

Biomarkers in Blood and Cerebrospinal Fluid for Monitoring and Differentiating Demyelinating Inflammatory Central Nervous System Disorders

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“In medicine, certainty is rare; responsibility lies in knowing how uncertain we are.” — Paraphrased from Sir William Osler

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ABSTRACT

Inflammatory demyelinating disorders of the central nervous system (CNS) are characterized by immune-mediated myelin injury and neuroaxonal damage. This thesis focused on multiple sclerosis (MS), neuromyelitis optica spectrum disorder (NMOSD), and myelin oligodendrocyte glycoprotein antibody-associated disease (MOGAD), which share clinical features but differ in immunopathology, prognosis, and treatment response. The aim was to evaluate soluble cerebrospinal fluid (CSF) and serum biomarkers reflecting neuroaxonal and astrocytic injury, blood-brain barrier dysfunction, and innate immune activation across these disorders.

The first two studies investigated serum neurofilament light (sNfL) concentrations in patients with relapsing-remitting MS (RRMS). In a prospective cohort (**Paper I**) of clinically stable patients switching from standard to extended-interval natalizumab dosing (n = 45), sNfL concentrations remained stable over 12 months, supporting maintained therapeutic efficacy without evidence of increased axonal injury. In a second prospective study (**Paper II**) including patients with active disease (n = 44), repeated sNfL measurements demonstrated moderate sensitivity and specificity for detecting inflammatory disease activity at the individual level,

supporting its role as a complementary monitoring tool. In **Paper III**, soluble biomarker data from patients with NMOSD and MOGAD were retrospectively retrieved from medical records. CSF glial fibrillary acidic protein (GFAP), particularly when combined with albumin quotient, robustly discriminated AQP4-IgG-positive NMOSD from the combined MS, seronegative NMOSD, and MOGAD groups. In **Paper IV**, a newly developed method for quantifying the intermediate filament protein alpha-internexin in CSF was applied, demonstrating elevated concentrations in MS and a strong correlation with NfL, supporting its role as a marker of axonal injury. In **Paper V**, elevated serum calprotectin distinguished NMOSD and MOGAD from RRMS, indicating distinct patterns of innate immune activation across these disorders.

In conclusion, biomarker profiles differ across inflammatory demyelinating disorders and reflect disease-specific pathobiology. Integrated biomarker assessment may improve diagnostic precision and individualized monitoring of disease activity.

Keywords: multiple sclerosis, neuromyelitis optica spectrum disorder, MOGAD, neurofilament light, calprotectin, alpha-internexin

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SAMMANFATTNING PÅ SVENSKA

Inflammatoriska neurologiska sjukdomar som drabbar hjärnan och ryggmärgen (centrala nervsystemet, CNS) kan ge allvarliga symtom med exempelvis känselbortfall och svaghet, och yttrar sig ofta i skov. Sjukdomar med inflammation och skada på myelin, det fett som omger, skyddar, och ger normal nervsignalering i nervtrådarna (axon), kallas *demyeliniserande* sjukdomar. Multipel skleros (MS) är den vanligaste inflammatoriska demyeliniserande neurologiska sjukdomen i CNS och drabbar ca 2 av 1000 personer i Sverige. De flesta av dessa insjuknar med skovvist förlöpande MS.

Två andra sjukdomar i samma grupp är neuromyelitis optica spektrum sjukdom (NMOSD) och myelin oligodendrocyt glykoprotein antikroppsassocierad sjukdom (MOGAD). Patienter med NMOSD har oftast en antikropp mot aquaporin-4 (AQP4), och patienter med MOGAD har en antikropp mot MOG. NMOSD och MOGAD är inte lika vanliga som MS, men skov i dessa sjukdomar kan ge svåra neurologiska bortfall.

Eftersom det utvecklats sjukdomsmodifierande behandlingar (disease modifying therapies, DMT), med olika effektivitet beroende på specifik sjukdom, så är det viktigt att snabbt kunna ställa rätt diagnos, och övervaka sjukdomsaktiviteten för att säkerställa stabilt tillstånd. Överlappande symtombild och utseende på magnetkameraundersökningar är en utmaning i detta avseende, men biomarkörer (i denna avhandling innefattande olika mätbara proteiner i cerebrospinalvätska (CSV) och blod) kan bidra till bättre precision. Syftet med avhandlingen var att utvärdera biomarkörer i CSV och blod vid dessa tre vanligaste inflammatoriska demyeliniserande sjukdomarna i CNS.

I delarbete I följdes totalt 70 patienter med skovvis MS under ett år när deras behandling med intravenöst natalizumab utvärderades med hjälp av mätning i blod av proteinet neurofilament light (NfL), en biomarkör för axonal skada. I denna grupp gjorde 45 patienter en utglesning av sin behandling från standardintervall var fjärde vecka till var sjätte vecka, medan de övriga (n = 25) redan hade utglesad behandling. I studien visade vi med regelbundna mätningar att nivåerna av NfL i blod låg stabilt utan tecken på förändringar över tid. Vi såg således inga tecken på ökad axonal nervskada hos patienter med skovvis MS som glesade ut sin behandling med natalizumab.

I delarbete **II** följdes 44 patienter med skovvis MS i samband med att de hade tecken på sjukdomsaktivitet (skov och/eller radiologiska tecken på aktivitet). De följdes under ett år med regelbundna mätningar av NfL i blod. Vi visade att nivåer av NfL initialt ökade, med högsta nivå efter 5,5 veckor från ett kliniskt skov, för att sedan gradvis normaliseras inom loppet av ett år. Vi jämförde nivåer av NfL med de koncentrationer som sågs hos stabila patienter i delarbete I, och med statistiska uträkningar kunde vi få fram gränsvärden för att bedöma sannolikheten att ett visst värde indikerade sjukdomsaktivitet. Vi kunde visa att ungefär fyra av fem patienter med sjukdomsaktivitet hade tecken på detta utifrån nivån på NfL, och att träffsäkerheten var bäst vid användning av NfL-nivåer som justerades för störfaktorer i form av ålder och body-mass-index (BMI).

I delarbete **III** inkluderades förutom patienter med MS (n = 33) även patienter med NMOSD (n = 26) och MOGAD (n = 29). Detta var en retrospektiv studie där vi identifierade patienter genom en diagnossökning för åren 2010 - 2022 i journalsystemet för Västra Götalands Region (VGR). Genom journalgranskning inhämtades data från laboratorieanalyser utförda i samband med utredningar, och nivåer av glial fibrillary acidic protein (GFAP), en markör för skada på stödjeceller (astrocyter), och albumin i CSV analyserades. I studien kunde vi visa att nivåer av GFAP var kraftigt förhöjda bland patienter med AQP4-antikropps-positiv NMOSD. Förhöjda nivåer av GFAP tillsammans med förhöjd nivå av albumin i CSV hade en hög förmåga att särskilja denna patientgrupp gentemot övriga diagnosgrupper.

I delarbete **IV** utforskades nivåer av biomarkören alfa-internexin (AINX) hos patienter med MS (n = 34). AINX är liksom NfL en biomarkör för axonal nervskada, men har inte tidigare kunnat nivåbestämmas på ett tillförlitligt sätt i CSV. Med hjälp av en nyutvecklad metod från Neurokemiska laboriet i Mölndal, kunde vi visa att nivåer av detta protein var förhöjda hos patienter med MS jämfört med friska kontrollpersoner, och att nivåerna korrelerade starkt med nivåerna av NfL.

Slutligen, i delarbete **V** mättes nivåer av kalprotektin, en biomarkör kopplad till det medfödda immunförsvaret, i CSV och blod hos patienter med MS, NMOSD och MOGAD. Kalprotektin var tydligt förhöjt i blod hos patienter med NMOSD och MOGAD jämfört med kontrollpersoner och patienter med MS, och indikerar aktivitet i det medfödda immunförsvaret hos dessa patienter.

LIST OF PAPERS

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

- I. Johnsson M, Farman HH, Blennow K, Zetterberg H, Malmeström C, Axelsson M and Lycke J. No increase of serum neurofilament light in relapsing-remitting multiple sclerosis patients switching from standard to extended-interval dosing of natalizumab. *Mult Scler* 2022 Vol. 28 Issue 13 Pages 2070-2080
- II. Johnsson M*, Stenberg YT*, Farman HH, Blennow K, Zetterberg H, Malmeström C, Sandgren S, Rosenstein I, Lycke J, Axelsson M and Novakova L. Serum neurofilament light for detecting disease activity in individual patients in multiple sclerosis: A 48-week prospective single-center study. *Mult Scler* 2024 Vol. 30 Issue 6 Pages 664-673
- III. Johnsson M, Eriksson K, Rosenstein I, Novakova L, Malmeström C, Lycke J, Sandgren S, Zetterberg H, Blennow K, Johansson K, Axelsson M. The value of CSF diagnostic and prognostic biomarkers in NMOSD and MOGAD in real-life use. *Mult Scler Relat Disord* 2025 Vol. 94:106302
- IV. Johnsson M*, Meda FJ*, Lycke J, Novakova L, Rosenstein I, Johansson K, Malmeström C, Zetterberg H, Kvartsberg H, Axelsson M. Cerebrospinal fluid alpha-internexin is increased in patients with multiple sclerosis and correlates strongly with neurofilament light protein. *Mult Scler Relat Disord*. 2025 Dec;104:106805.
- V. Johnsson M, Lycke J, Novakova L, Rosenstein I, Hafsteinsdottir B, Malmeström C, Axelsson M. Calprotectin Reveals Distinct Innate Immune Signatures in NMOSD, MOGAD, and Multiple Sclerosis. 2026 Jan Manuscript

* Shared first authorship

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ABBREVIATIONS

ADCC	Antibody-Dependent Cellular Cytotoxicity
ADEM	Acute Disseminated Encephalomyelitis
AHSCT	Autologous Hematopoietic Stem Cell Transplantation
AI	Artificial Intelligence
AINX	Alpha Internexin (α -internexin)
Alb	Albumin
ALS	Amyotrophic Lateral Sclerosis
AUC	Area Under the Curve
AQP4-IgG	Aquaporin-4 Immunoglobulin G
BBB	Blood-Brain Barrier
BMI	Body Mass Index
bnfL	Blood Neurofilament Light
CBA	Cell-Based Assay
CDW	Confirmed Disability Worsening
CEL	Contrast-Enhancing Lesion
CHI3L1	Chitinase-3-Like protein 1
CI	Confidence Interval
CIS	Clinically Isolated Syndrome
cNfL	Cerebrospinal fluid Neurofilament Light
CNS	Central Nervous System

CVS	Central Vein Sign
CXCL	Chemokine (C-X-C motif) Ligand
DIS	Dissemination In Space
DIT	Dissemination In Time
DMT	Disease-Modifying Therapy
EBV	Epstein-Barr Virus
EDA	Evidence of Disease Activity
EDSS	Expanded Disability Status Scale
EID	Extended-Interval Dosing
ELISA	Enzyme-Linked Immunosorbent Assay
EMA	European Medicines Agency
FLAIR	Fluid-Attenuated Inversion Recovery
GFAP	Glial Fibrillary Acidic Protein
HET	High-Efficacy Treatment
Ig	Immunoglobulin
IL	Interleukin
IPND	International Panel for NMO Diagnosis
IQR	Interquartile Range
JC	John Cunningham
KFLC	Kappa Free Light Chains
LETM	Longitudinally Extensive Transverse Myelitis

LP	Lumbar Puncture
MHC	Major Histocompatibility Complex
MOG-IgG	Myelin Oligodendrocyte Glycoprotein Immunoglobulin G
MOGAD	Myelin Oligodendrocyte Glycoprotein antibody-Associated Disease
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
NEDA	No Evidence of Disease Activity
NET	Neutrophil Extracellular Traps
NfL	Neurofilament Light
NMOSD	Neuromyelitis Optica Spectrum Disorder
OCB	Oligoclonal Bands
OSMS	Optico-Spinal Multiple Sclerosis
PIRA	Progression Independent of Relapse Activity
PML	Progressive Multifocal Leukoencephalopathy
PMS	Progressive Multiple Sclerosis
PPMS	Primary Progressive Multiple Sclerosis
PRL	Paramagnetic Rim Lesion
RAW	Relapse-Associated Worsening
RCT	Randomized Controlled Trial
RIS	Radiologically Isolated Syndrome

ROC	Receiver Operating Characteristics
RRMS	Relapsing-Remitting Multiple Sclerosis
SAW	Smouldering Associated Worsening
SD	Standard Deviation
SID	Standard-Interval Dosing
sNfL	Serum Neurofilament Light
SPMS	Secondary Progressive Multiple Sclerosis
S1P	Sphingosine-1-Phosphate
T25FW	Timed 25 Feet Walk
T2W	T2 Weighted
QA1b	CSF/Serum Albumin Quotient
9HPT	9-Hole Peg Test

1 INTRODUCTION

This thesis includes five papers that investigate biomarkers of disease activity in demyelinating inflammatory disorders of the central nervous system (CNS). These are characterized by inflammation and injury to myelin—the insulating sheath that surrounds nerve fibers (axons)—hence the term demyelinating. Many are familiar with multiple sclerosis (MS), but this group of disorders also includes the less common diagnoses myelin oligodendrocyte glycoprotein antibody-associated disease (MOGAD), and neuromyelitis optica spectrum disorder (NMOSD), the latter typically associated with the aquaporin-4 (AQP4) antibody. As the clinical activity of MS, NMOSD, and MOGAD mirror an underlying inflammatory disease mechanism, the activity is most often expressed as clinical relapses. Such fluctuations in disease activity can be captured by biomarkers that reflect cellular injury or immune activation.

The thesis builds on the long-standing and rich body of biomarker research in this field. The overall aim is to improve how we monitor and quantify disease activity, improve diagnostic accuracy, and deepen our understanding of disease mechanisms. The thesis begins with an overview of the background and the current research field in this area, followed by a description of the methods used in the included studies, a summary of the main findings, and a discussion of the results.

The first two papers focus on repeated (longitudinal) measurements of serum neurofilament light (sNfL), a biomarker of axonal injury, as a tool for monitoring disease activity in patients with MS. The first study addresses this primarily at the group level, whereas the second study further explores sNfL concentrations at the level of the individual patient. The third paper examines cerebrospinal fluid (CSF) biomarkers with the aim of improving diagnostic precision in NMOSD. The fourth and fifth papers have a more exploratory character, investigating biomarkers that have not previously been assessed in these disorders: α -internexin, and calprotectin.

1.1 THE IMPACT OF INFLAMMATORY DEMYELINATING DISORDERS OF THE CENTRAL NERVOUS SYSTEM

1.1.1 MULTIPLE SCLEROSIS (MS): CHRONIC DISEASE WITH HIGH PREVALENCE

MS is the most prevalent inflammatory demyelinating disease of the central nervous system (CNS) worldwide. As of 2020, an estimated 2.8 million individuals were living with MS globally, an increase across all world regions compared with earlier estimates.¹ Global prevalence is currently estimated at 36 per 100,000 population, and the pooled incidence rate is approximately 2 per 100,000 person-years. MS epidemiology varies substantially between regions, reflecting environmental influences, genetic susceptibility, and differences in surveillance quality. Europe reports the highest prevalence, followed by the Americas, while Africa and South East Asia report much lower figures.¹ A north–south gradient in MS prevalence has long been observed, with lower prevalence closer to the equator. MS is relatively common in Sweden (prevalence; 189 per 100,000).² The mean age at diagnosis is approximately 30 years, placing disease onset during a phase of life typically marked by career development and family formation. The diagnosis of a lifelong condition at this stage carries substantial physical, psychological, and social consequences for the individual, with secondary implications for healthcare utilization.^{1,3} In Sweden, the annual societal cost exceeds 6 billion SEK,⁴ underscoring the importance of early, effective treatment and monitoring as emphasized in the Swedish National Board of Health and Welfare (Socialstyrelsen) national guidelines for MS care.⁵ These guidelines provide the current evidence-based framework for monitoring care.⁵ Registry-driven surveillance has demonstrated real-world clinical improvements in MS care: the risk of converting from relapsing-remitting MS to secondary progressive MS (SPMS) decreased by 7% annually between 2005 and 2020. This improvement is likely attributable to earlier, biomarker-supported intervention with high-efficacy disease-modifying-therapy (DMTs).⁴

1.1.2 NMOSD AND MOGAD: LOW INCIDENCE, HIGH IMPACT OF RELAPSES

NMOSD is a rare disease worldwide, with prevalence estimates that vary between studies, ranging from 0.34 to 10 per 100,000 adult persons. Highest prevalence is reported in African and Asian populations,⁶ while an epidemiological study in Sweden, of whom three quarters were AQP4-IgG positive, reported a prevalence of 1.04 per 100,000 persons and a yearly incidence of 0.079 per 100,000 during 2007 to 2013.⁷ MOGAD is less investigated but has an estimated prevalence of 2 per 100,000 population.⁸ A yearly incidence of 0.37 per 100,000 adult persons has been reported for Region Västra Götaland, Sweden in 2019/2020.⁹

Despite their rarity, these conditions are characterized by a high risk of irreversible disability following acute attacks. Unlike MS, where disability often accrues through chronic progressive deterioration, disability in NMOSD is driven almost exclusively by acute clinical attacks.^{10, 11} These relapses are typically more severe than those seen in MS,¹⁰ and a single untreated or improperly managed attack in NMOSD can lead to permanent blindness or paralysis. While MOGAD patients often experience good motor recovery from attacks, they remain at risk for permanent bladder, bowel, or sexual dysfunction.⁸

1.1.3 WHY BIOMARKERS MATTER

Biomarkers are measurable indicators of biological processes and are essential tools in modern neurology. The evolution of diagnostic criteria to incorporate blood and cerebrospinal fluid (CSF) biomarkers has improved diagnostic precision and enables early and correct treatment in MS, NMOSD and MOGAD.^{8, 12, 13} In Sweden, quality indicators track the "time from debut to diagnosis" and "time from diagnosis to treatment start", metrics that are dependent on biomarker assessments.⁵ No single biomarker provides perfect diagnostic accuracy, nor is any test free from potential unintended clinical harm. The continued development of novel biomarkers and less invasive testing strategies therefore remains essential to improving patient care. This is also important on a global scale where disease epidemiology varies, and resources may be limited. Beyond diagnosis, biomarkers contribute to the understanding of disease processes, can aid prognostication and treatment monitoring, and may capture subclinical disease activity.¹⁴ Soluble biomarkers translate cell injury and immune activation into quantifiable signals, enable precision medicine, and are critical to modern neurological patient care.

1.2 HISTORICAL OVERVIEW: FROM CLINICAL SYNDROMES TO BIOLOGICAL ENTITIES

1.2.1 THE EMERGENCE OF MULTIPLE SCLEROSIS AS A CLINICOPATHOLOGICAL ENTITY DURING THE 19TH CENTURY

The conceptualization of multiple sclerosis as a distinct entity was formed during the 19th century, transitioning from isolated case observations to a medical category on its own. While Charles-Prosper Ollivier d'Angers provided early case reports of "myelitis" in 1824, the structural identification of the disease relied upon the pathological atlases of Robert Carswell (1838) and Jean Cruveilhier, who documented "gray degeneration" and "peculiar diseased states" of the spinal cord and brainstem.¹⁵ By 1863, Eduard Rindfleisch identified a morphological hallmark: the presence of blood vessels situated in the center of the inflammatory plaques.¹⁵

The nomenclature "sclérose en plaques disséminées" was formally introduced in 1866 by Edmé Vulpian, and later translated as "multiple sclerosis" in English-language medical literature. However, the definitive clinical and pathological synthesis is historically attributed to Jean-Martin Charcot through his landmark lectures in 1868. Charcot conceptualized the disease as being rooted in glial hypertrophy and the subsequent destruction of nerve fibers, a process he hypothesized occurred independently of vascular alterations. Diagnosis during this period was based on symptoms and neurological examination, such as "Charcot's neurological triad"—intention tremor, nystagmus, and scanning speech. In 1884, Pierre Marie formulated an influential hypothesis regarding infectious triggers, still, by the end of the century, the etiology of the disorder remained speculative.¹⁵

1.2.2 THE VARIANT CONCEPT OF DEMYELINATING DISEASE

The formal description of "neuro-myélite optique aiguë" by Eugène Devic and Fernand Gault in 1894 introduced a period of nosological uncertainty.^{16, 17} Devic intended the term to denote a unique clinical syndrome characterized by the simultaneous acute myelitis and optic neuritis.^{16, 18} Gault argued that it constituted a distinct "morbid entity," noting that the anatomical appearance of the lesions, often involving necrosis, did not follow the characteristic distribution of classical disseminated sclerosis.^{16, 17} In 1907, Peppo Acchioté proposed the eponym "maladie de Devic," yet many neurologists continued to regard the condition as a restricted variant of multiple sclerosis. During this era, most relapsing idiopathic demyelinating disorders of the CNS were traditionally classified as MS.¹⁷ Diagnostic ambiguity was further complicated by the observation of relapsing cases that challenged the original "monophasic" definition of Devic's syndrome.¹³ This uncertainty persisted throughout the first half of the 20th century, as clinicians lacked paraclinical markers to verify whether these syndromes represented a single unitary disease process or distinct biological entities.^{13, 16}

1.2.3 THE FORMALIZATION OF DIAGNOSTIC CRITERIA

The mid-20th century was defined by efforts to establish uniform terminology and objective requirements for the diagnosis of multiple sclerosis. The Schumacher Panel (1965) addressed the "lack of precision in diagnosis" by establishing six essential criteria to define "clinically definite multiple sclerosis".¹⁹ These criteria mandated "objective abnormalities on neurologic examination attributable to dysfunction of the CNS" and "evidence of involvement of two or more separate parts of the CNS" (dissemination in space).¹⁹ Temporal dissemination was strictly defined as two or more episodes of worsening separated by at least one month, or a slow progression over six months.¹⁹ The Poser Committee in 1983 introduced the category of "laboratory-supported" definite multiple sclerosis.²⁰ This framework allowed for the inclusion of paraclinical evidence, such as the analysis of CSF for oligoclonal IgG bands.²⁰ However, the underlying biological relationship between various phenotypes remained a subject of academic speculation.¹³

1.2.4 PARACLINICAL INTEGRATION

The late 20th century was characterized by an increasing use of paraclinical markers, especially magnetic resonance imaging (MRI) and CSF analysis.²¹ During this period, a significant nosological challenge was posed by "opticospinal multiple sclerosis" (OSMS), a phenotype prevalent in Asian populations that accounted for 15–40% of MS cases in Japan.^{17,22} Investigators questioned whether OSMS represented a regional variant of Western MS or was biologically identical to the syndrome described by Devic.¹⁷ In 1999, Wingerchuk and colleagues proposed formalized diagnostic criteria for neuromyelitis optica (NMO), which integrated clinical requirements with supportive paraclinical findings.²³ A hallmark radiological feature established during this era was the longitudinally extensive transverse myelitis (LETM) lesion, defined as an intramedullary signal abnormality spanning three or more contiguous vertebral segments.^{17, 22} This pattern diverged sharply from the short-segment, peripheral lesions typical of "Western" MS.¹⁷ Furthermore, laboratory investigations revealed that NMO was often characterized by a prominent CSF pleocytosis with neutrophils and a relative absence of the intrathecal IgG synthesis (oligoclonal bands) most often observed in MS.¹⁷ Despite these differentiating features, the relationship between NMO and MS remained controversial, as both disorders shared the common features of inflammatory demyelination. Consequently, the diagnosis of NMO remained anchored in clinical and radiological patterns rather than a defined biological mechanism.¹³

1.2.5 THE BIOMARKER REVOLUTION

The discovery of a specific serum autoantibody, NMO-IgG, in 2004 represented a milestone that defined NMO as an immunopathological biological entity.²² The subsequent identification of the astrocytic protein aquaporin-4 (AQP4) as the target of this antibody established NMO as a primary autoimmune astrocytopathy. This discovery prompted the publication of revised diagnostic criteria in 2006, which incorporated AQP4 IgG serology and coined the term NMO *Spectrum* Disorder to include patients with incomplete clinical presentations of NMO. This nomenclature was further unified and established by the 2015 International Panel for NMO Diagnosis (IPND) in the 2015 revised criteria.¹³

Parallel to the discovery of AQP4-IgG, antibodies targeting myelin oligodendrocyte glycoprotein (MOG-IgG), a protein on the surface of myelin sheaths and oligodendrocytes, were identified in patients with inflammatory

demyelinating disease,²⁴ including cases classified as seronegative NMOSD.²⁵ Early confusion regarding MOG antibodies resulted from low-specificity ELISA methodologies, but the development of cell-based assays (CBA) utilizing full-length human MOG protein allowed for the identification of a distinct entity in 2015: MOG antibody-associated disease (MOGAD).²⁶ MOGAD emerged as a primary demyelinating disorder clinically associated with ADEM, bilateral optic neuritis, and LETM, particularly in pediatric and AQP4-seronegative cohorts.^{8, 26} The 2018 consensus recommendations on MOG-IgG testing and interpretation,²⁷ and the 2023 international diagnostic criteria for MOGAD⁸ established its status as a distinct entity, separate from both MS and NMOSD. This biomarker-driven era has increased the need to exclude NMOSD/MOGAD in atypical presentations when applying the McDonald MS criteria, often prompting AQP4-IgG and MOG-IgG testing to prevent misdiagnosis.^{12, 28} By transitioning from topographically defined syndromes to biologically defined entities, the current framework facilitates precision medicine and improved therapeutic strategies.^{11, 12}

1.2.6 THE REMARKABLE PROGRESS IN MS TREATMENT

Throughout most of the 20th century, treatment of MS was largely confined to symptomatic management, high-dose corticosteroids for acute relapses, and the use of broad-spectrum immunosuppressants with limited evidence of efficacy.²⁹ The approval of Interferon beta-1b in 1993 marked the beginning of the disease-modifying era in relapsing–remitting multiple sclerosis (RRMS). Subsequent approvals of additional injectable therapies, including other interferon- β formulations and glatiramer acetate, enabled a reduction in relapse frequency in a substantial proportion of patients, although their overall efficacy was moderate (approximately a $\frac{1}{3}$ reduction in relapse rates).²⁹

These medications remained the mainstay of immunomodulatory treatment (platform therapy) in RRMS until the emergence of high-efficacy therapies (HETs) over the past 25 years. During this period, therapeutic development accelerated considerably with the introduction of oral and intravenous medications in addition to injectable therapies.²⁹ A current search of the European Medicines Agency (EMA) database identifies 17 centrally authorized immunomodulatory treatments for MS (excluding generics), representing nine mechanistically distinct pharmacological classes: type I interferons, glatiramer acetate, dihydroorotate dehydrogenase inhibition (teriflunomide), fumaric acid esters, sphingosine-1-phosphate (S1P) receptor

modulators, α 4-integrin blockade, anti-CD20 monoclonal antibodies, and immune reconstitution therapies (cladribine and alemtuzumab, not including autologous hematopoietic stem cell transplantation (AHSCT)).³⁰

A further review of DMTs in MS, NMOSD or MOGAD is beyond the scope of this thesis; however, one of the treatments for RRMS will be described briefly in the following section, as patients treated with this medicine were included in Paper I.

1.2.7 NATALIZUMAB AND EXTENDED DOSING

The introduction of natalizumab in 2004 was a milestone in MS treatment. Natalizumab represented the first approved monoclonal antibody therapy for RRMS and works by blocking α 4 β 1-integrin on leukocytes, preventing leukocytes from crossing the blood-brain barrier (BBB) and thereby reduces inflammatory activity within the CNS. In the pivotal AFFIRM trial, natalizumab significantly reduced relapse rates (by 68%) and MRI activity compared with placebo, establishing its high efficacy profile.^{31, 32} Although well tolerated and with few reported adverse effects during the study,³¹ treatment was soon after its introduction complicated by observations of progressive multifocal leukoencephalopathy (PML), a John Cunningham (JC) virus-mediated opportunistic infection.³³ Although rare, PML carries substantial morbidity and mortality, which led to temporary market withdrawal. However, it was reintroduced in 2006 with strict risk mitigation strategies, including risk stratification based on JC virus serostatus, treatment duration, and prior immunosuppression.³⁴ With the implementation of these strategies, the original standard-interval dosing (SID) regimen of 300 mg every four weeks remained the standard of care. However, the persisting risk of PML led to increasing interest in alternative dosing strategies.

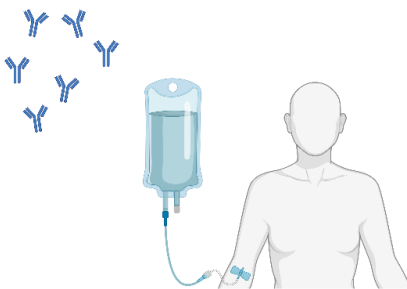


Figure 1 Natalizumab monoclonal antibody treatment in RRMS

The SID of 300mg every four weeks was originally based on pharmacokinetic and pharmacodynamic analysis showing that more than 80% saturation of the $\alpha 4$ integrin receptors on leukocytes was achieved and that receptor occupancy was 90 percent.³² Later data from pooled studies confirmed the maintained $\alpha 4$ -integrin receptor occupancy of at least 70-80% during SID.³⁵ However, subsequent studies suggested that lower receptor occupancy may be sufficient to inhibit leukocyte trafficking across the BBB.^{36, 37} Moreover, although treatment interruptions of more than 8 - 12 weeks were associated with increased risk of rebound activity,³⁶⁻⁴⁰ observational and later randomized data indicated that a moderate increase in interval dosing (extended-interval dosing: EID), typically every 5–6 weeks, maintains clinical efficacy while further reducing PML risk.⁴¹⁻⁴⁴ These findings established EID (typically six-week intervals) as a commonly used regimen in clinical practice.

Although no evidence of increased disease activity was observed with six-week EID, concerns remained that low-grade inflammatory activity or ongoing neurodegeneration could escape detection with conventional clinical assessment and standard MRI monitoring.⁴⁵⁻⁴⁷ At the time of publication of Paper I, only a limited number of studies had evaluated biomarkers of neuroaxonal injury in patients switching from SID to EID. While these studies reported stable serum concentrations of the neuroaxonal injury biomarker sNFL, they were constrained by relatively short follow-up periods and/or infrequent sampling.^{44, 48, 49}

1.3 MULTIPLE SCLEROSIS, NMOSD, MOGAD: SIMILARITIES AND DIFFERENCES

Within “the universe” of inflammatory demyelinating disorders of the CNS, MS, NMOSD, and MOGAD constitute the three principal disease entities. They have considerable similarities in clinical features, and at first presentation, clinical and radiological features may be insufficient to reliably distinguish between these disorders. This diagnostic uncertainty is clinically relevant, as disease course, prognosis, and therapeutic responses differ substantially.

1.3.1 CLINICAL OVERLAP AND DIAGNOSTIC CHALLENGES

1.3.1.1 CLINICAL PRESENTATION AND DISEASE CHARACTERISTICS

Patients with MS have a female predominance (approximately 2:1 ratio) and typically present between 20 and 40 years of age.^{1, 3} This overlaps with MOGAD, in which adult onset commonly occurs in the third to fourth decade. However, MOGAD shows a bimodal distribution, as paediatric onset is frequent and often manifests as acute disseminated encephalomyelitis (ADEM).⁸ In contrast, NMOSD typically presents later, around 40 years of age, and paediatric onset is rare. NMOSD shows a marked female predominance, whereas MOGAD has an approximately equal sex distribution.^{8, 10}

The clinical course in MS often includes both relapsing and progressive mechanisms,⁴⁷ whereas for NMOSD and MOGAD, a progressive course independent of clinical attacks is considered atypical and is categorized as a diagnostic red flag.^{8, 12, 13} Relapses in inflammatory demyelinating disorders reflect focal CNS inflammation producing acute neurological deficits. Relapses typically evolve over hours to a few days and reach a peak within days to weeks. In MS, attacks are heterogeneous and typically involve unilateral optic neuritis, partial transverse myelitis, sensory disturbances, brainstem syndromes (e.g., diplopia, internuclear ophthalmoplegia), motor weakness, or cerebellar dysfunction; multifocal presentations are common, and recovery is often incomplete but variable.¹²

AQP4-IgG-positive NMOSD is characterized by severe, attack-related syndromes predominantly affecting the optic nerves, spinal cord, and specific brain regions with high aquaporin-4 expression, such as the area postrema (area postrema syndrome). Attacks are typically more destructive than in MS and are associated with poorer recovery.¹⁰ In contrast, patients with MOGAD generally exhibit better recovery after a relapse despite substantial variability in relapse severity,⁸ but long term disability may be similar to those with MS. MOGAD presents both monophasic and relapsing courses and a wide phenotypic spectrum. In adults, optic neuritis (60%) and myelitis (21%) are the most common presenting phenotypes.⁵⁰ Other manifestations include ADEM, cortical encephalitis, brainstem or cerebellar syndromes,⁸ and meningitis.⁵¹ Patients that are seronegative for both AQP4-IgG and MOG-IgG (Double-Negative NMOSD; DN-NMOSD) represents a heterogeneous group defined by clinical criteria rather than a unifying biomarker, with symptom profiles overlapping both AQP4-IgG-positive NMOSD and MOGAD.¹⁰ DN-NMOSD is associated with high diagnostic uncertainty and risk of misdiagnosis.⁵² Cohort studies suggest that patients with DN-NMOSD are more frequently male and of Caucasian ethnicity, and that they may experience better visual recovery following optic neuritis compared with patients with AQP4-IgG-positive NMOSD.⁵³ However, interpretation is limited by rarity, treatment effects, and referral bias. Sex distribution, ethnicity, relapse frequency, and disability accrual remain incompletely defined.¹⁰

For consistency with Papers III and V, DN-NMOSD will hereafter denote seronegative NMOSD. The optimal classification and terminology for this entity, however, remain under discussion. See Table 1 for an overview of clinical and demographic characteristics in the three most common inflammatory demyelinating diseases in the CNS.

Table 1 Features of MOGAD, MS, and AQP4-IgG-positive NMOSD. Adapted from Banwell et al. *Lancet Neurol* 2023;22:268–82

	MOGAD	MULTIPLE SCLEROSIS	AQP4-IgG-POSITIVE NMOSD
PEDIATRIC ONSET	Frequent	Infrequent	Extremely rare
SEX DISTRIBUTION	Females = Males	Females > Males (after puberty)	Females >>> Males
DISEASE COURSE	Monophasic or relapsing	Relapsing and / or progressive	Most often relapsing
OPTIC NERVE	<p>Often severely impaired</p> <p>Typically favourable</p> <p>Frequently bilateral and anterior at onset, longitudinally extensive, and involving the optic nerve sheath</p> <p>Moderate to severe oedema is typical and can be associated with haemorrhage</p>	<p>Mild to moderately impaired</p> <p>Typically favourable</p> <p>Typically unilateral, anterior, short optic nerve lesions that do not involve the optic nerve sheath</p> <p>Mild oedema can occur but severe oedema with haemorrhage is rarely seen</p>	<p>Often severely impaired</p> <p>Risk for poor recovery</p> <p>Bilateral or unilateral at onset, often posterior, and frequently longitudinally extensive; chiasmal and optic tract involvement might be present</p> <p>Oedema and associated haemorrhages are less common than in MOGAD</p>
SPINAL CORD	<p>Severe</p> <p>Excellent motor recovery after treatment</p> <p>Risk for residual sphincter and erectile impairment despite good motor recovery</p> <p>Single or multiple longitudinally extensive lesions, grey matter involvement leading to the H-sign and conus lesions are characteristic</p>	<p>Mild to moderate</p> <p>Often good but risk for motor impairment during progressive phase of disease</p> <p>Risk of bladder impairment, especially during progressive phase of disease</p> <p>Often multiple focal cord lesions; often posterior and involving only a portion of the cross-sectional area of the cord; conus rarely involved</p>	<p>Severe</p> <p>Risk for poor recovery or worsening motor impairment with relapses</p> <p>Variable residual bladder impairment</p> <p>Single longitudinally extensive lesion, which commonly involves entire transverse diameter of the cord and might have bright spotty lesion appearance; conus rarely involved</p>
BRAIN	<p>Clinical presentation</p> <p>Brain MRI</p> <p>Qualitative MRI lesion features</p> <p>Typical MRI lesion locations</p> <p>MRI contrast enhancement pattern</p> <p>Resolution of T2-hyperintense lesions on MRI</p> <p>Silent MRI lesion accrual</p> <p>Residual T1-hypointense lesions</p> <p>Oligoclonal bands in CSF but not in serum</p>	<p>Focal or polyfocal neurological deficits common; encephalopathy or seizures are rare</p> <p>Multifocal T2-hyperintense white matter lesions</p> <p>Ovoid or round, well demarcated T2 lesions; Dawson's fingers, S-shaped or U-fibre lesions; central venic sign; smouldering or slowly evolving lesions</p> <p>Periventricular and corpus callosum, juxtacortical, cortical, white matter, and infratentorial</p> <p>Ovoid, ring, or open-ring lesion enhancement pattern</p> <p>Complete resolution is infrequent</p> <p>Frequent</p> <p>Frequent</p> <p>Extremely frequent</p>	<p>Area postrema symptoms, hiccups, hypersomnolence, or focal neurological deficits</p> <p>Might be normal in optic neuritis or myelitis presentations</p> <p>Multifocal T2 lesions most common in AQP4-rich regions; lesions can appear linear and along corticospinal tract or medulla</p> <p>Peri-third and peri-fourth ventricle, splenium of corpus callosum, internal capsule, and white matter</p> <p>Patchy, cloud-like lesion enhancement pattern; pencil-thin pattern of the ependymal surface of lateral ventricles</p> <p>Might be present</p> <p>Infrequent</p> <p>Might be present</p> <p>Infrequent</p>

1.3.1.2 CLINICAL COURSE IN MS

MS has been extensively studied epidemiologically, partly because of its relatively high prevalence and the long-standing use of standardized diagnostic criteria. Over time, terminology to describe clinical events and subtypes in MS has been established. Most patients with MS (approximately 85%) have a course at disease onset characterized by a relapsing-remitting pattern (RRMS). The first clinical episode suggestive of CNS inflammatory demyelination, in a patient who does not yet fulfill diagnostic criteria for MS, is termed clinically isolated syndrome (CIS). Typical CIS presentations include for example optic neuritis, sensory deficits, brainstem syndromes, or partial transverse myelitis, or other symptoms reflecting focal inflammatory lesions within the CNS.⁵⁴

Natural history cohorts from the era prior to DMTs demonstrated that most patients with RRMS eventually transitioned to a disease course characterized by gradual and irreversible disability progression, so called secondary-progressive MS (SPMS).^{55, 56} A subset of patients, approximately 10–15%, have primary progressive MS (PPMS), defined by gradual disability worsening from disease onset without preceding relapse.⁵⁴ Commonly reported features of progressive MS (PMS) include gradually worsening gait impairment, increasing lower limb spasticity and weakness, progressive cerebellar ataxia, and cognitive decline (particularly slowed information processing). For research purposes, several attempts have been made to define SPMS on the basis of disability scales, most commonly the Expanded Disability Status Scale (EDSS), the most widely used disability measure in MS.^{57, 58} However, in clinical practice, the diagnosis of SPMS remains largely retrospective and based on sustained disability progression independent of relapses rather than on a strict EDSS threshold. Lower rates of conversion from RRMS to SPMS in recent years are presumed to be due to earlier diagnosis and the introduction of DMTs.⁵⁹ Importantly, patients with SPMS may have superimposed relapses during a progressive course.⁵⁶

More recently, the concept of progressive disease has undergone re-evaluation, as evidence indicates that disability accumulation frequently occurs independently of overt relapse activity in patients with RRMS.⁶⁰ This phenomenon, referred to as progression independent of relapse activity (PIRA), reflects ongoing pathological processes that are not captured by relapses alone,^{61 62, 63} and has been demonstrated to be the main contributor to disability accumulation in patients with RRMS.⁶¹ Moreover, PIRA, often defined as a 6-month confirmed EDSS increase occurring during a relapse-free

period, is relatively common; approximately one quarter of patients in a large cohort followed from a first demyelinating event developed PIRA, with a median time to PIRA of about seven years.⁶⁴ Although PIRA represents an EDSS-based, confirmed measure of progression, it does not fully capture the broader progressive clinical worsening that some patients experience. This is driven by underlying disease mechanisms of chronic low-grade compartmentalized inflammation in the CNS, often referred to as smouldering disease in MS.⁴⁷ Smouldering-associated worsening (SAW) has therefore been proposed as an umbrella term encompassing not only PIRA, but also more subtle and often under-recognized manifestations of progression, including fatigue, cognitive decline, subtle motor deterioration, bladder, bowel or sexual dysfunction, and worsening detectable only with more sensitive clinical tests such as the 9-Hole Peg Test (9HPT) or the Timed 25-Foot Walk (T25FW).⁶⁵ Accordingly, detection of SAW requires more sensitive composite disability measures, such as EDSS-Plus (EDSS combined with 9HPT and T25FW), in addition to refined radiological and soluble biomarker monitoring.⁶⁵ Thus, MS is increasingly regarded as a continuum in which relapsing and progressive mechanisms coexist from early disease stages, contributing to permanent disability through both relapse-associated worsening (RAW) and SAW.⁴⁷ A schematic representation of these mechanisms across the disease course is provided in Figure 2.

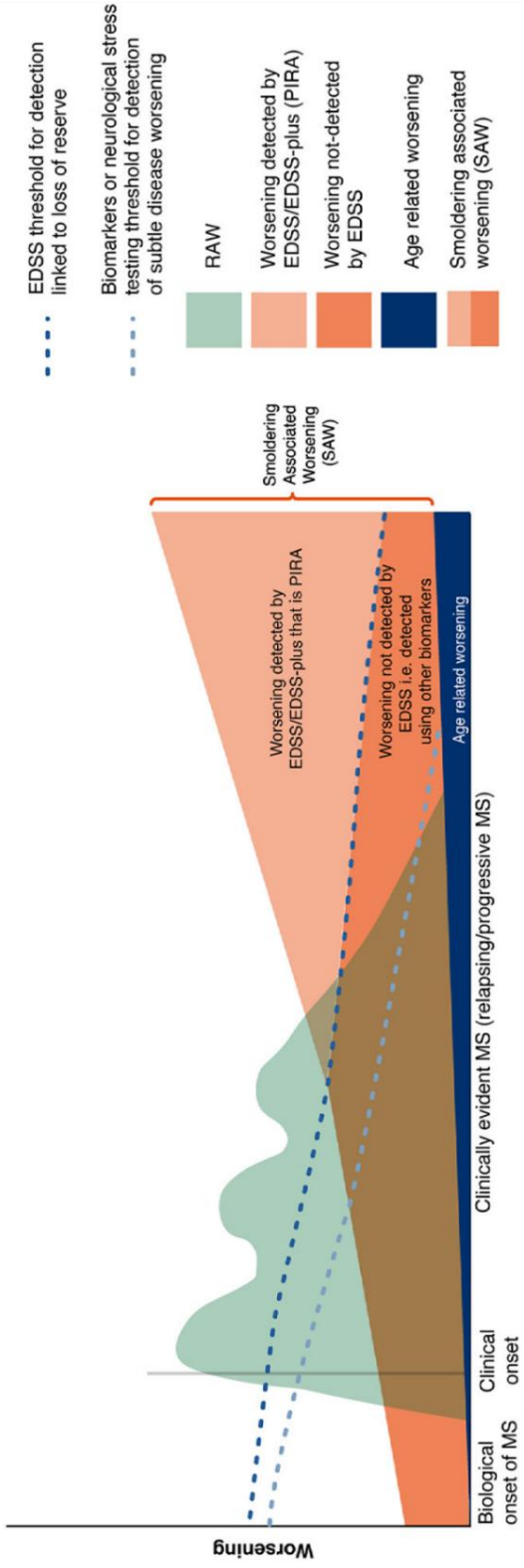


Figure 2 Multiple domains of disability accumulation in multiple sclerosis. Reproduced from Scalfari et al., *Annals of Neurology*, 2024, under the Creative Commons Attribution 4.0 License (CC BY 4.0). EDSS= Expanded Disability Status Scale, RAW=Relapse Associated Worsening, SAW = Smoldering Associated Worsening, PIRA = Progression Independent of Relapse Activity

1.3.1.3 CURRENT DIAGNOSTIC CRITERIA AND LIMITATIONS OF ANTIBODY ASSAYS

For MS, the 2017 revised McDonald criteria were applied for patient inclusion in this thesis,²⁸ while new criteria were published in 2025 (2024 revised McDonald criteria).¹² Importantly, patients fulfilling the 2017 criteria will also meet the 2024 revised criteria.¹² MS diagnosis has traditionally required evidence of dissemination in space (DIS) and time (DIT), meaning inflammatory demyelinating lesions must occur in distinct CNS locations at different time points, thereby demonstrating chronicity. The 2017 criteria introduced CSF oligoclonal IgG bands as evidence of DIT.²⁸ The 2024 criteria extend the 2017 framework by integrating new imaging and biological biomarkers to diagnose MS earlier. The optic nerve is now recognized as a fifth typical lesion location for DIS (alongside periventricular, juxtacortical, infratentorial, spinal). The criteria also allow kappa free light chains (KFLC) in CSF as an alternative to oligoclonal bands (CSF positivity) and advanced MRI features—central vein sign (CVS) and paramagnetic rim lesions (PRL)—as supportive evidence. Moreover, patients with atypical presentations or radiologically isolated syndrome (RIS) may fulfill diagnostic criteria.¹²

For MOGAD, 2018 consensus recommendations²⁷ regarding MOG-IgG testing and red flags for atypical phenotypes served as best available diagnostic criteria in Paper III, while formal criteria were published in 2023.⁸ For NMOSD, current diagnostic criteria date from 2015¹³ but are under revision (IPND 2025 Revised Classification). The new criteria are reported to classify MOGAD and AQP4-IgG-positive NMOSD as distinct *diseases*, whereas DN-NMOSD cases are proposed to be categorized by phenotypical and clinical character, as *syndromes*.⁶⁶ Current formal diagnostic criteria are presented in Figure 3 (MS), Table 2 (MOGAD), and Table 3 (NMOSD).

For AQP4-IgG-positive NMOSD, and MOGAD, accurate diagnosis depends heavily on assay methodology. Live cell-based assays outperform fixed assays for both AQP4-IgG and MOG-IgG,^{10, 67} and remains the reference standard assay.⁶⁶ Antibody titers may fluctuate over time, and seronegativity does not fully exclude disease, particularly when testing occurs remote from relapse.^{10, 68} Titrers may also be reduced by B cell-depleting therapy,⁶⁸ or plasmapheresis.⁶⁹ Patients evaluated for MOGAD should undergo CSF antibody testing, as isolated CSF MOG-IgG positivity may occur despite negative serum results.^{8, 70}

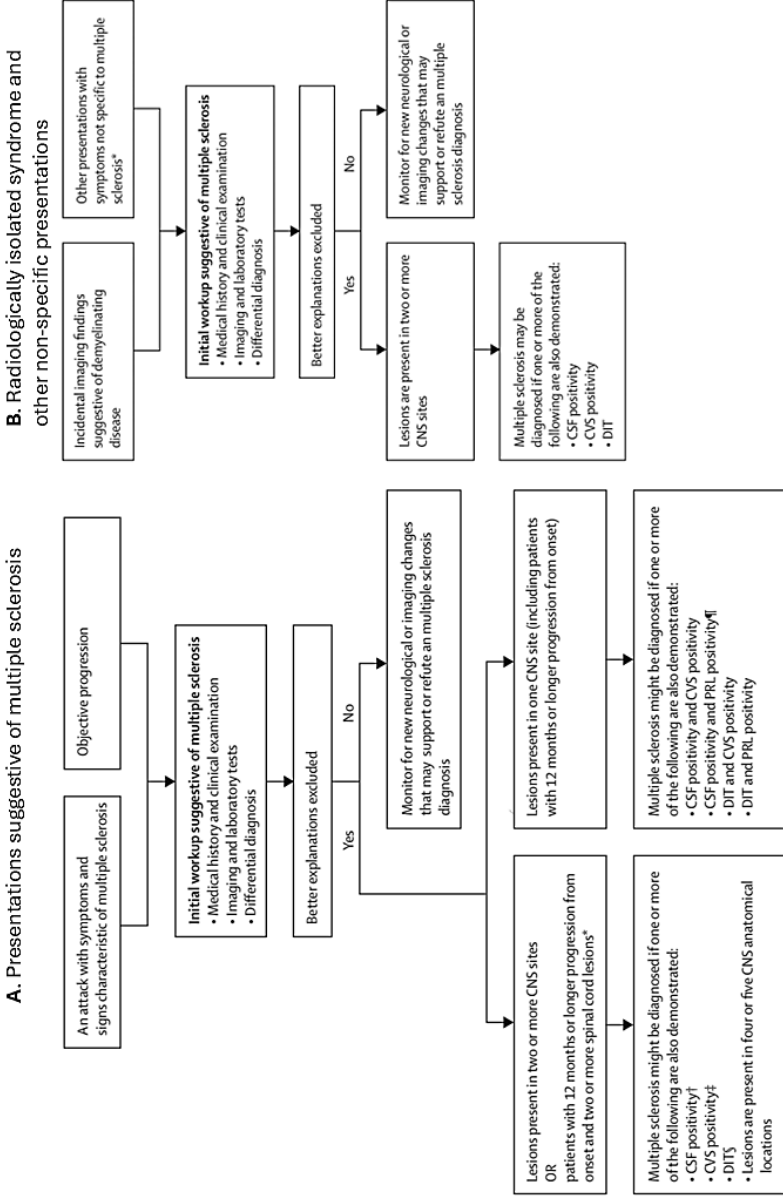


Figure 3 Diagnostic algorithm for diagnosing multiple sclerosis. CSF=cerebrospinal fluid. CVS=central vein sign. DIT=dissemination in time. PRL=paramagnetic rim lesion. † Presence of oligoclonal bands or kappa free-light chains $\ddagger \geq 6$ CVS

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*Table 2 The 2023 International MOGAD Panel proposed criteria. *When fixed CBA assay is used, a titer of $\geq 1:100$ is considered clear positive, while $\geq 1:10$ and $< 1:100$ is low positive. Reproduced with permission from Banwell et al. Lancet Neurol 2023; 22: 268–82*

Diagnosis of MOGAD (requires fulfilment of A, B, and C)			
(A) Core clinical demyelinating event	Optic neuritis Myelitis ADEM Cerebral monofocal or polyfocal deficits Brainstem or cerebellar deficits Cerebral cortical encephalitis often with seizures		
(B) Positive MOG-IgG test	Cell-based assay *: serum	Clear positive	No additional supporting features required
		Low positive	• AQP4-IgG seronegative AND • ≥ 1 supporting clinical or MRI feature
		Positive without reported titre	
Supporting clinical or MRI features	Optic neuritis	<ul style="list-style-type: none"> • Bilateral simultaneous clinical involvement • Longitudinal optic nerve involvement ($> 50\%$ length of the optic nerve) • Perineural optic sheath enhancement • Optic disc oedema 	
	Myelitis	<ul style="list-style-type: none"> • Longitudinally extensive myelitis • Central cord lesion or H-sign • Conus lesion 	
	Brain, brainstem, or cerebral syndrome	<ul style="list-style-type: none"> • Multiple ill-defined T2 hyperintense lesions in supratentorial and often infratentorial white matter • Deep grey matter involvement • Ill-defined T2-hyperintensity involving pons, middle cerebellar peduncle, or medulla • Cortical lesion with or without lesional and overlying meningeal enhancement 	
(C) Exclusion of better diagnoses including multiple sclerosis			

Table 3 The 2015 International consensus diagnostic criteria for neuromyelitis optica spectrum disorders. Reproduced from Wingerchuk et al., Neurology, 2015, under the Creative Commons Attribution 4.0 License (CC BY 4.0).

NMOSD diagnostic criteria for adult patients
<p>Diagnostic criteria for NMOSD with AQP4-IgG</p> <ol style="list-style-type: none"> At least 1 core clinical characteristic Positive test for AQP4-IgG using best available detection method (cell-based assay strongly recommended) Exclusion of alternative diagnoses
<p>Diagnostic criteria for NMOSD without AQP4-IgG or NMOSD with unknown AQP4-IgG status</p> <ol style="list-style-type: none"> At least 2 core clinical characteristics occurring as a result of one or more clinical attacks and meeting all of the following requirements: <ol style="list-style-type: none"> At least 1 core clinical characteristic must be optic neuritis, acute myelitis with LETM, or area postrema syndrome Dissemination in space (2 or more different core clinical characteristics) Fulfilment of additional MRI requirements, as applicable Negative tests for AQP4-IgG using best available detection method, or testing unavailable Exclusion of alternative diagnoses
<p>Core clinical characteristics</p> <ol style="list-style-type: none"> Optic neuritis Acute myelitis Area postrema syndrome: episode of otherwise unexplained hiccups or nausea and vomiting Acute brainstem syndrome Symptomatic narcolepsy or acute diencephalic clinical syndrome with NMOSD-typical diencephalic MRI lesions Symptomatic cerebral syndrome with NMOSD-typical brain lesions
<p>Additional MRI requirements for NMOSD without AQP4-IgG and NMOSD with unknown AQP4-IgG status</p> <ol style="list-style-type: none"> Acute optic neuritis: requires brain MRI showing (a) normal findings or only nonspecific white matter lesions, OR (b) optic nerve MRI with T2-hyperintense lesion or T1-weighted gadolinium-enhancing lesion extending over $> 1/2$ optic nerve length or involving optic chiasm Acute myelitis: requires associated intramedullary MRI lesion extending over ≥ 3 contiguous segments (LETM) OR ≥ 3 contiguous segments of focal spinal cord atrophy in patients with history compatible with acute myelitis Area postrema syndrome: requires associated dorsal medulla/area postrema lesions Acute brainstem syndrome: requires associated periependymal brainstem lesions

1.3.2 PATHOPHYSIOLOGICAL DISTINCTIONS

All disorders discussed in this thesis are widely accepted as having an autoimmune etiology, but MS and DN-NMOSD stand out as they are not strictly fulfilling the classical criteria for an autoimmune disease due to the absence of known specific autoantigens.⁷¹ MS is characterized by a complex interplay of adaptive immune responses, chronic compartmentalized inflammation, and progressive neurodegeneration. In contrast, AQP4-IgG-positive NMOSD represents a prototypical antibody-mediated astrocytopathy, in which antibodies target the AQP4 water channel expressed on astrocytic endfeet at the BBB. MOGAD is distinguished by antibody-mediated demyelination targeting MOG, with prominent innate immune activation. Patients with DN-NMOSD constitute a heterogeneous group, and accumulating evidence suggests that their pathobiology differs from that of AQP4-IgG-positive NMOSD, though the exact mechanism remains incompletely defined.⁶⁶

A better understanding of the underlying distinct pathophysiological mechanisms is important, for among other things, the tailoring of optimal treatments. For example, the development of natalizumab as a treatment for RRMS was mechanism-driven, and grew out of immunology work in the late 1980s-1990s on leukocyte adhesion and trafficking, combined with experimental autoimmune models of MS.³¹ Similarly, the prominent complement activation in AQP4-IgG-positive NMOSD constituted the basis for the development of the efficacious complement (C5) inhibitor eculizumab.⁷²

1.3.2.1 PATHOPHYSIOLOGY IN MS

MS is histopathologically characterized by multiple inflammatory plaques in the brain and spinal cord, typically organized around veins and showing confluent expansion. Lesions are most prominent in white matter, but grey matter involvement is evident from early disease stages.⁷³ In active plaques, breakdown of the BBB occurs and can be visualized on MRI as contrast-enhancing lesions (CELs). Active lesions contain abundant myelin-laden macrophages/microglia and lymphocytic infiltrates. In contrast to AQP4-IgG-positive NMOSD, astrocytes in MS are usually reactive rather than primarily destroyed.^{74, 75} CD8+ T cells are often the dominant lymphocyte population, displaying a cytotoxic and clonally expanded phenotype, while microglia/macrophages produce oxidative and inflammatory mediators that link inflammation to neuroaxonal injury. Demyelination is a core feature, but

axonal damage is also consistently present.⁷⁵ Chronic mixed active/inactive plaques show ongoing injury at the lesion edge with persistent innate immune activation, forming the pathological substrate of “smouldering MS” and PIRA.^{47, 76} See Figure 4 for the immunological mechanisms in MS.

MS is a complex disease with an incompletely understood cause, and its pathophysiological models have evolved over time. The classical view describes MS as an autoimmune disease driven by autoreactive lymphocytes targeting myelin components,⁷⁷ followed by microglial activation and chronic neurodegeneration.⁷⁸ Historically, competing frameworks include the “outside-in” model, emphasizing peripheral immune priming and CNS entry across the BBB—supported indirectly by therapies that block immune cell trafficking or deplete B cells—and the “inside-out” hypothesis, proposing a primary CNS process (e.g., early myelin or axonal injury) that secondarily induces autoimmunity.⁷⁹ The classical view of MS has been challenged. Epstein–Barr virus (EBV)–related immune dysregulation is now integrated into many models as a prerequisite for MS.⁸⁰ Nearly all patients with MS have prior EBV infection, and a landmark study demonstrated more than a 30-fold increase in MS risk after EBV infection.⁸¹ Axonal injury has been observed after EBV infection before later MS diagnosis,⁸² and 96 % of children with pediatric-onset MS show antibodies against EBV viral capsid antigen, supporting EBV as a trigger across age groups.⁸³

In MS, there are both genetic and environmental risk factors. Genetic factors are estimated to account for approximately 30% of overall susceptibility. A large genetic study suggested that part of the genetic susceptibility to MS rose in Eurasian steppe populations and spread into Europe through Yamnaya-related migration around 5,000 years ago.⁸⁴ MS is highly polygenic, with risk arising from the combined effects of many variants.⁸⁵ The strongest genetic association is in the major histocompatibility complex (MHC), particularly HLA-DRB1, encoding proteins central to immune function.⁸⁶ Outside the MHC, more than 200 susceptibility variants have been identified, mainly affecting peripheral immune cells but also implicating brain-resident microglia in directing autoimmunity toward the CNS.⁸⁵ Among environmental factors, smoking⁸⁷ as well as vitamin D deficiency⁸⁸ have been associated with increased risk and worse disease course, although evidence for other proposed factors remains less robust.

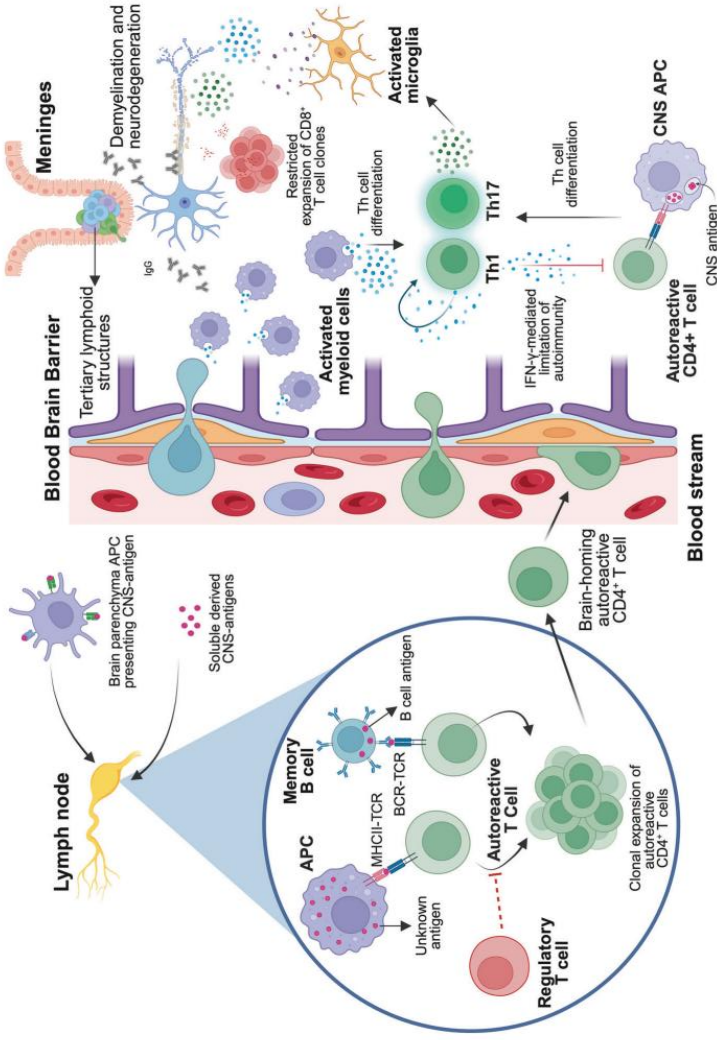


Figure 4 Immunological mechanisms in MS. Reproduced from Boutiaah-Benyaich et al., *Signal Transduction and Targeted Therapy*, 2025, under the Creative Commons Attribution 4.0 License (CC BY 4.0). APC, antigen-presenting cell; BCR, B-cell receptor; CNS, central nervous system; IFN- γ , interferon gamma; MHC-II, class II major histocompatibility complex; IgG, immunoglobulin isotype G; TCR, T-cell receptor; Th, T helper cell.

1.3.2.2 PATHOPHYSIOLOGY IN AQP4-IGG POSITIVE NMOSD

In the majority of NMOSD cases, pathogenesis is driven by immunoglobulin G1 (IgG1) autoantibodies targeting aquaporin-4 (AQP4) (AQP4-IgG-positive NMOSD), a water channel protein highly expressed on astrocyte endfeet.⁸⁹ The binding of AQP4-IgG leads to C1q engagement and triggering of the classical complement cascade and formation of the membrane attack complex, resulting in astrocyte lysis. Secondary mechanisms include antibody-dependent cellular cytotoxicity (ADCC) and AQP4 internalization, which induces astrocyte dysfunction independent of cell death.^{75, 89} Histopathological findings include loss of AQP4 immunoreactivity, vasulocentric deposition of activated complement, and marked infiltration of granulocytes—particularly neutrophils with extracellular traps (NETs) and eosinophils—into acute lesions. Demyelination and axonal injury occur as secondary downstream events of astrocyte destruction,⁸⁹ and chronic active lesions are absent.⁹⁰

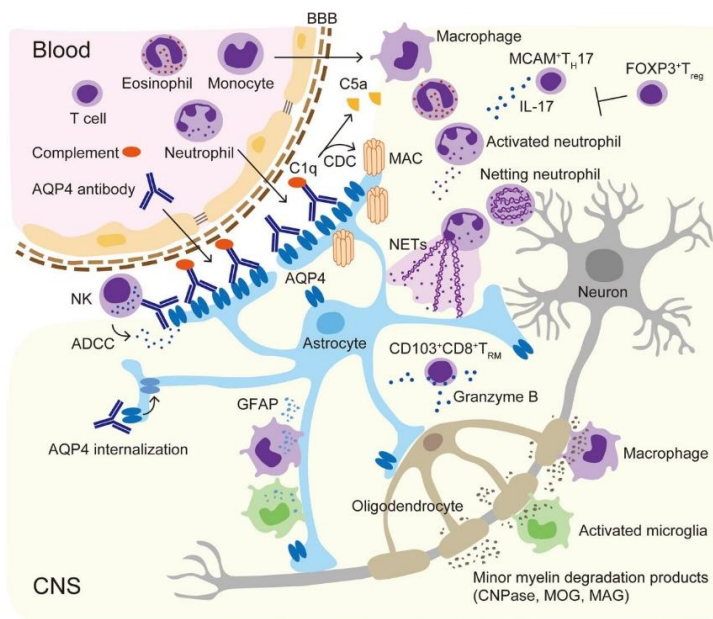


Figure 5 Immunopathology of AQP4-IgG-positive NMOSD. Reproduced with permission from Yanagimura et al., Clinical and Experimental Neuroimmunology, 2025. NET: neutrophil extracellular traps. ADCC: antibody-dependent cellular cytotoxicity. MAC: membrane attack complex. MAG: myelin-associated glycoprotein. NK: natural killer cell. CDC: complement-dependent cytotoxicity.

1.3.2.3 PATHOPHYSIOLOGY IN DN-NMOSD

DN-NMOSD constitutes a heterogeneous subgroup meeting clinical criteria despite the absence of AQP4-IgG and MOG-IgG. Disability accumulation is predominantly relapse-dependent,¹⁰ and proteomic profiles suggest DN-NMOSD is biologically more similar to MOGAD than to AQP4-NMOSD.⁹¹ Immune activity changes over time: in the acute phase, CD4+ T cells dominate around blood vessels, while tissue-resident memory T cells remain in the lesion and may trigger future relapses.^{75, 89}

1.3.2.4 PATHOPHYSIOLOGY IN MOGAD

MOGAD is characterized by primary antibody-mediated demyelination targeting MOG, a glycoprotein located on the outermost layer of the CNS myelin sheath and the surface of mature oligodendrocytes. Pathogenesis typically starts with the peripheral activation of MOG-specific B and T cells—frequently following an infectious prodrome—facilitating their transit across the BBB.⁹² The central mechanism involves the bivalent binding of IgG1 antibodies to MOG, triggering complement-dependent cytotoxicity, ADCC, and macrophage-mediated phagocytosis.⁷⁵ Notably, MOG-IgG engages complement less efficiently than AQP4-IgG, and significant injury results from direct disruption of the oligodendrocyte cytoskeleton independent of direct cell lysis.⁹² Histologically, MOGAD is defined by perivenous demyelination with ill-defined borders surrounding small vessels; these may merge into larger plaques but lack the radial expansion characteristic of MS.⁷⁵ Distinctive findings include the relative preservation of astrocytes and axons, contrasting with the necrotizing astrocytopathy of AQP4-IgG-positive NMOSD.^{75, 93} Furthermore, the inflammatory infiltrate is dominated by CD4+ T cells and macrophages, differing from the CD8+ T-cell predominance in MS.^{75, 92} Crucially, oligodendrocyte precursor cells are typically spared, facilitating a high potential for remyelination.⁹² MOGAD lacks the "smouldering" chronic active lesions of MS, reflecting that disability is predominantly relapse-mediated.⁸

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*Table 4 Adapted from Takai et al., Frontiers in Neurology, 2023, under the Creative Commons Attribution 4.0 License (CC BY 4.0). AQP4, aquaporin 4; GFAP, glial fibrillary acidic protein; MAG, myelin associated glycoprotein; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; MOGAD, MOG antibody-associated disease; MS, multiple sclerosis; NMOSD, neuromyelitis optica spectrum disorders; PVS, perivascular space; SEL, slowly expanding lesion. *Perivenous and confluent.*

Disease	MOGAD	MS	AQP4+NMOSD
Primary target	Myelin > Oligodendrocyte	Myelin, Oligodendrocyte	Astrocyte
Histopathology			
Lesion distribution	Mainly in white matter, the cerebral cortex and deep gray matter can also be involved	Mainly in periventricular and juxtacortical white matter, (cerebral cortex more common in the progressive phase)	Both white matter and gray matter, mainly in the spinal cord and optic nerves
Pattern of demyelination	Perivenous > Confluent or transitional*	Confluent (slowly expanding lesion in the progressive phase)	Secondary in the astrocyte lytic lesions, distal oligodendrogliaopathy
Lesion edge	Ill defined ~sharply defined	Sharply defined	Sharply defined
Damaged myelin proteins	MOG > or = others	Even, or MAG > others (in "Pattern III")	MAG > others
Oligodendrocyte	Relatively preserved	Partially loss ~regenerate	Loss
Astrocyte	Reactive	Reactive	Loss
Axon	Preserved	Relatively preserved, (degenerated in the progressive phase)	Damaged in various degrees
Site of complement deposition	Myelin, inside macrophage	Myelin, inside macrophage (in MS pattern II)	Vasulocentric (rim/rosette pattern)
Cellular infiltration			
Macrophage	Most conspicuous in the PVS and parenchyma	Most conspicuous in parenchyma, especially at the lesion edge	Most conspicuous in the PVS and parenchyma
T cells	CD4 dominant in the PVS	CD8 dominant in the PVS	CD4 dominant in the PVS (CD8 dominant in the chronic phase)
B cells	A small number in the PVS, occasional aggregates in the leptomeninges	A small number in the PVS (Ectopic lymphoid follicles in the progressive phase)	A small number in the PVS
Neutrophil/ Eosinophil	Mild ~Moderate	Rare	Mild ~Marked

1.3.3 MONITORING DISEASE ACTIVITY: CURRENT TOOLS AND THEIR LIMITATIONS

The landscape of disease-monitoring tools is evolving. Since the publication of Paper I, several biomarkers investigated herein have been incorporated into clinical practice, reflecting accumulating evidence of clinical validity. This development marks a shift toward more biologically informed and quantitative monitoring strategies. Nevertheless, disease monitoring has historically, and continues to rely on clinical assessment and radiological evaluation.

1.3.3.1 MAGNETIC RESONANCE IMAGING (MRI)

MRI has become the gold standard for monitoring of MS.⁹⁴ MRI contributes by detecting typical demyelinating lesions in periventricular, juxtacortical/cortical, infratentorial, spinal cord, and optic nerve regions, demonstrating dissemination in time (simultaneous enhancing and non-enhancing lesions or new T2 lesions), and identifying brain atrophy as well as specific markers such as the central vein sign (CVS) and paramagnetic rim lesions (PRL).⁹⁵ Routine monitoring in patients with RRMS is emphasized by the Swedish National Board of Health and Welfare national guidelines for MS care,⁵ and the Swedish MS Society recommends baseline MRI of both brain and spinal cord, with follow-up including brain MRI at 3–6 months, 6–12 months, and then annually, with possible extension to 18–24 months in stable disease.⁹⁶ While initial MRI scans can be diagnostic and convey prognostic information,⁹⁷ follow-up imaging focuses primarily on new, enlarging, or contrast-enhancing lesions (CELs) and atrophy progression. Importantly, MRI can detect subclinical radiological activity, and natural history studies from the era before DMTs demonstrated that new lesions on serial MRI occurred approximately five to ten times more often than clinical relapses.⁹⁸

MRI is essential in the investigative work-up for NMOSD and MOGAD, but unlike MS, there are no clear standardized recommendations for routine MRI monitoring and “silent” lesion accrual on follow-up MRI is uncommon.^{8, 10} Only 3% in a cohort of both pediatric and adult patients with MOGAD and AQP4-IgG-positive NMOSD had new silent MRI lesions at remission.⁹⁹ However, it is common for patients to have asymptomatic lesions in one region of the CNS while experiencing a clinical attack in another. Moreover, it has been reported that MRI can be normal at very early stages in a clinical attack and repeat MRI during the same attack should be considered if the clinical suspicion for NMOSD or MOGAD is high.^{8, 10}

1.3.3.2 CLINICAL ASSESSMENT AND THE EXPANDED DISABILITY STATUS SCALE

Besides radiological follow-up, conventional disease monitoring relies primarily on structured clinical evaluation and patient-reported symptomatology. A detailed medical history and a comprehensive neurological examination remain cornerstones of patient assessment and longitudinal follow-up.

A clinical relapse is defined as the occurrence of new or worsening neurological signs and symptoms persisting for more than 24 hours, in the absence of an alternative explanation. Fever and systemic infection must be excluded, as elevated body temperature can precipitate a so-called pseudo-relapse—a transient exacerbation of pre-existing neurological deficits without evidence of new focal inflammatory activity within the CNS.

For disability assessment, the Expanded Disability Status Scale (EDSS) is the most widely used instrument.⁵⁸ The scale spans from 0 (no disability and normal neurological examination) to 10 (death) (Figure 6). Most Swedish patients with MS have only minimal or moderate disability, as the mean EDSS score for patients in Sweden is reported to be 2.5.⁴ The term confirmed disability worsening (CDW) refers to a sustained increase in disability over ≥ 3 -6 months, and is mostly used for research purposes. EDSS is also used in the evaluation of disability in NMOSD and MOGAD.



Figure 6 Simplified description of the disability stages in the Expanded Disability Status Scale (EDSS), spanning from 0-10. Created by the author using Biorender.

1.3.3.3 THE NEDA CONCEPT IN MS

Over the years, there have been repeated efforts to define disease remission by combining conventional markers of MS activity. The concept of no evidence of disease activity (NEDA) has been developed to include no relapses, no CDW, and no MRI activity (NEDA-3), meaning no new or enlarging T2 lesions and no gadolinium-enhancing T1 lesions. The concept was intended to

move beyond relapse rate alone and provide a stricter definition of remission in the era of DMTs.¹⁰⁰ However, NEDA-3 is limited in that it does not include measures such as brain atrophy or cognitive decline, which led to proposals such as NEDA-4 that adds brain volume as a fourth measure.¹⁰⁰ Still, it has been argued that even more refined targets are needed, as the gradual worsening attributable to smouldering CNS processes may not be captured by either NEDA-3 nor NEDA-4. More recently, no evidence of inflammatory disease activity (NEIDA) and no evidence of smouldering disease activity (NESDA) have been put forward to incorporate cognition, patient-related outcomes, soluble biomarkers and more advanced imaging markers, in addition to previous measures.⁶⁵ Consistent with this, a 2025 therapeutic overview argues that near-complete suppression of relapses and new MRI lesions is now achievable with high-efficacy therapy, but progression can persist and is not adequately captured by current biomarker measures, motivating better markers of insidious progression.⁷⁶

1.3.3.4 THE NEED FOR RELIABLE BIOMARKERS

An ideal biomarker would:

- Reflect ongoing disease activity
- Capture subclinical pathology
- Be sensitive to treatment effects
- Be feasible in routine clinical practice

In MS, MRI has dramatically improved patient monitoring and conventional MRI is more sensitive than clinical examination alone for detecting subclinical inflammatory activity.⁹⁸ However, the correlation between clinical disability scales and MRI lesion load is only moderate,¹⁰¹ and conventional MRI metrics has limited sensitivity to detect dimensions of pathology that contribute to disability, such as cortical and deep grey-matter involvement, diffuse white-matter damage, and spinal cord pathology.¹⁰² In a large cohort study, one-fourth of patients with a clinical relapse showed no new T2 lesion or contrast-enhancing lesion on brain and spinal cord.¹⁰³ Moreover, MRI can be time-consuming and physically challenging for patients with severe disability. These limitations have stimulated interest not only in more advanced MRI techniques, but also — in parallel with major advances in assay technology — in soluble biomarkers that may complement clinical and radiological assessment. Such biomarkers have the potential to capture ongoing neuroaxonal injury and treatment effects that are not fully reflected by conventional MRI metrics.

1.3.4 BODY FLUID BIOMARKERS IN INFLAMMATORY DEMYELINATING DISEASE

A biomarker is an objectively measured and reproducible biological characteristic that reflects normal biological processes, pathogenic processes, or responses to a therapeutic intervention. Biomarkers are medical signs, distinct from symptoms, which are subjectively experienced by the patient. Depending on context, biomarkers can be used for diagnosis, disease monitoring, prognostication, or as outcome measures in clinical research.¹⁰⁴ Although not a formal classification used in the literature, soluble biomarkers in the context of this thesis can broadly reflect:¹⁴

- Tissue injury (e.g. axonal injury)
- Cell-specific pathology (neurons, astrocytes)
- Blood-Brain-Barrier (BBB) dysfunction
- Immune activation (adaptive vs innate)

1.3.4.1 INTERMEDIATE FILAMENTS AND AXONAL INJURY

Neurofilaments are proteins that act as scaffolding and support fibers in neurons. They are part of a group of structural proteins described in the 1960s named intermediate filaments due to their intermediate diameter (10nm) compared to the other major classes of cytoskeletal proteins; larger than actin (6nm) but smaller than microtubules (25nm).^{105, 106} The family of intermediate filaments have six classes, of which class III and IV are of interest in this thesis. Class III consists of peripherin, vimentin, and glial fibrillary acidic protein (GFAP), while class IV includes the triplet neurofilament light (NfL), medium (NfM), heavy (NfH), and a fourth protein called α -internexin (AINX) (Figure 7). The neurofilament triplet proteins were described and separated during the late 1970s,¹⁰⁷ and AINX was associated with these neurofilament proteins in 1985.¹⁰⁸

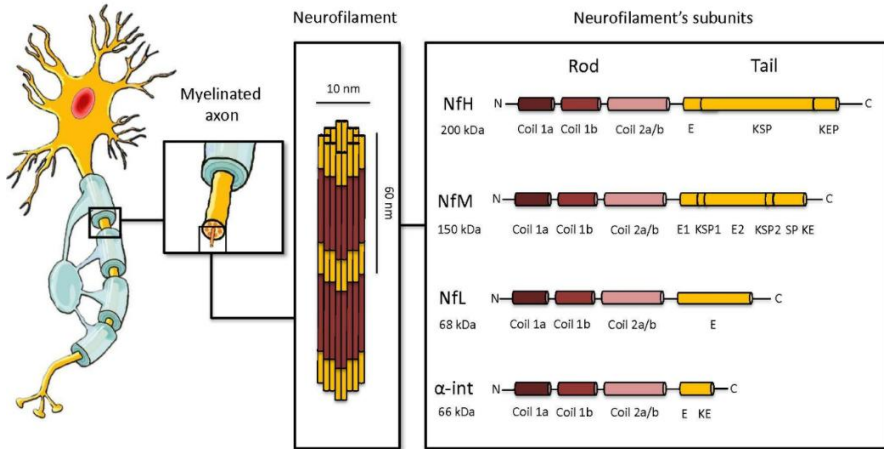


Figure 7 The basic structure of neurofilament triplet proteins and α -internexin. Reprinted with permission from Gaetani L et. al. "Neurofilament light chain as a biomarker in neurological disorders". *J Neurol Neurosurg Psychiatry*. 2019;90(8):870-81 (87).

NfL is particularly abundant in myelinated axons, not only functioning as structural support, but also implicated in axonal transport, conduction properties, and the regulation of axonal diameter.¹⁰⁹⁻¹¹³ AINX is closely related to the neurofilament triplet proteins. Rodent studies have demonstrated that AINX is widely expressed throughout the adult brain and spinal cord,¹¹⁴⁻¹¹⁶ although it has also been suggested to be predominantly expressed during developmental stages of the CNS,¹¹⁷ potentially acting as a precursor to the neurofilament triplet proteins.^{114, 116} In human protein studies, AINX has been reported to be expressed in the brain early in gestation, reaching steady-state levels by approximately 18 weeks.¹¹⁸ In adults, standardized human atlas resources show prominent AINX expression in CNS regions and in a subset of adrenal gland cells (Human Protein Atlas¹¹⁹ and Genotype-Tissue Expression¹²⁰). In addition to adrenal cells, AINX expression outside the CNS has been described in Leydig and spermatogonia cells in the testis,¹¹⁹ intestinal myenteric neurons,¹²¹ as well as in pancreatic neuroendocrine tumors.¹²² Immunohistochemical studies have demonstrated AINX in the human brain within the pathological inclusions characteristic of neuronal intermediate filament inclusion disease.¹²³⁻¹²⁵ The human protein atlases do not include specific annotated peripheral nerve categories. However, in a mice study, AINX was reported to be barely detectable in sciatic nerve compared with its abundant expression in corpus callosum, optic nerve, and spinal cord.¹²⁶

1.3.4.2 NFL AS A MEASURE OF AXONAL INJURY IN MS

Over the years, NfL has emerged as one of the most studied and clinically validated biomarker of axonal injury in MS,¹²⁷ and has increasingly been incorporated as an outcome measure in clinical trials.^{128, 129} During axonal injury, it is released into interstitial fluid and diffuses into CSF and blood. While not disease-specific, NfL is a marker for axonal injury in neurodegenerative disease.¹¹³ Elevated levels were first reported in the CSF of patients with amyotrophic lateral sclerosis (ALS) in 1996,¹³⁰ and soon thereafter were shown to be increased in association with relapse in patients with MS.^{131, 132} Subsequent studies showed that CSF NfL (cNfL) was reduced by effective DMTs,^{133, 134} and that increased concentrations at diagnosis were associated with worse prognosis.¹³⁵

The invasive nature of lumbar puncture (LP) to obtain CSF has limited the feasibility of repeated cNfL measurements. The development of highly sensitive immunoassays enabled detection of extremely low NfL concentrations in serum; in 2013, markedly elevated sNfL levels were demonstrated in patients with ALS (mean 95 ng/L) compared with controls (mean 4 ng/L).¹³⁶ Assay methodology was further refined with the introduction of the single-molecule array (Simoa) ultra-sensitive immunoassay platform.¹³⁷ The possibility of measuring NfL in blood expanded the applicability of NfL as a biomarker for axonal injury in MS. Although the concentrations of NfL are much lower in serum compared to CSF,¹³⁷ strong correlations between sNfL and cNfL were demonstrated,^{132, 137, 138} and previously established associations between elevated NfL levels and disease activity were replicated using sNfL.¹³⁸ Increased sNfL concentrations have also been observed in patients with CIS,¹³⁹ and are associated with brain and spinal cord atrophy.^{140, 141} Although cNfL concentration measurements seem to be more sensitive in capturing disease activity compared to sNfL,¹⁴² sNfL provides a practical and minimally invasive alternative suitable for disease monitoring.

Despite the relative ease of obtaining sNfL through blood sampling, a clear age-dependent increase in sNfL limits interpretation at the individual level, and fixed cut-off values are insufficient for clinical use.¹⁴³ To overcome this, large cohorts of healthy individuals have been used to establish age-stratified reference limits.¹⁴⁴ However, several factors influence sNfL levels and should be considered in interpretation. While age is the strongest confounder, higher body-mass index (BMI) is associated with lower levels, and impaired renal function may increase sNfL concentrations.¹⁴⁵ More advanced reference

models have incorporated both age and BMI into statistical models, allowing calculation of Z-scores that better distinguish physiological variation from disease-related elevations.¹⁴⁵ See Figure 8 for an example of how the Basel sNfL online app, based on large cohorts of healthy individuals,¹⁴⁵ can be utilized to retrieve a Z-score calculation for a hypothetical patient with a sNfL of 12, aged 30 years and a BMI of 20.¹⁴⁶ The resulting Z-score value of 1.8 means that this individual's sNfL concentration is 1.8 standard deviations (SD) above modeled normal age-and BMI-adjusted reference levels.¹⁴⁶

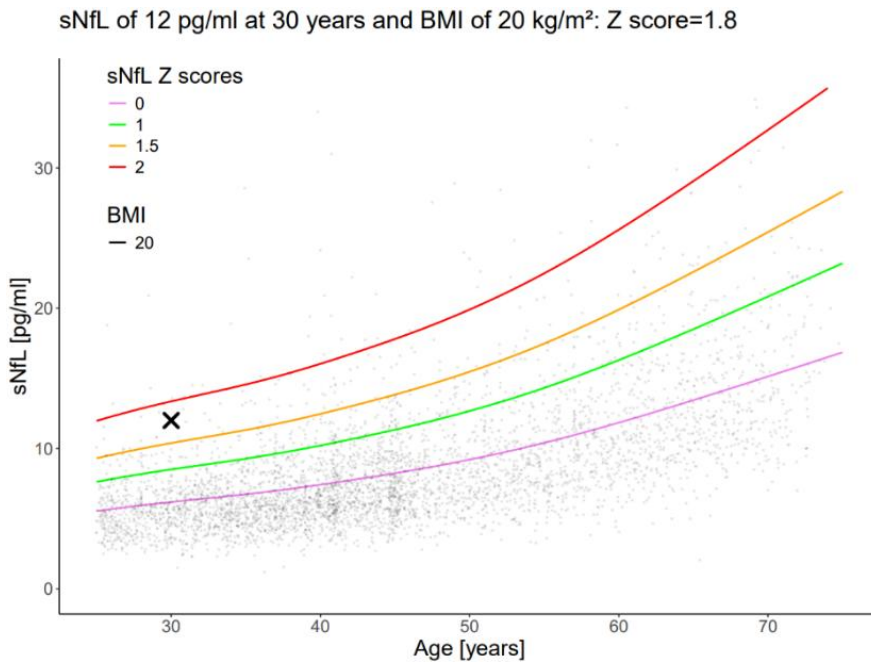


Figure 8 The online Basel-sNfL reference app for calculating the Z-score for a hypothetical person aged 30 years, a body-mass index (BMI) of 20, and a sNfL concentration of 12, resulting in a Z-score of 1.8 as indicated by the cross in the figure. (<https://shiny.dkfbasel.ch/baselnflreference/>) Reproduced with permission from dr J Kuhle, Department of Neurology, University Hospital Basel, Switzerland.

1.3.4.3 ASTROCYTIC BIOMARKERS AND BLOOD–BRAIN BARRIER DYSFUNCTION

Astrocytes are the main structural and homeostatic glial cells of the CNS. Beyond metabolic and synaptic support, astrocytes contribute critically to maintenance of the BBB through specialized endfeet that enwrap cerebral microvessels and regulate vascular permeability. In CNS injury, astrocytes become activated in what is called astrogliosis.¹⁴⁷

GFAP is the major intermediate filament protein of astrocytes and forms the cytoskeletal backbone of these cells. Upregulation of GFAP is a hallmark of reactive astrogliosis, which occurs in response to inflammatory and degenerative CNS injury.¹⁴⁷

The BBB is a highly specialized neurovascular interface composed of endothelial tight junctions, pericytes, and astrocytic endfeet. It regulates molecular and cellular trafficking between blood and CNS tissue. In inflammatory demyelinating diseases, BBB disruption permits entry of immune cells, antibodies, and inflammatory mediators into the CNS parenchyma. The CSF/serum albumin quotient (QAlb) is a well-established quantitative marker of BBB permeability; elevated QAlb reflects impaired barrier integrity and is widely used in the diagnostic work-up of neuroinflammatory disorders, including NMOSD and MS.¹⁰

Consistent with the established astrocytic activation and gliosis in chronic MS lesions, increased concentrations of CSF GFAP, correlating with disease progression, has been measured in patients with MS.¹⁴⁸⁻¹⁵⁰ Subsequently, elevated GFAP concentrations in serum has been linked to PIRA.¹⁵¹

In AQP4-IgG-positive NMOSD, characterized by aquaporin-4 antibody-mediated astrocytopathy, astrocytic injury is central to pathogenesis. Accordingly, increased QAlb and levels of CSF GFAP have been demonstrated in AQP4-IgG-positive NMOSD, reflecting the primary astrocytic target of the disease.¹⁰

1.3.4.4 INNATE IMMUNE MARKERS AND CALPROTECTIN

Innate immune activation is a central component of MS, NMOSD, and MOGAD. Fluid-based biomarkers reflecting myeloid, astrocytic, and granulocytic activity provide complementary information to antibody testing and may help distinguish the inflammatory characteristics of these disorders.¹⁵²

Among established innate-associated markers, microglial/macrophage-derived proteins such as Chitinase-3-Like protein 1 (CHI3L1, or YKL-40) are linked to chronic active lesion biology (e.g., slowly expanding and paramagnetic rim lesions) in MS.¹⁵³ Conversely, in NMOSD and MOGAD, several innate pathways track more closely with acute attack biology. Complement activation is central: CSF C5 and C5a rise in acute MOGAD and NMOSD (but not MS).¹⁵² IL-6, an innate cytokine, is markedly elevated in AQP4-IgG-positive NMOSD and MOGAD and supports a neutrophil- and Th17-skewed inflammatory milieu.^{92, 154} Increased levels of granulocyte activation markers (GAMs) in CSF further characterize neutrophil-driven pathology in NMOSD and MOGAD.^{89, 152}

Calprotectin (S100A8/S100A9), a neutrophil- and monocyte-derived alarmin that amplifies innate immune responses.^{155, 156} It is established as a biomarker in inflammatory bowel disease and systemic infection,^{157, 158} and elevated serum levels correlate with systemic inflammation.^{158, 159} In the CNS, CSF calprotectin rises in bacterial infection,^{160, 161} and the expression of a calprotectin subunit is seen in active MS lesions.¹⁶² However, studies of serum levels in MS are rare but suggest that levels may increase during periods of disease activity, although findings remain inconsistent.¹⁶³⁻¹⁶⁶ CSF calprotectin has been examined in only one study, which reported no overall difference compared with controls but higher detectability in the early phase following relapse onset.¹⁶⁷

2 AIMS OF THE THESIS

Despite the remarkable progress in biomarker research during the last decades, several knowledge gaps remain and form the basis for the studies included in this thesis. The overall research questions can be summarized:

- How reliably do blood-based biomarkers reflect disease activity at the individual patient level?
- How should biomarkers be interpreted longitudinally in real-world clinical settings?
- Can biomarkers distinguish between clinically overlapping inflammatory demyelinating disorders?
- Do different biomarkers reflect distinct biological processes across diseases?

The aim of this thesis was to evaluate soluble biomarkers reflecting neuroaxonal injury, astrocytic pathology, and innate immune activation in inflammatory demyelinating diseases of the CNS, with a particular focus on their clinical utility for diagnosis and disease monitoring. Collectively, the studies were designed to improve the precision of disease activity assessment and to enhance diagnostic accuracy across the spectrum of CNS demyelinating disorders.

Table 5 Knowledge gaps and objectives

Paper	Knowledge gaps	Objective/Aim
I	Despite increasing use of extended-interval dosing of natalizumab in patients with RRMS, its effect on subclinical axonal injury is not sufficiently investigated. Conventional clinical and MRI-based monitoring may fail to detect axonal damage during treatment modifications.	To determine whether switching from SID to EID of natalizumab is associated with changes in sNfL levels, as a marker of neuroaxonal injury.
II	Although sNfL is well established as a group-level marker of disease activity in RRMS, its utility for monitoring individual patients remains unclear. There is a lack of studies describing the sNfL temporal dynamics in high-frequency longitudinal sampling during disease activity.	To characterize the temporal kinetics of sNfL following inflammatory activity, to compare the performance of different sNfL-derived variables for capturing disease activity.
III	NMOSD and MOGAD can pose a diagnostic challenge due to clinically overlapping symptoms, and similar relapse presentation as in patients with RRMS. There is insufficient real-world data on non-antibody biomarkers to differentiate these disease entities.	To evaluate CSF biomarkers, with a focus on GFAP, in patients with NMOSD, MOGAD and RRMS.
IV	A new method for quantifying the neuronal intermediate filament α -internexin in CSF was developed and described in 2025. There is a lack of studies on α -internexin in patients with MS.	To validate the new method and assess levels of α -internexin in patients with RRMS compared to controls. The objective was also to examine the correlation between CSF AINX and NfL in patients with MS.
V	MS, NMOSD, and MOGAD involve innate immune activation. Calprotectin is a soluble biomarker for innate immune activity and can be measured in blood and CSF. There is limited data on the levels of calprotectin in patients with MS, and no studies on calprotectin levels in patients with NMOSD or MOGAD.	To measure the concentration of calprotectin in serum and CSF in patients with MS, NMOSD, and MOGAD. The study aimed to identify disease-specific immune signatures and assess associations with recent relapse activity.

3 METHODS

This section outlines the methodological framework that was used in the five studies of this thesis.

3.1 STUDY DESIGN AND PATIENT COHORTS

The research presented herein is based on prospective and retrospective observational studies utilizing real-world data. All studies were conducted at the MS Center, Sahlgrenska University Hospital, Gothenburg, Sweden. In Study III and V, participants with NMOSD and MOGAD from the entire Region Västra Götaland were eligible for inclusion. See Table 8 for an overview of Study I-V.

All patients with MS were diagnosed according to the 2017 McDonald criteria²⁸ and were consecutively enrolled. Patients with NMOSD fulfilled the 2015 international diagnostic criteria,¹³ while patients with MOGAD met the 2018 consensus recommendations for diagnosis and evaluation.²⁷ In Paper V, patients fulfilled the 2023 MOGAD diagnostic criteria.⁸

3.1.1.1 PAPER I

This was a prospective, single-center observational study conducted over 12 months, with inclusion between October 2019 and June 2021. Eligible patients had received natalizumab (300 mg intravenously every 4–6 weeks) for ≥ 1 year without relapse or new/enlarging MRI lesions within 6 months prior to baseline. Patients were consecutively enrolled after informed consent.

Participants were allocated according to dosing interval: one cohort switched from 4-week to 6-week dosing (EID4–6), while the other continued extended dosing (5–6 weeks; EID5/6). Serum sampling was performed longitudinally, with MRI at baseline, 24, and 48 weeks and EDSS assessment at baseline and week 48. Clinical and demographic data were retrieved from electronic medical records.

A total of 73 patients with RRMS were included; however, three patients were excluded due to concomitant neurological conditions, leaving 70 patients for analysis: 45 in the EID4-6 subgroup and 25 in the EID5/6 subgroup.

Baseline demographic and clinical characteristics are presented in Table 6. The median age was 44 years (IQR; 36-50) and there were no statistically

significant differences between groups in regard to age, sex distribution ($p=0.052$), disease duration, and disability as measured by the EDSS.

Table 6 Patient demographic and clinical characteristics in paper I. Values are the mean (range) unless indicated otherwise. Reproduced from Johnsson et al., Multiple Sclerosis Journal, 2022, under the Creative Commons Attribution 4.0 License (CC BY 4.0).

Characteristics	EID4-6	EID5/6
Patients, <i>N</i>	45	25
Sex, female/male; <i>N</i> (%)	40/5 (89%/11%)	17/7 (68%/22%)
Age, years	43 (25–73)	45 (23–61)
BMI, kg/m ²	24.6 (16–48.3)	27 (20.6–56.2)
Median EDSS score	2 (2.0; 0–4.5)	2 (2.1; 0–6.5)
Disease duration, years	13.4 (3–42)	11 (2–27)
NZ treatment, years	5 (1–11)	5.8 (1–12)
Interval from previous MS relapse to baseline, years	7.4 (1–17)	6.4 (2–16)
DMTs before NZ	1.8 (0–4)	1.5 (0–2)
Patients treated with 4-week SID, <i>N</i>	45	0
Patients treated with 5-week EID, <i>N</i>	0	11
Patients treated with 6-week EID, <i>N</i>	0	14
JC virus antibody positivity, <i>N</i>	0	18

RRMS: relapsing-remitting multiple sclerosis; EID: extended-interval dosing; EID4-6: patients switched from treatment at 4-week intervals to treatment at 6-week intervals; EID5/6: patients treated at 5- or 6-week intervals; BMI: body mass index; EDSS: Expanded Disability Status Scale; NZ: natalizumab; MS: multiple sclerosis; DMT: disease-modifying treatment; SID: standard-interval dosing; JC virus: John Cunningham virus.
Values are the mean (range), unless indicated otherwise.

3.1.1.2 PAPER II

This prospective single-center study included participants between September 2017 and January 2021. Patients with RRMS or CIS were eligible if they had a current relapse and/or CELs on MRI. RRMS patients with asymptomatic CELs were also included, and patients could be treated or untreated with DMT. Baseline was defined as the first serum sampling following relapse or MRI activity. Other neurological diseases were exclusion criteria. The study included 44 participants with evidence of disease activity. EDSS score and brain MRI were done at baseline, 24, and 48 weeks, and patients were followed with longitudinal sNfL sampling across 48 weeks. MRI of spinal cord was performed in 23 of 44 patients at baseline. A separate control cohort of natalizumab-treated RRMS patients ($n=66$) (Paper I: EID4-6 and EID5/6 cohorts) with NEDA-3 served as a reference group. Patients in the control group were older than the NEDA-3 controls but had similar disability (median EDSS; 2.0). When analyzing the data, patients were classified as active (clinical relapse and/or radiological activity: new or enlarging T2 lesion or CEL) or stable (NEDA-3 cohort from paper I). Subgroups in the active cohort comprised A) patients with CIS or RRMS and clinical relapse but no

radiological activity (n = 13), B) patients with CIS or RRMS with clinical relapse and radiological activity (n = 27), and C) patients with RRMS without clinical relapse but with radiological activity (n = 4). Baseline demographic and clinical characteristics are shown in Table 7.

Table 7 Patient demographic and clinical characteristics. Reproduced from Johnsson et al., Multiple Sclerosis Journal, 2024, under the Creative Commons Attribution 4.0 License (CC BY 4.0).

	CIS/RRMS study cohort (N=44)	NEDA-3 controls (N=66)	
Age, median (IQR)	35 (27–40)	45 (37–52)	$p < 0.001$
Sex, female, N (%)	31 (71)	54 (82)	$p = 0.165$
Years since MS diagnosis, median (IQR)	0 (0–8)	10 (6–16)	$p < 0.001$
Time in days from relapse onset or CEL detection to sampling, mean (SD)	22 (20)	NA	
Body mass index (kg/m ²), median (IQR)	24 (21–30) ^{N=29}	25 (19–56) ^{N=60}	$p = 0.746$
EDSS, median (IQR)	2.0 (1.5–3.5) ^{N=44}	2.0 (1.0–2.5) ^{N=66}	$p = 0.121$

CIS: clinically isolated syndrome; RRMS: relapsing-remitting multiple sclerosis; NEDA-3: no evidence of disease activity; CEL: contrast-enhancing lesion; EDSS: Expanded Disability Status Scale; IQR: interquartile range; NA: not applicable; SD: standard deviation; MS: multiple sclerosis.

3.1.1.3 PAPER III

This retrospective observational study included patients from the entire Region Västra Götaland, Sweden. Electronic medical records (Melior) (2010–2022) were systematically searched using ICD codes for neuromyelitis optica (G36.0), myelitis (G04.9), and optic neuritis (H46.9), identifying 1,400 potential cases. In parallel, historical positive anti-MOG (n=29) and anti-AQP4 (n=14) test results from the Sahlgrenska University Hospital immunology laboratory were reviewed. After diagnostic verification, 55 unique patients fulfilled criteria for NMOSD or MOGAD (Figure 9). Subgroups comprised AQP4-IgG-positive NMOSD (n=19), DN-NMOSD (n=7), MOGAD (n=29). Control groups with active RRMS (sampled within one month of relapse) (n=19), and stable RRMS (sampled ≥ 6 months after relapse or MRI activity) (n=14) were included.¹⁶⁸ Patients with NMOSD and MOGAD were sampled during relapse. Demographic and clinical variables, including age, sex, MRI findings, EDSS at onset and sampling, and relapse history, were extracted from medical records. Groups differed in age distribution, with AQP4-IgG-positive-NMOSD patients being older, while sex distribution reflected the known female predominance in NMOSD.

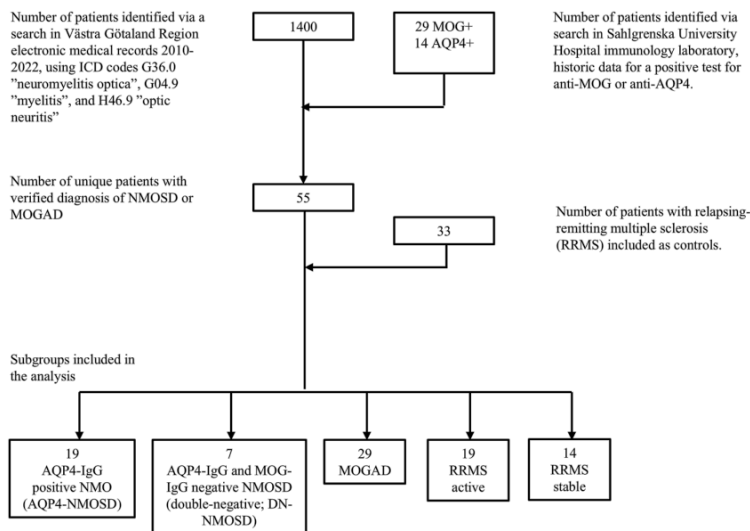


Figure 9 Study flow chart in Paper III. Reproduced from Johnsson et al., *Multiple Sclerosis Journal*, 2024, under the Creative Commons Attribution 4.0 License (CC BY 4.0)

3.1.1.4 PAPER IV

In this observational study, 34 patients with RRMS were included, of whom 24 patients had previously participated in a prospective study investigating biomarker levels before and after initiation of fingolimod (Novakova et al. *Mult Scler*, 2017).¹⁶⁹ Pre-treatment CSF samples collected between 2011 and 2014 within this study were retrieved from the local biobank. In addition, 10 RRMS and 8 control CSF samples were randomly selected from biobank material obtained during diagnostic evaluations between 2018 and 2021. Controls comprised four volunteers and four individuals undergoing diagnostic evaluation; all had normal neurological examination, brain and/or spinal MRI, and CSF findings. Individuals with concomitant neurological, ophthalmological, or inflammatory disease were excluded. Clinical data (age, sex, disease duration, EDSS, DMTs) were extracted retrospectively from medical records.

3.1.1.5 PAPER V

In this observational study, we included a subset of patients from Study III with a diagnosis of AQP4-IgG-positive NMOSD (n=10), DN-NMOSD (n=6), MOGAD (n=9) whom had serum and CSF samples stored at the Sahlgrenska Virology Department (See section "Sampling, assays, and analytical platforms"), and from RRMS (n=10) and controls (n = 8) in Study IV.

Table 8 Overview of study design and cohorts in Papers I-V

Study	Inclusion	Design	Participants	n	Biomarker(s)	Outcome
I	2019-2021	Prospective single-center cohort	RRMS	70	sNFL	Change in sNFL after switching dosing interval
II	2017-2021	Prospective single-center cohort	RRMS CIS	40 4	sNFL	Sensitivity, specificity, ROC, MRI disease activity
III	2010-2022	Retrospective observational	AQP4-NMOSD DN-NMOSD MOGAD RRMS RRMS HC	19 7 29 33 34 8	GFAP Qalb cNFL AINX cNFL	Diagnostic discrimination (ROC/AUC)
IV	2011-2014 and 2018-2021	Retrospective observational	RRMS RRMS HC	34 34 8	AINX cNFL	Difference MS vs HC; correlations with MRI, EDSS, inflammatory markers
V	2010-2022 (NMOSD/MOGAD) 2018-2021 (RRMS/HC)	Retrospective-observational	AQP4-NMOSD DN-NMOSD MOGAD RRMS HC	10 6 9 10 8	Calprotectin in CSF and blood	Diagnostic discrimination (ROC); relapse correlations

3.2 STATISTICAL METHODS

3.2.1 STATISTICAL ANALYSIS

The study of quantifiable soluble biomarkers is advantageous in that descriptive data and comparisons between groups can be described and visualized using statistical measures. In paper I, statistical analyses were performed in collaboration with a professional statistical consulting firm. In Papers II-V, all statistical analyses were conducted by the thesis author. Statistical analysis was done using the following software programs: Excel 365 (paper I-V), SAS 9.4 (paper I), SPSS version 23 or 27 (paper I-II), GraphPad Prism 9.3 (paper I-II) and R version 4.3.2 (paper III-V).

In univariable descriptive statistics, median and interquartile range (IQR) were used to describe distribution in non-normal data, while mean and standard deviation (SD) were used for normally distributed data. Categorical data are presented as counts and percentages. Non-parametric comparisons between groups were performed with Fisher's Non-Parametric Permutation test¹⁷⁰ or the Wilcoxon rank-sum test (Mann-Whitney U test). For correlation analyses, Pearson's correlation analysis and the Shrout-Fleiss reliability random test was used in paper I, and Spearman correlation analysis was used in paper II-V.

Longitudinal sampling of sNfL concentrations, meaning repeated sampling over time, necessitates statistical methods for within-group paired sampling. In paper I, Fischer's Non-Parametric Permutation Test¹⁷⁰ was used for this purpose, and the Wilcoxon signed-rank test was used in paper II, and IV.

In paper I, III, and IV, analysis of covariance (ANCOVA) was performed to calculate age-adjusted sNfL concentrations. In paper II, the compared groups (active versus stable patients) differed significantly in age, making this method for age-adjustments unsuitable. Since a substantial age-dependent increase of sNfL is mainly seen after the age of 50, only patients aged less than 50 years were included in the analysis when comparing raw absolute sNfL levels. In addition, so called Z-scores were derived from the online application created by Benkert et al.¹⁴⁵ to calculate age- and BMI-adjusted normative values. The Z-score value describes how an individual patient's sNfL concentration is related to the age- and BMI-adjusted mean level in a large group of healthy controls and is measured in terms of SD from the mean.

3.2.2 STATISTICAL METHODS THAT RELATE TO DIAGNOSTIC TESTS

All studies in this thesis evaluate soluble biomarkers and use tests to distinguish between patients with and without disease activity, or between different diagnostic groups. Several statistical terms are used frequently and warrant brief description. They relate to the statistical performance of diagnostic tests and include *sensitivity*, *specificity*, *positive predictive value (PPV)*, *negative predictive value (NPV)*, and the *area under the curve (AUC)* value, derived from a *receiver operating characteristics (ROC) curve* analysis.

It is important to acknowledge both the possibilities and the limitations of such statistical analyses. As in any evaluation, the purpose of a test must be clearly defined. The objective may be to confirm or exclude disease, to assess the added value of a test given existing information, to serve as a threshold tool to guide clinical action, or to monitor disease over time. Relevant questions also include when the test should be applied and whether its performance applies equally across different subpopulations. Test performance is often assessed univariately, that is, whether the test alone can discriminate between diseased and non-diseased individuals. However, its value must be interpreted in relation to other available information, such as clinical symptoms, MRI findings, and additional laboratory results. Furthermore, cut-off values are often required to define when a test is considered positive or negative. This is particularly relevant for assays that produce a continuous result, such as biomarker concentrations (e.g. NfL, GFAP, or calprotectin).

3.2.2.1 SENSITIVITY AND SPECIFICITY

To calculate sensitivity, specificity, PPV, and NPV, the numbers of *true positives*, *false positives*, *true negatives*, and *false negatives* must be known. A true positive represents a positive test result in an individual with disease, whereas a false positive represents a positive test in an individual without disease. Correspondingly, a false negative is a negative test in an individual with disease, and a true negative is a negative test in an individual without disease.¹⁷¹ In this thesis, *true positive* means a positive test in a person with disease activity (Paper II) or with a confirmed diagnosis of AQP4-IgG positive NMOSD (Paper III). See Table 9 for definitions of commonly used statistical terms.

Table 9 Statistical terms that relate to diagnostic tests

Statistical term	Can be interpreted as	Definition
Sensitivity	The probability of a positive test in a person with the disease.	$\frac{\text{True positives}}{\text{True positives} + \text{False negatives}}$
Specificity	The probability of a negative test in a person without the disease.	$\frac{\text{True negatives}}{\text{True negatives} + \text{False positives}}$
PPV (positive predictive value)	The probability of a person having the disease when the test is positive.	$\frac{\text{True positives}}{\text{All positives}}$
NPV (negative predictive value)	The probability of a person not having the disease when the test is negative.	$\frac{\text{True negatives}}{\text{All negatives}}$

A diagnostic test is most useful when it demonstrates both high sensitivity and specificity, but increasing sensitivity often occurs at the expense of specificity, and vice versa. While sensitivity and specificity are generally regarded as intrinsic properties of the test and are independent of disease prevalence, PPV and NPV may be advantageous from a clinical perspective, as they incorporate disease prevalence in the studied population. Higher prevalence generally increases PPV, which is intuitive. Conversely, low prevalence generally lowers PPV, because in a population with few diseased individuals, the large number of non-diseased individuals may generate a substantial number of false positives, even when specificity is high. Moreover, PPV and NPV are only applicable to an individual patient if that patient belongs to a population with a similar disease prevalence as the population in which these measures were calculated. This is particularly relevant when evaluating tests intended to distinguish a disease with very low prevalence (NMOSD or MOGAD) versus one with much higher prevalence (MS).¹⁷¹

3.2.2.2 CUT-OFF VALUES AND TEST ACCURACY

ROC analysis was used to evaluate how well a biomarker can distinguish between two groups (for example, patients with disease activity versus patients without disease activity (Paper II), or patients with AQP4-IgG-positive NMOSD versus patients with other inflammatory demyelinating disease. (Paper III)). Originally developed by radar engineers during World War II for distinguishing between enemy aircrafts and noise, this statistical method is now used widely to evaluate diagnostic tests.¹⁷² In ROC analysis, the true disease status of each individual (a binary outcome, e.g., active disease vs stable disease) is compared with a continuous test variable (e.g., biomarker concentration).

To determine an optimal cut-off value, all possible thresholds of the biomarker are evaluated. In practice, the observed biomarker values are commonly used as candidate cut-offs. For each threshold, individuals with values above the cut-off are classified as test-positive and those below as test-negative. Sensitivity (true positive rate) and $1 - \text{specificity}$ (false positive rate) are then calculated based on the entire study population, for whom the true outcome is known. Each cut-off therefore produces one pair of sensitivity and false positive rate values, which are plotted in a coordinate system. The resulting ROC curve illustrates the trade-off between sensitivity and specificity across all possible decision thresholds. An optimal cut-off can be defined in several ways, depending on the clinical context. In Paper II, the optimal cut-off was defined as the point closest to the upper left corner of the ROC plot, corresponding to the shortest distance to perfect classification (100% sensitivity and 100% specificity). In Paper III, the Youden index (sensitivity + specificity - 1) was used, and the cut-off that maximized this index was selected.

ROC curve (Figure 10) analysis was used to evaluate the discriminatory performance of the biomarker. AUC was calculated as a global measure of diagnostic accuracy, yielding a value between 0 and 1. An AUC of 0.5 indicates no discriminatory ability (corresponding to the reference line), whereas higher values indicate increasing discriminatory performance. The AUC can be interpreted as the probability that a randomly selected individual with the outcome of interest (e.g., disease activity) has a higher biomarker value than a randomly selected individual without the outcome.¹⁷¹

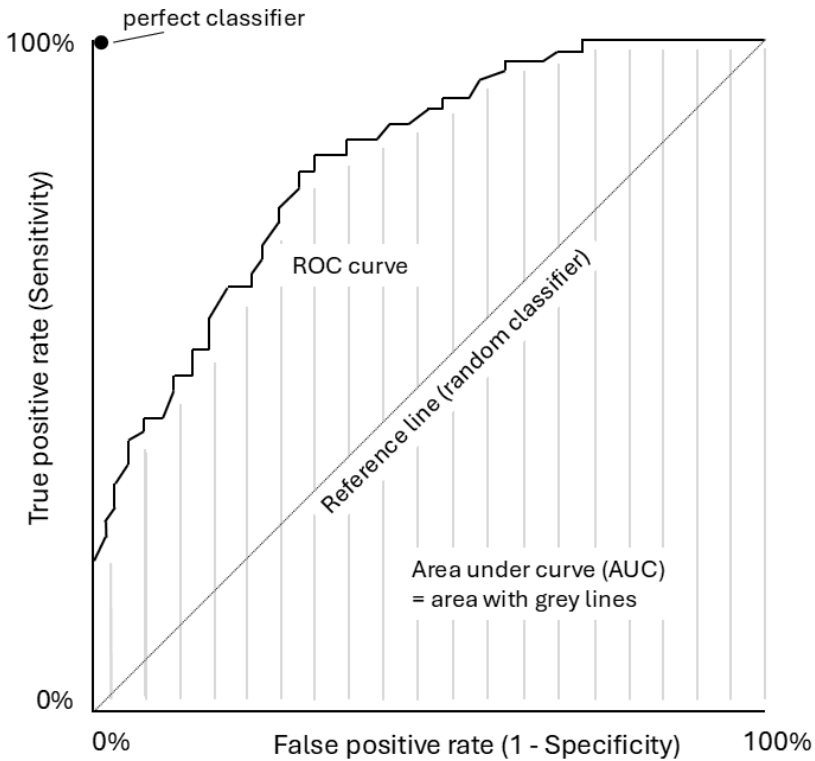


Figure 10 Example ROC curve. Created in PowerPoint by the thesis author.

To assess whether combining biomarkers improved discriminatory performance (Paper III: CSF GFAP and Qalb), multivariable logistic regression models were constructed including the selected biomarkers as independent variables and the clinical outcome as the dependent variable. Predicted probabilities derived from these models were then calculated for each individual. These probability estimates, reflecting the combined contribution of all included biomarkers, were subsequently used to generate ROC curves. The AUC of the combined model was compared with the AUCs of individual biomarkers to evaluate whether integration of markers provided incremental diagnostic value.

3.3 SAMPLING, ASSAYS, AND ANALYTICAL PLATFORMS

In **Study I**, peripheral venous blood was obtained immediately prior to natalizumab infusion. Samples were collected in three sets of paired 5 mL serum and plasma tubes. After clotting (serum) and centrifugation, samples were aliquoted and stored at -80°C until analysis. Sampling was performed at baseline and at each infusion throughout the 12-month follow-up period.

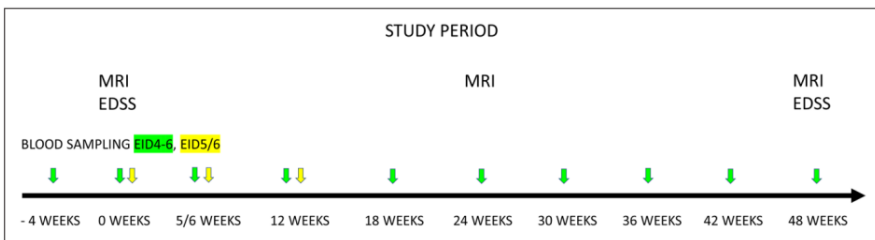


Figure 11 Study design and sampling in Paper I, Reproduced from Johnson et al., *Multiple Sclerosis Journal*, 2022, under the Creative Commons Attribution 4.0 License (CC BY 4.0)

In **Study II**, serum samples were collected at relapse onset (baseline) and at follow-up visits at weeks 0, 2, 4, 8, 16, 24, and 48.

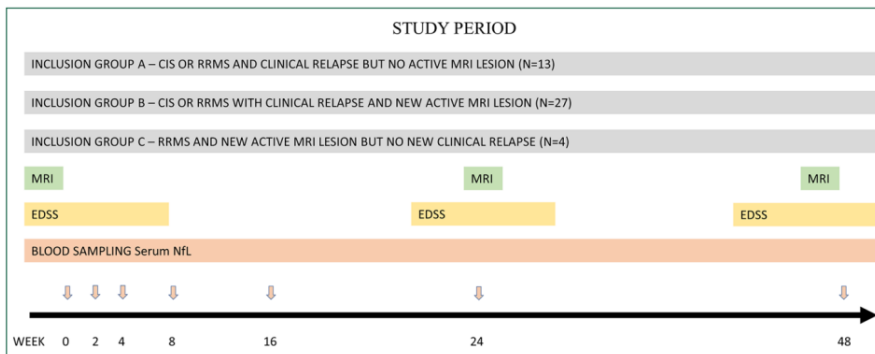


Figure 12 Study design and sampling in Paper II. Reproduced from Johnson et al., *Multiple Sclerosis Journal*, 2024, under the Creative Commons Attribution 4.0 License (CC BY 4.0)

In **Study III**, no additional biological samples were collected. Clinical and laboratory data were retrospectively retrieved from electronic medical records.

In **Study IV**, stored CSF samples were retrieved from the biobank and sent to the Clinical Neurochemistry Laboratory, Mölndal, for AINX analysis, as described separately. Results from routine CSF biomarkers (CSF cell count, IgG index, IgM index, Oligoclonal bands, Qalb) were extracted from medical records, while other CSF biomarkers analyzed within the previous study by Novakova et al. in 2017 (NfL, GFAP, CXCL13, YKL40, Neurogranin, Chitotriosidase) were included in the analysis for this subset of patients.¹⁶⁹

In **Study V**, patients with NMOSD and MOGAD previously identified in Study III were cross-referenced with the Department of Virology sample registry to identify available stored material. Residual CSF and serum samples obtained during routine diagnostic investigations (2010–2022) had been stored at -20°C at Sahlgrenska University Hospital and were retrieved for analysis. CSF and serum samples from patients with RRMS and healthy controls previously included in Study IV served as comparator groups. Calprotectin concentrations were analyzed as described separately. Additional CSF and blood biomarker data were extracted retrospectively from electronic medical records.

3.3.1 CSF NFL

All cNfL data were retrieved retrospectively either from laboratory reports in electronic medical records (Paper III) or from existing datasets generated in previously conducted studies (Papers IV and V). LPs were performed at the MS Center at Sahlgrenska, and in a minority of patients (Paper III) at other clinics within county hospitals in the Västra Götaland Region.

The analyses were performed at the Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal. NfL concentration was measured using a sensitive sandwich ELISA method (NF-light® ELISA kit; UmanDiagnostics AB, Umeå, Sweden). Intra- and inter-assay coefficients of variation were below 10%, and the lower limit of quantification (LLoQ) of the assay was 31 pg/mL.

3.3.2 CSF GFAP

All CSF GFAP data were retrieved retrospectively either from laboratory reports in electronic medical records (Paper III) or from existing datasets generated in previously conducted studies (Papers IV and V). The analyses were done at the Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal.

An in-house sandwich ELISA was used to analyze GFAP in CSF. Microtiter plates were coated with hen anti-GFAP IgG to capture antigen, followed by rabbit anti-GFAP IgG and detection using biotinylated goat anti-rabbit IgG with an avidin–biotin–peroxidase complex. The assay range was 32–16,000 pg/mL, with higher concentrations quantified after dilution. Absorbance was measured at 490 nm and concentrations calculated using log–log transformation or a four-parameter fit. Inter- and intra-assay precision were determined using pooled CSF. CSF samples from healthy volunteers served as reference values.¹⁴⁸

3.3.3 SIMOA: SERUM NFL AND CSF AINX

Conventional ELISA, as used for the measurements of NfL and GFAP in CSF, is not sensitive enough to quantify the low levels of NfL in blood. A new method for the detection of very low-abundance proteins in blood were presented in 2010: Single-Molecule-Array (Simoa), sometimes also called digital ELISA.¹⁷³ Simoa is based on the same principles as conventional ELISA but uses a different detection strategy that allows measurement of single protein molecules. Target proteins are captured on microscopic beads coated with specific antibodies, forming enzyme-labeled immunocomplexes similar to a sandwich ELISA. At very low protein concentrations, most beads carry either one or no target molecule, and the beads are then distributed into arrays of femtoliter-sized wells, where each well isolates a single bead in a very small reaction volume. After addition of a fluorogenic substrate, enzymatic activity on beads carrying a target molecule generates a fluorescent signal. The signal is read digitally by counting the proportion of fluorescent and non-fluorescent wells which enables a markedly higher sensitivity compared with conventional ELISA and allows quantification of proteins in blood at subfemtomolar concentrations.

3.3.3.1 SERUM NFL

Measurements were performed at the Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal by board-certified laboratory technicians blinded to clinical data. To reduce inter-assay variability, baseline and follow-up samples from each participant were analyzed in parallel on the same assay plate using a single reagent batch. Samples from healthy controls were randomly distributed across plates. All procedures were conducted at room temperature.

Serum NfL concentrations were quantified using the Simoa® NF-light™ Advantage Kit on the HD-X Analyzer (Quanterix, Billerica, MA, USA). Samples, internal quality controls, and calibrators were thawed at room temperature. The RGP reagent was mixed for 30 minutes at 800 rpm and heated to 30°C. Calibrators, samples, and quality controls were vortexed for 30 seconds at 2000 rpm and subsequently centrifuged for 10 minutes at 4000 × g. All materials were loaded onto the analyzer and measured using a fourfold dilution. Intra- and inter-assay coefficients of variation were 10%.

3.3.3.2 AINX

CSF AINX concentrations were measured using an in-house Simoa immunoassay developed at the Clinical Neurochemistry Laboratory, University of Gothenburg.¹⁷⁴ The assay employs a two-antibody sandwich format, with a monoclonal capture antibody coupled to paramagnetic beads and a biotinylated detector antibody targeting distinct epitopes within the core region of AINX. Recombinant human AINX served as calibrator, and quantification was performed on the Simoa HD-X platform according to validated laboratory procedures.

Analytical validation demonstrated high sensitivity, with a working range of approximately 0.14–140 ng/L and a lower limit of quantification below 0.2 ng/L. Intra- and inter-assay variability remained below predefined acceptance thresholds (<15%). The assay showed acceptable dilutional linearity, spike recovery, and resistance to repeated freeze–thaw cycles and short-term storage at room temperature or 4°C. Blood contamination at clinically relevant levels did not materially influence measured concentrations. All CSF samples were analyzed in randomized order with laboratory personnel blinded to clinical data.

3.3.4 AQP4 AND MOG ANTIBODY ASSAYS

The most recent guidelines for the diagnosis of AQP4-IgG positive NMOSD and MOGAD recommend live CBA for the detection of antibodies,^{8, 10} due to higher sensitivity and specificity compared to fixed CBA and ELISA.⁶⁷ Both live and fixed CBA use detection by immunofluorescence, but the main difference is that live CBA uses living cells that express native AQP4 or MOG on cell surfaces, which preserves conformational epitopes and patient IgG binds as it would in vivo.⁶⁷ However, live CBA requires maintaining transfected live cells and is more resource dependent, and was not available.

For the analysis in patients in the studies included in this thesis, serum AQP4 and MOG antibodies were analyzed semi-quantitatively using indirect immunofluorescence on AQP4-transfected and MOG-transfected cells (fixed CBA), respectively (EU-90; Euroimmun, Lübeck, Germany) at the Laboratory for Clinical Immunology, Clinical immunology and transfusion medicine, Sahlgrenska University Hospital. MOG-IgG titers ≥ 10 are reported as positive, and AQP4-IgG titers are reported as negative or positive. For this CBA, a sensitivity of 68% has been reported in the literature.¹⁷⁵ In 5 patients with AQP4-IgG-positive NMOSD, serum samples were analyzed by Wieslab Diagnostic Services (SVAR Life Science, Malmö, Sweden).

3.3.5 CALPROTECTIN

Calprotectin in CSF and blood were measured with the Gentian GCAL® Calprotectin Immunoassay kit (Gentian Diagnostics AS, Moss, Norway), which is a particle-enhanced turbidimetric immunoassay (PETIA), run on the Optilite® automated analyzer (The Binding Site Group Ltd, Birmingham, UK). The assay was performed according to the manufacturer's instructions for serum and according to standard operating procedures at the laboratory for clinical immunology at Sahlgrenska University Hospital. In brief, patient serum is mixed with reagent containing microparticles (immunoparticles) coated with anti-calprotectin antibodies. Calprotectin in the sample binds to these antibodies and forms immune aggregates, which makes the reaction mixture more turbid (cloudy). The Optilite measures the reduction in transmitted light through the cuvette (i.e., increased turbidity). The change in signal is proportional to the calprotectin concentration, and the final concentration is obtained by comparison to a calibration curve.¹⁷⁶ The level of quantification (LoQ) was 0.3mg/L and all samples were above limit of detection.

3.3.6 OTHER BIOMARKERS MENTIONED IN THIS THESIS

Biomarker	Main function and association	Assay
CSF Oligoclonal IgG bands and IgG index	Intrathecal synthesis of immunoglobulins by the clonal expansion of plasma cells.	Isoelectric focusing followed by immunoblotting
CSF Oligoclonal IgM bands and IgM index		
Neutrophils in blood	Nonspecific marker of systemic inflammation	Routine complete blood counts with differential performed on an automated hematology analyzer
Chitotriosidase	Marker of activated microglia/macrophages and innate immune activation. ¹⁷⁷	ELISA
Neurogranin	Postsynaptic protein and marker of synaptic injury/dysfunction. ¹⁷⁸	ELISA
CXCL13	B-cell-attracting chemokine and marker of intrathecal B-cell activity. ⁴⁶	ELISA
CHI3L1 (YKL-40)	Glycoprotein associated with microglial/macrophage activation and chronic active CNS inflammation. ¹⁷⁹	ELISA
T-Tau	Marker of neuronal cell body injury. ¹⁸⁰	ELISA
CSF/serum albumin quotient (QAlb)	The CSF/serum albumin quotient is a marker of BBB integrity. ¹⁸¹	Calculated from paired CSF and serum albumin concentrations measured by immunonephelometry

3.4 NEUROIMAGING

All MRI of brain and spinal cord were performed within clinical routine.

In Paper I and II, 3.0 Tesla scanner was used to perform MRI of brain and spinal cord including T2-weighted images, fluid-attenuated inversion recovery (FLAIR) images, diffusion-weighted imaging (DWI), and T1 with gadolinium contrast.

New MRI activity disease activity was defined as a new CEL or new or enlarged T2 lesion.

3.5 ETHICAL APPROVALS AND CONSIDERATIONS

All study participants participated voluntarily. In Studies I, II, IV, and V, written informed consent was obtained from all participants prior to inclusion. In Study III, participants were informed about the study by letter and were given the opportunity to decline participation or request further information. This opt-out procedure was approved by the relevant ethics committee and considered appropriate since the study was retrospective and based on previously collected clinical data and samples.

All data were pseudonymized prior to analysis and stored securely to prevent unauthorized access. Only authorized researchers had access to coded datasets.

MRI and LP were performed as part of routine clinical investigation and not for research purposes. New blood samples were collected prospectively only in Studies I and II. The potential risks associated with blood sampling are minor and are considered proportionate to the potential scientific and clinical value of the studies. In Studies IV–V, analyses were conducted retrospectively on stored frozen serum and CSF samples. Therefore, the additional burden to participants was limited.

Ethical approvals:

- Study I The study was approved by the Regional Committee for Medical Research Ethics, Gothenburg (EPN-460-13) and the Swedish Ethical Review Agency (DNR 2020-04900).
- Study II The Regional Ethics Review Board in Gothenburg, Sweden approved the study (Dnr 1133-16).
- Study III The study was approved by the Regional Ethics Review Board in Gothenburg, Sweden (Dnr 2022-04,293-01, Dnr 460-13, and Dnr 223-15).
- Study IV The regional ethical review boards in Uppsala, Sweden (Dnr 2005:253) and Gothenburg, Sweden, (Dnr 460-13) approved the study.
- Study IV The regional ethical review boards in Stockholm, Sweden (2022-04293-01) and Gothenburg, Sweden, (Dnr 460-13) approved the study.

4 RESULTS

4.1 PAPER I: SERUM NFL IN EXTENDED NATALIZUMAB DOSING INTERVAL

This study investigated longitudinal sNfL concentrations in patients with RRMS that extended the interval of natalizumab treatment (EID4-6) or already had an extended dosing interval (EID5/6). One patient in the EID5/6 cohort experienced a relapse with a new non-enhancing MRI lesion, while no other patients had a relapse or radiological disease activity during the study period. In the EID4-6 cohort, mean EDSS did not change significantly from baseline to 48 weeks ($p = 0.68$); although three patients were considered to convert to secondary progressive MS. Overall, NEDA-3 was achieved in 66 of 70 patients (94%).

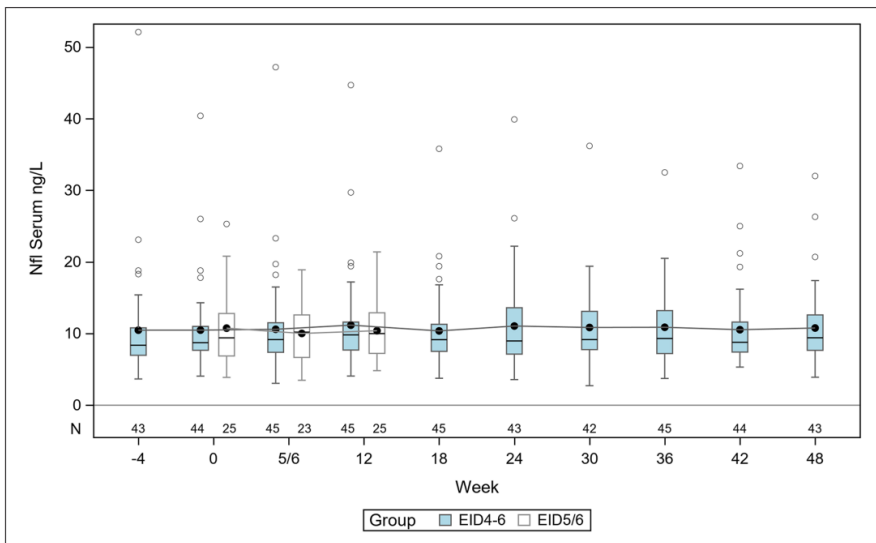


Figure 13 Serum NfL over time in EID4-6 and EID5/6 RRMS patient cohorts. Reproduced from Johnsson et al., *Multiple Sclerosis Journal*, 2022, under the Creative Commons Attribution 4.0 License (CC BY 4.0).

The primary finding was that serum sNfL concentrations remained stable over time in patients switching from standard- to extended-interval dosing. (Figure 13) Mean and median sNfL levels did not increase at any time point during the 12-month follow-up, and no systematic temporal trend was observed.

Moreover, there was no significant difference in the baseline mean sNfL between EID4-6 and EID5/6 (-0.28 ng/L, 95% confidence interval (CI) $=-2.97-2.70$).

Inter-and intraindividual variability in sNfL concentrations was low in both cohorts. In the EID4-6 group, the mean age-adjusted individual range was 4.9 ng/L (95% CI = 3.92–5.88). Three participants had sNfL concentrations above +2SD from mean sNfL (mean; 10.8ng/L, 2SD; 22.6ng/L) in the EID4-6 cohort; all were in the oldest quartile of the patient cohort, but none had increasing levels of sNfL during the study period, or radiological or clinical evidence of disease activity. The correlation between sNfL and age ($R=0.48$, $p<0.001$) and intra-individual variability is visualized in Figure 14.

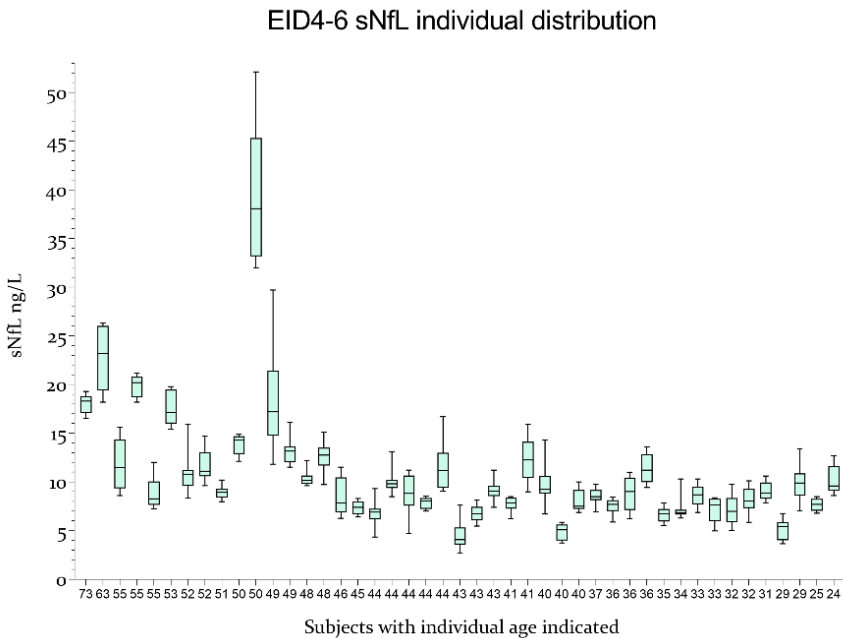


Figure 14 Serum NfL concentration distribution in individual patients. Reproduced from Johansson et al., *Multiple Sclerosis Journal*, 2022, under the Creative Commons Attribution 4.0 License (CC BY 4.0).

Overall, these results indicate that extending natalizumab dosing intervals does not lead to increased axonal damage as reflected by sNfL, and they highlight the low variability of sNfL at both the individual and group level under conditions of sustained disease stability.

4.2 PAPER II: SERUM NFL LEVELS DURING MS RELAPSE ACTIVITY

In this study, we repeatedly measured sNfL concentrations in 44 participants with either RRMS (n=40) or CIS (n=4) and evidence of clinical or radiological disease activity. At inclusion, 61% of patients had both a clinical relapse and CEL, 30% of patients had a clinical relapse but no CEL, and 9% of patients had CEL(s) but no clinical relapse. Nine patients had evidence of new disease activity after baseline. Nine patients had evidence of new disease activity after baseline.

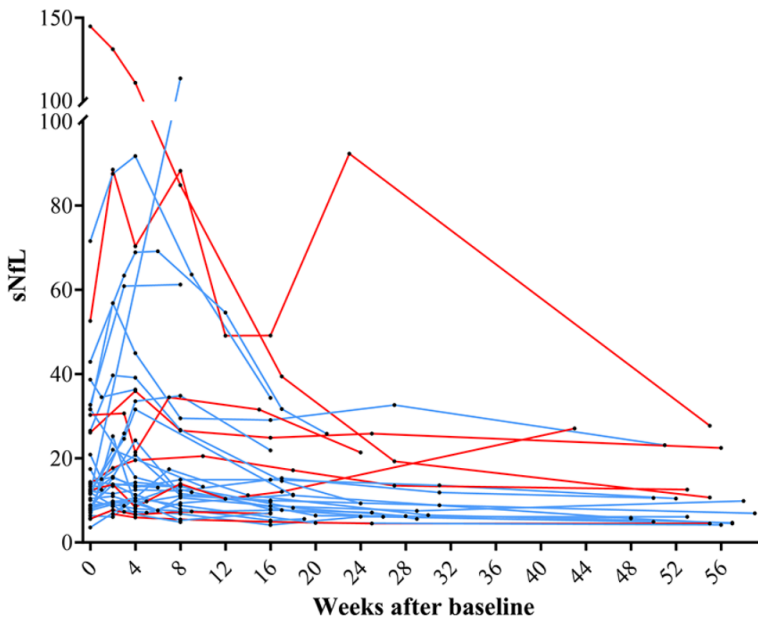


Figure 15 sNfL trajectories in individual patients with CIS or RRMS with clinical or radiological evidence of disease activity. Baseline (week 0) is first serum sampling. Blue lines indicate individual patients without disease activity during follow-up, and red lines indicate individuals with clinical or radiological evidence of new disease activity after baseline. Reproduced from Johnsson et al., Multiple Sclerosis Journal, 2024, under the Creative Commons Attribution 4.0 License (CC BY 4.0).

The median sNfL at baseline was 12.4 ng/L (IQR; 8.1-26.1), increased to 14.6 ng/L two weeks after baseline ($p=0.001$), with a subsequent gradual decline (Figure 15). Substantial heterogeneity was observed in individual sNfL trajectories, and it was only patients with relapses with concomitant CELs that had statistically significant increase in sNfL after baseline (Figure 16).

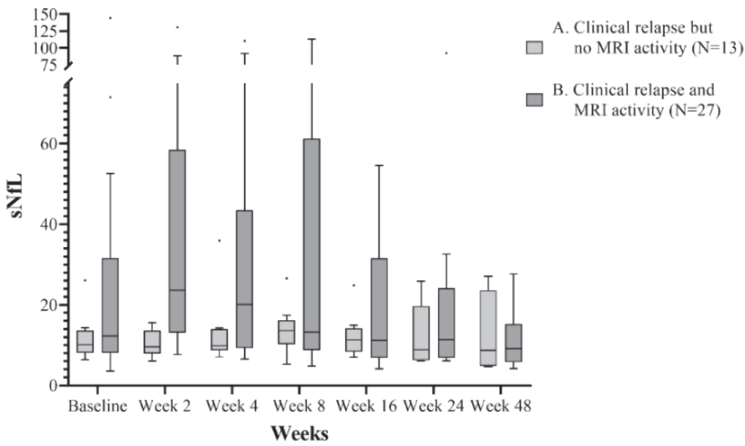


Figure 16 sNfL levels in subgroups. Reproduced from Johnsson et al., *Multiple Sclerosis Journal*, 2024, under the Creative Commons Attribution 4.0 License (CC BY 4.0).

In patients with a clinical relapse, sNfL concentrations reached a peak after a median of 5.5 weeks after relapse symptom onset, which is visualized in Figure 17.

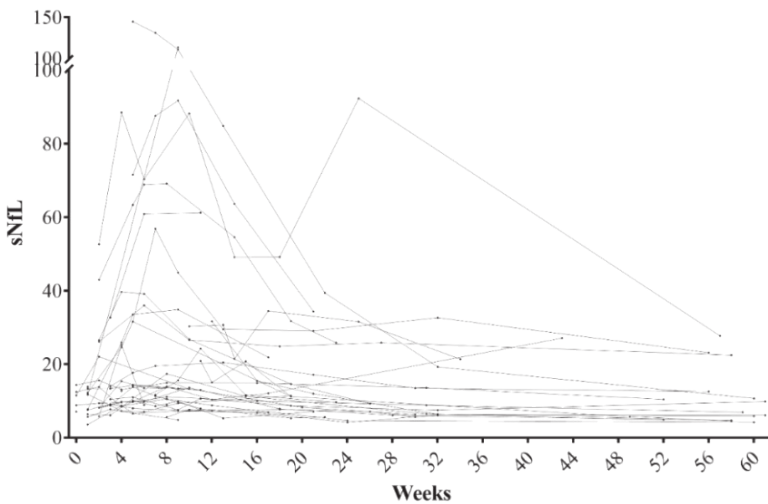


Figure 17 sNfL trajectories in patients with clinical relapses. Baseline is set to relapse symptom onset. Reproduced from Johnsson et al., *Multiple Sclerosis Journal*, 2024, under the Creative Commons Attribution 4.0 License (CC BY 4.0).

Analyses were performed to evaluate the sensitivity and specificity of sNfL for detecting disease activity (Table 10). The cohort with NEDA-3 (n = 66) in Paper I served as stable controls. Median baseline Z-score in NEDA-3 patients was 0.76 (IQR; 0.36-1.37), compared to 1.77 (IQR; 1.17-2.65) in patients with disease activity. Based on AUC values, age- and body mass index-adjusted sNfL Z-scores provided better discrimination than raw concentrations. A baseline Z-score threshold slightly above one SD from normative values yielded a moderate sensitivity (81%) and specificity (70%).

Table 10 sNfL variables and calculations of sensitivity and specificity for the detection of disease activity Reproduced from Johnsson et al., Multiple Sclerosis Journal, 2024, under the Creative Commons Attribution 4.0 License (CC BY 4.0).

sNfL variable	AUC	Cut-off	Sensitivity	Specificity	PPV	NPV	OR (95% CI) ^a	
							Cut-off used as dichotomous factor variable	Per unit of measurement
Raw absolute sNfL at baseline ^b	0.72 (<i>p</i> =0.0010)	>10.0 ng/L	60%	82%	77%	71%	2.8 (1.3–6.4)	1.1 (1.0–1.1)
sNfL range across the study period	0.76 (<i>p</i> =0.0001)	>7.7 ng/L	59%	93%	82%	79%	17.5 (5.7–53.4)	1.2 (1.1–1.3)
Z-score at baseline ^b	0.78 (<i>p</i> <0.0001)	>1.1	81%	70%	64%	85%	10.0 (4.0–26.0)	3.4 (2.0–5.8)
Z-score at week 5.5 after relapse ^{b,c}	0.84 (<i>p</i> <0.0001)	>1.3	82%	71%	63%	87%	11.3 (4.3–30.0)	4.7 (2.5–8.9)
Z-score at baseline in patients with CELs ^b	0.81 (<i>p</i> <0.0001)	>1.1	83%	70%	56%	90%	11.5 (3.9–34.4)	3.8 (2.1–6.8)
Z-score at baseline in patients with relapse but no CELs	0.72 (<i>p</i> =0.0140)	>0.8	77%	54%	26%	94%	5.8 (1.2–28.4)	2.9 (1.2–7.1)

AUC: area under the curve; sNfL: serum neurofilament light; PPV: positive predictive value; NPV: negative predictive value; OR: odds ratio; CI: confidence interval; CEL: contrast-enhancing lesion.

If not specified otherwise, all patients in the study cohort and all controls were included in the analyses.

^aOdds ratio for a patient being part of the study cohort with inflammatory disease activity versus controls.

^bIn controls, the individual mean sNfL was used to calculate the state variable.

^cIn patients with a clinical relapse (*N*=40).

4.3 PAPER IV: AINX CONCENTRATIONS IN PATIENTS WITH MS

In Paper IV, we used a novel method to quantify concentrations of AINX in patients with RRMS and healthy controls CSF.

AINX concentrations were significantly higher in RRMS (median; 1.19 ng/L, IQR 0.69-2.34) compared with controls (median; 0.44 ng/L, IQR 0.36-0.51) ($p < 0.001$) (Figure 18), and correlated with markers of disease activity, including CELs ($R = 0.60$; $p = 0.0002$) and new T2 lesions lesions ($R = 0.42$; $p = 0.013$).

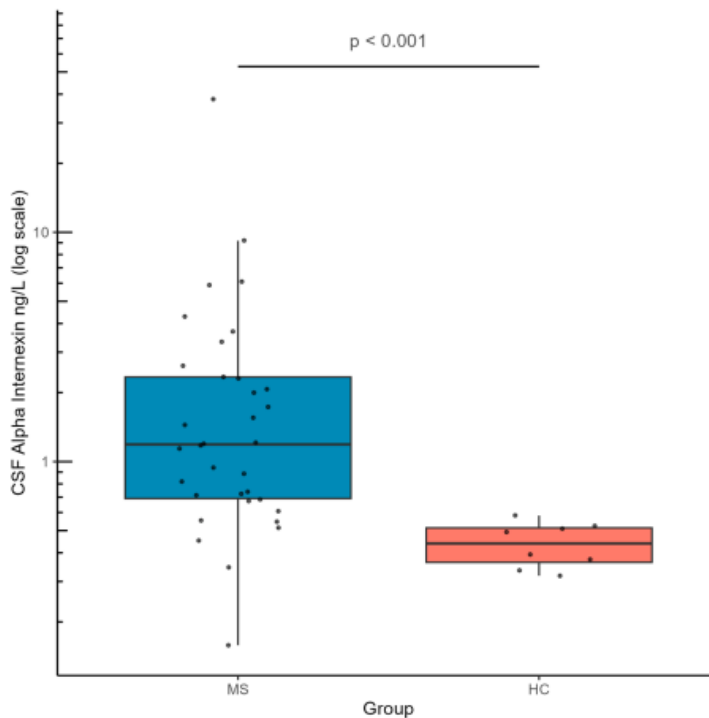


Figure 18 CSF α -internexin in patients with MS and controls. Reproduced from Johnson et al., *Multiple Sclerosis and Related Disorders*, 2025, under the Creative Commons Attribution 4.0 License (CC BY 4.0).

A strong correlation was observed between AINX and cNfL ($R = 0.9$; $p < 0.001$) (See Figure 19). Consistent with the pattern seen for cNfL, AINX levels decreased following escalation of DMT in the subgroup of patients who switched from platform therapy to fingolimod ($p = 0.0254$).

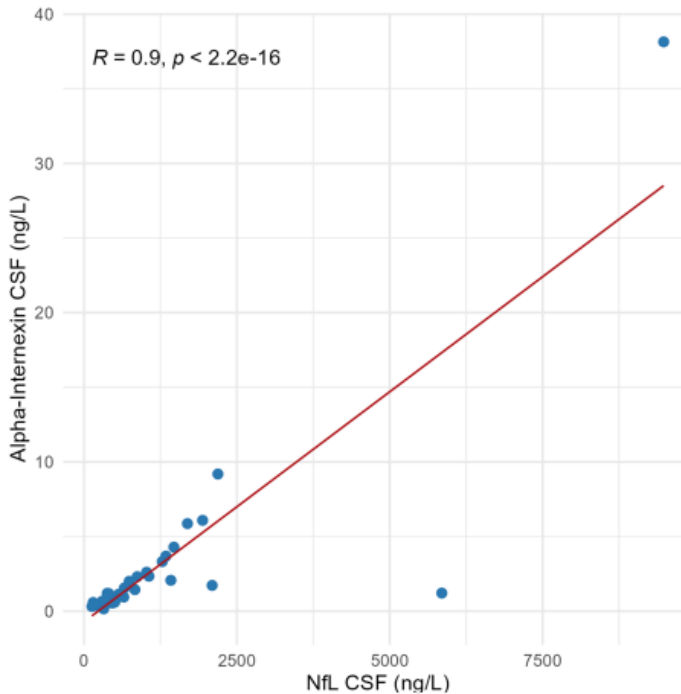


Figure 19 Correlation between CSF α -internexin and NfL. Reproduced from Johansson et al., *Multiple Sclerosis and Related Disorders*, 2025, under the Creative Commons Attribution 4.0 License (CC BY 4.0).

Retrospectively, we identified one extreme outlier with a disproportionately elevated cNfL concentration relative to CSF AINX (NfL: 5852 ng/L; AINX: 1.21 ng/L). According to the medical records, this patient had a documented peripheral nerve injury (radiculopathy secondary to disc herniation), which likely contributed to the markedly elevated cNfL level. No other patient had documented clinical evidence of peripheral nerve injury.

4.4 PAPER III AND V: BIOMARKERS FOR IMPROVED DIAGNOSTIC ACCURACY IN NMOSD AND MOGAD

4.4.1 PAPER III:

In this retrospective observational study, we expanded the scope and included patients with AQP4-IgG-positive NMOSD, DN-NMOSD, MOGAD, and RRMS. CSF GFAP concentrations were markedly elevated in AQP4-IgG-positive NMOSD (median 2470 ng/L) compared with the other diagnostic groups ($p < 0.001$; Figure 20). BBB dysfunction, assessed by the QAlb, was likewise more pronounced in AQP4-IgG-positive NMOSD.

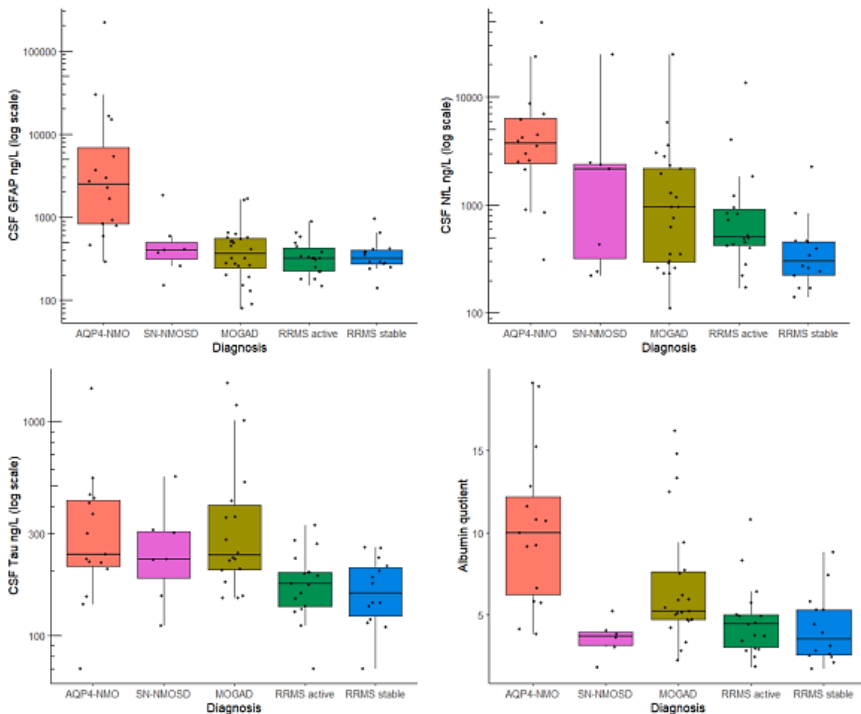


Figure 20 GFAP, NfL, Tau, and Albumin quotient in disease categories AQP4-NMOSD, DN-NMOSD, MOGAD, and RRMS. Reproduced from Johansson et al., *Multiple Sclerosis and Related Disorders*, 2025, under the Creative Commons Attribution 4.0 License (CC BY 4.0).

In patients with DN-NMOSD, MOGAD, or RRMS, the median CSF GFAP concentration was 330 ng/L, and no individual exceeded 2050 ng/L. In contrast, none of the patients with AQP4-IgG-positive NMOSD had CSF GFAP concentrations below 290 ng/L. ROC curve analysis demonstrated a high discriminatory performance of CSF GFAP for distinguishing AQP4-IgG-positive NMOSD from the other diagnostic groups (AUC 0.92; 95% CI 0.84–1.00). The optimal cut-off value (>715 ng/L) yielded a sensitivity of 81% and a specificity of 92%.

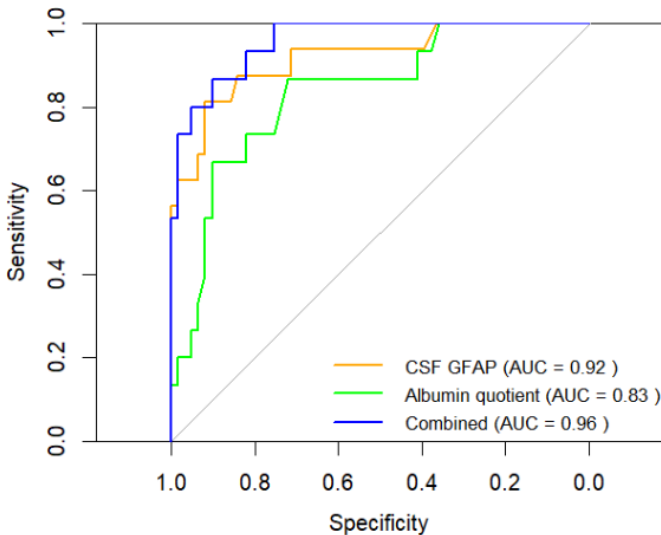


Figure 21 Reproduced from Johnsson et al., *Multiple Sclerosis and Related Disorders*, 2025, under the Creative Commons Attribution 4.0 License (CC BY 4.0).

Combining GFAP with QAlb to differentiate AQP4-NMOSD versus the other diagnostic groups combined, increased diagnostic specificity for AQP4-NMOSD. The combined biomarker model outperformed either marker alone (the combined biomarker model resulted in an AUC of 0.96) (Figure 21) supporting the concept that astrocytic injury and blood–brain barrier disruption represent complementary pathological features of AQP4-NMOSD. In contrast, DN-NMOSD showed a biomarker profile that was more similar to RRMS than to AQP4-NMOSD. GFAP levels were lower, and BBB disruption was less pronounced.

4.4.2 PAPER V

In this observational study, CSF and serum calprotectin were measured in AQP4-NMOSD (n=10), DN-NMOSD (n=6), MOGAD (n=9), RRMS (n=10), and healthy controls (n=8). Serum calprotectin was significantly elevated in AQP4-NMOSD, DN-NMOSD, and MOGAD compared with RRMS and controls (p<0.001) (See Figure 22).

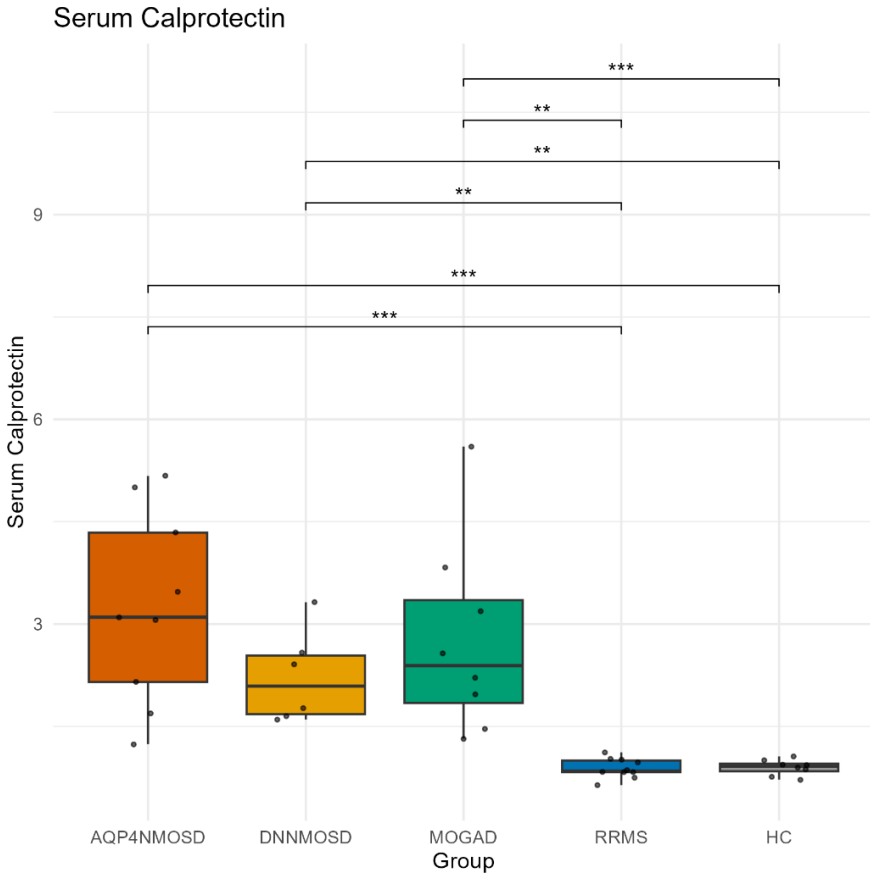


Figure 22 Serum calprotectin concentrations across diagnostic groups.

CSF calprotectin was detectable in all samples but was significantly lower in MOGAD compared to NMOSD and RRMS (median; 0.23 mg/L, IQR 0.22-0.31) ($p=0.025$) (See Figure 23). However, CSF calprotectin did not distinguish NMOSD from RRMS. In RRMS, both CSF and serum calprotectin correlated inversely with time since last relapse.

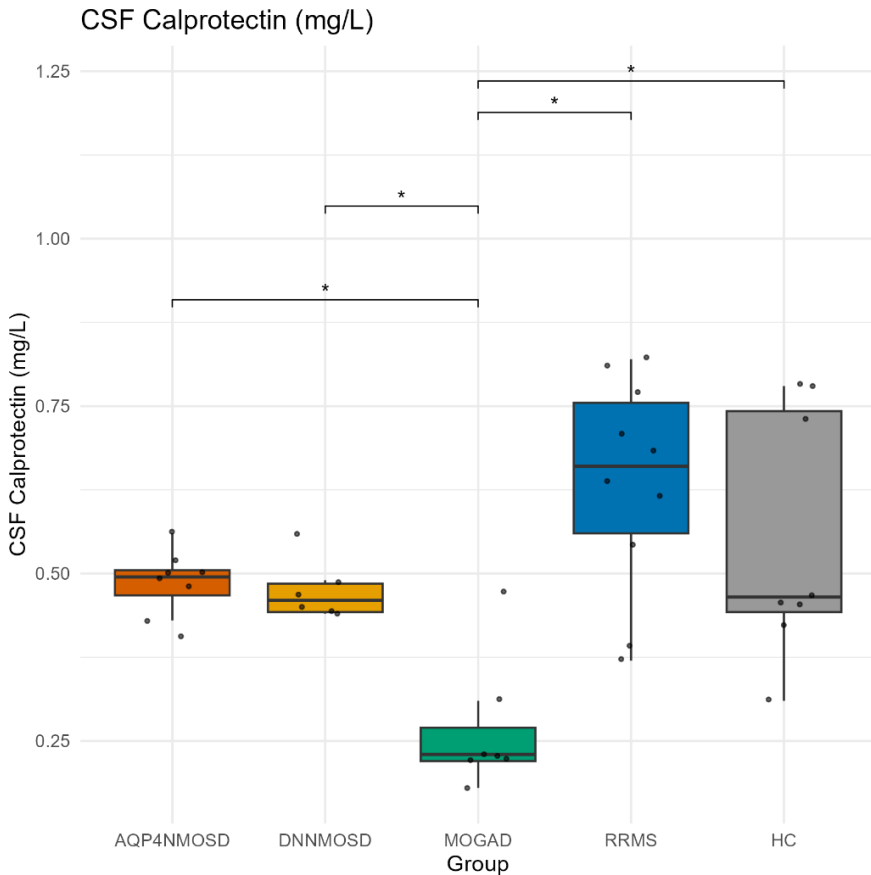


Figure 23 Cerebrospinal fluid calprotectin concentrations across diagnostic groups

5 DISCUSSION

This thesis focuses on soluble biomarkers that reflect different parts of inflammatory CNS disease: axonal injury (NfL, AINX), astrocyte damage (GFAP), BBB disruption (QA1b), and the innate immune system (calprotectin). Across the papers, a common theme is that biomarkers can add information beyond clinical assessment and MRI, but they also have important limits, especially when used for decisions in single patients.

A second overarching theme is context: the same biomarker value can mean different things depending on age, body mass index (BMI), comorbidities, timing relative to relapse, and ongoing treatment. This is particularly clear for NfL, where population-level associations are strong, yet the individual-level diagnostic performance is not perfect even when using age- and BMI-adjusted reference values (Z-scores).

5.1 AXONAL INTEGRITY DURING EXTENDED DOSING NATALIZUMAB TREATMENT

A key worry when extending natalizumab dosing interval was whether this could permit subclinical inflammatory activity not captured by routine monitoring. In Paper I, we addressed this by longitudinally measuring sNfL, a well-established marker of neuroaxonal injury, and found no evidence of increased axonal injury in patients switching to EID, as reflected by stable sNfL concentrations. Parallel to our study, the efficacy of EID was investigated by research groups worldwide. Prior to our publication, several studies had reported preserved clinical efficacy of EID based on relapse and MRI outcomes,^{42, 43, 182, 183} and some had assessed sNfL at baseline and after six months,¹⁸⁴ and at baseline and 12 months after dosing extension.⁴⁴ However, early data on cNfL kinetics suggests that repeated sampling every 3–6 months is required to capture inflammatory disease activity,¹³² an approach later reflected in more recent sNfL monitoring recommendations.¹⁸⁵ In Paper I, we applied frequent sampling, and our findings provide strong support for preserved axonal integrity when switching from SID to 6-week EID.¹⁸⁶ These results were subsequently corroborated by additional studies examining sNfL in patients switching to 6-week EID.^{187, 188} Taken together, there is now robust group-level evidence indicating that EID does not confer a statistically significant risk of increased neuroaxonal injury. This also illustrates how sNfL, as an established biomarker of neuroaxonal damage, can serve as a valuable

tool in clinical studies. Collectively, these findings have contributed to the adoption of 6-week EID as common clinical practice.

The question of the optimal natalizumab dosing interval remains relevant. Although a detailed discussion lies beyond the primary scope of this thesis, it is noteworthy that in one cohort of 50 patients with RRMS, sNfL levels remained stable in most individuals during the first eight weeks after natalizumab cessation, suggesting no immediate surge in neuroaxonal injury.⁴⁸ In efforts to refine treatment strategies, personalized intravenous interval dosing guided by natalizumab trough concentrations has recently been evaluated in several cohorts,^{44,189} demonstrating that maintaining levels around 10 µg/mL—and possibly even ~5 µg/mL—may preserve efficacy while allowing extended intervals.¹⁸⁹ However, the evaluation of optimal and safe dosing intervals with respect to neuroaxonal integrity is evolving in parallel with developments in treatment delivery, including the approval of subcutaneous natalizumab.⁴⁰ As the field continues to refine individualized dosing strategies, sensitive biomarkers such as sNfL are likely to play an increasingly important role in monitoring subclinical disease activity. Longitudinal sNfL assessments may therefore contribute meaningfully to future efforts aimed at balancing efficacy, safety, and treatment burden in natalizumab-treated patients.

Paper I also contributes data on inter- versus intra-individual variation during long-term stable natalizumab treatment. Most patients showed only minor fluctuations over time, and variability between patients was greater than variability within the same individual. This supports the practical view that sNfL is best interpreted in relation to a patient's own previous values whenever possible, rather than as a single isolated measurement. It also highlights the relevance of individualized interpretation approaches—such as age-adjusted reference ranges or z-score-based normalization—an aspect that leads into the next section.

5.2 SERUM NFL EVALUATION IN THE INDIVIDUAL PATIENT

In Paper II, we evaluated sNfL levels in 44 patients with RRMS or CIS during clinical and/or radiological activity. Consistent with prior work, sNfL reflected inflammatory disease activity at the group level.¹⁸⁵ However, rather than re-establishing this association, our primary aim was to assess its utility for detecting disease activity in the individual patient and to characterize its temporal dynamics.

Peak sNfL concentrations occurred at a median of 5.5 weeks after relapse onset, in line with the known biological kinetics of NfL release and clearance over a 3–6 month window.^{185, 190} Importantly, this increase was largely driven by patients with CELs, which aligns with established association between sNfL and radiological activity.¹⁴³ One recent study similarly reported elevations only in relapses accompanied by CELs.¹⁹⁰ In contrast, patients with clinical relapse without CELs showed minimal group-level sNfL dynamics. Thus, some patients experienced clinical relapses with stable sNfL and MRI findings. These events may represent less axonally destructive inflammatory episodes, but they also highlight a conceptual limitation: there is currently no perfect gold standard for defining “true” inflammatory activity. In one cohort of 637 clinical relapses, one-fourth were not associated with new T2 lesions or CELs in the peri-event window.¹⁰³ Moreover, even in the presence of CELs, sNfL demonstrated only moderate sensitivity—consistent with other reports.^{138, 191}

At the individual level, sNfL therefore has imperfect sensitivity. In our active cohort, only about two-thirds had peak sNfL values above commonly used upper reference limits.¹⁴⁴ Nevertheless, sNfL measurements impact clinical decision-making.¹⁹² In our study, Z-scores performed better than absolute cut-offs, and have in other studies been shown to outperform raw concentrations in predicting disease activity,¹⁹³ and treatment response.¹⁹⁴ This has contributed to its increasing adoption and the recent introduction of pediatric reference Z-scores.¹⁹⁵ However, even when applying age- and BMI-adjusted Z-scores, sensitivity and specificity were moderate. A baseline Z-score slightly above 1 provided reasonable discrimination, whereas higher Z-scores (e.g., ≥ 1.5) have been associated with increased future relapse risk in other cohorts (e.g., Benkert et al.).¹⁴⁵ These findings are not contradictory: lower thresholds may optimize detection of concurrent activity, whereas higher thresholds may better predict future risk. Context and intended use—diagnostic versus prognostic—are therefore critical.

Importantly, sensitivity and specificity must be interpreted in light of the study design. In our analysis, approximately 40% of individuals were classified as having active disease (44 active vs 66 NEDA-3 controls). This prevalence does not necessarily reflect a real-world outpatient setting and influences predictive values. Thus, these performance metrics should be viewed as approximate guides rather than universally applicable clinical constants. In addition, patients with MS tend to have elevated baseline sNfL compared with healthy controls, even during clinical quiescence. In our NEDA-3 controls, the median baseline Z-score was 0.76. Interpretation should thus not rely on “normalization to zero,” but rather on deviation from individualized baselines and prior values. A single measurement must always be interpreted alongside clinical and MRI findings, in line with recent consensus recommendations.¹⁸⁵

In summary, sNfL demonstrates meaningful group-level association with inflammatory activity and moderate diagnostic accuracy at the individual level. Its principal clinical value lies in longitudinal monitoring and risk stratification rather than binary decision-making based on a single measurement. Despite the substantial body of literature, optimal interpretation in the individual patient remains an evolving area, underscoring the need for further prospective studies integrating clinical, radiological, and biochemical data.

5.3 AINX: A NEW BIOMARKER FOR MEASURING AXONAL INJURY

In Paper IV, we demonstrate that CSF AINX is increased in patients with MS compared with healthy controls ($p < 0.001$). Although AINX has been described for decades as an axonal structural intermediate filament protein, it has remained largely unexplored as a quantitative biomarker. The ability to reliably measure AINX at low concentrations represents a methodological advance and enabled the present evaluation of its clinical relevance in MS.

We found that CSF AINX correlated strongly with cNfL ($R = 0.9$, $p < 0.001$), which strongly supports the interpretation of AINX as a marker of axonal injury. Furthermore, AINX correlated with radiological inflammatory activity, and intrathecal inflammatory markers, and was reduced in patients switching from platform therapy to fingolimod, paralleling the established behavior of NfL. Together, these findings position AINX as a neuroaxonal biomarker reflecting acute inflammatory tissue damage in MS.

A key conceptual aspect of AINX concerns tissue specificity. Neuropathological data suggest that AINX is predominantly expressed in the CNS, in contrast to NfL, which is abundant in both CNS and peripheral nerves. However, while available data support CNS enrichment, CNS specificity cannot yet be regarded as definitively established. Studies of AINX expression in peripheral nerve tissue are scarce, and additional translational work is required before firm conclusions can be drawn. If CNS specificity is confirmed, AINX represents a candidate for differentiating CNS from peripheral axonal injury. In our dataset, the patient with concomitant radiculopathy displayed a disproportionately high NfL/AINX quotient, consistent with peripheral NfL release without a corresponding rise in AINX. Such observations align with a potential advantage of AINX over NfL in clinical scenarios where confounders such as peripheral nerve injury may influence blood NfL concentrations. The clinical utility of AINX would likely be even greater if a sensitive blood-based assay were developed, as differentiation between central and peripheral sources of NfL can be particularly challenging in serum or plasma.

5.4 GFAP: A MARKER OF ASTROCYTOPATHY IN AQP4-IGG- POSITIVE NMOSD

In Paper III, we examined CSF biomarkers in a real-world cohort of patients with AQP4-IgG-positive NMOSD, DN-NMOSD, MOGAD, and RRMS, assessing the diagnostic performance of GFAP alone and in combination with QAlb. We confirmed previous studies demonstrating increased CSF GFAP levels in patients with AQP4-IgG NMOSD,^{154, 196} and high levels of GFAP demonstrated strong discriminatory accuracy compared to other diagnostic groups. Importantly, combining GFAP with QAlb further improved diagnostic performance (AUC 0.96), underscoring the complementary value of astrocytic injury and barrier dysfunction markers. Although GFAP demonstrated high discriminatory accuracy, interpretation must consider disease prevalence. In (NMOSD) low-prevalence settings such as Sweden, MS remains statistically more likely than NMOSD despite strong biomarker separation, underscoring the role of GFAP as a complementary biomarker.

Our study extended previous non-antibody biomarker research, and we also included MOGAD and DN-NMOSD in direct comparison. Notably, the biomarker profile of DN-NMOSD appeared more aligned with MOGAD or

MS than with AQP4-IgG-positive NMOSD, supporting the concept that seronegative cases may lack the profound astrocytopathy characteristic of AQP4-IgG-mediated disease.¹⁹⁷

Since the publication of Paper III, increasing attention has shifted toward serum GFAP as a less invasive biomarker,^{198, 199} and GFAP Z-scores—derived from MS research—have been proposed to account for age-related variation,²⁰⁰ although not yet validated for NMOSD or MOGAD. Moreover, the GFAP/NfL ratio has been suggested to further enhance discrimination between primary astrocytopathy and secondary axonal injury patterns.²⁰⁰ In our cohort, AQP4-NMOSD also showed elevated cNfL, reflecting substantial secondary axonal damage during severe attacks, which may partially reduce the discriminatory separation based on GFAP/NfL ratio calculation.

Serum GFAP has been evaluated as a monitoring biomarker in AQP4-IgG-positive NMOSD, but has not yet been found to correlate with time to relapse.²⁰¹ This supports our conclusion that, given the relapse-driven nature of NMOSD, the primary clinical utility of GFAP lies in the acute diagnostic setting rather than in longitudinal relapse prediction. When antibody testing is delayed, unavailable, or inconclusive, markedly elevated CSF GFAP may provide important supportive evidence for AQP4-IgG-positive NMOSD and facilitate timely treatment decisions.

5.5 CALPROTECTIN IN CSF AND SERUM

In Paper V, we found that serum calprotectin levels were elevated in patients with NMOSD and MOGAD compared with patients with RRMS and healthy controls. This supports the notion of systemic innate immune activation in these disorders and indicates that serum calprotectin may have diagnostic potential, although the small sample size means that the magnitude of these differences should be interpreted cautiously. In contrast, the relatively low CSF calprotectin levels observed in NMOSD, and particularly in MOGAD, were unexpected, since the neutrophil-associated inflammatory features of these disorders¹⁵² might have suggested increased CSF calprotectin concentrations. These findings indicate that CSF calprotectin may not be a reliable marker of granulocyte-driven pathology in NMOSD or MOGAD. While the sample size was too small to allow firm conclusions regarding associations with disease activity, serum and CSF calprotectin levels in RRMS were inversely correlated with time since last relapse, in agreement with the only previous study of CSF calprotectin in RRMS.¹⁶⁷

5.6 STRENGTHS AND LIMITATIONS

5.6.1 STRENGTHS

A major strength of this thesis is the prospective study design in Paper I and II, with predefined sampling schedules and systematic clinical and MRI follow-up. In particular, the studies with repeated serum sampling at short intervals allowed us to evaluate biomarker dynamics over time within the same individual, rather than relying only on cross-sectional comparisons. This longitudinal approach increases clinical relevance, since biomarkers are intended to support monitoring in individual patients.

Another important strength is that the data are derived from real-world clinical practice. Patients were recruited from a tertiary MS center and reflect routine care rather than highly selected trial populations. This improves generalizability and makes the findings directly applicable to everyday neurology practice. In addition, sampling in relation to relapse activity was carefully documented in several studies, which strengthens interpretation of biomarker levels in an inflammatory context.

The thesis also benefits from access to well-characterized biobank material and to biological samples from patients with rare diseases such as AQP4-IgG-positive NMOSD and MOGAD. Obtaining CSF and blood samples from these patient groups is challenging, and assembling sufficiently characterized cohorts requires long-term clinical follow-up and systematic record review. The availability of this material enabled comparative analyses across related but biologically distinct disorders, which strengthens conclusions regarding disease specificity and pathophysiological mechanisms.

The thesis also benefits from the use of well-validated laboratory methods, including ultrasensitive Simoa-based assays performed at accredited laboratories, but in addition also the implementation of novel analytical methods.

5.6.2 LIMITATIONS

Several limitations should be acknowledged. First, sample sizes were relatively small, particularly in the rare disease groups such as AQP4-IgG-positive NMOSD, DN-NMOSD, and MOGAD. Although this reflects the low prevalence of these disorders, small cohorts reduce statistical power and limit the robustness of subgroup analyses.

Second, not all studies were prospective. Paper III was a retrospective study, and Paper II included partly retrospective group comparisons. Such designs increase the risk of selection bias and incomplete documentation. Missing data also represent a limitation. For example, spinal MRI was not available in all patients in Paper II, which may have influenced associations between MRI findings and biomarker levels.

Third, Papers III and V were primarily cross-sectional with single time-point sampling, limiting conclusions regarding temporal biomarker dynamics and causality.

From a statistical perspective, conclusions regarding sensitivity and specificity in Paper II must be interpreted within the studied population. Predictive values depend on disease prevalence, and since the cohorts were enriched rather than population-based, PPV and NPV may not be directly generalizable to routine clinical settings.¹⁷¹

Preanalytical and analytical factors may also have influenced results. In Paper III, samples from patients with NMOSD and MOGAD were collected and stored at the virology department before analysis, introducing potential preanalytical variability. In Paper V, calprotectin was quantified using a turbidimetric assay validated for serum and plasma but not fully analytically validated for CSF. Matrix-related effects and differences in sample handling may therefore influence absolute concentrations and limit comparability.

Finally, in Papers III and V, fixed CBA were used for AQP4-IgG and MOG-IgG detection. These have lower sensitivity than live CBA,^{67, 175} the current reference standard, and may have contributed to misclassification in the DN-NMOSD group.

6 CONCLUSION AND FUTURE DIRECTIONS

This thesis builds on the long-standing biomarker tradition in inflammatory demyelinating disease, with Gothenburg playing a central role.

In Paper I, we found no evidence of increased axonal injury, as measured by sNfL, after extending natalizumab dosing in RRMS. The 6-week interval is now established in clinical routine, although the optimal interval remains a topic of studies. The field is moving toward individualized dosing based on drug concentration, and sNfL will likely remain central in defining safe lower exposure limits without compromising disease control.

In Paper II, we confirmed the association between sNfL and clinical and radiological activity but also demonstrated its limitations. Sensitivity at the individual level is moderate, even when using age- and BMI-adjusted Z-scores, and some patients with relapse or CEL show no marked elevation. sNfL should therefore be viewed as complementary to clinical assessment and MRI. Its main value lies in longitudinal monitoring and risk stratification rather than single-measure decision-making. As sNfL is now routinely measured, accumulating real-world data will further clarify its clinical role.

As seen with NfL, development of a sensitive blood-based assay for AINX would represent an important advance, enabling longitudinal studies and larger cohorts. Given its correlation with NfL and proposed CNS specificity, AINX may help distinguish central from peripheral axonal injury—for example in MS with concomitant neuropathy, suspected orthopedic myelopathy, or traumatic brain injury with extracranial confounders.

Finally, despite antibody-based diagnostics in NMOSD and MOGAD, non-antibody biomarkers remain relevant. Our data show that CSF GFAP robustly differentiates AQP4-IgG-positive NMOSD from MS and MOGAD, while DN-NMOSD appears heterogeneous and warrants further study.

USE OF GENERATIVE AI

Generative AI tools, including ChatGPT, were used in this thesis for grammar corrections and suggestions on language improvements.

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