Diagnostic, Prognostic, and Disease activity Biomarkers in Multiple Sclerosis

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To Maria, Jonathan, and Benjamin

"Science is objective. And in my view, we cannot take any experimental results seriously except in the light of good explanations of them"

- David Deutch

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ABSTRACT

Multiple sclerosis (MS) is the most common immune-mediated disease of the central nervous system. While benign cases exist, if left untreated, MS results in the compounding accumulation of disability. In the last two decades, various highly effective disease-modifying therapies (DMTs) have evolved, precipitating significant improvements in prognosis. The prompt diagnosis of MS and initiation of DMT are therefore essential to reduce the risk of disability. The gold standard for diagnosing and monitoring MS is currently magnetic resonance imaging (MRI), but since MS pathophysiology is multifaceted, there is a growing necessity for the use of various biomarkers.

Research concerning fluid biomarkers in MS has rapidly evolved in recent decades. Biomarkers are now used to increase diagnostic precision, to make prognostic predictions that may influence treatment decisions, and to monitor treatment response. In this thesis, we have evaluated the clinical utility of cerebrospinal fluid (CSF) neurofilament light (NfL), intrathecal kappa free light chain (KFLC) index, and immunoglobulin (Ig) M synthesis (ITMS) in retrospective real-world cohorts.

In study **I**, we included 757 individuals with relapsing-remitting MS (RRMS) with determination of CSF NfL (cNfL) between 2001 and 2018. We demonstrated that cNfL reflects both clinical and radiological signs of inflammatory disease activity, as well as treatment response. The sensitivity and specificity of cNfL to detect disease activity were 75% and 98.5%,

respectively. High cNfL at the onset of MS predicted the progression to meaningful disability milestones, such as secondary progressive MS (hazard ratio [HR] 2.5, 95% confidence interval [CI] 1.4–4.2, p=0.001).

In study **II**, KFLC index had a higher diagnostic sensitivity than IgG oligoclonal bands to distinguish MS (n=223) from controls (n=104) and had comparable diagnostic specificity. DMT did not influence the level of KFLC index, and it was not affected by demographic factors or associated with other degenerative or inflammatory CSF biomarkers.

In study **III**, we demonstrated the ability of ITMS as a disease severity biomarker to predict early disease activity and disability worsening in RRMS. The intrathecal fraction of IgM exhibited a moderate association with evidence of disease activity within 24 months of diagnosis (adjusted HR [aHR] 3.7, 95%CI 2.7-5, p<0.001). For the first time, we showed that combining ITMS with cNfL substantially increased the magnitude of the predicted risk of severe MS disease course (for expanded disability status scale ≥6 & $cNfL^{+}$/IgM-$index^{+}$: aHR 8.2, 95% CI 2.3-30, p<0.001).

In study **IV**, high KFLC index (>100) at MS onset was predictive of cognitive impairment, as determined by serial single-digit modalities tests (SDMT; aHR 10.5, 95% CI 2.2-50.8, p=0.003; median time to SDMT reduction 7 years).

In summary, we showed that CSF biomarker data retrieved from real-world RRMS cohorts had diagnostic and prognostic utility. Our data support the inclusion of cNfL, KFLC-index, and ITMS in the routine diagnostics and evaluation of suspected RRMS.

Keywords: multiple sclerosis, fluid biomarkers, cerebrospinal fluid, diagnosis, prognosis

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SAMMANFATTNING PÅ SVENSKA

Multipel skleros (MS) är den vanligaste immunmedierade sjukdomen i centrala nervsystemet. Godartade fall finns, men även dessa kommer utan behandling i de flesta fall få en ackumulering av olika funktionshinder. Under de senaste två decennierna har flera effektiva sjukdomsmodifierande terapier (disease modifying therapies, DMT) utvecklats, vilket lett till betydande förbättringar av prognosen av skovvis förläpande MS (relapsing-remitting, RRMS). Snabb diagnos och initiering av DMT är därför väsentligt för att minska risken för framtida funktionsnedsättning. För att diagnostisera och övervaka MS, används ofta magnetisk resonanstomografi, men eftersom MS-patofysiologin är mångfacetterad finns det ett växande kliniskt behov av andra biomarkörer. Forskning om lösliga biomarkörer inom MS har utvecklats snabbt under de senaste decennierna. Biomarkörer i ryggvätska och blod används numera för att öka diagnostisk precision, för att göra prognostiska förutsägelser som kan påverka behandlingsval, och för att övervaka behandlingssvar. Syftet med denna avhandling var att utvärdera den kliniska nyttan av att bestämma nivån av neurofilament light (NfL), kappa fria lätta kedjor (kappa free light chains, KFLC) samt immunglobulin (Ig) M (intrathecal IgM synthesis, ITMS) i cerebrospinalvätska (CSV) från den ordinarie kliniska populationen av RRMS så kallade ”real-world” kohorter vid Sahlgrenska Universitetssjukhuset.

I delarbete **I** inkluderade vi 757 personer med RRMS som genomgått diagnostisk utredning inklusive analys av CSV NfL (cNfL) mellan 2001-2018. Vi visade att cNfL reflekterar både kliniska och radiologiska tecken på inflammatorisk sjukdomsaktivitet, samt även behandlingssvar. Sensitiviteten och specificiteten av cNfL för att detektera sjukdomsaktivitet var 75 % respektive 98,5 %. Högt cNfL vid MS-debut förutspådde utveckling av sekundär progressiv MS (hazard ratio [HR] 2.5, 95 % konfidens intervall [CI] 1.4–4.2, p=0.001).

I delarbete **II** hade KFLC-index jämförbar specificitet, samt högre sensitivitet än oligoklonala IgG band för att skilja MS-patienter från kontroller. Varken DMT eller demografiska faktorer påverkade nivån av KFLC-index. Det sågs ingen koppling mellan KFLC-index och andra degenerativa eller inflammatoriska CSV-biomarkörer.

I delarbete **III** visade vi förmågan av ITMS att förutsäga tidig sjukdomsaktivitet och försämring av funktionshinder vid RRMS. Den intratekala fraktionen av IgM visade ett måttligt samband med tecken på sjukdomsaktivitet inom 24 månader från diagnos (justerad HR [aHR] 3,7, 95 % CI 2,7-5, p<0,001). För första gången visade vi att ITMS i kombination med förhöjda nivåer av cNfL vid MS-debut förutspådde försämring av funktionshinder så som behov av hjälpmedel vid gång (EDSS ≥ 6 (för cNfL+/IgM-index+: aHR 8,2, 95 % CI 2,3–30, p<0,001).

I delarbete **IV** fann vi att höga nivåer av intratekal KFLC produktion vid MS-debut förutsäger kognitiv funktionsnedsättning, vilket fastställdes med Single Digit Modalities Test (SDMT) (aHR 10.5, 95 % CI 2.2–50.8, p=0,003; median tid till SDMT reduktion 7 år).

Sammanfattningsvis kunde vi visa att biomarkörer som analyserats i den ordinarie MS vården var diagnostiskt och prognostiskt användbara. Våra studier stödjer att cNfL, KFLC-index och ITMS inkluderas i den kliniska diagnostiken och utvärderingen av misstänkt RRMS.

תקציר בעברית

טרשת נפוצה היא המחלה האוטואימונית השכיחה ביותר במערכת העצבים המרכזית. בעוד שקיימים מקרים שפירים, ככל שהמחלה אינה מטופלת, היא עלולה לגרום להצטברות גוברת של נכות. בשני העשורים האחרונים פותחו תרופות בעלות יעילות גבוהה המשנות את מהלך המחלה, ואשר מביאות לשיפורים משמעותיים בפרוגנוזה. לכן, אבחון מהיר והתחלת טיפול הינם חיוניים להפחתת הסיכון לנכות. טרשת נפוצה מאובחנת כיום בדרך כלל על ידי הדמיית תהודה מגנטית, שהינה גם הכלי הנפוץ ביותר לניטור הטיפול. מכיון שהפתופיזיולוגיה של טרשת נפוצה הינה רב-גונית, ישנו צורך הולך וגובר בשימוש בסמנים ביולוגיים שונים.

המחקר הנוגע לסמנים ביולוגיים נוזליים בטרשת נפוצה התפתח במהירות בעשורים האחרונים. סמנים ביולוגיים משמשים כיום להגברת דיוק האבחון, לביצוע תחזיות פרוגנוסטיות שעשויות להשפיע על החלטות הטיפול, ולניטור תגובת הטיפול. בתזה זו, הערכנו את התועלת הקלינית של נוירופילמנט לייט (neurofilament light – NfL), אינדקס שרשראות קאפה קלות (kappa free light chain index – KFLC), ואימונוגלובולין M (intrathecal immunglobulin M synthesis – ITMS) בנוזל השדרתי.

במאמר **I**, כללנו 757 חולי טרשת נפוצה התקפית-הפוגתית עם מדידת ריכוז NfL בנוזל השדרתי תוך כדי תהליך האבחון בין השנים 2001-2018. הדגמנו ש NfL משקף סימנים קליניים ורדיולוגיים של פעילות מחלה דלקתית, כמו גם תגובה לטיפול. הרגישות והסגוליות של NfL לזיהוי פעילות המחלה היו 75% ו- 98.5%, בהתאמה. רמות גבוהות של NfL חזו את התקדמות המחלה לאבני דרך משמעותיות של נכות, כגון טרשת נפוצה מתקדמת משנית.

במאמר **II**, ל KFLC index הייתה רגישות אבחנתית גבוהה יותר מרצועות אוליגוקלונליות כדי להבדיל בין טרשת נפוצה מקבוצת ביקורת בעוד שהסגוליות האבחנתית הייתה דומה. אופן הטיפול כמו גם גורמים דמוגרפיים לא השפיעו על רמת KFLC index. לא מצאנו קשר בין KFLC index וסמנים ביולוגיים ניווניים או דלקתיים אחרים בנוזל השדרתי.

במאמר **III**, הדגמנו את היכולת של ITMS כסמן ביולוגי של חומרת המחלה, לחזות פעילות מחלה מוקדמת והחמרת נכות בטרשת נפוצה התקפית-הפוגתית.
נצפה קשר חזק בין ITMS עם עדות לפעילות המחלה בתוך 24 חודשים מהאבחנה.
בפעם הראשונה, הראינו ששילוב ITMS עם NfL העלה באופן משמעותי את גודל הסיכון החזוי למהלך טרשת נפוצה חמורה.
במאמר **IV**, רמות גבוהות מאוד של KFLC index ניבאו ליקוי קוגניטיבי.
לסיכום, בתזה זו אנו מדגימים את התועלת האבחנתית והפרוגנוסטית של סמנים ביולוגיים בנוזל השדרתי. הנתונים שלנו תומכים בהכללה של סמנים אלו באבחון והערכה שגרתיים כאשר ישנו חשד לטרשת נפוצה.

LIST OF PAPERS

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

I. Rosenstein I, Axelsson M, Novakova L, Blennow K, Zetterberg H, Lycke J. Exploring CSF neurofilament light as a biomarker for MS in clinical practice; a retrospective registry-based study. *Mult Scler.* 2021:13524585211039104.

II. Rosenstein I, Rasch S, Axelsson M, Novakova L, Blennow K, Zetterberg H, Lycke J. Kappa Free Light Chain Index as a Diagnostic Biomarker in Multiple Sclerosis: A Real-World Investigation. *Journal of neurochemistry* (2021). Epub 2021/09/04. DOI: 10.1111/jnc.15500.

III. Rosenstein I, Rasch S, Axelsson M, Novakova L, Blennow K, Zetterberg H, Lycke J. Increased intrathecal neurofilament light and immunoglobulin M predict severe disability in relapsing-remitting multiple sclerosis. *Frontiers in immunology.* 2022;13:967953.

IV. Rosenstein I, Axelsson M, Novakova L, Rasch S, Blennow K, Zetterberg H, Lycke J. High levels of kappa free light chain synthesis predict cognitive decline in relapsing-remitting multiple sclerosis. *Frontiers in immunology.* DOI: 10.3389/fimmu.2023.1106028.

CONTENT

Abbreviations

AEA	An Example Abbreviation
aHR	Adjusted Hazard Ratio
Alb	Albumin
BBB	Blood Brain Barrier
bNfL	Blood Neurofilament Light
CD	Cluster of Differentiation
CDMS	Clinically Definitive Multiple Sclerosis
CDW	Confirmed Disability Worsening
CH13L1	Chitinase-3-Like protein 1
CI	Cognitive Impairment
CIS	Clinically Isolated Syndrome
cNfL	Cerebrospinal fluid Neurofilament Light
CNS	Central Nervous System
CXCL	Chemokine (C-X-C motif) Ligand
DIS	Dissemination In Space
DIT	Dissemination In Time
DMT	Disease-Modifying Therapy
ECTRIMS	European Committee for Treatment and Research in MS
EBV	Epstein-Barr Virus
EDA	Evidence of Disease Activity

EDSS	Expanded Disability Status Scale
ELISA	Enzyme-Linked Immunosorbent Assay
FLAIR	Fluid-Attenuated Inversion Recovery
FS	Functional System
FSS	Functional System Score
Gd+	Gadolinium-enhancing
GFAP	Glial Fibrillary Acidic Protein
HADS	Hospital Anxiety and Depression Scale
HR	Hazard Ratio
IEF	Isoelectric Focusing
IF	Intrathecal Fraction
Ig	Immunoglobulin
IL	Interleukin
IPS	Information Processing Speed
ITGS	Intrathecal IgG Synthesis
ITMS	Intrathecal IgM Synthesis
KFLC	Kappa free light chains
LP	Lumbar Puncture
LS	Lipid-Specific
MADRS	Montgomery-Åsberg Depression Rating Scale
MOGAD	Myelin Oligodendrocyte antibody Associated Disease
MRI	Magnetic Resonance Imaging

MS	Multiple Sclerosis
NEDA	No Evidence of Disease Activity
NfL	Neurofilament Light
NMOSD	Neuromyelitis Optica Spectrum Disease
OCB	Oligoclonal Bands
OCMB	Oligoclonal IgM Bands
ONID	Other Neuro-Inflammatory Diseases
PIRA	Progression Independent of Relapse Activity
PMS	Progressive Multiple Sclerosis
PPMS	Primary Progressive Multiple Sclerosis
Q	Quotient
RAW	Relapse-Associated Worsening
RCT	Randomized Controlled Trial
RIS	Radiologically Isolated Syndrome
RRMS	Relapsing-remitting Multiple Sclerosis
SC	Symptomatic Controls
SDMT	Single Digit Modalities Test
SMSreg	Swedish Multiple Sclerosis Registry
SPMS	Secondary Progressive Multiple Sclerosis
TH	T Helper
TNF	Tumor Necrosis Factor
T2W	T2 Weighted

DEFINITIONS IN SHORT

Clinical MS relapse	Neurological signs and symptoms lasting at least 24 hours and that cannot be explained by another cause.
Confirmed disability worsening (CDW)	An increase in EDSS score from baseline sustained between two follow-up visits separated in time by no less than six months (1.5 point if EDSS at baseline was 0, 1 point if EDSS was between 1 and 5, 0.5 points if the baseline EDSS≥5.5).
No Evidence of Disease Activity – 3	No clinical relapses; no CDW within 6 months (6 – CDW), and no new T1 gadolinium-enhanced lesions or new/newly enlarging T2-weighted lesions on MRI

1 INTRODUCTION AND THEORETICAL FRAMEWORK

Prologue

In May 1868, Jean-Martin Charcot (1825-93), a French physician regarded as the father of neurology, delivered a series of seminal lectures in which he described a condition "not yet officially recognized." In these lectures, of which we are fortunate to have preserved hand-written manuscripts, Charcot coined the term "sclérose en plaques disséminées," which translates to "multiple sclerosis" (MS) in English, thereby recognizing MS for the first time as a distinct nosological entity.[1] Since then, ongoing and intense scientific enquiry into the anatomical and pathophysiological basis of MS has revealed intricate details that have culminated in the ability to diagnose and treat MS with high efficacy.

However, MS had existed long before Charcot's official description of it, and for many years thereafter, no effective treatments or reliable diagnostic tools were available. It is remarkable that Charcot and others were able to offer such an accurate description of the pathological characteristics of MS without access to the modern tools available to MS clinicians today. With the advancement of neuroimaging and immunotherapy in the early 1990s, MS clinicians were starting to be able to offer their patients methods of prompt diagnosis and effective therapy. However, even today, the etiology of MS is still not fully known, which is staggering given the massive advancements in biomedical research and technology.

Nevertheless, developments in the understanding of the underlying complex pathophysiology of MS and the capacity to offer an extensive array of diverse

treatments with a broad range of effectiveness and adverse effect profiles have made it even more crucial to continue developing methods to diagnose MS rapidly and accurately and to monitor the effects of a given therapy. Charcot would most likely have been astonished by the various methods that are now routinely used to diagnose and monitor MS patients and by the striking effectiveness of disease-modifying therapies (DMT) in halting disease activity and in delaying or fully inhibiting disability progression.

This doctoral thesis focuses on a branch of MS-related research that has grown exponentially in the past several decades. Fluid biomarker research involves the exploration and discovery of new and relevant molecules in the pathophysiology of MS, the association of these molecules with meaningful clinical outcomes, the validation of these findings in other independent cohorts, and finally, the implementation in routine clinical diagnostic and follow-up procedures. The biomarkers investigated in the current thesis lie within that spectrum, most likely between the validation and implementation phases.

Over the years, the Neurochemistry Laboratory at the Sahlgrenska University Hospital in Mölndal has been instrumental in the discovery and development of various clinically useful fluid biomarkers. Early on, the laboratory had made some of these biomarkers available for clinical routine determinations. Consequently, all of the test results analyzed in this project were collected consecutively during clinical routine investigations throughout many years. In addition, since the early 2000s, clinical and demographical data regarding individuals with MS have been prospectively collected and registered in the Swedish MS registry (SMSreg).

As a result, we have had a unique opportunity to examine the clinical utility of the investigated biomarkers in a real-world setting. In that sense, this project

serves as a quality control for the analyses routinely performed by our Neurochemistry Laboratory. Fluid biomarkers such as those examined in this current thesis already play an important role in clinical decision-making, and their importance will certainly continue to expand and develop in the upcoming years. The following is an introductory summary of the most relevant clinical aspects of this thesis but is not a comprehensive review of MS.

1.1 Background

Multiple sclerosis (MS) is regarded as an immune-mediated disease that involves variable degrees of inflammation, demyelination, and axonal degeneration.[2] These neuropathological processes are central in causing the clinical phenotypes of MS, and if untreated, the course of the disease entails the increasing accumulation of physical and cognitive disability.[3,4] The primary prerequisite for a diagnosis of MS is the demonstration of central nervous system (CNS) lesion dissemination in time (DIT) and space (DIS) based on a combination of clinical features, magnetic resonance imaging (MRI) findings, and cerebrospinal fluid (CSF) analysis.[5]

MRI is the gold standard to support the clinical diagnosis of MS and to monitor disease activity due to its high sensitivity in detecting new MS lesions.[6] MS clinicians typically use the expanded disability status scale (EDSS) to evaluate the degree of disability in patients.[7] EDSS is a validated clinical tool based on routine neurological examinations and is a method to quantify disability in MS.

In the past 25 years, various highly effective DMTs have evolved for the treatment of MS.[8,9] All DMTs have immunomodulatory effects, which lead to reduced disease activity as well as an altered clinical course, thereby improving prognoses.[10] The early diagnosis of MS and initiation of DMT are therefore vital to reduce the risk of the development of permanent disability.

Since MS's pathophysiology is multifaceted, there is a mounting necessity for various biomarkers to improve diagnostic precision, tailor treatment regimens, monitor treatment responses, and facilitate the early prediction of a more aggressive disease course and future disability worsening. Molecular fluid biomarkers that can be measured in the CSF and blood have become a promising adjunct tool in the increasingly expanding toolkit of MS biomarkers. Their advantages are that they are easily accessible and quantifiable, which renders them attractive complements to other clinical and MRI measures, as they add more objective information pertaining to the heterogeneous nature of the disease. Although research regarding fluid biomarkers in MS has grown considerably in recent years, there is an unfulfilled need to validate the clinical utility of some of the most promising biomarkers, particularly in unselected cohorts of patients undergoing routine clinical investigations in real-world settings.

1.2 Epidemiology

Multiple sclerosis (MS) is the most common immune-mediated disease of the CNS and is a primary cause of non-traumatic neurological disability in young adults.[11,12] According to the Swedish National Board of Health and Welfare, it is estimated that MS affects around 1,000 people every year, and about 18,576 patients are actively registered in the SMSreg, with a coverage of an estimated 84%.[13] The prevalence of MS in Sweden was estimated to be 188.9/100,000 (95% CI 186.1–191.7), 113.4 (95% CI 110.3–116.5) for men and 263.6 (95% CI 258.9–268.3) for women, which are among the highest nationwide prevalence estimates in the world.[14] The risk of developing MS appears to increase with increasing northern latitude in both men and women in Sweden.[14]

The average incidence of MS in Sweden between 2001 and 2008 was estimated to be 10.2 per 100,000, which is substantially higher than earlier regional

approximations of 4.3 to 6.4.[15] The incidence and prevalence of MS appear to be increasing worldwide, both in developing and developed countries.[16] The reason for this increase is not fully clear, but a methodological effect may exist, as more mild cases are now easily discovered due to improvements in diagnostic tools. MS affects more women than men,[17] with an estimated female-to-male ratio of approximately 2.35:1 in Sweden.[14] The onset of MS typically occurs between 20 and 40 years of age, although about 10% of patients experience their first demyelinating event before the age of 18.[18,19]

1.3 Pathophysiology

The neuropathological hallmark of MS is the presence of multifocal demyelinating plaques within the brain and spinal cord.[20,21] Variable degrees of inflammation, demyelination, and axonal degeneration are believed to be the main causes of the clinical symptoms of MS.[2] Nevertheless, the cause of MS still remains unknown. The most generally recognized hypothesis is that MS starts as an autoimmune inflammatory process driven by autoreactive lymphocytes.[22-24] Subsequently, microglial activation and chronic neurodegeneration may take place.[25] However, this classical division between early inflammatory and late degenerative disease has recently been challenged by data supporting the notion that the period with the greatest inflammatory activity early in the disease course is also the period with the greatest neuroaxonal loss and that the rate of this loss decelerates in later stages of the disease.[26-29]

Autoreactive B and T lymphocytes are removed via central tolerance, either in the thymus in the case of T lymphocytes, or bone marrow in the case of B lymphocytes.[24] Cell-mediated and intrinsic peripheral tolerance normally inhibits these autoreactive lymphocytes from causing disease, although some cells may evade these mechanisms and be released into circulation. Peripheral

tolerance may fail due to diminished regulatory T cell function or the autoreactive lymphocytes' resistance to suppression.

T lymphocytes that are associated with MS pathophysiology include cluster of differentiation (CD) 8+ T cells and CD4+ T helper (TH) 1 and TH17 cells.[2] Cytokines that are produced and released by these T lymphocytes may contribute to the evolution of MS pathogenesis.[30] Some DMTs used in MS owe their efficacy partly to their ability to shift T cell differentiation from TH1 and TH17 to TH2 phenotypes, which are believed to convey a milder inflammatory reaction.[2]

MS has been historically conceived of primarily as a T cell-mediated disease. However, in recent years, the role of B lymphocytes in MS's pathophysiology has been gradually recognized and described. CSF-specific immunoglobulin G (IgG) oligoclonal bands (IgG-OCBs) are the result of immunoglobulins produced and secreted by B lymphocytes and have been incorporated into the MS diagnostic criteria intermittently for many years.[31] B lymphocytes are known to produce and secrete proinflammatory cytokines, including IL-6, lymphotoxin-α, and tumor necrosis factor (TNF)-α. Consequently, DMTs focused on B-cell depletion may reduce the inflammation promoted by the activity of CD4+ and CD8+ T lymphocytes.[31,32]

B-cell clusters within the CNS can be found in the meninges of MS patients, and a larger proportion of these infiltrates corresponds to the amount of cortical lesions as well as the extent of neurodegeneration and clinical disability.[31,33] B lymphocytes have also been suspected to provide a reservoir for the Epstein-Barr virus (EBV).[34] In MS, B lymphocytes are implicated in antigen presentation to T lymphocytes and in the production of cytotoxic molecules that may harm oligodendrocytes.[31,35] Consequently, B cell-depleting

monoclonal DMTs, such as rituximab, ocrelizumab, and ofatumumab, have emerged as effective treatment options for MS and are now widely used.

Microglia are abundant in the CNS, and their function may vary between pro- and anti-inflammatory states.[36] Although they were previously associated primarily with genetic leukoencephalopathies[37] and other neurodegenerative diseases,[38,39] their role in MS pathophysiology has been increasingly recognized.[40] Microglia are believed to play a role in both acute and chronic MS lesion formation, but they have also been demonstrated to contribute to remyelination and neuronal repair.[36,41,42]

Activated microglia may also be found in the periphery of smouldering or slowly expanding lesions[41] and have been increasingly implicated in the pathogenesis of progressive MS, as they may facilitate neurodegeneration.[43] Microglia express Bruton tyrosine kinase (BTK), which has precipitated a growing interest in BTK inhibitors as potential therapeutic options for both relapsing-remitting MS (RRMS) and progressive MS (PMS).[36]

RRMS is mainly characterized by acute inflammatory activity accompanied by blood-brain barrier (BBB) breakdown, often demonstrated by contrast-enhancing plaques revealed via MRI scans.[44] Acute MS lesions typically contain infiltrates of B and T lymphocytes, plasma cells as well as macrophages gathered around a central vein.[45] MS was previously considered to be a primarily white matter disease. However, deep gray matter and cortical involvement have now been described in all MS disease course categories and have been associated with the worsening of disability.[46-48] While RRMS is mostly influenced by peripheral immune responses directed at the CNS, PMS may be predominantly dependent on inherent immune processes within the CNS.[49-51]

1.3.1 Risk factors

MS is a multifactorial disease, and it is believed that both environmental and genetic factors interact in a complex fashion to influence the function of autoreactive lymphocytes. Genetic factors that appear to contribute to the risk of developing MS include a variation involving the HLA-DRB1 locus.[52-54] More than 150 single nucleotide polymorphisms (SNPs) related to the risk of developing MS have been described using genome-wide association studies.[55] However, most of these SNPs confer only an exceptionally small risk individually. Many of the SNPs are found in the proximity of other genes that are related to immune function, generally in regulatory regions rather than coding ones.[56] Important environmental risk factors that are believed to influence the evolution of MS are low vitamin D levels[57-59] as well as exposure to EBV infection prior to the development of MS.[60-62] Smoking[63,64] and obesity[65,66] are also known to be potentially modifiable environmental risk factors.

EBV belongs to the family of herpes viruses. Most people are exposed and infected with EBV during early childhood, in which case it typically causes a mild or asymptomatic infection. Infection in adolescence and early adulthood often manifests itself as infectious mononucleosis.[61] EBV may remain latent in host B cells after a primary infection,[62] and seropositivity for EBV antibodies and a history of infectious mononucleosis have been associated with the risk of developing MS.[60] Recently, a large study confirmed that EBV infection almost invariably precedes MS and that EBV may be a necessary risk factor for the development of MS.[62] However, EBV seropositivity is exceptionally high in the general population, and most people with a previous EBV infection never develop MS. Consequently, the role of EBV in the pathogenesis of MS

is most likely complex and multifactorial and may depend upon interactions between EBV and certain environmental and genetic risk factors.[67,68]

Smoking has been associated with the conversion from clinically isolated syndrome (CIS) to MS[64] as well as the transition to secondary progressive MS (SPMS).[63,69] In addition, the efficacy of several DMTs has been demonstrated to be reduced by smoking.[70,71] Passive exposure to smoking has been associated with an increased MS risk as well.[72] Several studies have suggested that the effects of smoking on MS risk may be potentiated by genetic factors. In patients who had HLA-DRB1*15:01 but not HLA-A*02, smoking was proven to contribute to MS risk at a rate of 41%.[73]

1.4 Clinical course and prognosis

The pattern and course of MS have been historically classified into several distinct clinical subtypes: clinically and radiologically isolated syndromes (CIS/RIS), RRMS, SPMS, and primary progressive MS (PPMS) (Figure 1).[74] The vast majority of patients with MS, specifically about 85%, have a relapsing-remitting course of disease, which usually presents in young adults with a CIS, such as optic neuritis, brainstem involvement (e.g., diplopia or internuclear ophthalmoplegia), or spinal cord involvement (myelitis).[75] RRMS is typically highly responsive to DMTs, and it is a major challenge to predict

which patients have an increased risk of further relapses and severe disability and to detect disease activity during ongoing treatment.

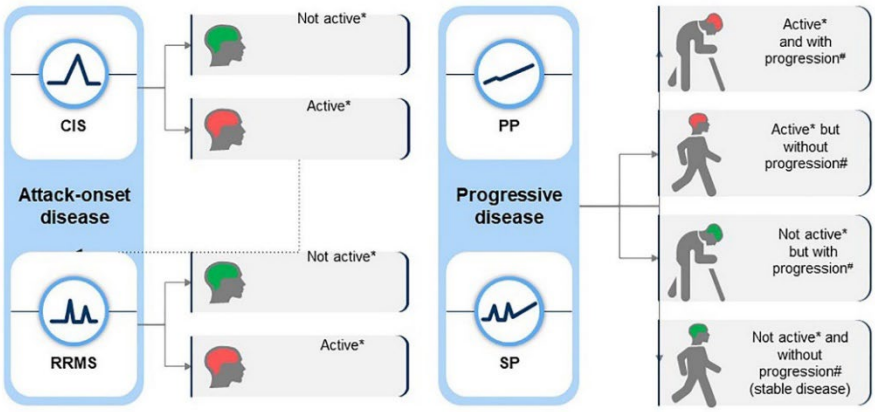

Figure 1. Multiple sclerosis phenotypes based on Lublin 2013.[74]

CIS – clinically isolated syndrome; RRMS – relapsing-remitting multiple sclerosis; PP – primary progressive; SP – secondary progressive. Reproduced with permission from Giovannoni et al.[76] *Copyright © 2022, © SAGE Publications*

Molecular biomarkers are highly valuable in that regard. The remaining 10% to 15% of adult patients have the primary progressive MS subtype, which manifests as slow and continuous build-up of disability from disease debut.[77] Some patients with RRMS may develop an insidious worsening of disability that is independent from inflammatory relapse activity after a period of time, in which case it is classified as SPMS.[51] Lublin et al.[74] define "active MS" as the occurrence of either a clinical relapse or MRI activity over a specified period of time (Figure 1).

1.4.1 Smouldering MS and progression independent of relapses

New approaches have recently been proposed to characterize and define the clinical course of MS.[78] A considerable number of patients with MS experience sustained clinical worsening despite not having any clinical relapses or exhibiting other signs of inflammatory disease activity in MRI scans or blood/CSF tests. This has been recently referred to as progression independent of relapse activity (PIRA) or smouldering MS, in contrast to relapse-associated worsening (RAW).[79-81] Therefore, it has been suggested that MS is most likely mediated by a primary smouldering process associated with superimposed inflammatory disease activity.[76] The concept of smouldering MS has been supported by a growing body of imaging and pathology data and has therefore led some experts to urge the MS community to expand the spectrum of MS pathology beyond "no evident inflammatory disease activity" and concentrate on other pathological hallmarks in the CNS to delay or avoid the slow worsening of disability that may characterize MS.[76]

1.4.2 Severe, highly active, or aggressive multiple sclerosis

A subset of RRMS patients eventually develop a highly active disease, characterized by recurrent and severe relapses within a relatively short time, new/enlarging MRI MS-lesions, and rapid disability accumulation. Over the years, many attempts have been made to reach a consensus regarding the definition of "aggressive MS," the majority of which are summarized in Table 1. This has proven to be a difficult task, and a recent effort in 2018 by a focused workshop of the European Committee for Treatment and Research in Multiple Sclerosis (ECTRIMS) regarding aggressive MS did not manage to reach a consensus regarding a new, more data-driven definition.[82] It is necessary to identify patients who are at risk of a more severe disease early in the disease

course, as highly effective DMTs initiated early in the disease course may halt intensive inflammatory activity, thus preventing disability accrual and clinical worsening.[83,84] Fluid biomarkers with prognostic properties may serve as important tools for objectively identifying such patients.

Table 1. Historical and contemporary attempts to define aggressive or highly active multiple sclerosis.

Definition	Citation	Author
Malignant MS	"A disease with a rapid progressive course, leading to significant disability in multiple neurologic systems or death in a relatively short time after disease onset."	Lublin et al.[85]
Ever malignant MS	"Patients that reached an EDSS of 6.0 within 5 years from onset."	Gholipor et al.[86]
Aggressive MS	"Patients that reached an EDSS of 6.0 within 5 years from onset" (Form 1). "Patients that reached an EDSS≥6.0 at the age of 40years" (Form 2). "Patients who entered SPMS phase within 3years after RRMS onset" (Form 3).	Menon S et al.[87] Menon S et al.[88]
Aggressive onset MS	"MS patients with (a) ≥2 relapses in the year after onset and ≥2 Gd+ lesions on brain MRI scan or (b) one relapse within 1 year after onset if it results in sustained baseline EDSS score of 3.0 along with ≥2 Gd+ lesions."	Kaunzner et al.[89]
Aggressive MS	"RRMS with one or more of the following features: (a) EDSS score of 4.0 within 5 years of onset. (b) Multiple (≥2) relapses with incomplete resolution in the past year. (c) ≥2 MRI scans showing new or enlarging T2 lesions or Gd+ lesions despite treatment. (d) No response to therapy with one or more DMTs for up to 1year."	Rush et al.[90]
Aggressive relapsing–remitting MS	"≥2 relapses or an EDSS increase ≥2 points in the 12 preceding months, ≥1 Gd-enhancing lesion and baseline EDSS between 2.5 and 5.0."	Edan G et al.[91]
Highly active MS	"Failure of conventional treatment and ≥1 severe relapses and/or incomplete recovery from clinically significant relapses and ⩾1 Gd+ lesion of diameter ≥3mm or accumulation of ≥0.3 T2 lesions/month in two consecutive MRI 6–12 months apart."	Saccardi et al.[92]
Aggressive MS	"Reaching an EDSS ≥6.0 within 10 years of disease onset."	Tintore et al.[93] Malpas et al.[94]

MS: multiple sclerosis; EDSS: Expanded Disability Status Scale; SPMS: secondary progressive multiple sclerosis; RRMS: relapsing multiple sclerosis; MRI: magnetic resonance imaging; Gd+: gadolinium-enhancing lesions; DMT: disease-modifying treatment.

Table adapted and modified from Iacobaeus et al.[82]

1.4.3 Cognitive impairment, affective disorder, and fatigue in MS

Up to 70% of MS patients exhibit some degree of cognitive impairment (CI).[95] CI appears to be common not only in the late, progressive stages of the disease, but at the onset of MS as well.[96,97] However, there is some evidence that RRMS patients perform better cognitively compared to patients with PMS.[98,99] Attention, executive function, abstract conceptualization, short-term memory, word recall, and information processing speed (IPS) are cognitive domains that are commonly affected in MS.[97,100] MS-related CI has been proven to correlate with the severity of brain pathology and lesion burden in MRI scans.[97,101-103]

For instance, corpus callosum and thalamus atrophy on MRI as well as the persistence of T1 black holes have been associated with CI,[103-108] and both gray and white matter atrophy appear to be involved.[104-108] One of the most frequently affected domains in MS-related CI is IPS,[100,109] assessed with the single-digit modalities test (SDMT).[110] CI often exerts significant effects on quality of life[111] and might be counteracted with effective DMTs.[112,113] It is therefore vital to assess and predict the risk of CI development in the early stages of MS.

Depression is known to negatively affect cognitive performance, particularly executive function, concentration, memory, and attention.[114-116] CI has been demonstrated to correlate with depression and lack of social support independent of the degree of physical disability.[117] Furthermore, MS-related fatigue is a major, frequently "invisible" symptom of MS, which might interact with cognition. It is estimated that approximately 75% to 85% of MS patients may experience MS-related fatigue that interferes with daily life activities and influences quality of life.[118-120] Despite its relatively high incidence and major

impact, the etiology of MS-related fatigue remains unknown.[121] Studies attempting to explore the relationship between fatigue and CI have been inconsistent.[122-125] Thus, the complex interaction between anxiety, depression, physical and mental fatigue, and CI must still be elucidated.

1.5 Diagnosis

MS is a clinical diagnosis. The main prerequisite for making the diagnosis is the demonstration of CNS lesion dissemination in time and space, based upon a combination of clinical features, MRI findings, and CSF analysis.[5] The McDonald diagnostic criteria include specific clinical, MRI, and CSF findings required for the demonstration of the dissemination of MS lesions in time and space (Table 2).[5] For patients who present with insidious neurological progression suggestive of PPMS, the McDonald criteria require evidence of one year of disease progression plus two of the three following criteria:[5]

- Dissemination in space in the brain based upon one or more T2 lesions in at least one MS typical area (periventricular, juxtacortical, or infratentorial)
- Dissemination in space in the spinal cord based upon two or more T2 lesions in the cord
- Positive cerebrospinal fluid findings with isoelectric focusing evidence of oligoclonal bands or elevated IgG-index

Table 2. 2017 McDonald criteria for the diagnosis of relapsing-remitting multiple sclerosis

Number of clinical attacks	Number of lesions with objective clinical evidence	Additional data needed for the diagnosis of multiple sclerosis
≥2 clinical attacks	≥2	None[a]
≥2 clinical attacks	1 (and clear-cut historical evidence of a prior attack involving a lesion in a distinct anatomic location)[b]	None[a]
≥2 clinical attacks	1	Dissemination in space demonstrated by an additional clinical attack implicating a different central nervous system (CNS) site or by MRI
1 clinical attack	≥2	Dissemination in time demonstrated by an additional clinical attack or by MRI or CSF specific oligoclonal bands
1 clinical attack	1	Dissemination in space demonstrated by an additional clinical attack implicating a different CNS site or by MRI AND Dissemination in time demonstrated by an additional clinical attack or by MRI OR Demonstration of CSF-specific oligoclonal bands

Abbreviations: CSF = cerebrospinal fluid; MRI = magnetic resonance imaging.

If the 2017 McDonald criteria are fulfilled and there is no better explanation for the clinical presentation, the diagnosis is multiple sclerosis. If multiple sclerosis is suspected by virtue of a clinically isolated syndrome but the 2017McDonald criteria are not completely met, the diagnosis is possible multiple sclerosis. If another diagnosis arises during the evaluation that better explains the clinical presentation, the diagnosis is not multiple sclerosis.

[a]No additional tests are required to demonstrate dissemination in space and time. However, unless MRI is not possible, a brain MRI should be obtained in all patients for whom the diagnosis of multiple sclerosis is being considered.

[b] A clinical diagnosis based on objective clinical findings for two attacks is most secure. Reasonable historical evidence for one past attack, in the absence of documented objective neurologic findings, can include historical events with symptoms and evolution characteristic of a previous inflammatory demyelinating attack; at least one attack, however, must be supported by objective findings. In the absence of residual objective evidence, caution is necessary.

1.5.1 MRI in the diagnosis and monitoring of MS

The diagnostic criteria for MS rely heavily on the demonstration of MS-typical lesions in brain and spinal cord MRI scans.[5] Due to its high sensitivity and specificity in detecting MS lesions as well as inflammatory disease activity manifesting as new/enlarging T2 lesions and contrast-enhancing lesions, MRI has become the gold standard for the diagnosis and monitoring of MS.[6] Diagnostically, if a patient has no prior neurologic history, DIS and DIT can be achieved by using either MRI or laboratory criteria (Table 3).

Table 3. 2017 McDonald Criteria for the Demonstration of Dissemination in Space and Time by MRI in a Patient with a Clinically Isolated Syndrome

Dissemination in space

- Can be demonstrated by one or more T2-hyperintense lesions[a] that are characteristic of multiple sclerosis in two or more of four areas of the central nervous system:
  - Periventricular
  - Cortical or juxtacortical
  - Infratentorial brain regions
  - Spinal cord

Dissemination in time

- Can be demonstrated by the following:
  - The simultaneous presence of gadolinium-enhancing and non-enhancing lesions[a] at any time
  - A new T2-hyperintense or gadolinium-enhancing lesion on follow-up MRI, with reference to a baseline scan, irrespective of the timing of the baseline MRI

MRI = magnetic resonance imaging.

[a] Unlike the 2010 McDonald criteria, no distinction between symptomatic and asymptomatic MRI lesions is required.

Adpated and modified from Oh, Continuum Neurology, © American Academy of Neurology.

MRI measures also convey important prognostic information. Baseline MRI measures of gadolinium-enhancing and spinal cord lesions were both associated with more pronounced disability worsening and progression to SPMS after 15 years.[126] The core indicators of inflammatory disease activity on MRI are the T2 lesion load as well as cortical atrophy.[127,128] MRI lesion load is typically evaluated on T2-weighted, fluid-attenuated inversion recovery (FLAIR), and contrast T1-weighted MRI sequences. The extent of cortical atrophy, in addition to the occurrence of so-called "black holes," is measured with T1-weighted sequences and may correlate better with the degree of disability compared with lesion load. While not regularly evaluated in clinical practice, spinal cord atrophy is also associated with worse neurological function.[129-131]

The central vein sign on MRI scans has recently emerged as an imaging measure with considerable diagnostic utility in MS.[132] Central veins within white matter lesions are frequently possible to visualize when appropriate susceptibility-based MRI sequences are used. These lesions are believed to be indicative of perivenular inflammation and demyelination, a pathological hallmark of MS lesions.[133-135]

Several studies have previously demonstrated that more than 50% of all chronic MS lesions are either active or mixed active/inactive.[21,136,137] These lesions, classified as smouldering or slowly expanding lesions, are distinguished by a gradual growth in size and slowly progressive loss of tissue. They are frequently seen in MS patients with a prolonged duration and course of the disease as well as in progressive phenotypes. In pathological studies, these lesions have been characterized by rims of microglia and macrophages with iron accumulations and a distorted appearance, activated microglia and macrophages at the edge, and a slowly ongoing demyelination and tissue

loss.[21,136-138] These rims may persist over years,[138-143] even if they may also gradually disappear.[144]

As of today, no agreement has been achieved concerning the most appropriate MRI technique to visualize and detect smouldering lesions. A technique oriented around the detection of these lesions on T2- and T1-weighted images has been recently described.[145] Smouldering lesions may have differential-diagnostic significance, as they are commonly seen in MS but not in neuromyelitis optica spectrum disorders (NMOSD)[146,147] or in cerebrovascular diseases.[148]

1.6 CSF analysis in MS

CSF analysis has been recognized as an important test for the evaluation of MS for many years. It is well known that CSF findings atypical for MS, such as elevated protein concertation>100 mg/dL or pleocytosis with >50 cells per mm,^{3} may assist in ruling out MS.[149] Conversely, although not specific for MS, the detection of CSF-specific IgG-OCBs has been known for many years to increase diagnostic certainty.[150,151]

In the latest 2017 revisions of the diagnostic McDonald Criteria for MS, the international panel on the diagnosis of MS has concluded that:

> "although CSF examination is not mandatory in some cases (e.g. patients with a typical CIS supported by characteristic MRI findings, unequivocal demonstration of DIS and DIT, and an absence of atypical clinical or imaging features), the threshold for CSF examination should be low to increase diagnostic confidence. CSF examination is strongly recommended in the following situations: when clinical and MRI evidence is insufficient to support a diagnosis of MS, particularly if

> initiation of DMTs is being considered; when there is a presentation other than a typical CIS, including a progressive course at onset (PPMS); when clinical, imaging, or laboratory features are atypical of MS; and in populations in which MS is less common (eg, children, older individuals, or non-white populations)."[5]

1.6.1 Fluid biomarkers

Generally, biomarkers may be defined as any characteristic that can be objectively measured and that confers objective knowledge about a normal or abnormal biological function.[152] In MS, fluid-based biomarkers may assist in diagnosis, prognostic prediction, and evaluation of treatment response (Figure 2). Various biomarkers are advantageous in the sense that they frequently reflect different aspects of the diseases' pathophysiology. Numerous promising fluid biomarkers have emerged as a result of extensive research during the last several decades.

A detailed and extensive description of the most essential definitions and characteristics of fluid biomarker research has been provided elsewhere.[153] Briefly, fluid biomarker research typically involves a discovery and exploratory phase, which encompasses an investigation of the usefulness of a particular or several biomarkers, the validation of the findings in further independent studies, and finally implementation in the clinical routine. However, the borders between these different phases are not always well-defined and are occasionally intertwined.

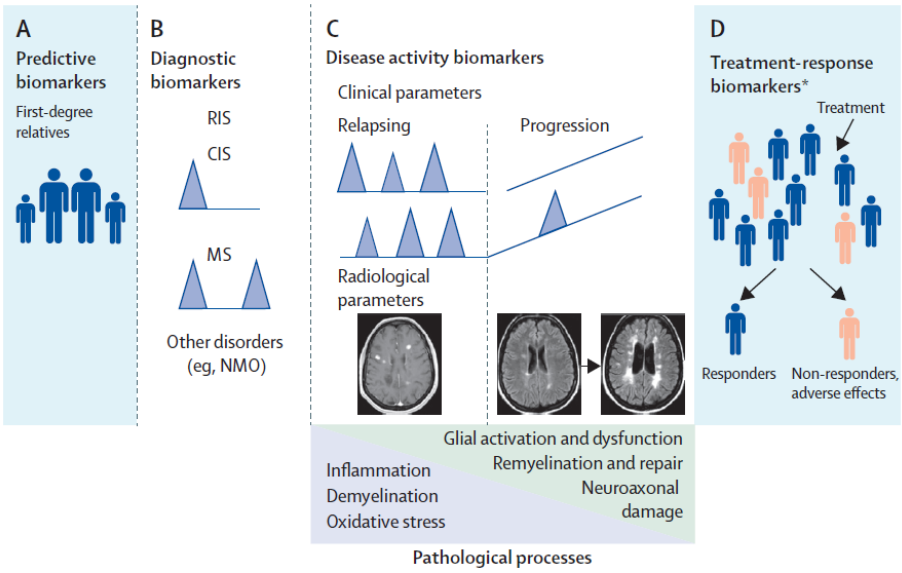

Figure 2. Types of biomarkers in MS: (A) Predictive biomarkers, (B) diagnostic biomarkers, (C) disease activity biomarkers, (D) treatment-response biomarkers
RIS=radiologically isolated syndromes. CIS=clinically isolated syndromes. MS=multiple sclerosis. NMOSD=neuromyelitis optica spectrum disease. Figure reproduced with permission from Comabella et al.[153] Copyright © 2014 Elsevier Ltd. All rights reserved.

As previously mentioned, it is highly desirable to be able to predict the risk of clinically meaningful disease worsening in the future, as this information may influence treatment strategy at baseline, a feat that may be achieved with various biomarkers.[154] Table 4 presents a summary of studies investigating the predictive prognostic value of various fluid biomarkers.

Table 4. A selection of CSF biomarkers and their association with prognosis in MS.

Biomarker	Disease course (n)	Major findings	Authors
IgG-OCBs	CIS (1047)	Conversion to CDMS	Kuhle et al.[155]
Intrathecal IgG synthesis	CIS/RRMS (673)	Higher risk and shorter time to EDSS worsening during four-year follow-up period	Gasperi et al.[156]
LS-OCMB	CIS (24)	LS-OCMB is related to early increase in lesion load and brain atrophy	Margraner et al.[157]
LS-OCMB	RRMS (81)	Higher risk of reaching EDSS 4 and conversion to SPMS	Thangarajh et al.[158]
ITMS	CIS/early MS (150)	ITMS is a strong independent risk factor for early conversion to MS	Pfuhl et al.[159]
ITMS	RRMS (530)	Higher risk and shorter time to first relapse and initiation of high-efficacy DMT	Oechtering et al.[160]
ITMS	CIS/RRMS (193)	Higher risk of EDSS≥4, EDSS≥6, and conversion to SPMS	Monreal et al.[161]
KFLC-index	RRMS (88)	High KFLC-index predicts early MS disease activity.	Berek et al.[162]
KFLC-index	RRMS (52)	High KFLC-index predicts EDSS≥3 and DMT escalation	Castillo-Villalba et al.[163]
cNfL	RRMS (95)	Higher risk of conversion to SPMS in patients with cNfL>386 ng/L	Salzer et al.[164]

cNfL	RRMS (41)	High cNfL levels are predictors of NEDA-3 status after four years	Hakansson et al.[165]
cNfL/IgG-OCBs	RIS (75)	cNfL>619 ng/L and IgG-OCBs predict conversion from RIS to CIS and CDMS	Matute-Blanch et al.[166]
CSF-CXCL12 and CSF-CXCL13	RRMS (52)	CSF-CXCL12 and 13 are elevated in MS CSF and in MS lesions	Krumbholz et al.[167]
CSF-CXCL13	CIS/RRMS (91)	CSF-CXCL13 levels predict conversion to MS	Brettschneider et al.[168]
CSF-CXCL13	CIS/RRMS (466)	CSF-CXCL13 levels predict MS, relapse rate, and new lesions	Khademi et al.[169]
CSF-CXCL13	RRMS (40)	CSF-CXCL13 associates with cortical thinning and high CSF leukocyte count	Puthenparampil et al.[170]
CSF-CH13L1	CIS (60)	Conversion to CDMS	Comabella et al.[171]
CSF-CH13L1	CIS (813)	CSF-CHI3L1 levels predict conversion to MS and increased disability	Canto et al.[172]

Abbreviations: IgG=immunoglobulin G; OCB=oligoclonal bands; CIS=clinically isolated syndrome; RIS=radiologically isolated syndrome; MS=multiple sclerosis; CDMS=clinically definitive MS; RRMS=relapsing-remitting MS; EDSS= expanded disability status scale; LS-OCMB=lipid-specific oligoclonal IgM bands; ITMS=intrathecal IgM synthesis; DMT=disease-modifying therapy; SPMS=secondary progressive MS; KFLC=kappa free light chain; CSF= cerebrospinal fluid; cNfL=CSF neurofilament light; CXCL=chemokine (C-X-C motif) ligand; CH13L1= chitinase-3-like protein 1.

Table adapted and modified from Iacobaeus et al.[82]

1.6.2 Fluid biomarkers included in the current thesis

As mentioned above, the diagnosis of MS necessitates a combination of signs and symptoms suggestive of MS, as well as findings from MRI and CSF analyses, to fulfil the McDonald diagnostic criteria.[5] Aside from establishing a prompt and accurate diagnosis, one of the main challenges faced by MS clinicians counselling people with MS relates to making prognostic predictions that may assist in risk-benefit decisions when selecting appropriate DMTs. A broad range of DMTs are now approved and are available for the treatment of MS. All DMTs have been demonstrated to reduce clinical and radiological disease activity, as well as disability accrual.[173]

However, DMTs vary considerably in terms of efficacy as well as adverse-effect profiles. Due to the highly heterogeneous interindividual nature of the MS disease course, it is vital to predict which patients will benefit most from the early initiation of highly efficacious DMTs. To date, the number of MRI lesions and the presence of intrathecal IgG synthesis, as well as neurofilament light (NfL), are considered to be the most important biomarkers in terms of prognostic value.[174,175]

1.6.2.1 The Qualitative and quantitative determination of intrathecal IgG production

Immunoglobulin production by activated mature B cells is a hallmark of MS. Over the years, various qualitative and quantitative laboratory methods have been developed to assess the intrathecal fraction of immunoglobulin synthesis. Qualitative analysis of CSF for IgG-OCBs using isoelectric focusing (IEF) has, for many years, been considered to be the most important CSF test in the context of MS diagnostics, especially when considering uncertain presentations. The role of CSF analysis recently became even more central, when it was integrated into the 2017 McDonald criteria by the International

Panel on Diagnosis of Multiple Sclerosis.[5] In patients with a typical CIS and clinical or MRI demonstration of DIS, the presence of CSF-specific IgG-OCBs may signify DIT, therefore allowing an early diagnosis of MS.[5]

This update of the McDonald criteria has resulted in increased sensitivity to MS diagnosis, although at the cost of somewhat lower specificity.[176,177] The immediate consequence of the latest revisions is that many patients presenting with a first demyelinating event are now receiving an MS diagnosis earlier in the course of the disease, rendering them eligible for treatment with effective DMTs. In addition to their high diagnostic worth, the presence of IgG-OCBs is known to confer prognostic information, predicting a second demyelinating event in CIS patients,[155] and conversion to clinically definitive MS (CDMS) after optic neuritis.[178]

In addition to IgG-OCBs, the intrathecal fraction of IgG may be assessed quantitatively and is a commonly used diagnostic test in MS.[156,159,179] Over the years, various methods and mathematical formulas have been deployed to optimally estimate the intrathecal IgG synthesis (ITGS) and differentiate it from IgG diffusing into the CNS from peripheral blood via the BBB.[180] One such method, namely the linear IgG-index, is defined as the CSF/serum IgG concentration quotient (Q_{IgG}) divided by the CSF/serum albumin concentration quotient (Qalb).[181,182] Qalb is a widely accepted measure of BBB dysfunction, as albumin is synthesized strictly outside the CNS.[183] Similarly to IgG-OCBs, ITGS has been demonstrated to predict clinical worsening in RRMS.[156]

1.6.2.2 Intrathecal kappa free light chain synthesis

Kappa and lambda light chains are small polypeptides that function as subunits of immunoglobulin antibodies (Figure 3). In intact immunoglobulins, kappa and lambda light chains are bound to heavy chains via disulfide bonds and noncovalent interactions.[184,185] In the course of inflammation, activated B

lymphocytes synthesize intact immunoglobulins, and an excess of kappa and lambda free light chains (FLC) are secreted.[186] The molecular weight of FLCs is approximately 24 kD, and they consist of two immunoglobulin domains, a constant region that specifies the isotype (kappa or lambda), and a variable domain. When bound in an intact immunoglobulin, the variable light chain domain is part of the immunoglobulin antigen binding site. However, its function in the free forms is not entirely clear.[187]

The analysis of IgG-OCBs via IEF is associated with numerous limitations. These include time-consuming manual handling and the potential for subjective interpretation. In recent years, the quantitative assessment of intrathecal kappa free light chains (KFLC) has emerged as an attractive method to measure the increased intrathecal immunoglobulin production that is frequently seen in MS[188,189] and has therefore become a potential substitute for the routine determination of IgG-OCBs.[179,190]

Similarly to quantitative estimations of other immunoglobulins, intrathecal KFLC synthesis may be assessed with several different metrics.[191] The most commonly used formulas to calculate the intrathecal fraction of KFLC are the linear index formula and the nonlinear hyperbolic reference range formula according to Reiber.[192] This nonlinear method was intended to reduce the risk of false positive and false negative interpretations associated with the linear index, taking into consideration the individual Qalb value.[193]

However, none of the aforementioned methods have been proven superior in terms of clinical utility, and depending on laboratory preferences, different formulas are used world-wide. Similar to IgG-OCBs, the analysis of intrathecal KFLC synthesis has been demonstrated to confer valuable prognostic

information regarding the risk of future disease activity[162] and clinical worsening,[163] in addition to its role in diagnosis.

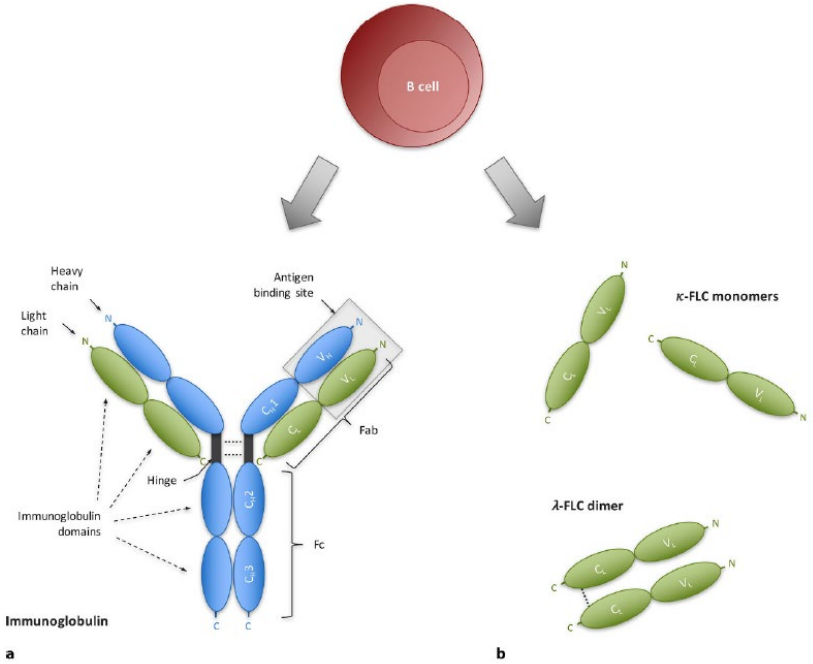

*Figure 3. Illustration of the structure of immunoglobulins and free light chains. B lymphocytes produce (**a**) intact immunoglobulins and (**b**) an excess of kappa and lambda free light chains (FLC). Both immunoglobulins and FLC serve as biomarkers for B cell activity.*
CH constant heavy chain domain, CL constant light chain domain, Fab fragment antibody binding, Fc fragment crystallisable, FLC free light chain, VH variable heavy chain domain, VL variable light chain domain. Figure reproduced with permission from Hegen et al.[194] Copyright © 2022 Hegen et al.

1.6.2.3 Intrathecal IgM synthesis

Immunoglobulin production by B lymphocytes in peripheral tissues is frequently characterized by the process of class-switching, in which the initial synthesis and secretion of IgM eventually transitions into IgG production.[195] Conversely, in the CNS, some active B lymphocytes continue secreting IgM without ever switching to IgG.[196] This is believed to occur as a result of somatic

hypermutation.[197] A growing body of knowledge regarding the role of intrathecal IgM synthesis (ITMS) in MS suggests that it may serve as a disease severity biomarker, signifying a more aggressive MS disease course.[198]

Various studies have previously revealed that ITMS in patients with CIS reflects an increased risk of converting to CDMS.[157,159,199-204] However, results from other studies with large study populations have been contradictory.[156,205-208]

Similar to IgG and KFLC synthesis, various methods to quantify the presence of ITMS have been developed and applied.[198] The most commonly used formulas for the estimation of ITMS are the linear IgM-index, the intrathecal fraction of IgM (IgM_{IF}) according to Reiber's formula,[156,159,203] and the qualitative detection of oligoclonal IgM bands (OCMB).[208,209] Some laboratories have promoted the use of lipid-specific OCMB as a particularly sensitive method to detect intrathecal IgM production.[157,210] However, it remains to be determined which of these methods estimates ITMS in the most reliable manner.

1.6.2.4 Neurofilament light

Neurofilament light (NfL) is a biomarker of acute and chronic neuroaxonal injury and loss. Since its discovery, NfL has gradually become the most promising fluid biomarker for the evaluation of disease activity, prognosis, and treatment response in relation to MS.[211] Moreover, due to its high sensitivity to neuroaxonal damage, NfL has been increasingly used as an outcome measure in clinical trials. Neurofilaments are a group of abundant structural scaffolding proteins which, in conjunction with microtubules and microfilaments, constitute the neuronal cytoskeleton.[211] They are found explicitly in nerve cells and are therefore highly specific to nerve cell damage.

With ongoing axonal damage, the neurofilament light subunit leaks into the extracellular space (Figure 4).[212] Using immunoassays, NfL can be quantified in CSF, and with ultrasensitive methods even in blood, and can thus function as a useful biomarker reflecting both acute and chronic axonal damage in various neurodegenerative diseases, including MS.[211] Accumulated data suggest that CSF NfL (cNfL) reflects disease activity[213] and therapeutic response in MS.[214]

However, due to the relatively invasive nature of lumbar punctures (LP), and the fact that many patients perceive it as unpleasant, the clinical utility of serial NfL measurements for the detection of disease activity has been limited. The development of ultrasensitive immunoassays enabled determinations of exceptionally low NfL concentrations in blood (plasma or serum),[215] opening the door for NfL to become a potential biomarker for clinical practice. Several studies have proven that the associations previously found between cNfL and clinical/MRI measurements[216,217] still apply to blood NfL (bNfL) determinations.[218,219]

Although the correlation between bNfL and cNfL is high,[219] the sensitivity of NfL in detecting disease activity in RRMS appears to be higher in CSF.[165] In addition to their ability to reflect ongoing inflammatory disease activity, cNfL[164] and bNfL[220,221] have been demonstrated to predict clinical worsening and disease progression over time, as well as brain and spinal cord atrophy.[222,223] The most important studies regarding NfL and MS are summarized in Table 5.

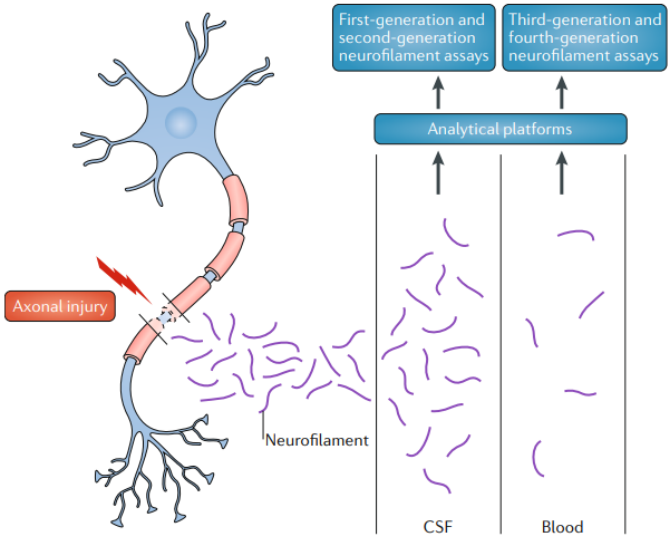

Figure 4. Neurofilament release after axonal damage. When an axon is damaged, cytoskeletal proteins, including neurofilaments, are released into the extracellular space and subsequently into the CSF and, at lower concentrations, into the blood. First-generation (immunoblots) and second-generation enzyme-linked immunosorbent immunoassays can detect neurofilaments in the CSF but have limited sensitivity for detection in the blood. Third-generation (electrochemiluminescence) and, in particular, fourth-generation (single-molecule array) immunoassays can reliably measure blood levels of neurofilament light and detect subtle longitudinal changes in disease and in healthy controls. Figure reproduced with permission from Khalil et al.[211] Copyright © 2018, Springer Nature Limited

Table 5. Key neurofilament studies in multiple sclerosis

Study	Study design	Tissue analyzed	Participants	Association with disease activity	Diagnostic or prognostic relevance	Relevance as drug response marker
Lycke et al.[212]	RCT, two years, acyclovir versus placebo	CSF (Rosengren et al., 1996)	60 patients with RRMS and 11 healthy controls	NfL associated with relapse rate and EDSS; levels increased for ~3 months after relapse	NfL levels higher in patients with RRMS than in healthy controls	No influence of acyclovir on NfL levels
Malmeström et al.[216]	Cross-sectional[a] observational	CSF (Rosengren et al., 1996)	66 patients with MS and 50 healthy controls	NfL increased during relapse	NfL levels higher during relapse in patients with RRMS than during remission; levels equal in patients with RRMS in remission and SPMS; levels higher in all patients than in healthy controls	Not applicable
Gunnarsson et al.[224]	Longitudinal[a], observational, 6 to 12 months	CSF (UmanDiagnostics NF-light ELISA)	83 patients with RRMS, 9 with SPMS and 28 healthy controls	NfL higher in patients with MS during relapse within 3 months than in remission	Not done	NfL levels reduced after natalizumab treatment (independent of relapse); less effect in SPMS
Disanto et al.[225]	Cross-sectional[a], observational	CSF and serum (Disanto et al., 2017)	48 patients with CIS, 62 with RRMS, 3 with SPMS, 16 with PPMS and 13 with RIS	Correlation between CSF and serum levels of NfL; increased serum levels of NfL in patients with enhancing MRI lesions or a higher MRI lesion load	Not done	Not applicable

	Longitudinal[a], observational (baseline, 7.5 months and 18 months)	Serum (Disanto et al., 2017)	14 patients with CIS, 185 with RRMS, 27 with SPMS, 20 with PPMS and 254 healthy controls at baseline (87 at 1 year)	Serum NfL levels independently higher in older patients, during relapse, in patients with higher EDSS scores, and in untreated patients	NfL levels higher in RRMS and progressive MS than in healthy controls; associated with increased risk of relapses and EDSS worsening over next 1 to 2 years	Baseline NfL levels higher in patients who started natalizumab or rituximab than in patients who started fingolimod or injectables; levels lower after disease-modifying therapies
Piehl et al.[226]	Cross-sectional[a], observational	CSF and serum (UmanDiagnostics NF-light ELISA and Gisslen et al., 2015)	33 patients with RRMS, 3 with SPMS, 3 with PPMS, and 27 controls with other neurological diseases	Correlation between CSF and serum levels of NfL; CSF NfL levels higher in patients with MS with recent relapse	CSF levels of NfL higher in patients with MS than healthy controls, higher in patients with RRMS than patients with SPMS, and higher during a relapse than during remission	Not applicable
	Longitudinal[a], observational (baseline, 12 months and 24 months)	Serum (Gisslen et al., 2015)	243 patients with RRMS	Plasma NfL levels correlated with MSSS and annual relapse rate	Not done	Plasma NfL levels lower after fingolimod treatment, sustained at 24 months

CIS, clinically isolated syndrome; CSF, cerebrospinal fluid; EDSS, Expanded Disability Status Scale; MS, multiple sclerosis; MSSS, Multiple Sclerosis Severity Score; NfL, neurofilament light chain; PPMS, primary progressive MS; RCT, randomized controlled trial; RIS, radiologically isolated syndrome; RRMS, relapsing–remitting MS; SPMS, secondary progressive MS. In cross-sectional studies, neurofilament levels were determined at a single time point; in longitudinal studies, levels were determined at multiple time points. Table adapted from Khalil et al.[211]

1.6.3 Fluid biomarkers not included in the current thesis

Other CSF biomarkers that have not been directly studied within this thesis or that have only been briefly mentioned have previously indicated valuable prognostic information. Glial fibrillary acidic protein (GFAP), a biomarker of astrogliosis,[227] has been associated with disease progression.[228,229] CSF GFAP has been routinely measured as part of clinical routine investigations at Sahlgrenska University Hospital, but CSF levels appear to be less useful in the context of RRMS. Therefore, we opted not to focus on this particular biomarker. Both chitinase 3-like proteins 1 and 2 (CHI3L1/2) and chemokine (C-X-C motif) ligand 13 (CXCL13) may reflect inflammatory disease activity and have also displayed promising prognostic value.[169,171,172,208,230] Although these biomarkers appear to be promising and useful, they have not been included in our routine CSF samplings and were therefore not included in the current thesis.

1.7 Treatment of MS

Since the early 2000s, the number of effective DMTs for the treatment of MS has steadily grown. As of today, around 16 different disease-modifying immunomodulatory therapies exist and confer important beneficial effects, particularly for patients with RRMS, but recently even for patients with PMS (Figure 5). These include interferon beta preparations, glatiramer acetate, dimethyl- and diroximel fumarate, natalizumab, alemtuzumab, sphingosine-1-phosphate receptor modulators (e.g. fingolimod, ponesimod, ozanimod, and siponimod), teriflunomide, cladribine, and B-cell depleting agents (e.g., rituximab, ocrelizumab, and ofatumumab). These agents have all been proven to decrease the relapse rate and slow the accumulation of MS lesions in MRI scans to various extents.[10] Another important and highly effective treatment option that has emerged in recent years, particularly for the treatment of highly

aggressive MS irresponsive to DMTs, is autologous hematopoietic stem cell transplantation.[231,232]

The selection of a specific treatment is typically individualized according to measures of disease activity and patient values and preferences. The response to DMTs is usually monitored via clinical follow-ups, new or contrast-enhancing lesions on MRI, and the onset or progression of sustained disability.[233,234] In that respect, fluid biomarkers, such as bNfL, have been increasingly recognized for their ability and usefulness in monitoring disease activity in treated patients.[218,219] However, CSF biomarkers remain crucially important due to their diagnostic and prognostic value, which may guide treatment decisions.

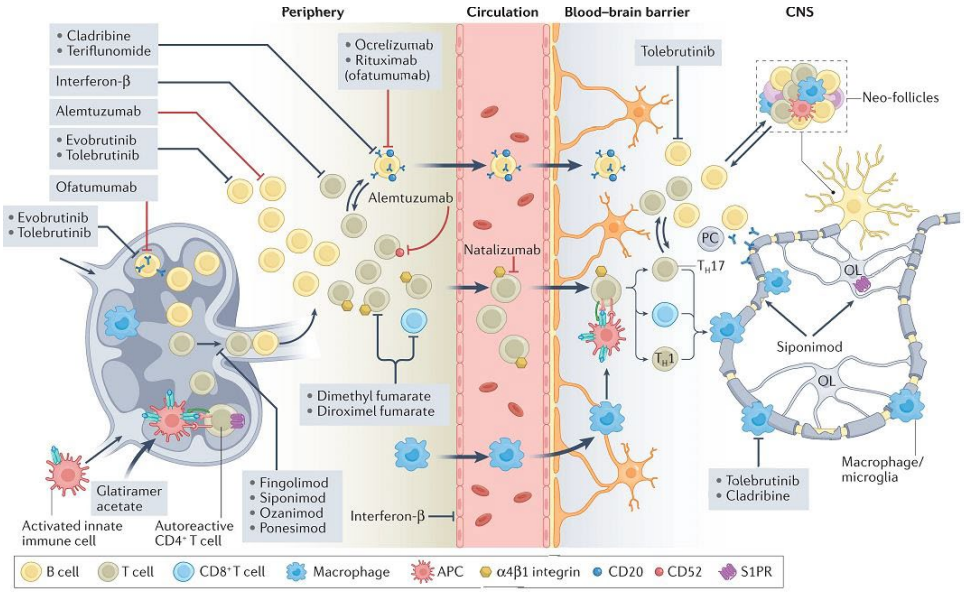

Figure 5. Overview of the immunopathogenesis and targets of available disease-modifying therapies in MS. The therapies depicted are subdivided into monoclonal antibodies (red lines) and pharmacological agents (black lines). APC, antigen-presenting cell; OL, oligodendrocyte; S1PR, sphingosine 1-phosphate receptor; T_H cell, T helper cell. Reproduced with permission from Bierhansl et al.[235] Copyright © 2022, Springer Nature Limited.

1.8 Research and study design considerations

1.8.1 Real-world data

All studies included in this thesis are based on real-world data. The concept of real-world data in biomedical research has become prevalent in recent years. A consensus concerning the exact definition of real-world data is still currently lacking, but it generally refers to data and evidence generated from sources other than traditional clinical trials.[236] Most experts agree that these types of healthcare-related data are usually gathered from electronic health records, claims and billing data, insurance agencies, and product and disease registries. Classical randomized controlled trials (RCT) have been widely accepted as a gold standard in biomedical research due to their ability to reduce bias and improve the accuracy of clinical experimentation.[237]

However, it has also become apparent in recent years that results from RCTs are limited, as they may not always reflect real-life clinical situations due to strict inclusion and exclusion criteria and a highly controlled setting. These limitations might also be applied to prospective cohort studies. Both RCTs and prospective studies are often difficult to design and are expensive to perform, which often limits their follow-up time periods. Since the disability worsening or progression that is associated with MS often develops gradually over the course of many years, RCTs and prospective studies with relatively short follow-up durations of up to five years might overlook these important clinical outcomes. In that sense, studies based on retrospective observational real-world data can complement and address these limitations. However, it is important to recognize that studies based on retrospective real-world data are themselves often limited by the quality of the data and the risk of confounding and selection bias.

1.8.2 The Swedish Multiple Sclerosis Registry

The SMSreg is a publicly funded nationwide register in which prospective data regarding MS patients in Sweden are collected. In the mid-1990s, a collaboration between all Swedish neurological university clinics was established to develop a joint structure for the registration of patients with MS. This effort culminated in a database platform that was originally envisioned to facilitate and improve patient-related clinical follow-up but that also enabled to locally manage both quality control and operational follow-up. The SMSreg was publicly introduced in 2001 and has since then provided data for over 180 scientific reports.

Data in the SMSreg may be registered by authorized physicians and nurses. Common variables included are baseline data, visits, EDSS, date of MS onset

and diagnosis, all DMT exposures, exacerbations, including the type of relapse and whether patients were given high-dose corticosteroids, MRI variables, laboratory analyses, functional rating scales, quality of life scores, work capacity, and rehabilitation. While inclusion in the SMSreg is not mandatory, coverage is high and is estimated at around 80% of all Swedish people with MS.

1.9 Statistical considerations

1.9.1 Sensitivity, specificity, and cut-off values

A diagnostic test is a test that has the ability to discriminate individuals who have a certain condition or who meet a given outcome from those who do not. A clinically useful diagnostic test must have high sensitivity and specificity. The sensitivity of a given diagnostic test is defined as the proportion of individuals for whom the outcome is positive who are correctly identified by the test:

$$Sensitivity = \frac{number\ of\ true\ positives}{number\ of\ true\ positives + number\ of\ false\ negatives}$$

The specificity of a diagnostic test is the proportion of patients for whom the outcome is negative who are correctly identified by the test:

$$Specificity = \frac{number\ of\ true\ negatives}{number\ of\ true\ negatives + number\ of\ false\ positives}$$

Sensitivity, specificity, and other measures, such as the positive predictive value and the negative predictive value, may be easily calculated using contingency tables. An ideal clinical diagnostic test would need to have sensitivity and specificity levels that are near 100%. However, for most clinical tests, high sensitivity might come at the expense of low specificity, and vice

versa. Depending on the circumstances, researchers might prioritize sensitivity over specificity, or vice versa.

For example, if a given condition has serious health consequences but can be treated with a particular drug x, drug x may in turn have detrimental side-effects. In such a case, one would wish to ensure that no false positives will receive drug x. In such a case, it may be reasonable to prioritize specificity over sensitivity. In contrast, in a condition that has high mortality but that can potentially be cured if it is discovered early, sensitivity might be prioritized to minimize false negatives.

A diagnostic test that is based on a continuous measurement may have different cut-off values that can be considered for discrimination between groups of patients according to a given outcome. Various methods have been developed for the calculation of the optimal cut-off value, and researchers might opt to use a given method depending on whether it is desirable to prioritize sensitivity over specificity or vice versa. However, if no preference is specified, researchers might choose to use the Youden's index (J):

J = sensitivity + specificity – 1

When the test is perfect, the maximum value of J is 1, whereas the minimum value is typically 0 when the test has no diagnostic value. A graphical representation of sensitivity against 1 – specificity is called a receiver operating characteristic (ROC) curve. The optimal cut-off value typically lies closest to the 1.0 point. The ability of two variables to diagnose an outcome can be compared using ROC curves with corresponding area under the curves. Calculations of cut-off values, sensitivity and specificity analyses, and ROC curves were performed in studies **I** and **II** as described below.

1.9.2 Cox proportional hazards model

A proportional hazards model is a type of model that is frequently used in survival analyses. It associates the passage of time before an event occurs to one or more predictive/explanatory covariates to determine the hazard rate of a particular event that may occur. In the Cox regression model, the effect of the covariates is to multiply the hazard function of the covariates. In other words, two units of observation have a ratio of their hazards that is constant and that depends on covariate values. This type of modelling was primarily used in studies **III** and **IV** as described below.

2 AIMS

The overall purpose of this doctoral project was to evaluate and validate the clinical utility of various routinely used blood and CSF soluble biomarkers in the diagnostic workup of MS, the monitoring of disease activity, and prognostic predictions.

In study **I**, we aimed to explore the clinical utility of cNfL as a disease activity and treatment response biomarker in RRMS and to assess its prognostic value.

In study **II**, we evaluated KFLC-index as a diagnostic biomarker in MS and examined whether it has the potential to improve diagnostic accuracy and eventually replace the determination of IgG-OCB.

The purpose of study **III** was to evaluate ITMS and its prognostic ability to identify patients with a severe MS disease course and to assess whether the prognostic prediction would be improved if ITMS was combined with cNfL.

Study **IV** aimed to investigate whether high levels of intrathecal KFLC synthesis at MS onset may be predictive of future cognitive impairment.

3 METHODS

3.1 Study design

All four studies included in this doctoral project share a common study design. Studies **I**, **III**, and **IV** are observational retrospective longitudinal cohort studies of patients with early RRMS based on demographic, clinical, and laboratory data that were prospectively collected and stored in a dedicated national MS registry (see section 1.8.2), as well as archived data of biomarker concentrations at the Neurochemistry Laboratory. In study **II**, we retrospectively retrieved prospectively collected data concerning patients undergoing clinical routine MS investigations and follow-ups. Patients were sorted into an MS group and a control group, as described below.

3.1.1 Data sources

For the purpose of this doctoral project, three data sources were combined to identify eligible patients: the Swedish Multiple Sclerosis Registry (SMSreg, http://www.msreg.net),[238] archived data regarding biomarker concentrations analyzed at the Neurochemistry Laboratory, and electronic health records of patients at Sahlgrenska University Hospital.

3.1.2 Patients and controls

An overview of all study cohorts is presented in Table 6. All MS patients included in this project were registered and identified in the SMSreg. In study **I**, RRMS patients (n=757) who had cNfL prospectively analyzed as part of the diagnostic workup in our MS center between 2001 and 2018 were retrospectively identified.[239] Patients who had a follow-up LP (n=157) were assessed for their treatment responses, and some were treatment-naïve (n=43), some had initiated a first-line treatment (n=44, interferon-β n=10, glatiramer-acetate n=4, teriflunomide n=7, and dimethyl-fumarate n=23), and some had

switched to a second-line therapy (n=70, natalizumab n=49, fingolimod n=10, rituximab n=5, and alemtuzumab n=6).

In study **II**, patients (n=343) who had prospectively determined KFLC in CSF and serum between May 2013 and February 2020 were retrospectively retrieved.[179] After exclusion, the study population was comprised of 327 patients: CIS/RIS (n=20), RRMS (n=161), PPMS, (n=19), SPMS, (n=23), other neuro-inflammatory disease controls (ONID, n=29, acute disseminated encephalomyelitis n=1, acute unspecified myelitis n=8, neurologic Lyme disease n=5, chronic inflammatory demyelinating polyneuropathy n=2, neuro-inflammatory disease not otherwise specified n=7, myelin oligodendrocyte glycoprotein associated disorder (MOGAD) n=3, aquaporin-4 associated NMOSD n=2, autoimmune encephalitis n=1), and patients classified as symptomatic controls (SC). These patients had MS-suspected symptoms, but the diagnostic work-up was negative (n=75).[5] All controls were pooled into one group (n=104), and patients with CIS/RIS and MS formed the MS study group (n=223). All patients with MS fulfilled the 2017 McDonald criteria.[5]

For the purpose of study **III**, we identified all patients included in study **I** who, in addition to the analysis of cNfL, also had information about ITMS.[240] Data about clinical relapses, EDSS,[7] follow-up MRIs, and exposure to DMTs were collected. All patients (n=457) fulfilled the 2017 revised McDonald criteria at the time of diagnosis.[5] Patients with analyses of serum and CSF IgG, IgM, and cNfL were also included in the study. The study population was restricted to patients who had their diagnostic investigation within 12 weeks after the first demyelinating event. Further inclusion criteria were an available baseline MRI scan and a follow-up period with a minimum of 24 months.

In study **IV**, we derived a cohort of patients with RRMS[5] (n=77) from study **II**, undergoing a routine clinical investigation between 2013 and 2018 after the

first demyelinating event. Patients included had determination of KFLC-index, longitudinal testing of SDMT, and a minimum follow-up time of four years. Excluded from the study were patients (n=84) in whom SDMT was not prospectively followed. Due to the possible interference of depression with SDMT assessments, patients were screened for concurrent depression with the Montgomery-Åsberg depression rating scale (MADRS)[241] and the Hospital Anxiety and Depression Scale (HADS).[242] Four patients were excluded due to coexisting depression. Disability was determined annually with EDSS.[7]

Table 6. Overview of the four study cohorts included in the thesis.

Study	Patients	N	Age (years), Mean ± SD	Sex (female), n (%)	Biomarker	Study outcomes
I.	RRMS	757	36.5 ± 11.3	517 (68.3)	cNfL	Clinical relapse; MRI disease activity; EDA-3 (ROC); EDSS≥3; SPMS; Treatment response
II.	Cohort	327			KFLC-index	Sensitivity; Specificity; Diagnostic accuracy; MRI disease activity; Treatment response; Correlation with other CSF biomarkers
	• CIS/RIS/MS	• 223	• 41 ± 13	• 123 (65.4)		
	• Controls	• 104	• 43 ± 14	• 68 (67.3)		
III.	RRMS	457	36.7 ± 11.1	316 (69)	ITMS	EDA-3; EDSS≥3; EDSS≥6
					cNfL	
IV.	RRMS	77	35.3 ± 11.1	54 (70.1)	KFLC-index	SDMT reduction ≥8 points
					cNfL	
					CSF Tau	

Abbreviations: CSF = cerebrospinal fluid; MS = multiple sclerosis; RRMS = relapsing-remitting multiple sclerosis; cNfL= CSF neurofilament light; EDA-3 = evidence of disease activity-3; ROC = receiver operating characteristic; EDSS = expanded disability status scale; SPMS = secondary progressive multiple sclerosis; KFLC = kappa free light chains; ITMS = intrathecal immunoglobulin M synthesis; SDMT = single-digit modalities test.

3.2 CSF biomarker analysis

3.2.1 Cerebrospinal fluid neurofilament light

All cNfL analyses were gathered at the Department of Neurology at the Sahlgrenska University Hospital in Gothenburg, Sweden. They were then analyzed by certified laboratory technicians at the Clinical Neurochemistry Laboratory at the Sahlgrenska University Hospital in Mölndal. Two different methods were used for CSF NfL analyses between 2001 and 2018. The first method was an in-house ELISA with a lower limit of detection of 250 ng/L,[243] which was later improved to 125 ng/L.[244] The second method was a more sensitive sandwich ELISA method (NF-light ELISA kit; UmanDiagnostics AB, Umeå, Sweden) with a lower limit of quantification of 31 ng/L.

The two first methods are based on the same reagents and result in comparable concentrations for measurements > 250 ng/L. The third method linearly correlates with the earlier methods (r = 0.90), but gives higher NfL levels than the previous two. To correct for this discrepancy, the ratio between the two mean values was used to recalculate the concentrations measured by the first in-house ELISA method to normalize values across the two methods.[245] Mean values of measured NfL levels were calculated for analyses from the in-house methods jointly (n=284, mean=1099), and the UmanDiagnostics method separately (n=630, mean=1868). The ratio between the two mean values (1.7) was then used to recalculate the levels measured by the in-house ELISA method. The adjusted concentrations of NfL measured with the two methods were compared by a Mann-Whitney U test which indicated no significant differences in distribution between the adjusted results of the two methods (p=0.17).

Age-adjusted upper limits of the reference range applied in clinical practice at our institution were used to stratify patients into two groups, those with increased cNfL concentrations and those with normal cNfL concentrations. These upper limits are as follows: <380 ng/L (<30 years), <560 ng/L (30-39 years), <890 ng/L (40-60 years), and <1850 ng/L (>60 years). These reference values are based on NfL determinations from 120 healthy control subjects without histories, symptoms, or signs of neurological or psychiatric disorders, using the upper 95% percentile as the cut-off. They had neither any significant systemic disorder nor diabetes mellitus or high BMI. Previous or current tobacco smoking was unknown.

3.2.2 Intrathecal immunoglobulin synthesis

Paired CSF and serum samples were consecutively collected and analyzed as part of the routine diagnostic work-up. Serum and CSF levels of KFLC were measured using the N Latex FLC kappa kit on an Atellica NEPH 630 instrument (Siemens), following the instructions of the manufacturers. The KFLC-index was calculated using the equation [(CSF KFLC / serum KFLC) / (CSF albumin / serum albumin)]. CSF- and serum albumin and IgG levels were analyzed using the IGG-2 and ALBT2 Reagent cassettes on a Cobas c module instrument (Roche).

The ratio between CSF and serum albumin was determined by dividing CSF albumin (mg/L) by serum albumin (g/L). The IgG-index was calculated by dividing the ratio of CSF IgG to serum IgG by the ratio of CSF albumin to serum albumin. Analyses were conducted by board-certified lab technicians who were blinded to the clinical status, following strict quality control and run approval procedures. The reference range for the hyperbolic formula was determined using Reiber's formula: $KFLC_{IF} = KFLC_{LOC}/KFLC_{CSF}$ x 100 or (1-$KFLC_{LIM}/KFLC_{ratio}$) x 100, where $KFLC_{LOC}$ = ($KFLC_{ratio}$ – $KFLC_{LIM}$) x $KFLC_{serum}$, and $KFLC_{LIM}$ = (3.27 x [Qalb + 33] – 8.2) x 10^{3}.[192]

An in-house method involving 7.7% polyacrylamide gels and silver staining was utilized to determine CSF-specific IgG OCBs. Samples of paired patient serum and CSF were run on adjacent lanes, and CSF-specific OCBs were identified as additional bands in the gamma-zone which were not present in the corresponding serum sample. To ensure quality, a positive CSF sample with known CSF-specific OCBs was included on each gel.

IgM levels in both serum and CSF were measured using a cobas c module instrument (Roche) with the ALBT2, IGM-2, and IGM-C reagent cassettes. The IgM-index was determined by dividing the ratio of CSF IgM (mg/L) to serum IgM (g/L) by Qalb. An IgM-index greater than 0.1 was considered increased.[246,247] Alternatively, the intrathecal fraction of IgM (IgM_{IF}) was calculated according to Reiber's formula.[248] CSF-specific oligoclonal IgM bands were identified by agarose gel electrophoresis (Hydrasys 2 system, Sebia) followed by an in-house immunoblotting method. The proteins were blotted onto a polyvinylidene fluoride membrane, and IgM was detected using a polyclonal goat anti-human IgM antibody conjugated with alkaline phosphatase (Sigma).

Paired patient serum and CSF samples were analyzed side by side, and CSF-specific IgM bands were defined as IgM bands present in CSF which were not present in the matching serum sample. OCMB≥2 were considered positive. On each gel, a CSF sample with known oligoclonal IgM bands was included as quality control.

3.3 Study endpoints and definitions

3.3.1 No Evidence of Disease Activity-3

NEDA-3 (no evidence of disease activity-3), defined as no clinical relapses, no confirmed disability worsening (CDW) within 6 months (6-CDW), and no new

T1 gadolinium-enhanced lesions/new/newly enlarging T2-weighted [T2W] lesions), has become an important and meaningful clinical goal for the treatment of MS.[249-251] In addition, NEDA-3 has become an important secondary endpoint in clinical trials.[252,253]

A clinical relapse is defined as neurological signs and symptoms that persist at least 24 hours and that cannot be explained by another cause.[5] CDW is defined as an increase in EDSS score from baseline sustained between two follow-up visits separated in time by no less than six months (1.5 point if EDSS at baseline was 0, 1 point if EDSS was between 1 and 5, and 0.5 points if the baseline EDSS≥5.5). In study **I,** we assessed cNfL's ability to detect ongoing disease activity according to NEDA-3 criteria. In study **III**, we assessed the prognostic ability of ITMS alone or in combination with cNfL to predict NEDA-3 status at follow-up.

3.3.2 Disability worsening

Disability worsening in MS is often measured with the EDSS.[7] This method is based on the routine neurological examination and classifies disability into eight Functional Systems (FS) by assigning a Functional System Score (FSS) to each of these functional systems. It comprises an ordinal rating system ranging from 0 (no neurological disability) to 10 (death due to MS) in 0.5-increment intervals (from EDSS 1 onwards). The determination of EDSS≥6 is depends to a high extent on walking ability and the need of walking aids.[254]

EDSS has become the most widely used method to quantify disability in MS and describe disease worsening and progression, both in clinical practice and in clinical trials and research studies.[254] Nevertheless, the high inter-rater variability for EDSS is well-established.[255] The main disability outcomes used in this project are EDSS≥3 (**I & III**), EDSS≥6 (**III**), and conversion to SPMS

(**I**), which is defined as steadily increasing objectively documented neurological disability of one year or more independent of relapses.[5]

3.3.3 Score of SDMT as study endpoint

In study **IV**, patients completed the SDMT[256] within six months after their diagnosis and, thereafter, annually by an experienced MS nurse. They were expected to be clinically stable, and a new version of the SDMT was used each time to minimize learning bias. Reduced SDMT values of at least eight points or more[257] at follow-up compared to the baseline were considered significant, provided that no recovery in SDMT scores to less than eight points compared with the baseline could be seen within at least one year.

3.4 Statistical methods

Since all investigated fluid biomarkers were non-normally distributed, nonparametric tests were used in most analyses comparing whole-value concentrations. In studies **I** and **II**, the influence of age, sex, and disease duration on the levels of cNfL and KFLC-index was investigated by quantile regression analyses. The Mann-Whitney U test was used for comparisons of two groups, such as cNfL concertation in patients with relapse vs. no relapse in study **I**, to compare CIS/RIS/MS and controls in study **II**, and to compare cNfL and KFLC-index in patients with and without MRI activity in studies **I** and **II**, respectively.

The Kruskal-Wallis test and false discovery rate (FDR) test and the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli were used for comparisons of multiple groups, such as cNfL levels between different relapse-types (**I**), and to compare KFLC-index in different disease courses (**II**). The correlation of cNfL with the number of contrast-enhancing lesions, as well as

correlations between KFLC-index and other inflammatory and degenerative biomarkers, were calculated with the Spearman's rank correlation coefficient.

The ROC curve estimations were performed with the assumption of nonparametric distribution and devised to study the sensitivity and specificity of cNfL to detect clinical relapse, MRI activity, 6-CDW, and overall EDA-3 in study **I** and in order to study the diagnostic accuracy of KFLC synthesis compared with OCBs≥2 and IgG-index. A paired sample area difference under the ROC curves analysis was used to compare the different ROC curves. The Youden index was used to calculate sensitivity and specificity and to identify optimal cut-off values for cNfL, KFLC-index, and IgG-index.

A Wilcoxon matched-pairs signed rank test was used to analyze the treatment effects on cNfL concentrations in study **I** and in study **II** to compare KFLC-index at baseline and follow-up in the two treatment groups. The statistical significance was then determined using FDR two-stage step-up (Benjamini, Krieger, and Yekutieli). In study **II**, Reiberograms were constructed, and $KFLC_{IF}$ was computed using the online software found on www.albaum.it.[258] The measurement of agreement between the different methods to evaluate ITMS in study **III** was determined using the kappa statistic. Statistical significance was assumed at $p<0.05$.

3.4.1 Survival analyses and predictive models

To investigate the predictive value of cNfL in study **I,** Kaplan-Meier survival analysis was used where the dates of reaching the studied milestones or the date of the last visit in patients who did not reach milestones were used for censoring. Patients reaching EDSS milestones at baseline were not excluded. Statistical significance and hazard ratios (HR) were calculated by the log-rank test. In study **III**, the following endpoints were used to investigate the ability

of ITMS to predict a worse MS disease course: the association of ITMS with EDA-3 and the disability worsening endpoints EDSS≥3 and EDSS≥6.

These endpoints were used as time-dependent variables in Cox proportional hazards regression models, and the adjusted HR (aHR) along with corresponding 95% confidence intervals (CI) were calculated. For the EDA-3 endpoint, the following potential confounding factors were included in the model: age at debut, sex, disease duration at diagnosis, T2W lesion burden at the time of diagnosis, as well as exposure to DMTs (first-line/second-line). In the case of EDA-3, the total follow-up time was 24 months. Patients who exhibited EDA-3 during the follow-up period were censored at the time of the first signs of EDA-3. Those who fulfilled NEDA-3 at the end of the 24-month follow-up period were censored at 24 months.

In the analyses of disability worsening endpoints, patients were censored either at the time of reaching the investigated milestone or at the time of the last visit in case EDSS was maintained at <3 and <6, respectively. For the disability milestones, adjustments were made for age at the time of diagnosis, sex, disease duration, baseline MRI T2W lesion burden, exposure to DMTs, and whether subjects escalated therapy during the follow-up. The time to EDA-3, EDSS≥3, and EDSS≥6 was analyzed with Kaplan-Meier survival analysis and the logrank test. To investigate whether ITMS and cNfL have an additive predictive value, we then computed a new variable that combined positivity for IgM-index, IgM_{IF}, or OCMB and cNfL and performed Cox proportional hazards regression with the same endpoints and adjustments as above.

In study **IV**, we calculated the fourth quintile (KFLC-index=100.8) to identify the most appropriate cut-off value for KFLC-index for prognostic purposes. In agreement with our results as well as previous reports on the prognostic value

of KFLC-index,[162,259] we thereafter opted to dichotomise the cohort according to the cut-off value KFLC-index>100.

For the purpose of the Cox proportional hazards regression models, we computed a binary endpoint variable for SDMT reduction of ≥8 points at follow-up. Candidate predictor variables of SDMT reduction were identified as those variables that statistically significantly differed between those patients who had reduced SDMT at follow-up and those who did not (i.e., age, disease duration, baseline EDSS, baseline SDMT and brain MRI characteristics (T2-weighted [T2W] lesions)).

Sex, MRI Gd+ lesions, EDA-3 status at follow-up, and treatment strategy (first-line therapy from start, second-line therapy from start, and escalation from first- to second-line during follow-up) did not achieve statistical significance but were included in the models, as they are known potential confounders.[112,260,261] Exposure to high-dose corticosteroids prior to LP has been demonstrated to affect KFLC serum levels.[262] We adjusted for exposure to corticosteroids within 30 days prior to LP, as the influence of corticosteroids on the KFLC-index cannot be ruled out. We then tested KFLC-index as a log (2)-transformed continuous predictor variable.

The same analyses utilizing multivariable time-dependent models were performed independently and separately for cNfL and CSF Tau. We tested cNfL and CSF Tau as categorical nominal variables based on calculations of the 4th quintile cut-off value and as log (2)-transformed continuous variables. The 4th quintile for cNfL in our cohort was 910 ng/L, whereas for CSF Tau, it was 211 ng/L.

3.5 Ethical considerations

In accordance with its retrospective study design, this doctoral project concerns a retrospective review of data and statistical analysis of variables already

collected and stored in registers and archives as part of clinical routine investigations. As such, no physical procedures or interventions were performed as part of the data collection. Therefore, any potential physical or psychological risks of studying participants were considered to be low.

A breach of privacy could potentially be a risk, given that some data was collected via journal review. However, given that all MS patients were previously informed that their data would be registered in the SMSreg and given approval, this risk was deemed to be low. To counteract this risk, we minimized the number of researchers with access to data sources to only one person. Large databases may contain sensitive personal information about patients' health status. To minimize the risk of sensitive data leaking out, all databases were password-encrypted.

All individual data from the different sources were made anonymous to the authors via the replacement of the personal identity numbers by unique number codes for use in the studies. These number codes were discarded at the end of each study. Only aggregated data on large groups were reported. The risk of integrity and privacy intrusion must be weighed against the benefits of performing such studies. Patients arguably benefit extensively from improvements in their healthcare that are achieved via research, and the vast majority of MS patients who are followed-up at our centre indeed approve of their data being collected in a systematic and discreet manner in an MS registry and the use of this data for research purposes.

Most prospective studies and clinical trials involve some form of active intervention. Therefore, informed consent is always required in these cases. However, in retrospective registry-based studies, informed consent is not always mandatory. The SMSreg allows patients to opt out of research studies, although patients must actively act to do so. This solution appears to function

well, as the number of research studies that are being conducted annually using data from the SMSreg is over 100. Actively demanding informed consent from every patient for each specific study could potentially encumber individuals with exhaustive bureaucratic paperwork, leading many to opt not to participate in research projects.

The retrospective cohort analyzed in studies **I** and **III** included RRMS patients who had consented to be registered in the SMSreg. The studies have been approved by the Swedish Ethical Review Agency (Dnr: 2019-01199). The study material included in study **II** was retrieved from medical records regarding persons who underwent diagnostic investigation of suspected MS, recommended at the Sahlgrenska University Hospital.

In addition, we used CSF data from a sub-group of patients who also participated in research projects with an extended investigation of inflammatory and degenerative biomarkers (ethical approval Dnr 895-13).[213] They all gave their informed consent. For study **IV**, we used data regarding a subgroup of RRMS patients from study **II** who have all been registered in the SMSreg. Studies **II** and **IV** were approved by the Swedish Ethical Review Agency (Dnr: 2020-06851).

4 RESULTS

4.1 Diagnostic fluid biomarkers

4.1.1 KFLC-index as a diagnostic biomarker in MS

In study **II**, we found that patients with CIS, RIS, and MS had markedly higher KFLC-index levels (44.6, IQR 16–128) compared with a control group including ONID and SCs (2.19, IQR 1.68–2.98, $p < 0.001$, Figure 6).[179] RRMS patients had the highest median values for KFLC-index (54.7, IQR 21-143). The sensitivity and specificity of KFLC-index to distinguish CIS/RIS/MS patients from ONID and SC were 0.93 (95% CI 0.88–0.95) and 0.87 (95% CI 0.8–0.92) respectively (AUC 0.94, 95% CI 0.91–0.97, $p < 0.001$, Figure 7). KFLC-index and $KFLC_{IF}$ had similar accuracies in terms of detecting RIS/CIS/MS.

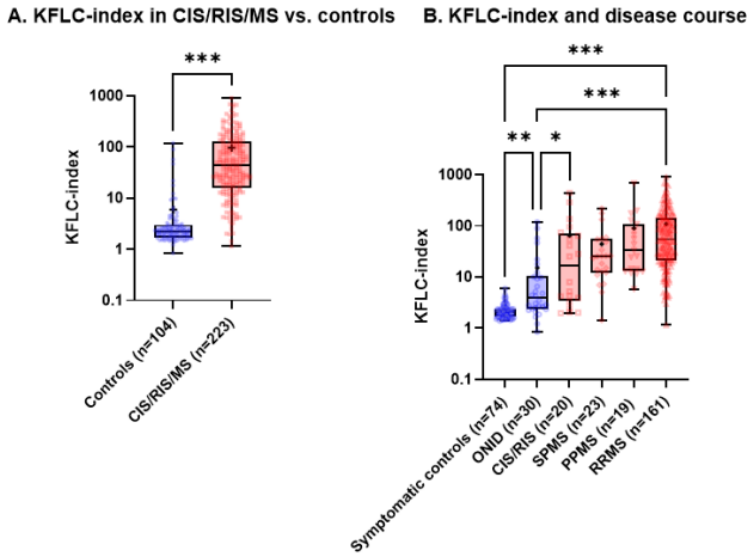

Figure 6. KFLC-index in patients with MS compared with controls. Box represents IQR. Bar indicates median, whereas + indicates mean. Abbreviations: SC = symptomatic controls; CIS = clinically isolated syndrome; RIS = radiologically isolated syndrome; SPMS = secondary

*progressive MS; PPMS = primary progressive MS; RRMS = relapsing-remitting MS. Adapted from Rosenstein et al.[179]©Wiley online library. * p<0.05 ** p<0.01 *** p<0.001.*

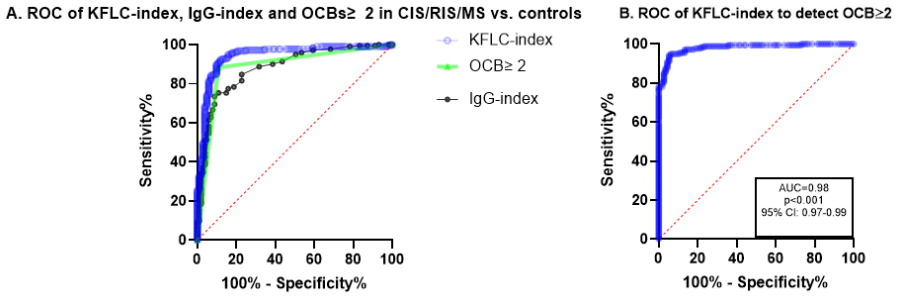

Figure 7. A. ROC curves of KFLC-index, OCB≥2, and IgG-index to detect CIS/RIS/MS in the study cohort (n=327); B.ROC of KFLC- index to distinguish patients who had OCB≥2 on IEF from those who did not (n=327). Adapted from Rosenstein et al.[179] ©Wiley online library.

4.2 Prognostic fluid biomarkers

4.2.1 Association of ITMS and risk of future disease activity

In study **III**, we investigated the ability of ITMS at MS onset to predict future disease breakthroughs within two years according to NEDA-3 criteria.[240] In this study, 178 patients (38.9%) exhibited EDA-3 within a follow-up of 24 months. The IgM-index and IgM_{IF} were both moderately associated with a higher EDA-3 hazard (aHR 2.3, 95% CI 1.6-3.4, p<0.001; and aHR 3.7, 95%CI 2.7-5, p<0.001, respectively) (Figure 8, Table 7), whereas OCMB demonstrated a fair association with a higher hazard for EDA-3 (aHR 1.4, 95%CI 1.04-2, p=0.03). In a univariable analysis, the median (95%CI) times for attaining an EDA-3 status in patients with IgM-index>0.1, IgM_{IF}≥0%, and OCMB were 15 (12.3-17.7), 14 (11.3-16.7), and 18 (16.8-19) months, respectively.

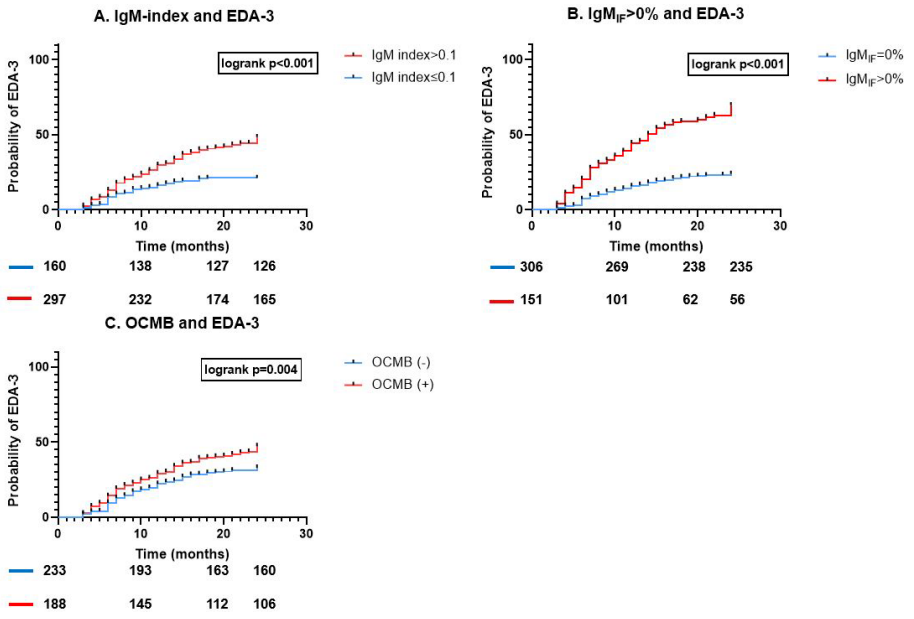

Figure 8. Time to EDA-3 for all IgM-metrics used to assess ITMS association of ITMS and time to EDA-3. Kaplan-Meier survival curves and results of the logrank test for A. IgM-index>0.1; B. IgM_{IF}>0% according to Reiber; and C. OCMB; and the probability of EDA-3 within 24 months from diagnostic LP. EDA: Evidence of Disease Activity; Ig: immunoglobulin; IF: intrathecal fraction; OCMB: oligoclonal IgM bands. Adapted from Rosenstein et al.[240]

Table 7. Unadjusted and multivariable Cox regression models for IgM metrics and prediction of 24-month NEDA-3 status, EDSS≥3, and EDSS≥6

Endpoint	Univariable model			Cox proportional hazards		
	HR	95% CI	*p* value	aHR	95% CI	*p* value
EDA-3						
• IgM-index>0.1	2.6	1.9-3.5	**<0.001**	2.3	1.6-3.4	**<0.001**
• IgM_{IF}>0%	3.9	2.8-5.5	**<0.001**	3.7	2.7-5	**<0.001**
• OCMB	1.6	1.2-2.2	**0.004**	1.4	1.04-2	**0.03**
EDSS≥3						
• IgM-index>0.1	1.6	1.1–2.3	**0.006**	1.9	1.3-2.8	**<0.001**
• IgM_{IF}>0%	1.1	0.8-1.6	0.5	1.4	0.9-2.1	0.06
• OCMB	1.2	0.8-1.7	0.3	1.4	0.9-2.1	0.07
EDSS≥6						
• IgM-index>0.1	1.6	0.8-2.9	0.2	2.1	1-4.4	0.05
• IgM_{IF}>0%	1.09	0.5-2.2	0.8	1.48	0.7-3	0.3
• OCMB	1.9	1-3.9	0.05	2.5	1.2-5.4	**0.01**

Abbreviations: HR- hazard ratio; aHR = adjusted hazard ratio; CI = confidence interval; EDA = Evidence of Disease Activity; Ig = immunoglobulin; IF = intrathecal fraction; OCMB = oligoclonal IgM bands; EDSS = expanded disability status scale.

Bold p-values indicate statistical significance (p<0.05). *Adapted from Rosenstein et al.[240] © 2022 Frontiers Media S.A. All rights reserved.*

4.2.2 Association of CSF NfL and ITMS with disability worsening

In study **I**, we explored the prognostic value of cNfL in a large, unselected cohort of RRMS patients followed up for 17 years.[239] In a univariable analysis, we found that patients with age-adjusted increased concentrations of cNfL at baseline had a higher hazard of progressing to EDSS≥3 (HR=1.9, 95% CI=1.4–

2.6, logrank p<0.001; median time to EDSS≥3 was 15 years) as well as transitioning to SPMS (HR=2.5, 95% CI=1.4–4.2, p=0.001; median survival undefined). Comparable results were obtained in a multivariable Cox regression proportional hazards analysis in study **III** (for EDSS≥3: aHR 2.5, 95%CI 1.7-3.6, p<0.001; and for EDSS≥6: aHR 3.1, 95%CI 1.5-6.4, p=0.003).

In study **III**, we investigated the ability of ITMS to predict disability progression, both on its own and in combination with cNfL.[240] In a multivariable analysis, patients with IgM-index>0.1 at baseline had a significantly higher hazard of reaching EDSS≥3 (aHR 1.9, 95%CI 1.3-2.8, p<0.001). However, when investigating EDSS≥6 as an endpoint, IgM-index displayed only a borderline significant higher risk (aHR 2.1, 95%CI 1-4.4, p=0.05). Nevertheless, RRMS patients who had OCMB exhibited a significantly higher risk of attaining EDSS≥6 (aHR 2.5, 95% CI 1.2-5.4, p=0.01).

Subsequently, we investigated the combination of ITMS and cNfL at baseline. Patients with increased cNfL levels and IgM-index>0.1 exhibited the highest risk of reaching EDSS≥3 and EDSS≥6 (aHR 4.6, 95%CI 2.6-8.2, p<0.001 and aHR 8.2, 95%CI 2.3-30, p<0.001 respectively; Figure 9). In addition, patients with cNfL+/OCMB+ displayed a statistically significant result as well (aHR 7.4, 95%CI 2.3-24.4, p<0.001).

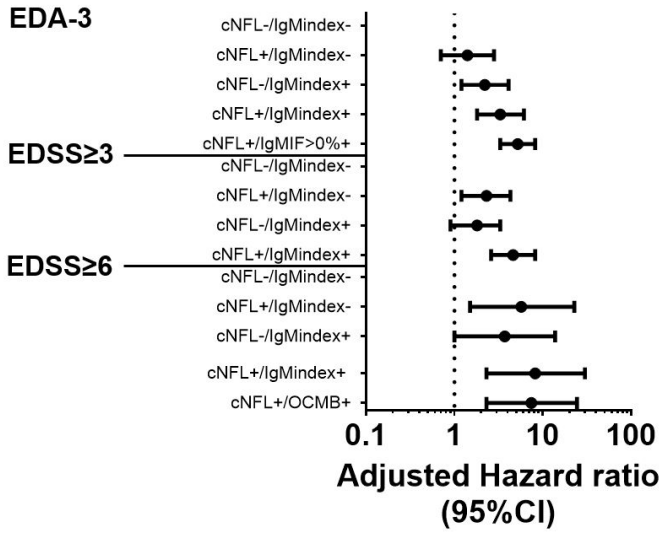

Figure 9. Combination of baseline cNfL and ITMS to predict EDA-3, EDSS≥3, and EDSS≥6. Forest plot with adjusted hazard ratios for risks of achieving EDA-3 status within 24 months, EDSS≥3, and EDSS≥6, stratified by combinatory possibilities of cNfL and ITMS. cNfL: cerebrospinal fluid neurofilament light; ITMS: intrathecal IgM synthesis; EDSS: expanded disability status scale; Ig: immunoglobulin; CI: confidence interval. Adapted from Rosenstein et al.[240]

4.2.3 High levels of KFLC-index predict cognitive impairment in RRMS

In study **IV**, we explored whether high levels of baseline KFLC-index may predict the SDMT score reduction at the time of follow-up.[263] The study population was dichotomized into patients with a high baseline KFLC-index (>100, N=31, 40%) and those with a low baseline KFLC-index (≤100, N=46, 60%). Eleven of 31 patients (35.5%) with a high baseline KFLC-index performed worse and therefore had a sustained reduction of the SDMT score by ≥8 points at the time of follow-up/censoring (mean time [years] ± SD, 6.5±2.5) compared with baseline (p=0.01).

In patients with a low baseline KFLC-index, 9 of 46 (19.6%) had a sustained reduction of the SDMT score (p=0.83). We found that KFLC-index>100 was strongly associated with a higher hazard of a sustained SDMT score reduction at follow-up (aHR 10.5, 95% CI 2.2–50.8, p=0.003) (Figure 10A). The median time to significant sustained SDMT reduction in patients with KFLC-index>100 was 7 years, compared with 9 years in patients with KFLC-index≤100. When testing KFLC-index as a log (2)-transformed continuous variable in univariable and multivariable models, we found a significant association with higher SDMT reduction hazard (HR 1.4, 95%CI 1.06–1.8, p=0.015; and aHR 1.4, 95%CI 1.02–1.9, p=0.037, respectively).

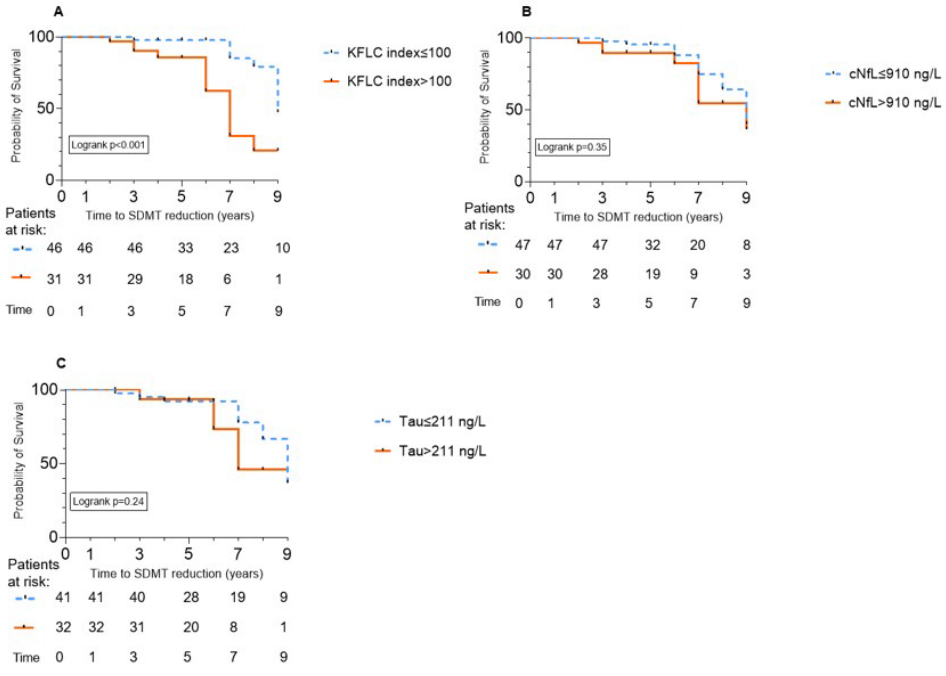

Figure 10. Kaplan-Meier survival curves indicating the time to information processing speed (IPS) worsening, as predicted by cerebrospinal fluid biomarkers in patients with relapsing-remitting multiple sclerosis. Adapted from Rosenstein et al.[263] *© 2023 Frontiers Media S.A. All rights reserved.*

4.2.4 CSF NfL and Tau did not predict SDMT worsening

In univariable and multivariable models, neither cNfL nor CSF Tau stratified by the 4th quintile (>910 ng/L for cNfL and >211 ng/L for CSF Tau) were significantly associated with a higher hazard of SDMT reduction at follow-up (Figures 10B and 10C).

4.3 Fluid biomarkers of disease activity

4.3.1 CSF NfL and clinical relapse

In study **I**, we investigated the association of cNfL with concurrent disease activity (i.e., within 90 days of clinical relapse onset) as well as radiological signs of inflammatory disease activity.[239] We found that patients with clinical relapse during sampling had 4.4 times higher median cNfL concentrations in comparison to patients without clinical relapse ($p<0.001$) (Figure 11A). When patients with concurrent relapse were divided into subgroups based on relapse phenotypes (ON, myelitis, infratentorial, supratentorial, and multi-focal relapses), cNfL levels were higher ($p<0.001$) among patients with all relapse phenotypes when compared to patients without concurrent clinical relapse (Figure 11B). An association between increasing cNfL concentrations with relapse severity was observed, with relatively lower levels with ON, and increasing through myelitis, infratentorial, and supratentorial relapses. The highest concentrations were observed in multifocal relapses.

4.3.2 CSF NfL and MRI

Subsequently, we investigated the association of cNfL with signs of inflammatory disease activity within MRI scans.[239] Patients who had contrast-enhancement lesions in MRI scans at the time of CSF sampling had 3.3 times higher ($p<0.001$) cNfL concentrations compared to patients with no MRI evidence of ongoing disease activity (Figure 11C), and cNfL increased alongside the number of contrast-enhancing lesions (Spearman's $\rho=0.523$, $p<0.001$, Figure 11D).

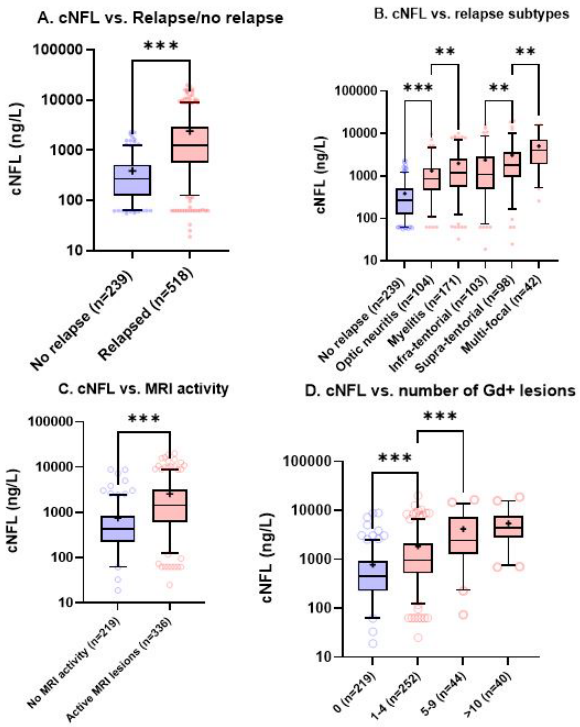

Figure 11. cNfL and concurrent inflammatory disease activity. A) cNfL levels in patients without concurrent relapse and those who were sampled at the time of a clinical relapse; B) distribution of cNfL across a spectrum of different relapse-types; C) cNfL in patients with or without MRI disease activity; D) cNfL in patients with different amounts of contrast-enhancing lesions on MRI. Box represents IQR. Bar indicates median, whereas + indicates mean.

*** <0.05 *** p<0.001; Adapted from Rosenstein et al.[239] © 2022 by SAGE Publications.*

4.3.3 Sensitivity and specificity of cNfL in detecting disease activity according to NEDA-3

Patients with EDA-3 at the time of sampling had five times higher cNfL levels compared to patients with NEDA-3 ($p<0.001$).[239] cNfL determinations stratified according to age-adjusted cut-off values demonstrated an overall sensitivity of 75% and a specificity of 98.5% for the detection of disease activity, or EDA-3. A cut-off value of 483.5 ng/L resulted in a sensitivity of

80% (95% CI 76.5-83.1) and a specificity of 80% (95% CI 74.2-85.1) in detecting EDA-3. A significant proportion of patients with ON (n=36, 35%) had normal levels of cNfL.

4.3.4 No correlation between KFLC-index and MRI

In study **II**, we demonstrated that KFLC-index did not significantly differ between RRMS patients who had contrast-enhancing lesions in MRI scans at the time of sampling compared with patients who did not exhibit signs of MRI activity.[179]

4.4 Fluid biomarkers and inflammatory treatment response

4.4.1 CSF NfL and treatment response

In a subgroup of patients in study **I** in which baseline and follow-up CSF samples were available, we compared cNfL concentrations in three groups as follows: patients who remained treatment-naïve, patients who received a first-line therapy, and patients who received treatment with a highly effective DMT (Figure 12).[239] Most baseline samples were obtained during a relapse (83.4%), whereas the majority of the follow-up samples were acquired in a stable phase (80%). The mean interval between LPs was 13.2 months (range 2-26).

In treatment-naïve patients who remained untreated at follow-up (n=43), the median baseline cNfL was essentially unchanged at the time of the follow-up (652 [IQR 346-1527] ng/L vs 523 [IQR 238-1894] ng/L, p=0.91). Follow-up cNfL (406.5 [IQR 250.5-648.5] ng/L) in patients who initiated a first-line therapy (n=44) was significantly lower (p<0.001) compared with the baseline (833 [IQR 518.5-1694] ng/L).

Patients who switched from a first-line (n=70) to a second-line therapy demonstrated marked reductions in cNfL (1554 [IQR 697.8-3182] ng/L vs. 328.5 [IQR 239.5-545.8] ng/L, p<0.001). Patients who switched to second-line treatment had significantly higher baseline cNfL than treatment-naïve patients (p=0.001) or those who received first-line therapy (p=0.04). No significant differences in cNfL levels between these treatment groups were observed at the time of follow-up.

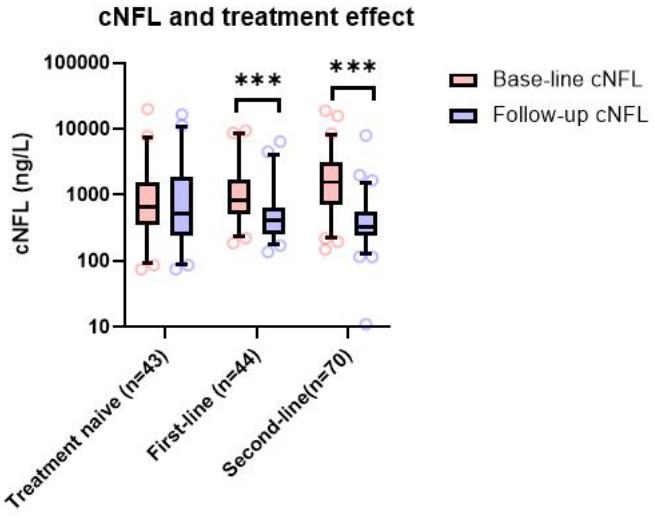

Figure 12. cNfL levels at baseline and follow-up in patients with RRMS who remained untreated, received a first-line treatment, or switched to a second-line therapy. The median bar indicates median; + indicates mean; and box indicates IQR. Adapted from Rosenstein et al.[239] © 2022 by SAGE Publications.

**** P<0.001*

4.4.2 No effect on KFLC-index from disease-modifying treatment

In study **II**, we performed a subgroup analysis to assess the effect of DMT on KFLC-index levels. Based on an analysis before and after 12 (fingolimod) or 24 (alemtuzumab) months after treatment, RRMS patients treated with fingolimod (n=20) or alemtuzumab (n=15) did not demonstrate any difference in terms of the KFLC-index levels.[179]

5 DISCUSSION

This doctoral thesis is comprised of four studies that all focus on commonly used CSF biomarkers that have long been components of the diagnostic clinical routine investigation of MS at the Sahlgrenska University Hospital in Gothenburg, Sweden. All of the material and real-world data included in these studies were collected during routine diagnostic investigations and follow-ups with MS patients in clinical practice. We aimed to investigate and map the clinical utility of these biomarkers according to their diagnostic, prognostic, and disease activity properties.

5.1 Intrathecal KFLC synthesis as a diagnostic biomarker in MS

The only biomarker investigated within the scope of this thesis with clear diagnostic properties in MS is intrathecal KFLC synthesis. In study **II**, we confirmed KFLC-index as an exceedingly valuable diagnostic biomarker in MS. In agreement with previous studies, our data imply that the sensitivity of KFLC-index to distinguish CIS/RIS/MS patients from controls is higher than IgG-OCBs and the specificity is similar.[186,188,191,264-268] Moreover, we validate the high diagnostic accuracy of KFLC-index to predict intrathecal immunoglobulin synthesis via IEF[269] as well as the contribution of KFLC-index to the identification of OCB-negative MS patients.[270]

The qualitative assessment of IgG-OCBs via IEF has long been the gold standard for estimating intrathecal IgG synthesis.[180] This method has recently gained increasing importance since its reincorporation into the 2017 revised McDonald criteria to potentially fulfill the criterion for DIT.[5] However, qualitative assessments have clear pitfalls, as they are nominal, labor-intensive,

and rater-dependent. In light of this, intrathecal KFLC synthesis offers an appealing quantitative alternative to qualitative assessments. Arguably, its main advantages relative to qualitative methods are that it is metric, fast, and easy to perform, and rater-independent, with high diagnostic sensitivity, high reproducibility, and greater cost-effectiveness.

Intrathecal kappa free light chain synthesis is the result of activated mature B lymphocytes producing intact immunoglobulins and excreting an excess of free kappa chains.[186] In that sense, and in contrast to IgG-OCBs or the IgG-index, intrathecal KFLC synthesis reflects the total sum of immunoglobulin production in the CNS, regardless of the isotype. This might partly account for the high sensitivity of KFLC-index in MS.

The cut-off KFLC-index>4.6 used in our analysis was the result of an ROC analysis and a calculation with the Youden index. It yielded a sensitivity of 0.93 and a specificity of 0.87 to discriminate CIS/RIS/MS from controls. The sensitivity was higher than IgG-OCBs, and the specificity was slightly lower. Notably, specificity largely depends on the cohorts' demographic and clinical characteristics.

In a country such as Sweden, with a relatively high prevalence of Lyme disease, neurological Lyme disease being an important differential diagnosis to intrathecal immunoglobulin synthesis, the specificity of KFLC-index (and IgG-OCBs) might be reduced in comparison with other regions where Lyme disease is less prevalent. Our real-world data thus reflect the clinical reality in our specific region. This highlights the need for further validating studies in different geographical populations.

Several previous studies have demonstrated that cut-offs of >5.0 discriminate MS from controls.[186,270-274] For example, one of the earliest cut-offs suggested was >5.9.[186] Thereafter, a large multi-centre study including 745 patients from 18 centres across Europe used Gaussian mixture modelling to define the cut-off for KFLC-index as >6.6.[267] More recently, the different KFLC cut-offs proposed in the literature were specifically assessed in the context of the current 2017 McDonald criteria.[190] The authors compared the proportion of patients with CIS and concordant IgG-OCBs, KFLC-index ≥5.9,[186] KFLC-index ≥6.6,[267] KFLC-index ≥10.61,[275] as well as the IgG-index.

The authors determined that overall, KFLC-index>5.9 and >6.6 performed similarly to OCBs but with a slightly higher accuracy.[190] This finding was recently further validated in a large multi-centre French study.[276] A recent systematic review and meta-analysis summarized data from 32 studies regarding the diagnostic properties of intrathecal KFLC synthesis.[277] The authors proposed a cut-off of 6.1 as the most discriminatory value. Moreover, a study focusing on diagnostic fluid biomarkers in patients with RIS recently demonstrated the ability of KFLC-index to differentiate MS from other differential diagnoses in patients presenting with white-matter changes in MRI scans.[278] Thus, we and others conclude that KFLC-index should be considered in the next revision of the McDonald criteria as an option for DIT.

A quantitative assessment of intrathecal immunoglobulins or free light chains may be performed with different quantitative mathematical, or qualitative methods (Table 8). In our analysis, we used the most commonly used method, namely the linear index formula. However, concerns have arisen regarding a potential limitation to this method, specifically that it may not reflect the true nature of the relationship between CSF/serum ratios of KFLC and albumin accurately, as this relationship might be hyperbolic as opposed to linear. The

evaluation of $KFLC_{IF}$ according to Reiber's formula[192] has the benefit of high sensitivity in detecting intrathecal Ig production.

In addition, this formula accounts for the difference in molecular sizes between the free kappa chain and albumin using a nonlinear function of KFLC diffusion into the CSF relative to the Qalb. Although $KFLC_{IF}$ in our analysis exhibited somewhat higher sensitivity than KFLC-index in identifying MS, it had lower specificity, and the overall diagnostic accuracy (AUC) was indistinguishable. Other studies have confirmed this as well.[191,279,280] For example, another study investigated and compared the diagnostic ability of CSF KFLC concentrations, the KFLC ratio, KFLC-index, and $KFLC_{IF}$.[279] Patients were dichotomized into low and high CSF KFLC subgroups based on median values. Methods and formulas adjusting for albumin demonstrated the highest diagnostic accuracy, predominantly in patients with lower intrathecal KFLC synthesis, and both KFLC-index and $KFLC_{IF}$ demonstrated similar diagnostic accuracy.

These results imply that in patients with high intrathecal KFLC synthesis, the influence of serum KFLC and Qalb is insignificant, but most likely not in patients with low intrathecal KFLC synthesis. To facilitate and enhance further research into the potential role of intrathecal KFLC synthesis, it will be important to achieve a consensus regarding the analytic method of choice. Thus, it is likely the KFLC-index will eventually be established as the gold standard for the quantitative assessment of intrathecal KFLC synthesis, and international efforts to establish a consensus are ongoing.

Table 8. A comparison of the most commonly used methods to estimate intrathecal immunoglobulin and free light chain synthesis and their advantages/disadvantages

Method	Advantages	Disadvantages
Linear index formula (quantitative)[281]	• Metric • High diagnostic sensitivity • Easy and fast to quantify • Rater-independent • Simple formula • Has not been shown to be inferior to other methods in terms of predicting clinically important prognostic outcomes • High reproducibility	• Concerns regarding reduced sensitivity at very high and very low CSF/serum ratios
Non-linear functions (quantitative: Reiber formula[248] Auer-Hegen formula[282])	• Metric • Easy and fast to quantify • Rater-independent • High sensitivity to detect intrathecal immunoglobulin synthesis • High reproducibility	• Moderate diagnostic accuracy • Lower specificity • Complicated formula (Reiber)
Isoelectric focusing (IgG) Gel electrophoresis and immunoblotting (IgM) (Qualitative)	• Most well-studied • Formally included in diagnostic criteria • High diagnostic sensitivity	• Nominal • Labour-intensive • Time-consuming • Rater-dependent

Abbreviations: CSF= cerebrospinal fluid; Ig= immunoglobulin.

5.2 Prognostic biomarkers

All three CSF biomarkers included in this thesis confer important prognostic information. In study **I**, we showed that age-adjusted increased concentrations of cNfL at diagnosis predicted an increased hazard of achieving the disability milestones EDSS≥3 and SPMS at the time of follow-up. Only a few studies have previously shown the predictive ability of cNfL in terms of disability progression.[164,283,284] The survival analysis in study **I** was limited by the lack of adjustment for other confounding factors, such as DMTs and MRI measures. Therefore, we conducted a more detailed analysis validating this finding in study **III**, in which we performed a multivariable cox regression adjusted for clinical and MRI variables, as well as exposure to DMTs, and obtained comparable results.

cNfL is a marker for neuroaxonal injury, a pathological process that characterizes many acute and chronic neurological conditions, including MS, and which is linked to permanent neurological disability. In that regard, neuroaxonal degeneration, often captured by high levels of cNfL, appears to be the most important factor in terms of influencing disability worsening.[285,286] The main conclusion that can be drawn from our findings above is that high levels of cNfL as early as the first demyelinating event reflect more extensive early neurodegeneration, which in turn increases the risk of worsening neurological function as time transpires. This initial neurodegeneration might later interact with normal aging processes, thereby leading to lower thresholds in the CNS of developing clinically overt neurological disability. Thus, similarly to other studies,[26-29] we have demonstrated that the extensive neuroaxonal loss that may occur early in the course of the disease is inextricably linked to chronic neurodegeneration and future disability worsening.

Due to the invasive nature of LP, cNfL might be less suitable for serial measurements to follow longitudinal disease activity in treated patients. However, it is well-suited as part of the diagnostic investigation in patients presenting with suspected MS, as it conveys important prognostic information.

In that regard, several studies have highlighted the potential of baseline and serial serum/plasma NfL measurements in tracking disability worsening in people with RRMS[220,225,287] and recently even progressive MS.[288] However, some other studies did not find longitudinal serum NfL measurements useful in tracking disability worsening in RRMS.[289]

In study **III**, quantitative and qualitative assessments of ITMS emerged as the ideal predictors of EDA-3 status within two years of the first demyelinating event. This ability of ITMS to predict future disease activity in CIS/early RRMS is well-known.[157,159,161,199-204] In our analysis, the agreement between the two quantitative methods, namely the index and the Reiber formulas, was acceptable and could even be further improved by the minimal adjustment of the IgM-index cut-off value (from 0.1 to 0.18).

However, the agreement between the quantitative methods with qualitative assessment was weak. This may depend on the technical and methodological difficulties associated with qualitative assessment. Nonetheless, judging by the predictive performance of the different methods regarding prognostic outcomes in our analyses, it is not clear which method is superior. Other studies have compared the different quantitative formulas and did not find any significant differences.[290,291]

The key finding in study **III** is that combining two robust prognostic biomarkers, such as cNfL and ITMS, with seemingly different pathophysiological implications doubles the prognostic value obtained by measuring these biomarkers separately. Since LP is often a part of the routine

clinical investigation in MS, and in light of our results, the determination of these biomarkers simultaneously makes clinical sense.

ITMS has recently emerged as a reliable disease severity marker in MS, and it appears to be useful in predicting the risk of future inflammatory disease activity, as well as clinical disability worsening. Contrary to the process of immunoglobulin class switching typically observed in the periphery, in which B cells initially secrete IgM and thereafter transition into IgG secretion, ITMS often persists in the CSF of MS patients. This phenomenon might be dependent on high degrees of somatic hypermutation in IgM-producing B cells in the CNS.[196]

OCMB is often comprised of immunoglobulins directed against myelin lipids.[210] This could mean that IgM may be involved in the pathogenesis of progression and influences degeneration. Due to its pentameric structure, IgM is the most effective immunoglobulin isoform with regards to complement binding,[292] and extensive complement activation may result in more severe tissue injuries.[293] Therefore, the presence of ITMS in the immune-mediated attack on myelin and axons may contribute to disability worsening.

The prognostic ability of increased NfL levels to predict the conversion from CIS or RIS to MS has been extensively studied and demonstrated.[166,217,294,295] CSF NfL has been shown to predict shorter time to CIS and CDMS conversion from RIS.[166] In our analyses, cNfL seemed to be the optimal predictor of disability progression, but compared to ITMS, it was less useful in predicting EDA-3 within two years, although the results were nevertheless significant.

Another important observation in studies **I** and **III** is the relatively low proportion of patients developing SPMS (n=55/754 [~7%]) or reaching EDSS≥6 (n=37/457 [~8%]). The observation period in both these studies is 2001-2018, a period during which major developments in effective DMTs

were made. A large proportion of patients have initiated highly efficacious DMTs already after the first demyelinating event, and many more switched during the follow-up. This highlights the major impact of highly efficacious DMTs early in the disease course on disability worsening and progression.

Intrathecal KFLC synthesis has, as mentioned above, been previously established as an excellent diagnostic biomarker in MS. In recent years, several studies have explored its prognostic properties, predominantly regarding disease activity and conversion from CIS to CDMS[162,190,259,275,296-298] and disability worsening.[163,297]

In study **IV**, we explored the ability of high levels of KFLC-index at the time of RRMS diagnosis to predict future CI as determined with serial SDMTs. We demonstrated that patients with KFLC-index>100 had a considerably higher hazard of SDMT reduction ≥8 points at the time of follow-up. Earlier studies have demonstrated that IgG-OCBs may be associated with cognitive decline,[299,300] but the ability of KFLC-index to predict decline in cognition has never been studied. High levels of KFLC-index are known to correlate with and predict the presence of IgG-OCBs.[179] Our data thus emphasise the notion that there is an association between early and extensive intrathecal immunoglobulin production and the risk of reduced IPS.

To determine the appropriate cut-off value for prognostic purposes, we opted to calculate the 4th quintile for KFLC-index in our cohort. The cut-off value of KFLC-index>100 was thereafter selected for our investigation. This method to calculate the most appropriate cut-off value for prognostic purposes was used in a previous study[162] that investigated the ability of high levels of KFLC-index to predict early disease activity. In another recent study, KFLC-index>58 was demonstrated to predict the risk of achieving EDSS≥3 and for escalating therapy to highly effective DMT.[163] However, the authors of that study opted

to stratify their cohort according to the most discriminative KFLC-index. Other studies investigating the prognostic value of intrathecal KFLC synthesis have utilized even lower cut-off values (>5.9, >6.6, and >10.6) for the prediction of future disease activity.[190,275,301] In all of the above cases, as well as in our own work, it is clear that high levels of KFLC-index at diagnosis are indicative of poorer prognosis.

Theoretically, high levels of intrathecal KFLC production may reflect an early and more prominent immune activation of mature B lymphocytes, hence resulting in a more pronounced tissue damage. It has been recently demonstrated that CXCL13 could differentiate MS patients with severe CI from those with mild CI or apparently cognitively normal profile.[302] Both CI and CXCL13 are related to gray matter damage,[303,304] and several studies have previously demonstrated an association of CXCL13 with signs of antibody production in the CSF.[170,305] Therefore, an association might be drawn between extensive B cell activation, intrathecal KFLC synthesis, and cognitive decline.

Accumulating evidence indicates that some highly efficacious DMTs may be useful in improving cognitive functions and/or preventing cognitive deterioration in RRMS,[112,306] and cognitive domains are now often used as secondary outcome measures in clinical trials.[112] Furthermore, SDMT is broadly considered to be exceptionally sensitive to detect changes in IPS in people with MS.[307] Therefore, we conclude that fluid biomarkers such as KFLC-index may play an important role for treatment decisions as well as for evaluating eligible patients for future clinical trials.

5.3 NfL as a disease activity biomarker

Since the first description of NfLs' clinical utility in MS,[212] its role as a sensitive biomarker reflecting disease activity in MS has been increasingly replicated and established.[211,216,218,219,229,308] This ability of cNfL to reflect ongoing disease activity in RRMS has been thoroughly investigated in study **I**. We validated cNfL as a biomarker for disease activity in RRMS[212,213,216,229,309] and treatment response[214,219,224] in a large real-world cohort of patients followed up in a single MS center for many years.

The key findings of this study regarding MS disease activity are as follows: 1) Baseline cNfL is increased across a range of clinical relapse phenotypes, with the lowest in optic neuritis and the highest in multifocal relapses; 2) cNfL concentrations are higher in patients with MRI activity, and cNfL increases with the growing number of contrast-enhancing lesions; 3) high cNfL concentrations are overall effective predictors of patients experiencing EDA-3 and may add additional information in patients with no signs of disease activity on MRI; and 4) cNfL concentrations are reduced to normal levels after treatment with effective DMTs.

The major advantages of NfL are that it can be easily and objectively measured and quantified; it is highly sensitive to neuroaxonal damage, and its concentration changes with worsening or improvement of the disease; all properties that make it an effective biomarker for neurodegenerative conditions, including MS. The fact that cNfL is significantly reduced by treatment with DMTs[214,224,309-311] has established it as a valuable outcome measure in clinical trials. However, NfL is known to have low diagnostic specificity because many other neurodegenerative conditions cause changes in its concentration.[312,313]

In addition, its use in the past has been limited by the need for repeated LPs. The emergence of ultrasensitive immunoassays allowed measurements of very low NfL concentrations in blood,[215,218,219] rendering NfL a potential biomarker for clinical practice. While measurements of NfL in CSF appear to be more precise than in blood,[165] the research focus in recent years has nevertheless shifted towards investigations of the clinical utility of bNfL, measured in serum or plasma. However, the application of bNfL on an individual basis in clinical practice has been limited by the growing evidence of it being affected by confounding factors, such as age,[314,315] peripheral nerve disease,[316,317] blood volume and body mass index (BMI),[318] and possibly even renal function.[319,320] Notably, BMI and blood volume do not appear to influence cNfL.[321]

Most importantly, a consensus regarding standardized normal cut-off values for bNfL that can be applied on an individual basis has yet to be achieved. Studies contributing to the growing body of literature on bNfL have utilized a broad variety of optimal cut-off values. Some research groups have promoted age-adjusted reference limits based on neurologically healthy individuals (upper 95th percentile in each arbitrary age category),[315] while others have focused on establishing standardized z-scores based on age and BMI.[322] However, these approaches are limited by intra-individual variability,[323,324] particularly among older age groups, prompting some to advocate longitudinal measurements rather than absolute cut-off values.[325-328]

While research concerning the use of NfL in CSF has been somewhat obscured by the growing interest in blood measurements, we still anticipate a clear role for determinations of cNfL in clinical practice, especially during the diagnostic LP together with other CSF biomarkers. Based on our data, we strongly believe that cNfL levels at diagnosis convey important prognostic information that should be taken into consideration when making treatment decisions.

5.4 Strengths and limitations

5.4.1 Study design

In this doctoral thesis, we retrospectively identified and analyzed real-world data obtained during routine clinical investigations and explored the clinical utility of CSF biomarkers in clinical practice. The main purpose of this study design was to assess how effectively the investigated biomarkers perform in the setting of a clinical reality. The biomarkers included in this project have all been incorporated into the diagnostic routine CSF sampling at our centre for many years. In that sense, our studies serve as a quality control for routinely used biomarkers and their clinical utility, analyzed at the Neurochemistry Laboratory at the Sahlgrenska University Hospital.

A retrospective study design is known to bear the risk of introducing selection bias. While this may influence cNfL and KFLC-index determinations for treatment response, it is less likely to concern evaluations of cNfL, KFLC-index, and ITMS predictive value, since the determination of these biomarkers has been incorporated into the diagnostic work-up and lab-routine at Sahlgrenska University Hospital for many years.

In addition, the retrospective design implies that the timing of MRI scans and clinical scoring was not wholly consistent between all study participants. Some patients had their MRI scans before 3 Tesla MRI was widely available. Over the years, multiple neurologists and radiologists have contributed to the clinical assessment of patients and reviewing MRI scans. The high inter-rater variability for EDSS is well known,[255] and the higher risk related to numerous evaluators most likely also influences classifying relapses in addition to the evaluation of MRI lesions.

Despite these limitations, we were still able to find meaningful associations between the investigated biomarkers and clinically meaningful disease outcomes. Our data thus support the high quality of the routine clinical assessments, as well as the usefulness of the biomarkers evaluated.
In addition, the cohorts in our studies were large in comparison with other monocentric studies, and the follow-up time was relatively long.

In study **III**, a qualitative analysis of OCMB was not available for all included patients, limiting the assessment of agreement with the quantitative analyses. Moreover, determination of LS-OCMB, which might be an even stronger prognostic biomarker, was not available at our laboratory.

In study **II**, we managed to gather data only regarding a limited number of ONID (n=29). As these conditions often are the critical differential diagnoses of MS and may be an important source of intrathecal KFLC synthesis, this could influence sensitivity and specificity calculations. However, this was a consequence of using real-world data obtained from diagnostic investigations.

5.4.2 Normalization between different cNfL immunoassays

As mentioned in section 3.2.1, two different immunoassays were used during the study periods to analyze cNfL. To be able to study cNfL in the whole cohort, we opted to correct for the differences between the assays calculating the ratio of means between the second method and its predecessor for the purpose of normalization. We then calculated a factor by which we could multiply cNfL values from the older assays and harmonize values across both methods.

This method of normalization was previously utilized in a study that focused on NfL in dementias.[245] The advantage of this method is that it allowed us to study a large, unselected cohort in an unbiased manner and compare cNfL

values measured during a period of 18 years. The normalization method is certainly an elegant solution for this problem, but it is not perfect, and it can never be as effective as using the same immunoassay for all measurements.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

The study of the clinical utility of fluid biomarkers in MS has been rapidly growing. Many biomarkers, such as some of the ones studied in this thesis, are transitioning from the validation phase into being established in clinical MS care. The results presented in this thesis reflect the clinical usefulness of cNfL, KFLC-index, and ITMS, analyzed as part of the diagnostic investigation. Therefore, we believe that determining these biomarkers in CSF as part of the diagnostic routine contributes important and useful information that MS clinicians can take into consideration when making decisions about treatment and follow-up.

However, the need to continue validating known biomarker candidates or to discover new promising ones is still not fully met. The newly developed technologies that recently made it possible to analyze NfL in blood have resulted in channelling much of the research focus into this particular attractive avenue. However, before clinical decisions based on individual bNfL concentrations can be made, normal reference values that can be applied on an individual-patient basis remain to be established.

To that end, various confounders, such as age, BMI, and other comorbidities, have to be taken into consideration. In addition, it remains to be determined when and how often bNfL should be sampled to evaluate subclinical disease. Some experts have advocated individual longitudinal measurements, but a clear threshold that reflects clinically meaningful increase in bNfL levels has yet to be defined.

Accordingly, the field will undoubtedly continue to evolve in this direction as international efforts are ongoing, first addressing the aforementioned challenges that still prevent bNfL from being widely used in patient care, and later continuing with the development of other blood-based biomarkers. Blood GFAP, a biomarker of astrogliosis, has shown some promise in this regard,[329,330] although the results to date have been somewhat less convincing.

Our finding regarding the additive predictive value obtained from combining two different biomarkers (**III**) must be validated in further independent cohorts. Other studies have explored combinatory alternatives as well,[208] and there is certainly potential in further exploring similar combinations.

It has recently been recognized that a substantial number of patients with RRMS experience disability worsening (progression) independent of relapse activity (PIRA) in contrast with relapse-associated worsening, even in the setting of high-efficacy DMTs.[79] Therefore, it will be important in the future to identify biomarkers that can early on predict the risk of such worsening. Moreover, PPMS is a relatively poorly understood and studied subtype of MS and biomarkers that enable the correct identification and monitoring of progressive MS are still largely lacking. Therefore, there is an unmet need to continue exploring molecules that may add vital information in that regard.

In study **IV,** we investigated the association of intrathecal KFLC synthesis with cognitive decline. It is of high interest to validate these findings and to further explore whether KFLC synthesis and/or other measures of intrathecal immunoglobulin synthesis are associated with other aspects of MS-related CI in addition to IPS. Moreover, further investigations into the prognostic ability of other CSF and blood biomarkers to predict future CI are warranted. In particular, the interaction of intrathecal immunoglobulin synthesis with other

biomarkers reflecting B cell activation, such as CXCL13, and the association of such interaction with cognitive function in MS should be further studied.

MS-related cognitive decline has thus far been difficult to capture and quantify. SDMT is admittedly the most reliable and sensitive measure of cognition in MS, but limitations with regards to improved performance with repeated exposure have been challenging. In that sense, digitally based neuropsychological testing batteries may have the potential to offer more standardized solutions for the longitudinal assessment of cognitive function in people with MS.[331] These adjunct tools may facilitate prospective data-gathering in conjunction with CSF and blood biobanks for the purpose of further exploring the prognostic value of fluid biomarkers in relation to CI.

Furthermore, MS-related CI, along with other disabling symptoms, such as motor disability, mental fatigue, disturbed sleep, and psychiatric comorbidity, are known to influence the working capacity of MS patients over time. Real-world data from the SMSreg, the Swedish Social Insurance Agency, and the Swedish Patient Register may be used to develop a neural network-based algorithm that, in conjunction with biomarkers and other measures, assists in predicting patients at risk for debilitating quality-of-life-lowering MS.

The incorporation of fluid biomarkers such as CXCL13 and CH13L1, as well as blood NfL and GFAP, in our clinical routine could further facilitate real-world data gathering. In addition, proteomic panel technologies[332] have made it possible to simultaneously analyze large amounts of candidate protein biomarkers in serum and CSF,[333] thus significantly enhancing the explorative discovery phase. These technologies are highly likely to result in discoveries of new biomarkers that may reflect different aspects of MS pathophysiology and that may add further clinically useful information.

These methods tend to generate large amounts of data, and it is highly plausible that in the future, deep neural networks will be incorporated into data analysis processes. Other technologies, such as high-resolution single-cell RNA sequencing and flow cytometry, make it possible to discriminate myeloid cell types in the CSF,[334] and circulating microRNAs are showing promise in MS diagnostics, although it remains unclear which specific microRNAs should be used, especially for the diagnosis of RRMS.[335]

Epilogue

The field of fluid biomarker research continues to grow and develop and will certainly do so in the near- and long-term future. Over the years, the MS unit at the Sahlgrenska University Hospital, in cooperation with the Neurochemistry Laboratory at Mölndal, has played a pivotal role in MS fluid biomarker research and development. It was here at the Sahlgrenska University Hospital that the first steps towards the clinical use of cNfL were made in the 1990s, and with it the establishment of routine biomarker measurements as part of the diagnostic work-up.

On a personal note, it was therefore highly meaningful for me as a doctoral student to partake in this exciting research field and to be able to contribute to the growing body of knowledge on fluid biomarkers in MS. The findings included in this thesis are by no means revolutionary. Rather, they are the result of meticulous data gathering, analysis, and processing for the main purpose of validating the utility of commonly-used fluid biomarkers in routine MS care. As a doctoral student, I was humbled by the research field I was attempting to contribute to and by the amount of knowledge I still am not in full control of.

I believe it will be evident for anyone who reads the articles on which this thesis is based that the quality of the work has gradually improved with every published study. Many would argue that precisely that is the main purpose of doctoral studies. If anything, I hope I could at least achieve continuous improvement, and I could only hope for further development as a researcher in the future.

Many challenges remain and will continue preoccupying MS researchers for years to come. The results in this thesis are only a small drop in the expansive sea of exciting data that are continuously emerging, but hopefully, they will mean that we as MS clinicians can use cNfL, KFLC-index, and ITMS in a more informed, objective, and clinically meaningful manner to provide our patients with a higher quality of care.

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9 APPENDIX

1. Correction to Table 1, section “treatment response”, from study **I.**

1. Table 1, Study I. Demographic and clinical characteristics population.

Demographic data	Patients (n=757)
Gender, number (%) Female Male	 517 (68.3%) 240 (31.7%)
Mean age, years (range)	36.5 (8-74)
Mean follow-up time, years (range)	8 (2-17)
Time from onset to diagnostic lumbar puncture, months (range)	38.2 (0-473.2)
Disability	Patients (n=754)
Mean baseline EDSS (range)	1.9 (0-8)
Mean EDSS at last visit (range)	2.1 (0-8)
MRI activity	Patients (n=555)
Days between LP and MRI, mean (range)	9.2 (0-42)
MRI brain+spinal cord/brain	296/259
Relapse	Patients (n=757)
Relapse/no relapse	518/239
Type of relapse % No relapse Optic neuritis Myelitis Infratentorial Supratentorial multi-focal	 31.6 13.7 22.6 13.6 12.9 5.5
Treatment response	Patients (n=157)
No DMT, n First line DMT Interferon-β Glatiramer acetate Teriflunomide Dimethyl fumarate Second/third line DMT Natalizumab Fingolimod Rituximab Alemtuzumab	43 44 11 4 9 20 70 49 10 5 6
Abbreviations: EDSS=expanded disability status score; LP= lumbar puncture; DMT= disease modifying therapy	