disease duration was 14.3 (range, 1-40 years), and median EDSS was 3.0 (range, 1.0-8.0).

The population from Oslo consisted of 21 patients (3 men and 18 women) with a mean age of 42 years (range, 27-65 years), mean disease duration was 5 years (range, 0.3-16 years), and median EDSS was 2.0 (range, 0-5.0). Fourteen had RRMS and 7 had PPMS.

The population from Stockholm constituted 13 patients (4 men and 9 women) with a mean age of 39 years (range, 25-52 years), mean disease duration of 3.2 years

(range, 1-14 years), and median EDSS of 1.0 (range, 0-4.5). Eleven had RRMS and two had SPMS.

6.1.2 MS patients with long-term follow-up (Papers I, II, III, and V)

Twenty-six patients (18 women and 8 men) were recruited from a previous study population of 66 MS patients (208). At their first assessment, the mean age was 41 years (range, 21-59 years) and mean disease duration was 11 years (range, 1-40 years). Each patient was assessed twice with a median interval of 9 years (range, 8-10 years). All had a clinically stable course and none had a relapse within 6 months prior to either assessment. At the first assessment, 16 patients were classified as RRMS and 10 as SPMS. At the second assessment, six additional patients were classified as SPMS. None of the patients were receiving disease-modifying treatment at the first assessment, but six were receiving immunomodulatory treatment at the second assessment (five interferon beta and one glatiramer acetate). Their median EDSS was 3.25 (range, 1.0-8.0) at the first assessment and 5.25 (range, 0-9.5) at the second. From this long-term follow-up cohort, 25 patients were examined for Paper I, 23 patients for Paper II, 26 patients for Paper III, and 20 patients for Paper V.

6.1.3 MS patients treated with immunosuppressive or second-line immunomodulatory treatment (Papers III, IV, and V)

6.1.2.1 Patients treated with immunosuppressive therapies (Papers III, IV, and V)

After publication of the OLYMPUS study (104), we modified the patient inclusion criteria for mitoxantrone treatment from the MIMS study (94), expanding the population to also include patients treated with rituximab. The cohort included 38 patients (19 women and 19 men); three had RRMS, 30 had SPMS, and five had PPMS. Fourteen patients had previously been treated with interferon beta and one with glatiramer acetate. At the pre-treatment assessment, the mean age was 49 years (range, 24-65 years), mean disease duration was 14 years (range, 2-29 years), and median EDSS was 6.0 (range, 3.0-8.0). After 12-24 months of immunosuppressive treatment (33 patients with mitoxantrone and 5 with rituximab), the median EDSS was 6.0 (range, 3.0-8.5). From this cohort, 20 mitoxantrone-treated patients (18 with SPMS and 2 with RRMS) were included in Paper III, and 10 of them (9 with SPMS, and 1 with RRMS) were re-examined post-treatment. In Paper IV, patients with RRMS (n = 3) were excluded, and Paper V included 22 mitoxantrone-treated patients (3 RRMS, 15 SPMS, 4 PPMS).

6.1.2.2 Second-line immunomodulatory treatment (Paper III)

Forty-one RRMS patients (14 men and 27 women) were assessed prior to natali-

zumab treatment. Five patients were previously untreated, whereas 36 had breakthrough disease with relapses or MRI activity while receiving other immunomodulatory treatment (23 with interferon beta, 8 mitoxantrone, 4 glatiramer, and 1 fingolimod). These patients comprised a subgroup of a previously described population (264). They were all regarded as highly active, and were scheduled to start monthly intravenous treatment with 300 mg natalizumab according to Swedish guidelines (96) At first assessment, their mean age was 36 years (range, 13-60 years) and mean disease duration was 8.5 (range, 0.5-26 years). At both assessments, their median EDSS was 3.5 (range, 0-6.5).

6.2 Control subjects

This thesis includes three major control populations; 82 healthy controls (HC), 32 spinal anaesthesia subjects (SAS), and 67 patients with systemic lupus erythematosus (SLE). Informed consent was obtained from all control subjects, and studies were approved by the regional ethical boards of Gothenburg and Uppsala.

The first cohort consisted of HC and SAS who were included in a cross-sectional investigation of the sAPP-Aβ pathway in MS (Paper II). The majority were recruited at the Sahlgrenska University Hospital, Gothenburg, Sweden. HC were also recruited from the MS centre at Akershus University Hospital, Oslo, Norway. The second cohort was a sub-group of HC from the first cohort, who were further investigated in a long-term follow-up setting (Papers I-V). The third cohort consisted of patients with SLE (Paper II) recruited at the Department of Rheumatology, Sahlgrenska University Hospital, Sweden.

6.2.1 HC and SAS included in the cross-sectional study of the sAPP-Aβ pathway (Paper II)

Our cross-sectional study included 114 HC and SAS (46 men and 68 women) with a mean age of 42 years (range, 18-82 years). The population consisted of 50 HC from the MS Centre, Gothenburg (208), 22 HC from the MS Centre, Oslo, and a mix of 42 HC and SAS from other departments at the Sahlgrenska University Hospital. The subjects included blood donors, students (recruited by advertisement), spouses of patients, and a group of neurologically healthy individuals undergoing lower limb surgery, which was previously described (265).

6.2.2 HC population with long-term follow-up (Papers I-V)

Twenty-eight HC (20 men and 8 women) were included from a previously described control population of 50 healthy blood donors and students (208). Their mean age

was 33 years (range, 18-53 years). They were subjected to lumbar puncture twice with a median interval of 9 years.

6.2.3 Control population with systemic lupus erythematosus (Paper II)

Sixty-seven SLE patients (10 men and 57 women) were included. Their mean age was 42 years (range, 17-75 years). They were recruited at the Department of Rheumatology, Sahlgrenska University Hospital, and were diagnosed according to the revised SLE criteria of The American Rheumatism Association (266). Twenty-six of these subjects were considered to fulfill the criteria for cerebral SLE (267, 268).

Table 1. Overview of the number of patients and control subjects from Papers I-V.

	MS patients				Control subjects		
	Long-term follow-up	Cross-sectional	Treated		Long-term follow-up	Cross-sectional HC/SAS	SLE
			Mx or Rx*	Nz**			
Paper I	25	0	0	0	28	0	0
Paper II	23	100	0	0	27	82/32	67
Paper III	26	0	20	41	28	0	0
Paper IV	0	0	35	0	14	0	0
Paper V	20	0	22	0	26	0	0

Mx, mitoxantrone; Rx, rituximab; Nz, natalizumab.
*Immunosuppressive therapies.
**Second-line immunomodulatory therapy.

Table 2. Demographics and clinical characteristics of the long-term follow-up cohort of patients (n = 25) and HC (n = 28) at baseline from Paper I.

Study group	**All MS**	**RRMS**	**SPMS**	**Healthy controls**
Subjects, n	25	15	10	28
Gender, F/M	18/7	14/1	4/6	8/20
Mean age (range), years	41 (21-59)	40 (21-59)	43 (27-52)	33 (18-53)
Median EDSS (range)	3.0 (1.0-8.0)	2.75 (1.0-4.5)	5.5 (2.0-8.0)	NA

NA, not applicable.

Table 3. Demographics and clinical characteristics of the long-term follow-up cohort of patients ($n = 23$) and HC ($n = 27$), and the cross-sectional cohort of patients ($n = 100$), HC/SAS ($n = 114$), and SLE ($n = 67$) at baseline from Paper II.

Group	MS	SLE	Healthy controls
Subjects, n	100	67	114
Gender, F/M	33/67	10/57	46/68
Mean age (range), years	42 (17-65)	42 (17-75)	42 (18-82)
Median EDSS (range)	3.0 (0-8.0)	NA	NA

Some patients and HC were initially included in the cross-sectional study and thereafter included in the long-term follow-up cohort.
NA, not applicable.

Table 4. Demographics and clinical characteristics of the long-term follow-up cohort of patients ($n = 26$) and HC ($n = 28$), and patients of the treated cohort (natalizumab $n = 41$, mitoxantrone $n = 20$) at baseline from Paper III.

Group	Subjects, *n*	Gender F/M	Age, Median (IQR), years	Baseline EDSS Median (IQR)
Study population at baseline				
RRMS	54	39/15	37 (32-46)	3.5 (2.0-4.5)
SPMS	33	17/16	53 (43-58)	6.0 (4.8-7.0)
Controls	28	7/21	40 (35-52)	NA
Subpopulation of natalizumab and mitoxantrone treated patients				
Natalizumab	29 RRMS	21/8	35 (30-39)	3.5 (2.0-6.0)
Mitoxantrone	9 SPMS 1 RRMS	3/7	45 (38-50)	6.0 (4.5-6.4)
Subpopulation analysed with immunoprecipitation and mass spectrometry				
RRMS	10	6/4	38 (32-47)	2.5 (0.0-6.0)
SPMS	11	6/5	54 (50-58)	6.5 (5.0-9.5)
Controls	10	2/8	41 (38-47)	NA

IQR, interquartile range; NA, not applicable.

Table 5. Demographics and clinical characteristics of the treated cohort (mitoxantrone or rituximab, *n* = 35) and 14 HC of the long-term cohort from Paper IV.

Group	MS patients	HC
Subjects, n	35	14
Gender, F/M	15/20	5/9
Mean age, (range), years	48 (22-65)	42 (31-61)
EDSS, median (range)	6.0 (3.0-8.0)	NA

NA, not applicable.

Table 6. Demographics and clinical characteristics of the long-term follow-up cohort of patients (*n* = 20) and HC (*n* = 26), and patients of the treated cohort (mitoxantrone, *n* = 22) from Paper V.

Group 1	**Subjects, *n***	**Gender F/M**	**Age***	**EDSS Baseline****
All MS	20	15/5	41.5 (21-59)	3.0 (1.0-8.0)
RRMS	9	9/0	33.7 (21-48)	3.0 (0-8.0)
SPMS	7	3/4	46.7 (41-52)	7.5 (2.0-8.0)
Transitory	4	3/1	50 (44-59)	3.25 (2.0-4.0)
Group 2	***n***	**Gender F/M**	**Age***	**EDSS Baseline****
All MS	22	11/11	45.2 (22-60)	6.0 (3.0-8.0)
RRMS	3	3/0	42.6 (37-50)	6.0 (4.5-6.5)
SPMS	15	7/8	46.1 (22-60)	6.0 (3.0-8.0)
PPMS	4	1/3	43.5 (34-52)	6.5 (6.0-7.5)
Group Controls	***n***	**Gender F/M**	**Age***	**EDSS Baseline****
HC	26	8/18	33.1 (18-60)	NA

*Data are mean (range), years.
**Data are median (range).

6.3 Clinical assessments, MRI, and serum and CSF sampling

Disease activity was measured by annual relapse rate (Papers I-V) or presence of Gd^+ T1-weighted images (Paper IV). A relapse was defined as patient-reported or objectively observed events typical of an acute inflammatory demyelinating event in the CNS, current or historical, with duration of at least 24 hours, in the absence of fever or infection (63). Neurological disability was determined by EDSS and its seven functional systems (152), and disease severity or progression was scored according to progression index (158) or MSSS (156).

The MRI investigations (Paper IV) were performed according to the standard MS protocols of Sahlgrenska University Hospital, Örebro University Hospital, and Umeå University Hospital. The sequences used in the standard program are T1, T1 with standard-dose gadolinium contrast, T2, DWI, and Flair. MRI scans were performed with 1.5 or 3.0 Tesla machines, using 3-mm slice thickness, and took place no more than three months from clinical neurological examination and lumbar puncture. Serum samples were obtained by venepuncture and CSF samples were obtained by lumbar puncture; samples were handled and stored according to the recommendations of Bio MS (269). The clinical assessments and the serum and CSF sampling were performed once in the subjects of the cross-sectional cohorts, and twice in subjects of the long-term follow-up and the treated cohorts, with intervals of 8-10 years and 12-24 months, respectively.

6.4 Assays

All biochemical analyses in the studies of this thesis were run at the laboratory of neurochemistry, Sahlgrenska University Hospital, Mölndal, Sweden. They were performed by experienced and certified laboratory technicians who were blinded to the clinical and MRI data. To minimize variation, baseline and follow-up samples were analysed side by side on each assay plate, using one batch of reagents. All intra assay coefficients of variation were < 10%.

6.4.1 Polyclonal NFL assay (Paper I)

NFL was measured by an in-house-developed sandwich ELISA (205). Rabbit polyclonal anti-NFL IgG was used as the secondary antibody. Bound secondary antibody was detected using peroxidase-conjugated donkey anti-rabbit IgG. The standard curve ranged from 125 to 16 000 ng/L. The assay sensitivity was 125 ng/L.

6.4.2 Monoclonal NFL assay (Paper IV)

NFL levels in CSF were determined by an enzyme-linked ELISA developed by the UMAN Diagnostics. CSF samples were incubated in pre-coated anti-NFL plates. As secondary antibodies, we used two monoclonal antibodies that are highly specific for NFL. Streptavidin horse radish peroxidase was used for detection. The assay sensitivity was 31 ng/L (209).

6.4.3 Glial fibrillary acidic protein assay (Papers I and IV)

GFAP was measured using an in-house developed ELISA, as previously described (270). Rabbit polyclonal anti-GFAP IgG was used as the secondary antibody. Captured secondary antibody was detected using peroxidase-conjugated donkey anti-rabbit IgG. The procedure was performed at room temperature. The sensitivity of the GFAP assay was 32 ng/L. The standard curve ranged from 32 to 16 000 ng/L.

6.4.4 CXCL13 (Papers IV and V)

CXCL13 was measured by an ELISA Human CXCL13/BLC/BCA-1 Immunoassay (R&D Systems Inc., Abingdon, United Kingdom) according to the manufacturer's instructions. In brief, this assay employs a sandwich enzyme immunoassay technique. A monoclonal antibody specific for CXCL13 was precoated on a microplate. HRP-labelled CXCL13 monoclonal soluble detection antibody was used. The quantification limit was 7.8 pg/mL, and CSF samples below that level were designated as 3.9 pg/mL.

6.4.5 Albumin ratio (Paper V)

Quantitative determinations of albumin in serum and CSF were performed by immunonephelometry on a Beckman Image Immunochemistry system (Beckman Instruments, Beckman Coulter, Brea, CA, USA). Briefly, the nephelometer measured the increased light scattered as a result of particles created after addition of anti-albumin antibodies to a solution. The albumin ratio was calculated as CSF albumin (mg/L)/serum albumin (g/L) × 1000.

6.4.6 IgG index, CSF-specific oligoclonal IgG bands (Paper V)

Quantitative determinations of IgG in serum and CSF were performed by immunonephelometry on a Beckman Image Immunochemistry system (Beckman Instruments, Beckman Coulter, Brea, CA, USA). The IgG index was calculated as CSF IgG (mg/L)/serum IgG (mg/L)/albumin ratio. OCB were visualized in CSF and serum by isoelectric focusing followed by silver staining, as previously described (271). Ocular quantification of CSF-specific OCB was performed independently by

two experienced researchers. Patients were divided into five different groups according to the number of OCB: 0, 1, 2-4, 5-10, and >10.

6.4.7 BACE1 (β-Secretase) activity (Paper II)

CSF BACE1 activity was measured using a highly sensitive and specific solution-based assay, as previously described (272). Briefly, the assay consisted of an enzymatic reaction of CSF with a biotinylated 15-mer optimized BACE1 substrate (biotin-KTEEISEVNFEVEFR) and the use of pepstatin A to block non-specific protease activity, followed by cleavage product detection using a neoepitope-specific antibody. The extent of BACE1 activity was quantified using recombinant BACE1 standards. The sensitivity of the assay was below 1.0 pM of recombinant BACE1.

6.4.8 *α*-sAPP and β-sAPP (Papers II and III)

The CSF α-sAPP and β-sAPP were determined using the sAPPα/sAPPβ Multiplex Assay for Meso Scale Discovery (MSD) platform (MSD, Gaithersburg, MD, USA), as described by the manufacturer. Briefly, this assay employed antibodies specific for the two fragments, run at the same time in two different electrodes coated with antibodies for 6E10 (an epitope within amino acids 3-8 of the Aβsequence) to capture α-sAPP, and a neoepitope-specific antibody on the C-terminal to capture β-sAPP. Both also had matching control electrodes. Both species were detected by the SULFO-TAG-labelled anti-APP antibody, p2-1. The sensitivity of this method was 120 pg/mL for α-sAPP, and 52 pg/mL for β-sAPP.

6.4.9 AβX-38, AβX-40, and AβX-42 (Papers II and III)

CSF AβX-38, AβX-40, and AβX-42 were determined using the MSD Aβ Triplex kit (Human Aβ peptide Ultra-Sensitive Kits), as described by the manufacturer. Briefly, this assay ran three samples at the same time on three attached electrodes. The N-terminus included 4G8 antibodies (an epitope within amino acids 18-22 of the Aβ sequence) as capturing antibodies, while the C-terminus included SULFO-TAG-labelled specific antibodies for detecting the different Aβ peptides. The method sensitivity was 6 pg/mL for AβX-38, 2 pg/mL for AβX-40, and 7 pg/mL for AβX-42.

6.4.10 Aβ1-42 (Papers II and III)

CSF Aβ1-42 was determined using the INNO-BIA AlzBio3 kit (Innogenetics, Ghent, Belgium) on the xMAP Luminex platform, as described previously (273). Briefly, one monoclonal antibody (4D7A3) was attached to a capturing microsphere, and a second antibody (3D6) was attached to the opposite end. Both ends included antibodies for establishing the sequence 1-42. The flourochrome phycoerythrin was

coupled to streptavidin to measure the amount of Aβ1-42 on each microsphere. The method was flow cytometric, allowing microspheres with different antibodies to be run at the same time. Each microsphere was separated based on different signals after laser excitation. The sensitivity of the method was 25 ng/L.

6.4.11 Immunoprecipitation and mass spectrometry (Paper III)

This analysis used immunoprecipitation (IP) followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) (274, 275). Briefly, aliquots of the monoclonal antibodies 6E10 and 4G8 were separately added to magnetic beads coated with sheep anti-mouse IgG (Life Technologies, Carlsbad) and incubated. 6E10 + 4G8 antibody-bead solutions and Tween in phosphate-buffered saline (PBS) were added to CSF samples. The solution was then transferred to a magnetic particle processor (KingFisher Flex; Thermo Scientific, Waltham, MA, USA) for automatic washing and elution. The collected supernatant was dried by speed-vac and redissolved in formic acid (FA) in acetonitrile (ACN). The MALDI samples were prepared with the seed layer method, and analysed by MALDI-TOFMS (Bruker AutoFLEX (Bruker Daltonics). Each sample was analysed in duplicate. The mass/charge ratio (m/z) used to identify the Aβ-peptide sequences represents the monoisotopic peak of the protonated molecule [M+ H^+].

6.4.12 Liquid chromatography and tandem mass spectrometry (Paper III)

To confirm the identities of the Aβ species, the immunoprecipitates (IP) were analysed by liquid chromatography (LC) combined with high-resolution tandem mass spectrometry (MS-MS) (276). Briefly, LC-MS/MS analysis was performed on a chromatographic system (GE Healthcare) using HotSep Kromasil C4 columns (G&T Septech) coupled to a mass spectrometer (Thermo Fisher Scientific). Collision-induced dissociation (CID) was used to obtain fragment ion data.

6.5 Statistics

Statistical inference was performed using SPSS software (several versions, updated over time), PASW Statistics 18 software (Paper IV), GraphPad Prism 5.00 (GraphPad Software, San Diego, CA, USA) (Paper III), and Excel 2007 (Microsoft Corporation, Redmond, WA, USA) (Paper III).

Normally-distributed data were analysed by parametric tests. Between-group comparisons were made using Student´s *t*-test, ANOVA, and ANCOVA. Within-subject differences over time were analysed by paired *t*-test. Correlation analyses were performed using Pearson correlation, linear regression analysis, partial correlation analysis, and one-way ANOVA. Data with skewed distributions were analysed using non-parametric tests. Between-group differences were analysed by Mann-Whitney U test, Kruskal-Wallis test, and non-parametric ANCOVA. The Wilcoxon ranked-sign test was used for within-subject differences over time. The correlations between variables were calculated using Spearman's rank correlation.

6.5.1 Multivariate analyses

In Paper III, multivariate analysis was used to differentiate two groups based on their biomarker profiles. Each marker formed an orthogonal projection, which combined to form an algorithm. This algorithm determines the direction (score vector) in the multivariate orthogonal space spanned by biomarkers that provides the best separation between the predefined groups. A stepwise variable reduction was performed until only the markers with positive contributions toward the separation were left, as judged by their relative contributions to the model. The calculations were implemented using the SIMCA P+ v. 12 software (Umetrics, Umea, Sweden).

Corrections for multiple comparisons were done using Bonferroni-correction. The data were reported as the mean when presumed to be normally distributed, otherwise the median was given. $P \leq 0.05$ indicated statistical significance and $P \leq 0.01$ highly significance.

7. Results

7.1 Paper I

7.1.1 Increased CSF GFAP levels in MS patients

With adjustment for age, increased CSF GFAP levels were recorded in patients compared to in HC ($P < 0.05$), whereas similar CSF NFL levels were observed in patients and HC. No significant differences were found between the RRMS and SPMS patients. GFAP and NFL levels were both correlated with age (both r = 0.50, $P < 0.01$) *(Figure 7)*.

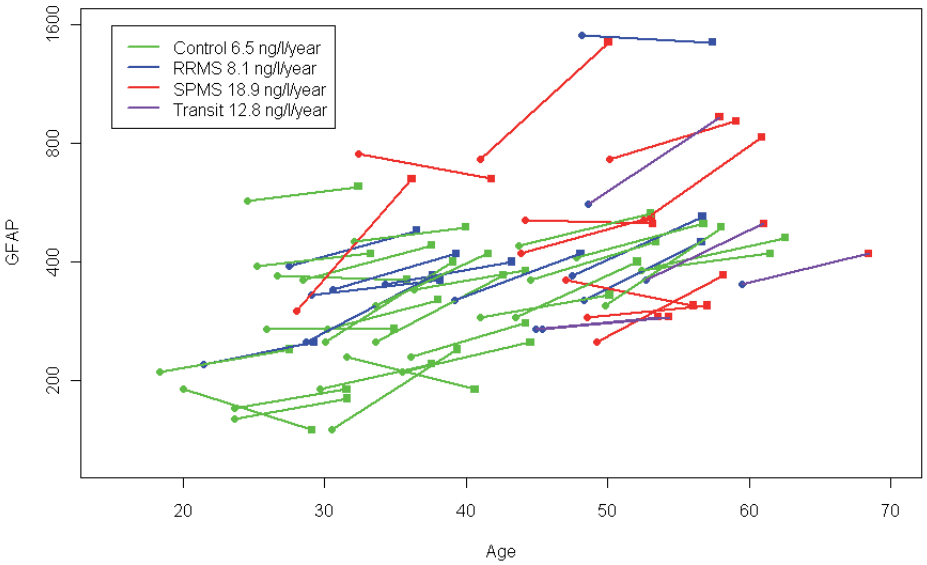


Figure 7
Individual GFAP levels at two occasions for patients with relapsing-remitting multiple sclerosis (RRMS, $n = 10$), secondary progressive multiple sclerosis (SPMS, $n = 10$), patients converting from RRMS to SPMS (Transit, $n = 5$), and healthy controls ($n = 28$). The first examination is indicated with circles and the second examination with squares. The mean annual GFAP increase is indicated for each group

7.1.2 CSF GFAP correlated with progression

When the data were corrected for age, CSF GFAP levels increased between the assessments ($P < 0.05$). This increase was most prominent in the SPMS group. The mean annual GFAP increases were 6.5 ng/L for HC, 8.1 ng/L for RRMS patients,

12.8 ng/L for patients converting from RRMS to SPMS, and 18.9 ng/L for SPMS patients. However, this progressive trend was not accompanied by significant between-group differences.

7.1.3 GFAP correlated with clinical disability and was closely related to neurological disability

GFAP levels correlated with neurological disability as measured by EDSS ($r = 0.51$, $P < 0.05$), and with disease severity or progression determined by MSSS ($r = 0.47$, $P < 0.05$). Dividing disability into the seven FS of EDSS revealed significant correlations between GFAP and the FS scores of pyramidal ($r = 0.51$, $P < 0.01$), bowel and bladder ($r = 0.51$, $P < 0.01$), and brain stem ($r = 0.53$, $P < 0.01$). EDSS ($P < 0.01$) and progression index ($P < 0.01$) increased between assessments, mainly driven by increases in the scores of SPMS and patients changing course from RRMS to SPMS during long-term follow-up.

7.1.4 GFAP had predictive value

GFAP level at the first assessment had predictive value for neurological disability (EDSS) 8-10 years later ($r = 0.45$, $P < 0.05$). This was most pronounced in the SPMS group ($r = 0.80$, $P < 0.01$). However, GFAP level could not predict the increase of EDSS between the two assessments. The group with high GFAP levels (>350 ng/L) had a higher risk of achieving high EDSS (>5.5), with an OR of 4.5.

7.1.5 NFL was not elevated in clinically stable MS but some patients showed signs of subclinical disease activity

NFL levels were not increased in MS patients compared to in HC; they had no relationship with disability or progression, and no prognostic value for disability development. Two patients had increased NFL levels on only one occasion, and three patients showed high levels on multiple occasions, indicating acute axonal damage not associated with relapse.

7.2 Paper II

7.2.1 BACE1 distinguished MS from other inflammatory disease and controls

MS patients had lower CSF BACE1 activity than controls ($P < 0.05$) and patients with cerebral SLE ($P < 0.01$) *(Figure 8)*. Patients with cerebral SLE had higher BACE1 activity than any other group ($P < 0.05$ for all comparisons). SPMS patients tended to have lower levels compared to other MS groups ($P < 0.07$). In MS, BACE1 activity correlated negatively with disease duration ($r = -0.26$, $P = 0.01$) and neurological disability as determined by EDSS ($r = -0.25$, $P < 0.05$).

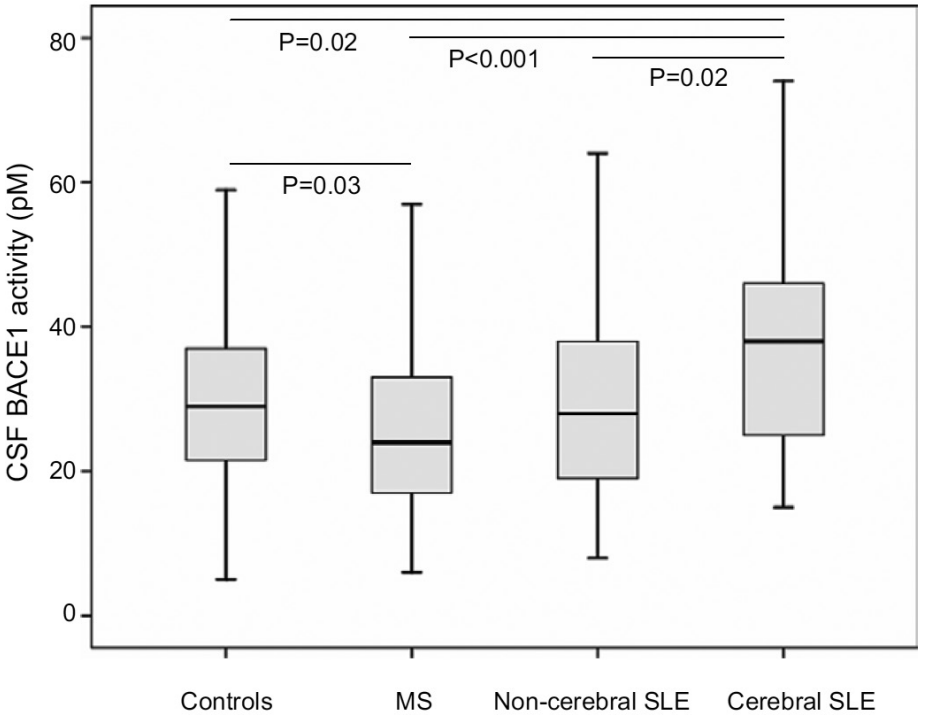


Figure 8
CSF BACE1 activity in patients with MS, patients with SLE with and without cerebral involvement, and controls.

7.2.2 BACE1 activity correlated with APP metabolites that were altered in MS

In MS patients, BACE1 activity correlated weakly with levels of sAPP/Aβ markers (Aβ42, α-sAPP, and β-sAPP). Subgroup analysis revealed stronger correlations in SPMS patients (Aβ42: r = 0.40, $P < 0.05$; α-sAPP: r = 0.66, $P < 0.01$; β-sAPP: r = 0.77, $P < 0.01$) than in RRMS patients (Aβ42: r = 0.38, $P < 0.01$; α-sAPP: r = 0.40, $P < 0.01$; β-sAPP: r = 0.26, $P < 0.05$). These correlations with Aβ42, α-sAPP, and β-sAPP, respectively, were also significant in non-cerebral (r = 0.54, r = 0.66, r = 0.64, all $P < 0.01$) and cerebral (r = 0.75, r = 0.55, r = 0.54, all $P < 0.01$) SLE. In controls, only a weak correlation was found between BACE 1 and β-sAPP (r = 0.19, $P < 0.05$).

The CSF Aβ42, α-sAPP, and β-sAPP levels were significantly reduced in patients with MS compared to in controls *(Table 7)*. However, the reductions in α-sAPP and β-sAPP were not specific to MS but were also seen in cerebral and non-cerebral SLE *(Table 7)*. Compared with MS patients in remission, patients with on-going

or recent relapse (within one month) had lower levels of α-sAPP [respectively, 476 ng/L (range, 265-1195 ng/L) and 711 ng/L (range, 186-1564 ng/L); $P < 0.01$] and β-sAPP [respectively, 239 ng/L (range, 146-559 ng/L) and 301 ng/L (range, 105-463 ng/L); $P < 0.01$].

Table 7. Demographics and biomarker concentrations in MS, cerebral SLE, noncerebral SLE, and controls

	MS ($n = 100$)	Cerebral SLE ($n = 26$)	Noncerebral SLE ($n = 41$)	Controls ($n = 114$)
Age, years	42 (17–65)	41 (19–75)	43 (17–75)	42 (18–82)
Gender, men/women	33/67	3/23	7/34	46/68*
α-sAPP, ng/mL	582 (186–1564)**	585 (142–1106)†	510 (234–1285)†	669 (238–1727)
β-sAPP, ng/mL	260 (105–559)‡	225 (110–395)†	210 (112–447)†	285 (93–615)
Aβ42, pg/mL	648 (220–1118)†	707 (345–1182)	632 (262–952)‡	722 (140–1132)

MS, multiple sclerosis; SLE, systemic lupus erythematosus.
Data are median (range).
*$P < 0.001$ versus MS, cerebral and noncerebral SLE.
**$P < 0.001$ versus controls.
†$P < 0.01$ versus controls.
‡$P < 0.05$ versus controls.

7.2.3 BACE1 activity decreased towards progressive disease course

BACE1 activity decreased over time in MS patients ($P < 0.05$) who were assessed twice with an interval of 8-10 years. This decrease was most pronounced in RRMS patients, whereas SPMS patients had constantly low activity. None of the markers showed prognostic abilities or relation to clinical disability (EDSS).

7.3 Paper III

7.3.1 The APP metabolites were decreased in MS patients

RRMS and SPMS patients had lower baseline CSF levels of AβX-38, AβX-40, AβX-42, α-sAPP, and β-sAPP than control subjects, while only SPMS patients showed lower Aβ1-42 level *(Figure 9)*. No significant differences were found between RRMS and SPMS patients. Since the study groups differed in age and gender distribution, these factors were corrected for in the analysis.

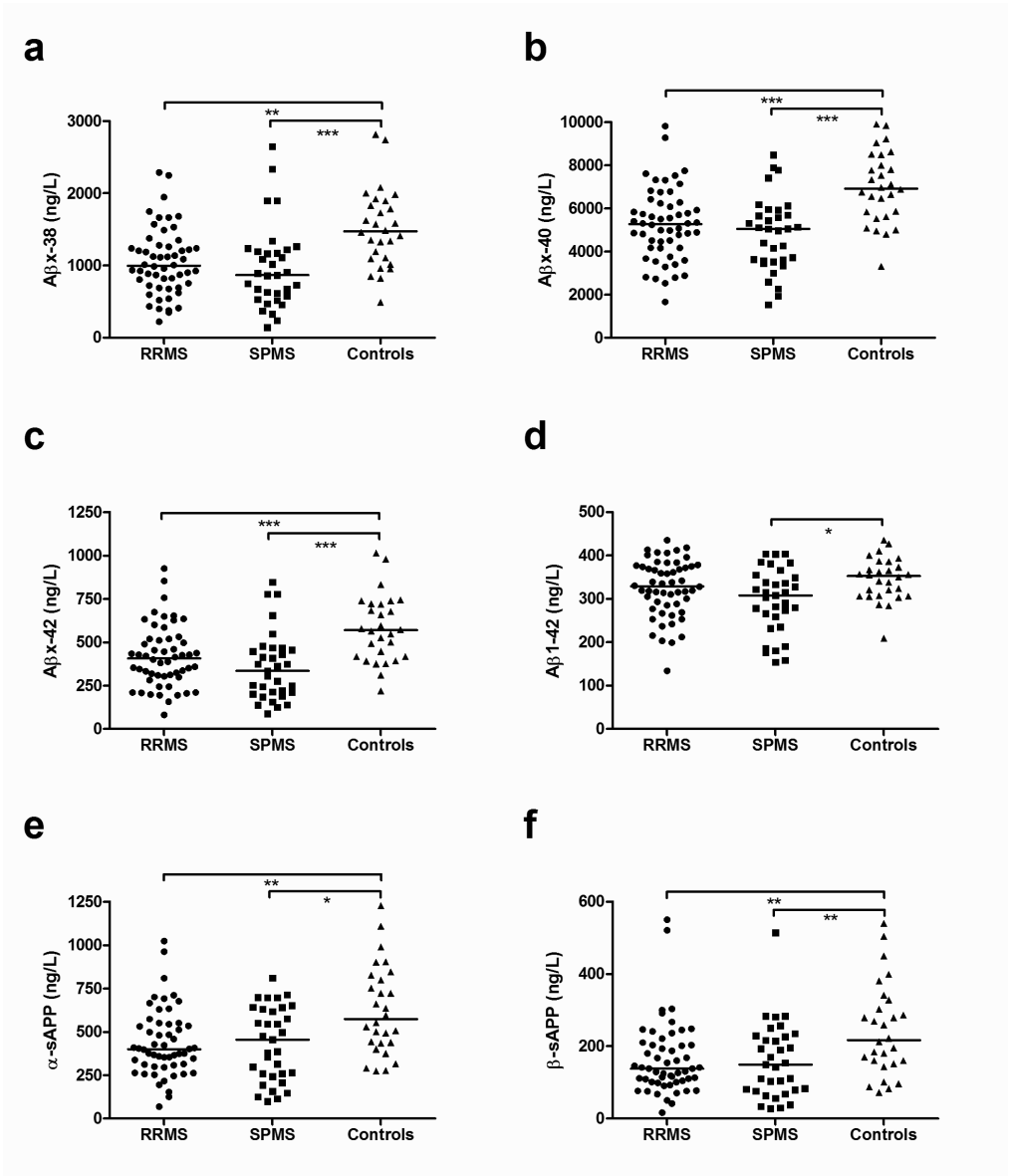


Figure 9
Baseline levels of biomarkers, as measured by immunoassays.
* P<0.05, ** P<0.01 and *** P<0.001

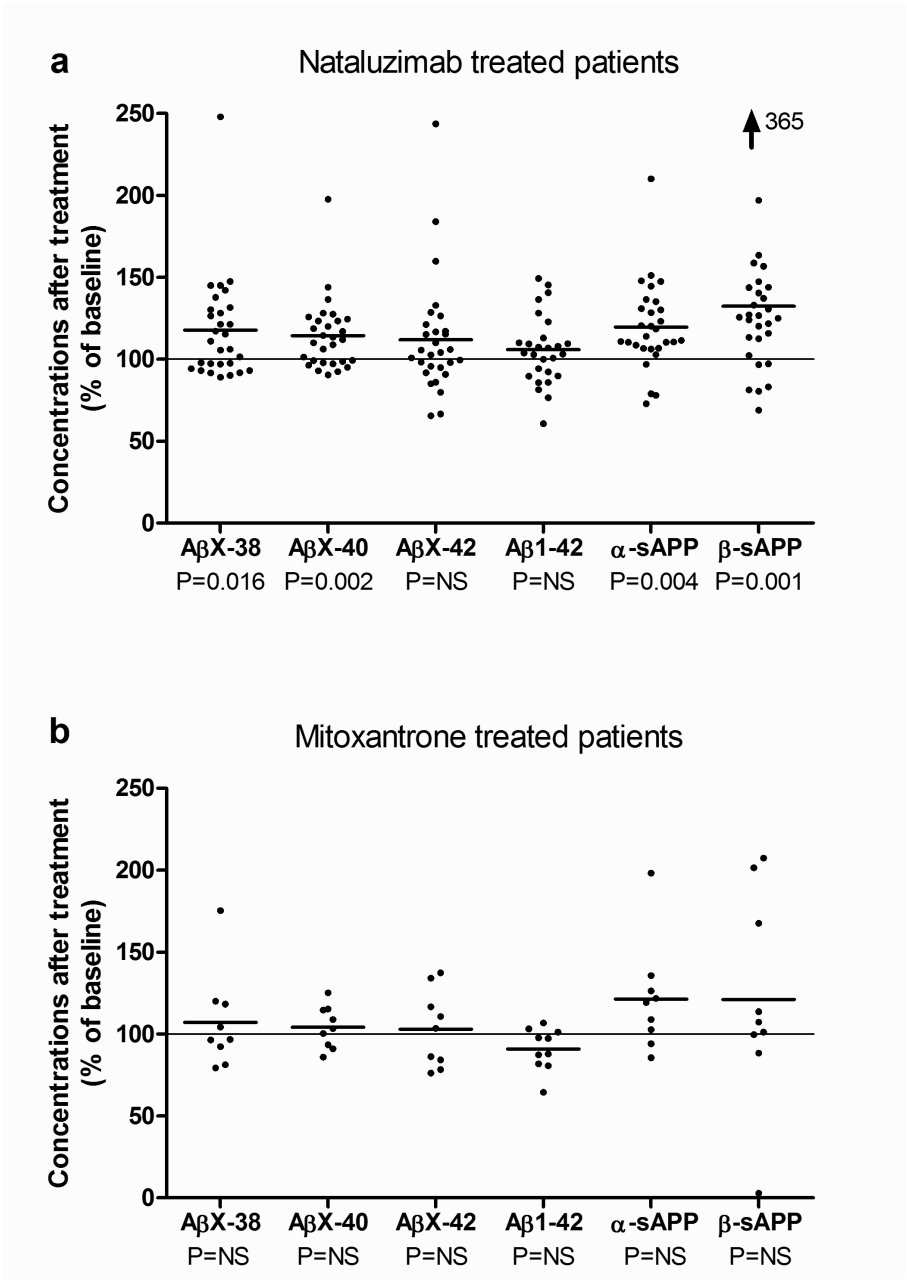


Figure 10
Changes in biomarkers after treatment.
Patients treated with natalizumab (Panel (a)) and mitoxantrone (Panel (b)). The number of patients analyzed varied between biomarkers, due to missing samples (Panel (a), $N = 27–29$; Panel (b), $N = 8–10$). The graphs show that follow-up concentrations normalized in relation to baseline levels (100%).

7.3.2 Natalizumab normalized the APP metabolite levels

The APP metabolite levels were normalized in patients treated with natalizumab, with only two exceptions (Aβ x-42 and Aβ 1-42), while no changes in APP metabolites were seen after treatment with mitoxantrone *(Figure 10)*. However, patients treated with natalizumab were younger than those treated with mitoxantrone ($P < 0.05$). Within the natalizumab-treated group, older patients had a lower absolute change in β-sAPP compared to younger patients ($r = 0.43$, $P < 0.05$). The age differences seen between the groups may have influenced the β-sAPP trajectories in the two treatment groups; however, the overall dynamic biomarker response after natalizumab treatment did not seem to be confounded by age, and the correlation between age and β-sAPP was not significant after multiple comparisons.

7.3.3 Progressive MS patients exhibited altered sAPP/Aβ metabolism

The CSF was analysed with IP-MS and mass spectrometry, and 18 different Aβ species were isolated. Only small univariate differences in the relative levels of individual peptides were observed between the disease courses. Multivariate analysis revealed distinctly different patterns of sAPP/Aβ between SPMS and controls, with an intermediate pattern observed in RRMS.

7.4 Paper IV

7.4.1 NFL levels were reduced by immunosuppressive treatment in progressive MS patients

The mean NFL level in patients with progressive MS was reduced following 12-24 months of mitoxantrone ($n = 30$) or rituximab treatment ($n = 5$), from 1780 ng/L (SD, 2018) to 870 ng/L (SD, 693) ($P < 0.01$). However, these reduced NFL levels in patients did not reach those found in HC [577 ng/L (SD, 326); $P < 0.05$] *(Figure 11)*.

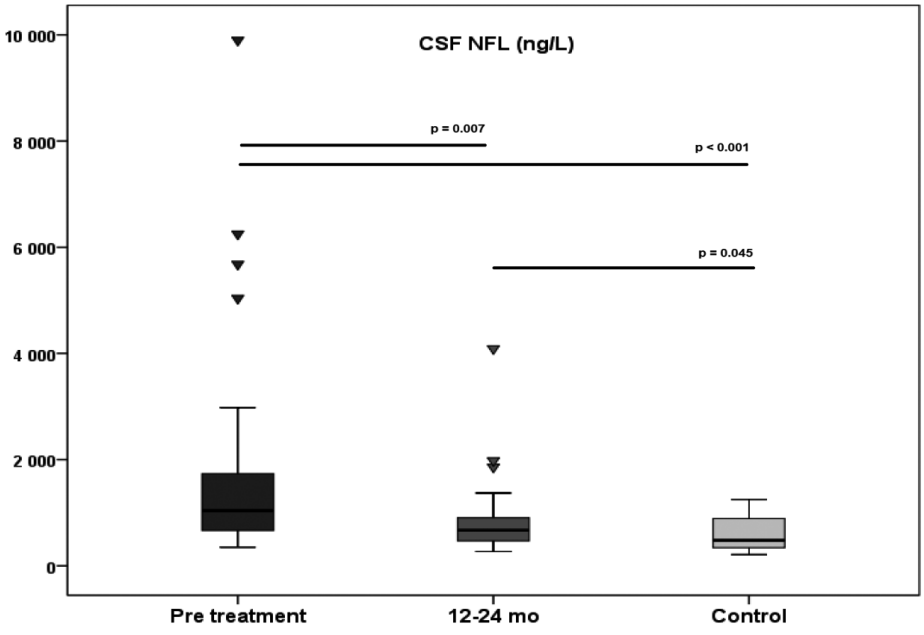


Figure 11
Neurofilament light (NFL) in cerebrospinal fluid (CSF) before and following mitoxantrone (n=30) or rituximab (n=5) treatment. NFL levels in the CSF were compared to levels obtained in 14 healthy individuals. Box indicates interquartile range, bar indicates median, and whiskers indicating 95% CI.

7.4.2 First-line disease-modifying therapies seemed to influence the NFL levels in progressive MS

In previously untreated patients (n = 20), the pre-treatment mean NFL level (2462 ng/L; SD, 2452) was significantly higher than in patients with on-going first-line disease-modifying therapies (DMT) (874 ng/L; SD, 434; n = 15; $P < 0.05$) *(Figure 12)*. Only the group of previously untreated patients showed significant NFL level reduction following 12-24 months of immunosuppressive treatment (P<0.01).

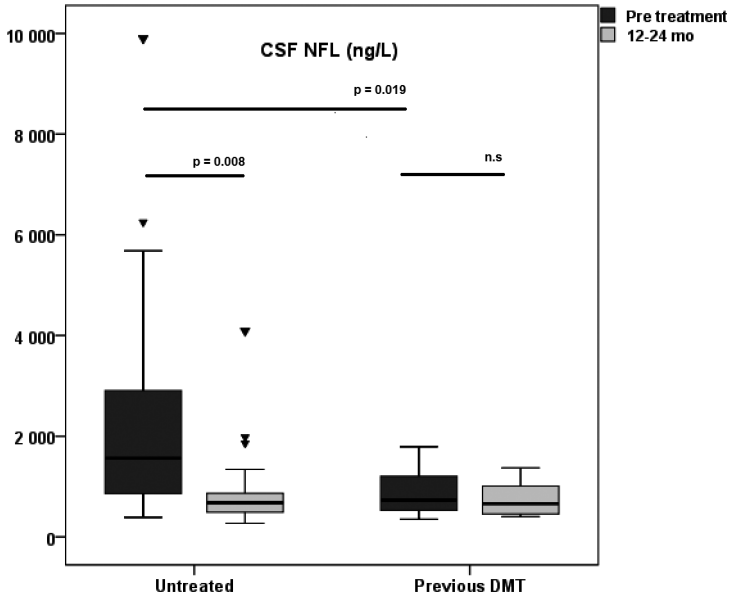


Figure 12
Neurofilament light (NFL) in cerebrospinal fluid (CSF) before and following 12-24 months of mitoxantrone or rituximab treatment in previously untreated patients (n=20) or patients with ongoing disease modifying therapies (DMT, n=15) at study baseline. Box indicates interquartile range, bar indicates median, and whiskers indicating 95% CI.

7.4.3 NFL levels seemed to influence Gadolinium enhancement on MRI in progressive MS

Progressive MS patients with Gd^+ lesions at study baseline (n = 12) presented with significantly higher pre-treatment NFL levels than patients without Gd^+ lesions on MRI (n = 23) [2925 ng/L (SD, 2884) vs. 1184 ng/L (SD, 1024); $P < 0.05$] (Figure 13). Only one patient with Gd^+ lesions had been previously treated. Although patients with Gd^+ lesions had the most marked NFL decrease [2925 ng/L (SD, 2884) vs. 889 (SD, 1041); $P < 0.01$], patients without signs of BBB damage on MRI also showed significantly reduced NFL levels following immunosuppressive treatment [1184 ng/L (SD, 1024) vs. 866 (SD, 449); $P < 0.05$].

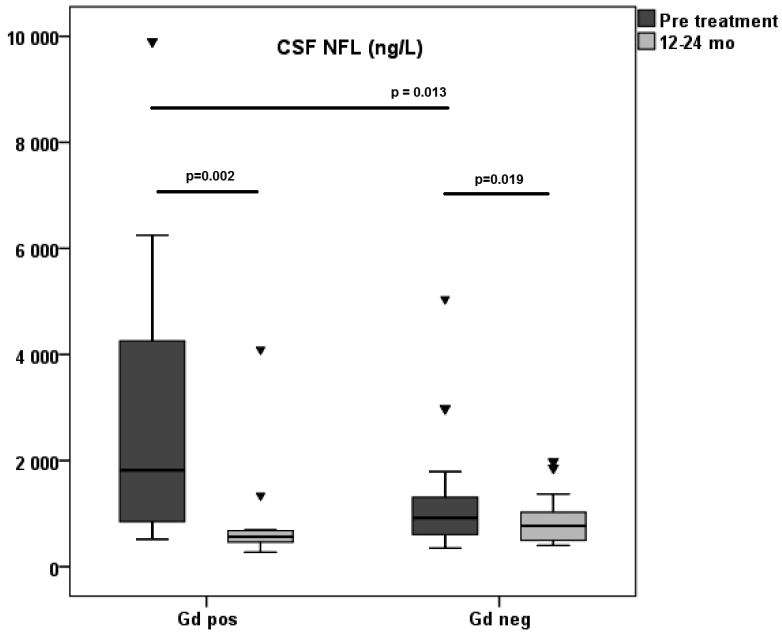


Figure 13
Neurofilament light (NFL) in cerebrospinal fluid (CSF) before and following 12-24 months of mitoxantrone or rituximab treatment in patients with (n=12) or without (n=23) pretreatment gadolinium enhancing lesions on MRI. Box indicates interquartile range, bar indicates median, and whiskers indicating 95% CI.

7.4.4 CXCL13 levels in CSF of progressive MS were normalized by immunosuppressive treatment

CXCL13 levels increased to above the ELISA detection limit were only found in 11 of the 70 CSF samples obtained from progressive MS patients. The mean pre-treatment CXCL13 level was significantly higher in MS patients (9.7 pg/mL) compared to in HC subjects (<7.8 pg/mL; $P < 0.05$). Moreover, the mean CXCL13 level at treatment follow-up was significantly reduced (4.4 pg/mL; $P < 0.01$). With one exception, all of the CSF samples with detectable CXCL13 levels also had high NFL levels (>1200 ng/L, i.e. 2 SD above the mean NFL level of HC).

7.4.5 GFAP levels were increased in progressive MS patients but unaffected by immunosuppressive treatment

GFAP levels were higher in progressive MS patients compared to HC [650 ng/L (SD, 240) vs. 490 ng/L (SD, 170); $P < 0.05$). Immunosuppressive treatment had no significant effect on GFAP levels at follow-up. However, mean GFAP level was

significantly higher in patients with pre-treatment Gd^+ compared to patients without signs of disease activity on MRI [803 ng/L (SD, 224) vs. 578 ng/L (SD, 216); $P < 0.01$].

7.4.6 NFL were correlated with CXCL 13, and GFAP in progressive MS

Pre-treatment CSF NFL levels were correlated significantly with CXCL13 ($r = 0.527$, $P < 0.01$) and GFAP levels ($r = 0.471$, $P < 0.01$). Following 12-24 months of immunosuppressive treatment, NFL levels were only correlated with GFAP levels ($r = 0.440$, $P < 0.01$). The pre- to post-treatment differences were significantly correlated between NFL and CXCL13 ($r = 0.475$, $P < 0.01$) and between NFL and GFAP ($r = 0.391$, $P < 0.05$).

7.5 Paper V

7.5.1 The OCB pattern changed over 8-10 years of follow-up

Of 18 patients examined twice with an interval of 8-10 years, 16 had ≥2 OCB and two had one IgG band selectively produced in the CSF. Between the two examinations, the OCB pattern changed in 15 patients. Without any isolated predisposing factor, the number of bands increased in four patients and decreased in six, three patients both gained and lost bands, and two had unchanged OCB number but with differences in intensity.

7.5.2 Immunosuppressive treatment did not affect the OCB

Of the 22 patients examined before and after 1-2 years of mitoxantrone treatment, the OCB counts or patterns changed in four patients (all with SPMS). Two exhibited increased OCB and two demonstrated both new and lost bands. OCB counts were not correlated with clinical course, EDSS, or MSSS.

7.5.3 CXCL13 levels were decreased following immunosuppressive treatment of progressive MS

CXCL13 was analysed at the second assessment in the long-term follow-up group, and at both pre- and post-treatment assessments, performed 24 months apart. Patients of the long-term follow-up group had CXCL13 levels similar to those of controls. Among progressive patients, the pre-treatment CXCL13 levels were significantly higher than in HC [13.8 (SD, 1.22) vs. 4.6 (SD, 2.6); $P < 0.01$]. After 2 years of mitoxantrone treatment, the mean CXCL13 level was significantly reduced [4.3 (SD, 1.9); $P < 0.01$] to the levels of HC.

7.5.4 Correlations between OCB, IgG synthesis, CXCL13, and clinical parameters

In the long-term follow-up group, the IgG index was correlated with the numbers of OCB at both the first ($r = 0.60$, $P < 0.01$) and second assessments ($r = 0.47$, $P < 0.01$). No significant relationship was found between OCB and EDSS in SPMS. However, in RRMS patients ($n = 9$), EDSS correlated both with the OCB counts ($r = 0.70$, $P < 0.05$), and with the changed numbers of bands between the assessments ($r = 0.68$, $P < 0.05$).

In mitoxantrone-treated patients, the number of OCB correlated with the IgG index at both pre-treatment ($r = 0.58$, $P < 0.01$) and post-treatment ($r = 0.45$, $P < 0.01$) assessments.

CXCL13 level correlated with the IgG index in the long-term follow-up group ($r = 0.51$, $P < 0.05$), as well as in the mitoxantrone-treated group ($r = 0.61$, $P < 0.01$). CXCL13 levels also correlated with the number of OCB ($r = 0.49$, $P < 0.05$) in mitoxantrone-treated patients. We found weak correlations of CXCL13 levels with EDSS ($r = 0.32$, $P < 0.05$) and with MSSS ($P = 0.32$, $P < 0.05$) in both investigated groups.

8. Discussion

8.1 General considerations regarding biomarkers in MS

Recent years have seen the development of a growing number of therapeutic agents for MS, some of which have been introduced or are currently awaiting approval. There remains an obvious need for objective and reliable biomarkers to assess disease activity, rate of progression, and therapeutic efficacy. Investigations of biomarkers in MS may also reveal important information about pathophysiological processes. Currently available evidence indicates an indisputable role of inflammation in the demyelination and axonal damage in early RRMS, whereas immune activity is less clearly involved in neurodegeneration in PMS. A set of biomarkers reflecting different aspects of MS immunopathogenesis could be useful for further exploring this subject.

MRI measurements are currently the dominant biomarkers in MS. Visualization of focal inflammation can be used to confirm clinical or detect sub-clinical disease activity (277). MRI is less useful in PMS; hence, there is a great need for new biomarkers reflecting the pathological processes of this stage. Unlike MRI, biochemical biomarkers in CSF have the potential to specifically and directly mirror the immunopathological processes, indicating which inflammatory or degenerative pathway is activated. Additionally, our knowledge of the autoaggressive immune attack on the CNS in RRMS is strongly based on the animal model of experimental autoimmune encephalomyelitis (EAE), but no such reliable model exists for PMS. Therefore, investigations of CSF biomarkers may be of particular importance for resolving PMS pathophysiology. The present thesis discusses the value of biochemical CSF biomarkers as a complement to clinical and MRI investigations in PMS, particularly regarding their potential for monitoring disease activity, disability progression, disease severity, prognosis, and therapeutic efficacy.

8.2 CSF biochemical biomarkers related to PMS disease activity

A biochemical biomarker of MS disease activity should reflect the occurrence of relapse or the appearance of new lesions. Accumulating numbers of inflammatory biomarkers, e.g. cytokines or chemokines (234, 278-281), and CNS parenchymal biomarkers (206, 207, 209, 236, 282-284) have been associated with increased disease activity in MS. However, only few studies have shown an association between on-going inflammation and signs of CNS damage (206, 285).

8.2.1 Neurofilament light protein

Several studies have reported elevated CSF levels of neurofilament proteins during all stages of MS, with increased levels associated with relapse (207-209, 282, 286) and with Gd^+ lesions on MRI (206). In the present study, we detected increased NFL levels in PMS patients with signs of increased activity on MRI (Paper IV). Increased NFL was also related to CXCL13, suggesting increased immune activity in these patients. In RRMS, the CSF NFL level increases 3- to 10-fold during relapse (208, 209), then declines to low levels within 3 months (208, 286). In Paper IV, we found that PMS patients had increased NFL levels prior to immunosuppressive treatment, whereas the RRMS and SPMS patients from Paper I exhibited NFL levels similar to those observed in HC. These differences between studies may be partly due to the differences in patient selection. In Paper I, patients were included based on their low clinical disease activity, with no relapse documented within 3 months prior to study enrolment. In contrast, the PMS patients eligible for immunosuppressive treatment in Paper IV had obvious disability progression, with several of them showing subclinical disease activity on MRI. Furthermore, the NFL immunoassay used in Paper I had lower sensitivity compared to the NFL ELISA used in Paper IV. Therefore, low-grade NFL changes that were detected in Paper IV may have been below the detection limit of the assay used in Paper I.

8.2.2 Glial fibrillary acidic protein

Previous studies have reported augmented CFS GFAP levels in RRMS, but these changes did not correlate with relapse rates (223). In Paper IV, we confirmed increased GFAP levels in PMS patients compared to in HC (208). Interestingly, we also found that patients with Gd^+ lesions on MRI prior to immunosuppressive treatment had significantly higher GFAP levels compared to Gd-negative patients, indicating that GFAP was partially influenced by disease activity.

8.2.3 CXCL13

Previous studies have shown elevated CXCL13 in MS, and that CXCL13 level correlates with disease activity in RRMS (234, 236). In Paper IV, we found increased CXCL13 levels in PMS compared to in HC; however, only 11 of the 35 PMS patients had CXCL13 levels above the detection limit of the assay. The highest CXCL13 levels were found in patients without previous DMT and with Gd^+ lesions on MRI. The correlation between CXCL13 and disease activity reported for RRMS also seemed to be present in PMS. A clinically stable group of RRMS and SPMS patients without relapses occurring within 3 months prior to assessment ($n = 20$, Paper V) exhibited CXCL13 levels similar to those found in HC; again, only a minority ($n = 3$) of the 20 patients in this study had elevated CXCL13 levels. This finding is in

contrast with previous reports describing elevated CXCL13 levels in considerably higher proportions of patients (234, 236). This difference may be due to the lower proportion of immune active patients in our study populations, which comprised mainly progressive cases. The low frequency of CXCL13-positive CSF samples in our studies implies that the use of CXCL13 as a biomarker for PMS may be limited.

8.2.4 BACE1 activity and sAPP/Aβ metabolites

In the first study of these biomarkers, we showed significantly reduced CSF BACE1 activity (Paper II) and lower CSF sAPP and Aβ metabolite levels (Papers II and III) in MS compared to in HC. The CSF concentrations of these biomarkers did not significantly differ between RRMS and SPMS. However, decreased levels of α- and β-sAPP were found in CSF of RRMS patients during relapse, compared to in stages of remission (Paper II), suggesting that clinical disease activity in RRMS seemed to reduce CSF levels of α- and β-sAPP.

8.3 Biochemical biomarkers in CSF reflecting disability development, progression, or severity of PMS

Ideally, biochemical biomarkers should predict and reflect disability progression and severity of MS. However, the search for suitable biomarkers is complicated by several unsolved questions regarding MS pathophysiology, as well as by the frequently weak reliability of clinical measurements of disability. Changes of MRI measurements are currently the dominant surrogate marker for MS progression. MRI can show correlations between T1 and T2 lesion volume (167), cerebral or cervical spinal cord atrophy (184, 185), and disability development in MS. However, few biochemical biomarkers in CSF have been convincingly associated with disability progression (209, 211, 223, 287) or with signs of degeneration on MRI (210).

In the present studies (Papers I-V), disability was evaluated by EDSS, which is the most widely used scale but has low inter- and intra-rater sensitivity (153, 154). To reduce EDSS variability in the present studies, EDSS scoring was performed by only a few experienced neurologists (154). Other limitations of EDSS include its bimodal character and the relative lack of linearity, which particularly affects the scoring of PMS. Since EDSS scores above 3.5 mostly rely on ambulation, patients may progress in disability without influencing the EDSS score. This makes EDSS unreliable, especially in studies of shorter duration. To some extent, this limitation was overcome by the long-term follow-up of patients in Papers I, II, and V.

Disease severity and disability progression rate were determined by MSSS and progression index, respectively. These methods are derived directly from the EDSS, and provided temporal and prognostic information about the patient.

8.3.1 Neurofilament light protein

We found no correlation between NFL and disability (EDSS) or progression (MSSS), and NFL showed no prognostic value (Papers I and IV). This was in contrast to the results of previous studies (209, 288), which obtained CSF for diagnostic reasons and found that NFL levels predicted disability assessed at 4 and 14 years later. However, the EDSS score from the diagnostic investigation was not reported, and hence the dependence of developed disease severity on early disability was not calculated. Nevertheless, these previous findings imply that the extent of early axonal damage predicts future disability. It should be noted that early disability is mainly dependent on the location of individual lesions, while disability during progression results from accumulated lesion volumes and the development of cerebral and spinal cord atrophy. Moreover, the NFL level in CSF is also dependent on the interval between relapse onset and lumbar puncture (208, 286). The patients from Paper I were subjected to long-term follow-up of 8-10 years; however, no predictive value of NFL was observed. As discussed in the previous section, this may have been due to the patient selection and the sensitivity of the NFL assay.

8.3.2 Glial fibrillary acidic protein

Our findings confirmed that patients with MS had increased GFAP levels compared to HC, and that the levels were particularly high in SPMS patients (208). Among MS patients, GFAP correlated to clinical disability (EDSS) and progression or severity (MSSS) (Paper I). Similar results were previously reported in patients with high EDSS (>6.5) (289). Here, we also found that GFAP had prognostic value for future disability development. The risk for high EDSS (>5.5) was increased (OR = 4.5) in patients with GFAP levels above 350 ng/L at the first assessment. However, this relationship was dependant on the EDSS found at the first assessment (Paper I).

In chronic neurological diseases, including PMS, sustained clinical disability is expected to be irreversible. High sustained EDSS can be achieved early in MS due to severe relapses, or can result from slow progression over several years. To compensate for this imbalance, disease duration is included in the calculations of progression index and MSSS, which should result in more accurate measurements of progression rate or disease severity. We found that GFAP also correlated with progression index and MSSS, and that the annual GFAP increase was twice as high in SPMS compared to RRMS.

In the long-term follow-up setting (Paper I), GFAP seemed to have both prognostic value and to be a biomarker for progression; however, this was not true in PMS patients subjected to immunosuppressive treatment during 12-24 months (Paper IV).

8.3.3 CXCL13

CXCL13 has been previously shown to exhibit a non-significant trend of correlating with clinical disability in RRMS (234). We found that CXCL13 weakly correlated with EDSS and MSSS in the long-term follow-up group and in patients about to start mitoxantrone treatment (Paper V). The prognostic value of CXCL13 was not measured because only a minority of the studied patients had detectable levels.

8.3.4 Intrathecal IgG synthesis

The clinical relevance of OCB and increased IgG index is unclear, apart from their diagnostic uses (290). In contrast to earlier studies (291), here we found that both the number of OCB and the change in OCB numbers were correlated with clinical disability (EDSS) in RRMS patients.

8.3.5 BACE1 activity and sAPP/Aβ metabolites

BACE1 activity and the sAPP/Aβ metabolites were found to be decreased in MS, and were negatively correlated with disability (EDSS) and severity (MSSS) in SPMS but not in RRMS. In RRMS, BACE1 activity decreased after 8-10 years of follow-up.

8.4 CSF biochemical biomarkers for discriminating between MS clinical courses

At some point, each of the investigated CSF biochemical biomarkers showed significantly higher (NFL, GFAP, CXCL13, OCB, and IgG index) or lower (BACE1 activity and sAPP/Aβ metabolites) levels in MS patients than in HC; however, none could differentiate between the clinical courses of MS. None of the studied biomarkers showed any distinct borders differentiating between RRMS and PMS, although some observations indicated a gradual change towards progressive course. GFAP levels tended to increase with disability progression, and the annual rate of GFAP increase seemed higher among SPMS patients than RRMS patients. Additionally, multivariate analysis revealed that the Aβ metabolite patterns were altered in MS patients, and that SPMS patients could be distinguished from HC based on the pattern of APP degradation products. RRMS patients seemed to constitute an intermediate group, but further discriminations were not possible with so few patients. These studies represent promising attempts to identify biomarkers that detect patients who are entering MS progression.

8.5 Biomarkers for determining therapeutic efficacy in MS

In MS, therapeutic intervention with immunomodulatory or immunosuppressive therapies can change or inhibit immune activity and, if successful, reduce damage and degenerative processes of the CNS. MRI evidence of lesion formation, lesion load, and atrophy development are used as end-points in MS trials, and no biochemical biomarkers have been accepted as surrogate markers for evaluating therapeutic efficacy in MS. Investigating biochemical biomarkers during therapeutic intervention could increase our knowledge of the possible mechanism of action of the applied treatments, as well as of the pathophysiology of the disease. Such biomarkers may also be able to serve as objective measures in conjunction with clinical and MRI measures for monitoring therapy efficacy.

In the present thesis, we investigated the effects of natalizumab (Paper III), mitoxantrone (Papers III, IV, and V) and rituximab (Paper IV) on a number of biomarkers. We also observed the treatment effects of first-line disease modifying treatment (DMT) (interferon beta and glatiramer acetate) in PMS (Paper IV).

8.5.1 Neurofilament light protein

In a previous study of relapsing MS (n = 83), 6-12 months of natalizumab treatment reduced NFL levels to those found in HC (264). Natalizumab treatment also affected NFL levels in 9 SPMS patients with prior superimposed relapses. In Paper IV, we showed that 12-24 months of mitoxantrone or rituximab treatment reduced CSF NFL to levels approaching those observed in age-matched HC subjects. This effect was not confined to patients with Gd+ activity on MRI, but was also found in patients with increased NFL levels in the absence of Gd+ lesions at treatment onset. Pre-treatment NFL levels from patients who were previously treated with DMT were significantly lower than those from patients who were untreated at study baseline, suggesting that first-line DMT might influence NFL levels in PMS.

8.5.2 Glial fibrillary acidic protein

In patients with PMS, immunosuppressive treatment with mitoxantrone or rituximab had no significant impact on GFAP levels. However, PMS patients with Gd+ activity on MRI had increased GFAP levels prior to immunosuppressive treatment. Their GFAP levels were reduced post-treatment, indicating a treatment effect only in active PMS.

8.5.3 CXCL13 and intrathecal IgG synthesis

PMS patients in Paper IV and SPMS patients in Paper V showed reduced CXCL13 levels after 12-24 months of immunosuppressive treatment; IgG production and

OCB were not affected (Paper V). To our knowledge, this result has not been previously shown.

We confirmed that the number and pattern of OCB were unchanged, even following potent immunosuppressive treatment (292, 293). Natalizumab treatment decreases IgG synthesis (294) and, to date, is the only treatment shown to reduce the proportion of OCB-positive patients (294, 295).

8.5.4 sAPP/Aβ metabolites

In RRMS patients, natalizumab treatment increased sAPP/Aβ metabolites (except Aβ 1-42 and x-42) towards normal levels. On the other hand, after 12-24 months of mitoxantrone treatment, 9 SPMS patients and 1 RRMS patient exhibited no changes in Aβ and sAPP metabolites.

8.6 Biochemical biomarkers for exploring the pathophysiology of progressive MS

8.6.1 Axonal degeneration

An early phenomenon in MS, axonal loss and degeneration is observed in all MS courses (112), and has been established as the major cause of cerebral and spinal cord atrophy and disability progression in MS (115, 296). The highest levels of neurofilament protein occur during clinical or MRI signs of activity (206, 285). However, high neurofilament levels have also been observed in a plethora of diagnoses and pathological processes, such as ischemic stroke (297); CNS infections (201) and other autoimmune CNS diseases (268); and neurodegenerative diseases, such as ALS (205) and atypical Parkinson disorders (PSP, CBD, MSA-C, and MSA-P) (203).

We observed significantly increased NFL levels in PMS, with the highest levels in those with Gd+ lesions on MRI (Paper IV). Elevated NFL levels were associated with increased CXCL13 levels. Previous reports have shown similar associations between inflammatory biomarkers (CXCL13, osteopontin and MMP9) and NFL levels (207). In a previous study, we observed normalized NFL levels following 6-12 months of natalizumab treatment of RRMS (264), and in Paper IV we showed that immunomodulation/suppression also reduced axonal damage in PMS. Overall, these findings indicate that the increased NFL release in CSF is largely mediated by the on-going CNS inflammation. However, it cannot be ruled out that other non-inflammatory degenerative mechanisms may also participate in PMS evolution.

8.6.2 Astrogliosis

Astrocyte-mediated gliosis is a physiological reaction that is secondary to all forms of CNS damage (298). In MS, GFAP is considered the morphological basis of astrogliosis, and is the major constituent of chronic MS lesions (219). GFAP is considered a valuable astrogliosis biomarker and is elevated in MS, with the highest levels occurring during SPMS (208). Our results imply that astrogliosis increases with MS progression, but this process seemed to be essentially unresponsive to immunosuppressive treatment (Paper IV).

8.6.3 The role of the B cell lineage in PMS (CXCL13, IgG index, and OCB)

In recent years, evidence has accumulated indicating the importance of B cells in MS pathogenesis. One important finding is the reduction of MS disease activity achieved by anti-CD20 therapy (299-301), which results in B cell depletion. However, despite the B cell eradication in both the periphery and the CNS (101), IgG production in the CNS was not affected (293). Additionally, ectopic B cell follicles have been found in the meninges of PMS patients (129, 130, 302), which are reportedly associated with a gradient of subpial cortical demyelination, neuronal loss, and cortical atrophy (130). These follicles are aggregates of B cells and plasma cells, and may explain the maintained CSF IgG production in OCB that is observed in PMS (1).

Increased intrathecal IgG synthesis, including presence of OCBs, is observed in >95% of MS patients and has diagnostic value (63, 228, 239, 290). In Paper V, we showed that the OCB patterns changed over the long-term follow-up, and were not related to disease course. This was in contrast to the widespread opinion that OCBs are stable over the course of MS, and represent an immunological “fingerprint” for each patient (290). This concept was based on a number of studies that have mostly been small, short in follow-up, and have used different methodologies for CSF OCB detection (247, 303-305). Our findings are consistent with the different OCB patterns found in different MS plaques from the same brain (306), and suggest a dynamic shift of clonally expanded cells of the B cell lineage over the course of MS. Another explanation could be the rearranged immunoglobulin genes with extensive mutations that have been observed in CSF B cells (307) and in the brains of MS patients (308). Such mutations may be antigen driven (309), and the exposure of new antigens following inflammation and degeneration might induce new mutations of immunoglobulin genes and possibly explain the altered OCB patterns.

We did not find that OCB or IgG production were influenced by immunosuppressive treatment (Paper V). This result was in line with reports from previous studies that OCB patterns were stable after potent immunomodulatory or immunosuppressive treatments, such as rituximab (293) and hematopoietic stem cell transplantation

(292). Only natalizumab treatment has been shown to induce OCB disappearance in MS (294, 295). Mitoxantrone did not alter IgG production, despite its powerful ability to depress both B and T cells. Mitoxantrone does not cross the BBB, which supports the suspicion that an autonomous and long-lived B cell/plasma cell population is compartmentalized in the CNS.

CXCL13 is a chemokine that can attract and maintain B cells within the CSF/CNS. Here we confirmed previous findings that the CXCL13 level in the CSF was associated with IgG synthesis (234). These results support a B cell dependence of IgG synthesis. Furthermore, the complexity of this system is illustrated by the fact that only CXCL13, and not IgG synthesis, was affected by mitoxantrone.

8.6.4 BACE1 activity and sAPP/Aβ metabolism

In Paper II, we illustrated for the first time that BACE1 activity and sAPP/Aβ metabolites are involved in MS pathophysiology. The β-pathway has been considered to be of special interest in studies of MS, since animal studies demonstrated that BACE1 cleaves neuregulin 1 from its membrane-bound form to stimulate myelination by oligodendrocytes. Demyelination and defective remyelination is a dominant feature in MS (148). Here we found reduced levels of BACE1 in MS patients compared to in controls and in patients with cerebral SLE used as inflammatory controls (Paper II); this difference was most pronounced in patients with long-term SPMS. While there is no clear direct parallel between myelination in mouse studies and remyelination in humans, these findings suggest one possible explanation for the altered oligodendrocyte function observed in late MS (250, 310). These results cannot be considered a general phenomenon due to CNS inflammation, since cerebral SLE demonstrated significantly increased CSF BACE1 levels.

We observed reduced levels of sAPP/Aβ in the CSF of MS patients (Papers II and III), which has also been recently confirmed by other groups (311, 312). Based on our findings and the available literature, we concluded that these reduced levels were a result of general neuroinflammation (258-261). Although it is unclear why these levels decrease during inflammation, possible explanations include decreased production, increased aggregation, or increased clearance. However, amyloid plaques are uncommon in MS (313). Neuronal Aβ release is related to synaptic activity (314), and CSF Aβ levels correlate to CSF levels of granins (315), which are released during regulated neuronal secretion. Since oligodendrocytes initiate myelination preferentially on active axons, the decreased Aβ levels might represent parts of a vicious circle of axon inactivation (316) and disturbed remyelination. Our findings in Paper III are consistent with a reversible neuronal impairment early in the disease (reversible reductions of sAPP/Aβ peptides in RRMS) and irreversibility during the progressive course (irreversible reductions of sAPP/Aβ peptides in SPMS).

9. Concluding remarks and future considerations

The pathophysiology of MS is complex, prominently featuring numerous active mechanisms, such as demyelination, astrogliosis, and axonal degeneration. It is obvious that no biomarker alone can describe this. The aim of this thesis was to investigate if biomarkers in the cerebrospinal fluid (CSF) of MS patients could provide new insights into the pathophysiology of MS progression, and if biomarker levels could reflect disease activity, disability progression, or therapeutic efficacy.

We found elevated levels of NFL, illustrated ongoing axonal damage, in PMS patients in fast progression; most pronounced in patients with Gd^+ lesions on MRI without first line DMT (Paper IV). This was in contrast to findings in clinically stabile patients at long-term follow-up, who had levels as HC (Paper I). We found thus that the axonal damage was determined by the disease activity regardless of disease course.

In long-term follow-up (Paper I) GFAP was confirmed as a biomarker reflecting disability and disease severity in PMS. GFAP also showed a prognostic value for future disability. These findings were in line with accumulating astrogliosis in PMS.

The increased levels of NFL and CXCL13 in PMS were associated with disease activity and the levels decreased after immune suppressive and immune modulatory treatment. These biomarkers are potential tools for monitoring therapeutic efficacy.

We found signs of B-cell activity in all stages of MS (Paper IV and V). CXCL13 was associated to disease activity and axonal damage and also to the IgG production in CNS and OCB. We illustrated, in opposite to the general view, changed number and pattern of OCB in CSF over time (Paper V). These findings illustrated an on-going and a dynamic B-cell activation in all courses of MS and a B-cell dependence of intrathecal IgG synthesis.

We showed for the first time an alteration of the sAPP/Aβ metabolism in MS (Paper II and III). This was illustrated both by the reduced activity of BACE1 and by the reduced levels of sAPP/Aβ metabolites in CSF and are possible signs of impaired neuronal activity in the CNS. The reduction of the sAPP/Aβ metabolism was reversible in the RRMS after immune modulating treatment but irreversible in SPMS after immune suppressive treatment. We concluded this as a potential sign of irreversibility of neuronal impairment late in the disease. In PMS the levels of degradation products was not only reduced but the pattern of metabolites was also changed compared to HC. This was visualized by mass spectrometry.

All data in the present thesis illustrated a relationship between CNS inflammation and neurodegeneration in all courses of MS; however, it cannot be ruled out that other non-inflammatory degenerative mechanisms may also participate in PMS evolution.

Investigated biomarkers in CSF provide important information about the pathophysiology in PMS, and may serve as complement to clinical and MRI measures for assessment of disease activity, progression, severity and therapeutic efficacy.

NFL has previously been investigated in a number of studies and its relevance as a disease activity marker was confirmed in these studies also in PMS. NFL could be evaluated for use as a biomarker for monitoring treatment effect and could be suggested as an endpoint in phase II clinical trials. By combining NFL, CXCL13, GFAP and evaluation of disease activity and MRI a more complete evaluation of treatment effects could be achieved. Further investigations are needed to evaluate the correlation between CSF biomarkers and degeneration parameters on MRI, as T1 lesion volume and atrophy. The sAPP/Aβ metabolism are warranted to be further investigated for evaluation of the clinical usefulness of these markers and further investigate the mass spect profiles of the Aβ metabolites in CSF on larger patient populations. Access to these biomarkers also in blood should be of clinical importance but demands further development of more sensitive analysing methods.

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