# Rituximab in multiple sclerosis - Fatigue, cognition, and efficacy

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# Rituximab in multiple sclerosis

## Fatigue, cognition, and efficacy

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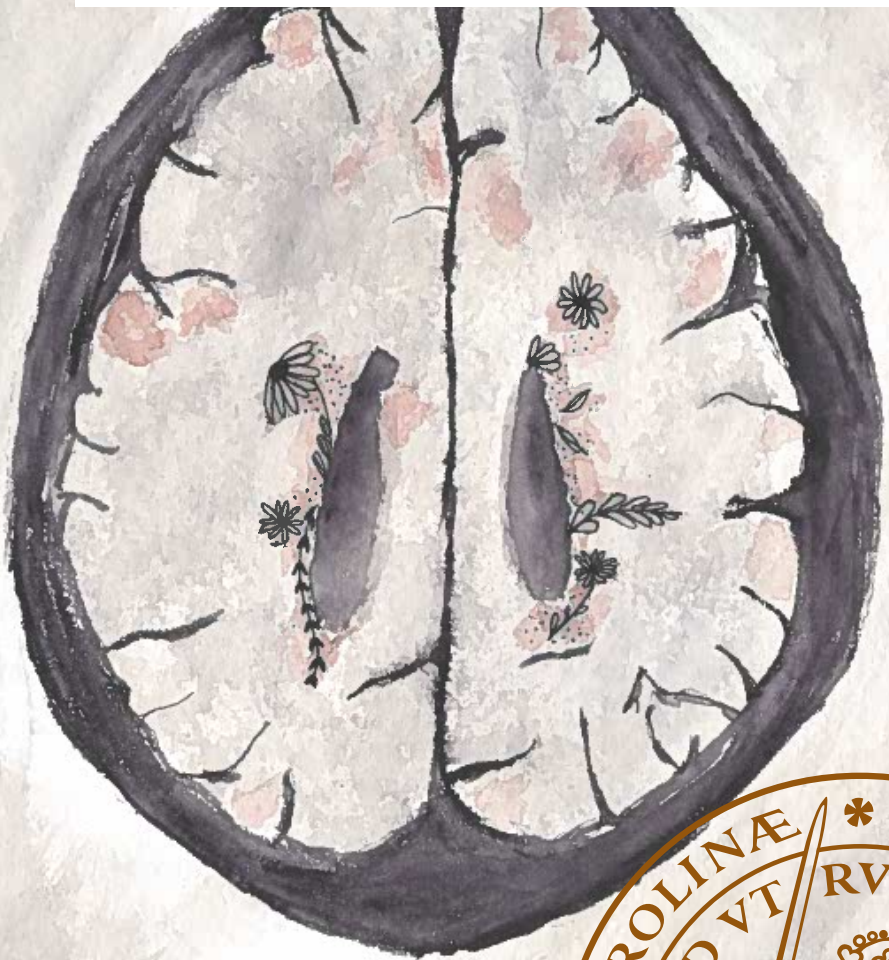

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Rituximab in multiple sclerosis

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## Fatigue, cognition, and efficacy

Johan Hellgren


LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 5th of December at 13.00 in in the Medicinhistoriska Museet, Bergaliden 20, Helsingborg

*Faculty opponent*
Professor Emeritus Sten Fredrikson, Department of Clinical Neuroscience, Karolinska Institute

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**Title and subtitle:** Rituximab in multiple sclerosis – Fatigue, cognition, and efficacy

**Abstract:**

**Background:** Multiple sclerosis (MS) is an inflammatory, demyelinating disease affecting the central nervous system. Fatigue and impaired information processing speed (IPS) are the most debilitating symptoms of MS. Natalizumab (NTZ) and rituximab (RTX), currently Sweden's two most-prescribed drugs for MS treatment, are known to almost completely ameliorate clinical and radiological relapse activity in patients with RRMS. However, the effects on fatigue and IPS are unclear. RTX remains an off-label therapy for MS.

**Methods:** This thesis comprises two study designs. The first is a retrospective observational study evaluating the safety and efficacy of RTX in 83 patients with MS at the Helsingborg General Hospital. The second is a cross-sectional multicenter study including 128 patients with relapsing-remitting MS, treated with either NTZ or RTX for at least 24 months. Fatigue, IPS, and levels of the astrocyte marker, glial fibrillary acidic protein (GFAP), and axonal damage marker, neurofilament light (NfL), in the cerebrospinal fluid and plasma were compared in both treatment groups. IPS was assessed using the Symbol Digit Modalities Test (SDMT) and fatigue, with the Fatigue Scale for Motor and Cognitive Functions (FSMC). Fatigue and IPS were explored in relation to GFAP and NfL.

**Results:** In the retrospective study, RTX significantly reduced relapses and new lesions on MRI. Serious adverse events were few. In the comparative cross-sectional study, no between-group differences were found regarding fatigue. Participants treated with NTZ performed significantly better on the SDMT than those treated with RTX. Plasma GFAP and NfL levels did not significantly differ between the treatment groups. Neither GFAP nor NfL exhibited significant associations with fatigue or IPS.

**Conclusion:** RTX is a safe and effective option for treating relapsing-remitting MS. The significantly better performance on SDMT of patients treated with NTZ may have been influenced by repeated exposure to the test, i.e., practice effects. Longitudinal controlled studies are needed to further evaluate the effects of high-efficacy disease modifying therapies on fatigue and IPS in MS.

**Key words:** Multiple sclerosis, rituximab, natalizumab, fatigue, information processing speed, GFAP, NfL, oligoclonal bands, kappa free light chain, patient reported outcome measures

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# Rituximab in multiple sclerosis

## Fatigue, cognition, and efficacy

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*To Emelie*

*"Insanity is doing the same thing over and over again and expecting results to change"*

*- Albert Einstein (attributed)*

# Table of Contents

# Abstract

**Background:** Multiple sclerosis (MS) is an inflammatory, demyelinating disease affecting the central nervous system. Fatigue and impaired information processing speed (IPS) are the most debilitating symptoms of MS. Natalizumab (NTZ) and rituximab (RTX), currently Sweden's two most-prescribed drugs for MS treatment, are known to almost completely ameliorate clinical and radiological relapse activity in patients with RRMS. However, the effects on fatigue and IPS are unclear. RTX remains an off-label therapy for MS.

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# Populärvetenskaplig sammanfattning

Multipel skleros (MS) är en inflammatorisk sjukdom i centrala nervsystemet (CNS). CNS är uppbyggt av många miljarder nervceller där varje nervcell består av en cellkropp med en nervtråd, ett axon, kopplat till sig. Runt varje axon finns en isolerande skida kallad myelin, vilken fungerar som gummiisoleringen runt en elkabel. Myelinet förbättrar nervledningshastigheten och säkerställer snabb kommunikation mellan olika delar av nervsystemet. En av de viktigaste skyddsmekanismerna för CNS är den barriär som finns mellan CNS och blodet, kallad blod-hjärn-barriären, bestående av bland annat blodkärlsväggen, men även en celltyp som heter astrocyter. Tillsammans bildar de en skyddsbarriär som hindrar bakterier och virus från att passera över till CNS och på så sätt orsaka skada.

Vid MS är kroppens immunförsvar, som är designat för att skydda oss från infektioner, felprogrammerat och angriper kroppens egna, friska organ. För att detta skall kunna uppstå krävs specifika förutsättningar, vissa påverkningsbara och andra inte. Vi vet att kvinnligt kön, vissa genetiska förutsättningar, brist på sol, låga D-vitaminnivåer, rökning och fetma ökar risken för sjukdomen. Att under livet blivit infekterad av Epstein-Barr-viruset, det virus som orsakar körtelfeber, verkar dessutom vara närmast en förutsättning för att kunna utveckla sjukdomen. När tillräckligt många av dessa riskfaktorer föreligger tippar immunförsvaret över och kroppens immunceller börjar angripa myelinet i CNS.

Sjukdomen kommer vanligen i skov. Det innebär att den kommer i attacker där kroppens immunceller tar sig över blod-hjärn-barriären och orsakar inflammatoriska skador för att sedan dra sig tillbaka. Efter en tid sker en ny attack där immunceller tar sig in i CNS och skapar nya skador. Dessa inflammatoriska attacker kallas för skov och leder till symptom i form av känselstörningar, svaghet eller koordinationsstörning. Därefter följer av en period av återhämtning, kallad remissionsperiod, när kroppen återhämtar sig från skovet och de symptom det orsakade. Resterna av skoven och de inflammatoriska skadorna kan ses på magnetkameraundersökningar (MR-undersökningar) som så kallade MS-plack. Under remissionsperioden har patienten möjlighet att återhämta sig från de neurologiska symptom som inflammationen orsakat. Symptomen skiljer sig åt beroende på var i nervsystemet skadan skedde. Efter hand återhämtar patienten sig allt sämre, och de neurologiska besvären blir allt fler och mer handikappande. Detta sjukdomsförlopp kallas skovvis förlöpande MS (relapsing-remitting multiple sclerosis, RRMS).

De senaste 20 åren har behandlingsmöjligheterna för RRMS ökat lavinartat. År 2004 lanserades läkemedlet natalizumab (NTZ) som sjukdomsmodifierande behandling mot RRMS. NTZ hindrar de vita blodkropparna, från att passera blod-hjärn-barriären. Därmed hindras cellerna från att nå CNS och minimerar skov och antal nya synliga plack på MR-undersökningar. Behandlingen ges som dropp eller

som injektion under huden på magen var fjärde till sjätte vecka. Några år senare, runt 2008, började intresset öka för att försöka angripa B-cellerna, då dessa är central del i sjukdomsförloppet. Intresset blev särskilt stort för en medicin som heter rituximab (RTX), som länge hade funnits på marknaden för behandling av ledgångsreumatism och vissa former av blod- och lymfkörtelcancer. RTX binder till en struktur på B-cellen som heter CD20 och eliminerar samtliga celler som bär strukturen CD20 i blodbanan. Detta har visat sig kraftigt minska inflammatorisk aktivitet vid MS. Behandlingen är mycket effektiv och släcker ut skoven och den mätbara sjukdomsaktiviteten på MR-undersökningar i stort sett helt och hållet. Behandlingen ges som dropp var sjätte till tolfte månad. Idag står RTX för drygt 60% av de aktiva behandlingarna mot MS, följt av NTZ som är näst vanligast med ca 10%. Detta trots att RTX är inte godkänt för behandling av MS enligt läkemedelsmyndigheterna, utan är en så kallad off-label behandling.

Trots effekten av NTZ och RTX på skov och MS-plack kvarstår vissa symptom hos patienter med RRMS. Sedan lång tid tillbaka har patienter med MS rapporterat att deras värsta symptom är en extrem trötthet, kallad fatigue, och nedsatt kognitiv förmåga. De mekanismer som orsakar dessa symptom är inte kända, och det finns ingen effektiv behandling.

Personalen på neurologimottagningarna i Lund, Helsingborg och Danderyd upplevde att patienterna som behandlades med NTZ tycktes må bättre och klagade mindre på trötthet och kognitiva besvär. Syftet med denna studie var att utforska om det fanns en skillnad mellan patienter som behandlades med NTZ respektive RTX gällande fatigue och kognition, samt granska säkerhets- och effektaspekterna av RTX.

För att vidare efterforska möjliga bakomliggande mekanismer till fatigue och kognitiv påverkan tittade vi även på blod- och ryggmärgsvätskeprover för att se om vi kunde finna några markörer som samvarierade med fatigue eller kognition.

Först gjorde vi en granskning av hur effektivt och säkert RTX är vid MS. Genom att använda Svenska MS-registret, ett nationellt kvalitetsregister för att förbättra MS-vården i Sverige, identifierade vi alla patienter med MS som behandlades med RTX vid Helsingborgs Lasarett. Genom journalgranskning tittade vi på hur effektiv behandlingen varit och vilka eventuella biverkningar som uppkommit. Vi identifierade 83 patienter som behandlades med RTX. Antalet skov och nya plack på MR-undersökningarna reducerades markant efter att de började behandlas med rituximab. Den årliga skovfrekvensen gick från 0.38 till 0.05. Det innebär att patienterna gick från att ha ett skov ungefär vartannat år till ett skov var 20e år. När det gällde säkerhetsaspekterna av RTX var de acceptabla. Biverkningarna var generellt lindriga. Vi såg fyra fall av sjukhuskrävande infektioner där ett fall var potentiellt livshotande. Våra resultat var likvärdiga på länsortssjukhus med de som tidigare publicerats från universitetskliniker, nämligen att behandlingen är både säker och mycket effektiv.

Nästa del i denna avhandling är en jämförelse mellan NTZ och RTX. Vi valde en tvärsnittsdesign på de tre efterföljande studierna. Det innebär att preparaten jämfördes vid ett specifikt tillfälle och ger således en ögonblicksbild av läget precis vid tvärsnittet.

Potentiella deltagare identifierades genom Svenska MS-registret. Deltagare rekryterades från neurologimottagningarna vid Helsingborgs Lasarett, Skånes Universitetssjukhus Lund och Malmö samt Danderyds sjukhus. Samtliga studiedeltagare lämnade skriftligt samtycke till deltagande. För utvärdering av fatigue och kognitiv nedsättning samt behandlingsnöjdhet valdes ett antal självskattningsskalor. Fatigue mättes med hjälp av The Fatigue Scale for Motor and Cognitive Functions (FSMC), behandlingsnöjdhet med Treatment Satisfaction Questionnaire for Medications och kognition utvärderades med testet Symbol Digit Modalities Test (SDMT). SDMT är ett så kallat substitutionstest. Den som testas får ett ark med 110 symboler framför sig. Det är nio olika symboler som alla har en korresponderande siffra, vilket framgår av en kodnyckel överst på pappret. Därefter gäller det för den som testas att ersätta så många symboler som möjligt med korrekt korresponderande siffra på 90 sekunder.

Sammanlagt rekryterades 128 deltagare (56 individer med NTZ-behandling och 72 med RTX-behandling). Vi fann ingen skillnad i fatigue, mellan behandlings-grupperna. Båda patientgrupperna var även mycket nöjda med sina behandlingar. Vi fann en statistiskt säkerställd skillnad i SDMT-resultat mellan grupperna, där patienterna som behandlades med NTZ presterade bättre än de som behandlades med RTX. Därtill kunde vi se att patienterna som behandlades med NTZ hade förbättrats tydligt på SDMT sedan behandlingsstart jämfört med de med RTX. Det var dessvärre enbart med SDMT vi kunde göra denna longitudinella jämförelse, eftersom det fanns SDMT-data från behandlingsstart vilket tyvärr inte var fallet med fatigue-skalan FSMC. En stor felkälla när det gäller denna skillnad i SDMT mellan grupperna är risken för inlärningseffekt hos NTZ-gruppen. Majoriteten av de studiedeltagare som behandlats med NTZ gjorde SDMT varje gång de fick sin behandling, alltså var 4-6 vecka. Med tanke på att deltagarna haft sin behandling i minst 24 månader, oftast betydligt längre än så, leder till en stor risk att de lärt sig hur man ska tackla detta test, trots att kodnyckeln byttes vid varje testtillfälle. Det innebär att det finns en risk att skillnaden mellan grupperna delvis kan förklaras av sagda inlärningseffekt.

Vi undersökte sedan biomarkörers relation till fatigue och SDMT-resultat. Av de 128 rekryterade deltagarna lämnade 121 blodprover (54 deltagare med NTZ, 67 med RTX), och 30 ryggmärgsvätska (16 med NTZ, 14 med RTX). I blodet mätte vi specifikt två markörer – glial fibrillary acidic protein (GFAP) och neurofilament light (NfL). GFAP är ett protein som utsöndras av astrocyterna, de cellerna som bland annat utgör en del av blod-hjärn-barriären. Nivåerna av detta protein stiger vid skada på astrocyterna eller ett tecken på att astrocyternas form förändras, deras funktioner ändras och de producerar signalsubstanser och ämnen som kan vara

skadliga för omkringliggande strukturer. GFAP har visat sig vara starkt kopplat till sjukdomsprogression. Höga nivåer samvarierar med en ökad risk för individen att utveckla svårare handikapp i framtiden. NfL är ett protein i axonen i nervcellerna där förhöjda nivåer signalerar nervsönderfall. Vi fann inga skillnader mellan NfL och GFAP i blod mellan behandlingsgrupperna.

I ryggmärgsvätska var GFAP-nivåerna högre hos den grupp som behandlas med NTZ, men det fanns ingen skillnad i NfL-nivåer. Ingen skillnad av GFAP-nivåer kunde hittas i blod. GFAP i ryggmärgsvätska är svårtolkat, och värdet av det resultatet är därför tveksamt. Speciellt då det rör sig om mycket få patienter.

Vi fann inga säkra samband mellan NfL, GFAP, SDMT och fatigue. Det verkar som att fatigue hos våra studiedeltagare inte beror på axonal skada som vi kan mäta med NfL, eller astrocytreaktivitet. Efter att behandling med högeffektiva läkemedel som NTZ och RTX påbörjas sker en närmast total elimination av inflammatorisk aktivitet vid sjukdomen. När det gäller orsaker till fatigue och kognitiv påverkan vid MS finns det sannolikt inte en enskild orsak. Troligen uppkommer dessa besvär som en konsekvens av inflammation och etablerade skador på myelin och nervceller. Även astrocytreaktivitet och en inkapslad inflammation bakom blod-hjärn-barriären, som våra nuvarande läkemedel inte biter på, bidrar sannolikt. Dessa skador leder tillsammans till påverkan på komplexa nätverk i hjärnan som är centrala för att kognitiv påverkan och fatigue ska uppkomma. I vår studie har deltagarna även genomgått en högupplöst magnetkameraundersökning. Dessa bilder har ännu inte analyserats, men skulle kunna ge ytterligare insikt i bakomliggande mekanismer, så som avkapslad inflammation bakom blod-hjärn-barriären, som bidrar till fatigue och kognitiv påverkan och i längden möjliggöra för att utvärdera behandlingseffekt mer långsiktigt.

Slutsatsen av denna avhandling är att rituximab som, trots att det är ett off-label preparat mot MS, är mycket effektiv och säker behandling. Vi har inte hittat några indikationer på att RTX är mindre effektivt mot RRMS än NTZ, trots att RTX inte är godkänt för RRMS. Detta är av vikt då RTX har en betydligt lägre kostnad än NTZ och andra högeffektiva MS-läkemedel. Resultaten från denna avhandling kan därför ge stöd för användning av rituximab vid MS i länder med begränsade ekonomiska resurser.

# List of published papers

*Paper I*
**Hellgren J**, Risedal A, Källén K. Rituximab in multiple sclerosis at general hospital level. *Acta Neurol Scand.* 2020 Jun;141(6):491-499. doi: 10.1111/ane.13225. Epub 2020 Feb 6. PMID: 31990978.

*Paper II*
**Hellgren J**, Strandberg MC, Källén K, Svenningsson A. A comparative study of fatigue and processing speed in patients with multiple sclerosis treated with natalizumab or rituximab. *Mult Scler J Exp Transl Clin*. 2024 May 26;10(2):20552173241252566. doi: 10.1177/20552173241252566. PMID: 38807848; PMCID: PMC11131408.

*Paper III*
**Hellgren J**, Ahlström I, Strandberg MC, Jonsson MF, Hansson O, Janelidze S, Svenningsson A, Källén K. Comparison of CSF biomarkers in multiple sclerosis patients treated with natalizumab and rituximab. *Mult Scler Relat Disord*. 2025 Apr 30;99:106479. doi: 10.1016/j.msard.2025.106479. PMID: 40345115.

*Paper IV*
**Hellgren J**, Strandberg MC, Hansson O, Janelidze S, Svenningsson A, Källén K. Plasma levels of glial fibrillary acidic protein and neurofilament light in patients with relapsing-remitting multiple sclerosis treated with natalizumab or rituximab. Manuscript under review.

# Author's contribution to the published papers

*Overall contribution*

The author of this thesis was involved in the conceptualization of the studies and their designs, writing parts of the ethical applications and biobank permits, designing information for study participants, drafting guidelines for blood and CSF samples, and approaching collaboration partners. Moreover, the author was responsible for participant recruitment, data collection from the Swedish MS-registry and medical records, ordering of materials for blood- and CSF analyses, performing lumbar punctures, and MRI referrals. The author was responsible for deciding the statistical analyses in dialogue with the statistician.

*Paper 1*

The author of this thesis was responsible for data collection from the Swedish MS-registry and medical files, statistical analyses, writing the primary manuscript, and assisting in the response to the reviewers.

*Paper 2*

The author of this thesis was the corresponding author, and was responsible for writing the primary manuscript, interpreting the statistical results, creating the selected figures, and co-writing the response to the reviewers.

*Paper 3*

The author of this thesis was the corresponding author, and was responsible for writing the manuscript, interpreting the statistical results, creating the selected figures, and co-writing the response to the reviewers.

*Paper 4*

The author of this thesis was the corresponding author, and was responsible for writing the primary manuscript, interpreting the statistical results, creating the selected figures, and manuscript submission to the journal.

# Abbreviations

| | |
|---|---|
| ADA | anti-drug antibodies |
| ARR | annual relapse rate |
| BBB | blood-brain barrier |
| CELs | contrast-enhancing lesions |
| CIS | clinically isolated syndrome |
| CNS | central nervous system |
| CSF | cerebrospinal fluid |
| CWD | clinical worsening of disease |
| DMT | disease modifying therapy |
| EDSS | Expanded Disability Status Scale |
| FSMC | Fatigue Scale for Motor and Cognitive Functions |
| G-CSF | granulocyte colony stimulating factor |
| GFAP | glial fibrillary acidic protein |
| IEF | isoelectric focusing |
| IPS | information processing speed |
| JCV | John Cunningham Virus |
| k-FLC | kappa-free light chain |
| LON | late onset neutropenia |
| LP | lumbar puncture |
| MRI | magnetic resonance imaging |
| MS | multiple sclerosis |
| NEDA | no evidence of disease activity |
| NfL | neurofilament light |
| NTZ | natalizumab |
| OCBs | oligoclonal bands |
| PIRA | progression independent of relapse activity |
| PML | progressive multifocal leukoencephalopathy |
| PMS | progressive multiple sclerosis |

| | |
|---|---|
| PPMS | primary progressive multiple sclerosis |
| PROMs | patient-reported outcome measures |
| RIS | radiologically isolated syndrome |
| RRMS | relapsing-remitting multiple sclerosis |
| RTX | rituximab |
| SDMT | Symbol Digit Modalities Test |
| SMSreg | Swedish Multiple Sclerosis registry |
| SPMS | secondary progressive multiple sclerosis |
| TSQM-10 | Treatment satisfaction questionnaire for medications - 10 items |

# 1 Preface

My career in research did not begin well. My first research project since high school was my bachelor's thesis. The project was conducted in the fall of 2014, during my fifth semester of medical school, and it was completely unrelated to neurology. It was a complete disaster from start to finish. Thereafter, I considered my master's thesis as something I simply had to complete to graduate medical school.

Four years later, I had almost forgotten the disappointing experience from my bachelor's thesis. My tenth semester of medical school was forthcoming, and with that, my master's thesis. I was interested in a project on autoimmune encephalitis, and I was directed to Prof. Kristina Källén, the then associate professor in neurology at Lund University, and senior consultant in neurology at Helsingborg General Hospital. She informed me that my project of interest was impossible as a masters project and that I should shelve it. However, she had another idea: a project on rituximab and multiple sclerosis. I gladly accepted, and my master's thesis became my first scientific paper.

After completing my master's thesis, Prof. Källén encouraged me to pursue a PhD and continue in the field of multiple sclerosis. She approached Prof. Anders Svenningsson, the then associate professor at Karolinska Institute to provide expert knowledge on MS, and together, they suggested a project: a comparison of the two most-used disease modifying therapies for MS in Sweden, natalizumab and rituximab, with fatigue as the primary outcome. We were interested in including 7T MRI scans in the project, and therefore, we needed expertise on the subject. Consequently, associate professor Maria Compagno Strandberg, Prof. Källén's former PhD student, was included in the research group.

The result of our efforts is the present thesis. I consider this a new beginning. An opportunity to expand my knowledge and expertise, and in the future, be able to share my knowledge with aspiring researchers, as my supervisors have shared their knowledge with me.

# 2 Introduction

## 2.1 History of multiple sclerosis

The earliest record of the demyelinating and neurodegenerative disease, multiple sclerosis (MS), is probably that of Saint Lidwina of Schiedam, Holland[1]. Lidwina, who lived in the 14th century, experienced recurring episodes of balance disturbance, focal weakness, and visual impairment, which began in her 16th year. Initially, she partially recovered between relapses. Over time, the remissions ceased, and the disease became progressively worse without periods of recovery. Lidwina's condition was considered as being bestowed upon by God to endure the pain of others.

A few centuries later, in the early 19th century, Sir Augustus d'Este, a cousin of Queen Victoria of England, experienced recurrent episodes of vision loss and lower extremity weakness, which eventually led to paraplegia[1]. A review of his diaries points to a diagnosis of relapsing-remitting MS (RRMS) with conversion to secondary progressive disease. In 1824, the French pathologist Charles-Prosper Ollivier d'Angers published a treatise, "On the spinal cord and its diseases," containing the first-known medical case report of MS. It described a young man with bladder and gait disturbance, which was aggravated in hot spas. Over time, the episodes became increasingly worse, until the disease entered a progressive course with continued worsening of gait and speech. In 1868, Jean-Martin Charcot, the pioneering French neurologist, presented three lectures wherein he described the condition of a relapsing-remitting neurological disease with specific neuropathological plaques, which he termed "multiple sclerosis." Charcot pieced together a puzzle based on the clinical and pathology works of colleagues, and thus, the disease had a name, and knowledge about MS could now be spread.

Subsequently, Charcot's students, Pierre Marie and Joseph Babinski, continued to report on the clinical presentations, manifestations, and pathological features of the disease. Epidemiological research on MS began shortly thereafter, and knowledge of the condition has since grown exponentially. The invention of magnetic resonance imaging (MRI) in 1981 revolutionized the understanding of MS, as the disease activity could now be visualized and evaluated in real-time. Currently, MRI is the most useful tool in the diagnosis and long-term evaluation of patients with MS[1].

## 2.2 Pathogenesis

MS is a neuroinflammatory disease, mainly affecting the myelin sheaths surrounding neurons in the central nervous system (CNS). An inflammatory attack, a relapse, disrupts the blood-brain-barrier (BBB) with an influx of autoreactive inflammatory cells damaging the myelin sheaths, and, to an extent, the neurons[2]. This is followed by a remission period associated with recovery. Most patients exhibit a relapsing-remitting disease course from onset. The cerebral cortex and deep grey matter structures in the brain are affected as well[3], with the cytoskeletal protein neurofilament light (NfL), a marker of axonal damage, correlating with relapse severity and CNS damage extent[4].

The pathogenesis of MS remains an enduring mystery. The cause of disease onset, CNS antigen targets of autoreactive immune cells, mechanisms of the relapsing-remitting course, and the intricate interplay between the innate and adaptive immune system leading to MS remain unclear.

However, a few things are established. For example, persons with *HLA DRB1*1501* genetic variations are more prone to develop MS, probably owing to its role in the antigen presenting system[5]. MS is more common in women, and some people may have gut flora that is inclined to produce CNS-reactive T-cells, further increasing the risks of MS[2, 6]. Moreover, an Epstein Barr virus (EBV) infection seem to be necessary for the development of MS, as those without an EBV infection have a minimal risk of developing MS[7, 8]. Among modifiable risk factors, smoking, obesity, and low vitamin D levels may contribute to the MS development[9-13]. The current hypothesis holds that an increase in the combined burden of these risk factors overwhelms the immune system of the person at risk, leading to MS development.

After the commencement of the neuroinflammatory process, B-cells, macrophages, memory B-cells, and microglia enable autoproliferation of, or act as antigen presenting cells for, autoreactive T helper 1 and 17 cells, further impelling inflammation[14]. Moreover, activated astrocytes may influence the process. Glial fibrillary acidic protein (GFAP), an astrocytic intermediate filament, is a marker for astrocyte reactivity and damage[15, 16]. The homeostatic functions of astrocytes are probably more extensive than currently understood. Astrocytes maintain the integrity of the BBB via their endfeet, regulate the extracellular ion concentration, and recycle neurotransmitters[17]. Studies have confirmed the strong correlation of GFAP with clinical disease progression, and the risk of conversion to secondary progressive MS (SPMS), suggesting that astrocytes effectuate this transition[18], although the underlying pathological mechanisms remain unclear.

Intrathecal IgG production by long-lived plasma cells and short-lived plasmablasts is the significant hallmark of MS[19-21]. These CSF antibodies are measured as elevated IgG-index, CSF oligoclonal IgG bands (OCBs), and elevated levels of kappa free light chain (k-FLC)[22, 23]. OCBs are observed in 95% of patients with MS;

however, what antigens they are directed against remains unknown. Although OCBs are not unique to MS, they are a strong supporting feature of its diagnosis[24, 25]. OCBs may be found in patients with neuroborreliosis and other CNS infections, neuromyelitis optica spectrum disorders, and myelin oligodendrocyte antibody-associated disease[26-29]. OCBs are considered evidence of long-standing CNS inflammation, and patients with MS with CSF OCB-positivity at diagnosis have a worse prognosis than those with CSF OCB-negativity[30-33]. OCBs are detected via isoelectric focusing (IEF) for IgG. During IEF, CSF and plasma samples concurrently obtained are separated on a gel and visually examined for the number of unique OCBs on the CSF plates[25]. Therefore, the evaluation of OCB numbers is highly subjective. Another IgG marker is the k-FLC index. k-FLC are secreted by plasma cells consequent to an excess of light chains components when producing immunoglobulins. The k-FLC index is an objective marker of intrathecal IgG-production compared to the subjective IEF-detected OCBs[34].

## 2.3 Diagnosis and clinical features

### 2.3.1 Epidemiology

MS is the most common non-traumatic disabling neurological disease in young adults, affecting approximately 2.8 million people worldwide[35]. It predominantly affects women, with a ratio of 3:1 (women to men)[36]. Moreover, it is more common in the Nordic countries, such as Sweden, than in equatorial countries. In Sweden, MS affects approximately 20 000 people, with an incidence of 10/100 000 persons/year[37], as against a global incidence of 2.1/100 000 persons/year[35, 38]. Moreover, the incidence is increasing for reasons hitherto unknown. Improved diagnostic criteria and diagnostic sensitivity, increased frequency of obesity, and increased willingness to investigate sensory symptoms as a possible MS symptom might be contributing factors for this increase[39, 40].

### 2.3.2 Diagnosing MS

MS diagnosis is based on the presence of demyelinating lesions disseminated in space and time. Evidence is required of an inflammatory activity in more than one part of the CNS, with a dissemination in time between the inflammatory relapses. From 1983 to 2001, the Poser criteria (based on medical history, clinical examination, CSF findings, and results from evoked potentials) were used to diagnose MS[41]. The collective results of these examinations would confirm or discount the presence of CNS lesions, possibly fulfilling the dissemination criteria.

In 2001, the McDonald criteria were introduced[42]. Based on the same principle as the Poser criteria, that is, dissemination in time and space, it includes MRI and

primary PMS-related criteria as well. The McDonald criteria have since undergone multiple revisions, with more emphasized being put on MRI findings. The most recent revised criteria, presented at the 2024 ECTRIMS Congress, enable a diagnosis based solely on MRI findings[43]. Moreover, k-FLC are to be considered interchangeable with CSF OCBs. These revisions enable earlier diagnosis and facilitate earlier treatment, even before the onset of any clinical symptoms.

### 2.3.3 Clinical and radiological features of MS

The evidence for RRMS can be obtained at three "levels" based on the amount of clinical and paraclinical data supporting the diagnosis:

First level - the radiological isolated syndrome (RIS): Incidental discovery of demyelinating lesions in the CNS on MRI in patients without symptoms or medical history suggestive of a neuroinflammatory disease. The risk of conversion to clinically definite MS (CDMS) in patients with RIS is 60–82%[44]. Available data suggest that treatment for patients with RIS is beneficial, as it is associated with better long-term prognosis[45, 46].

Second level - the clinical isolated syndrome (CIS): The patient has experienced an initial inflammatory attack with clinical symptoms; however, the dissemination criteria required for MS diagnosis are not fulfilled[47]. These patients are usually investigated using MRI to support a diagnosis of CDMS, examining evidence of previous inflammation (manifesting as white matter plaques) at distinct CNS locations. Lumbar puncture (LP) findings, such as CSF OCBs and elevated levels of k-FLC can support long standing neuroinflammation and is considered a substitute for dissemination in time. Currently, most patients with CIS are offered early immunomodulatory treatment; however, in some situations, they may be monitored clinically and with regular MRI follow-ups[48].

Third level: Fulfillment of the McDonald diagnostic criteria with the patient receiving an MS diagnosis. Most patients present with a subacute focal neurologic syndrome compatible with an inflammatory lesion in the CNS. This is termed a "relapse," and it is defined as a new onset of neurological symptoms persisting for at least 24 h, which cannot be attributed to confounding factors such as infections or metabolic imbalances[49]. This is the relapsing-remitting phase of the disease. After days to weeks, the inflammatory activity subsides, and the patient enters a remission phase with recovery from the neurological symptoms. Initially, patients regain full, or nearly full, neurological function post-relapse. In the absence of effective treatment, these relapses continue to occur, causing progressive neurological damage with disability accrual as a consequence. After 15–20 years, the relapse activity gradually ceases. In 60–80% of cases, the disease becomes progressively worse in the absence of relapse activity (SPMS)[50]. In 10–15% of cases, for reasons

hitherto unknown, the disease has a progressive course from the beginning (primary progressive MS [PPMS])[51].

White matter damage dominates the radiological picture of conventional MRI in MS. In the acute phases of an active disease, gadolinium-based contrast agents-enhancement can be observed in the active lesions. Once the activity ceases, the lesion becomes inactive, and it is observed as a gliosis scar on MRI[52]. MS lesions typically develop around the CNS veins, and a vein can often be observed in the middle of a lesion (central vein sign)[53]. Moreover, grey matter lesions are a common feature in the brains of patients with MS, affecting both the cortical and deep grey matter structures such as the thalamus[54]. Cortical atrophy frequently occurs in patients with MS[54-56]. For reasons unknown, some lesions retain a rim of low-grade active immune cells even in well-treated, clinically stable patients, called chronic active lesions. Chronic active lesions comprise of slowly expanding lesions and paramagnetic rim lesions. These do not exhibit contrast enhancement, and are suggested as possible culprit lesions in the conversion from RRMS to SPMS[57].

The McDonald criteria-based radiological criteria for dissemination in space, requires lesions in two of five (four until September 23rd 2025) different locations[58].

1) Periventricular lesions
2) Cortial/juxtacortical lesions
3) Brainstem or cerebellar lesions
4) Spinal cord lesions
5) Optic nerve lesions

In the 2024 revised McDonald Criteria, the optic nerve was presented as a fifth location[43], thus easing the diagnosis of dissemination in space. Moreover, multiple sclerosis might be diagnosed if only one location is affected, provided that one or more of a combination of CSF-positivity for k-FLC or OBCs, paramagnetic rim lesions, dissemination in time and six central vein sign lesions are present. The criteria for diagnosis on solely MRI findings in the absence of clinical signs or symptoms is also improved.

### 2.3.4 Expanded Disability Status Scale – EDSS

The EDSS was introduced by John Kurtzke in 1983. It is based on the Disability Status Scale (DSS), which was originally devised to evaluate the clinical efficacy of isoniazid in MS. The DSS was considered limited, as it comprised too few steps to be fully applicable in a clinical setting. Kurtzke later developed the expanded DSS (EDSS), which remains in use in clinical practice and MS research[59]. The EDSS ranks impairment in seven different neurological functions, commonly affected by MS: 1) visual, 2) brainstem, 3) pyramidal tract, 4) cerebellar, 5) sensory,

6) bowel and bladder, and 7) higher cerebral functions. Each functional system is graded 0–5 or 0–6, depending on the system, and a total value is assigned based on the results of a clinical examination and recorded medical history, ranging from 0–10. An EDSS score of 0–4 is based on the results in the individual functional systems, 4.5–5.5 is determined either by the individual functional systems or by the patient's walking ability, 6–9.5 is entirely determined by locomotion and self-care abilities, and 10 indicates death due to MS.

### 2.3.5 Fatigue in MS

Since the implementation of high-efficacy disease modifying therapies (DMTs) in MS, clinical relapses are rare, and new MRI lesions are seldom detected. The most common complaint currently is fatigue, an invisible symptom that significantly impacts quality of life[60]. Even in the 1990s, many patients reported fatigue as their most debilitating symptom when they experienced frequent relapses and conversion to SPMS[61]. However, fatigue lacks a uniform definition. An expert panel has summarized the main clinical features of fatigue as follows: "A subjective lack of physical and/or mental energy that is perceived by the individual or caregiver to interfere with usual and desired activities"[62]. Patients commonly describe fatigue as an overwhelming tiredness that does not resolve, even after sleep. Small activities require hours to days of rest. Along with cognitive impairment, fatigue is the main reason for sick leave and disability pension in patients with MS[63]. However, an unambiguous distinction between fatigue and cognitive impairment is still lacking. Presumably, an overlap exists between the conditions, leading to diagnostic challenges. Moreover, inverse causality between cognitive fatigue and cognitive impairment is probably present in many patients.

Currently, no effective treatments are available for fatigue. Patients experiencing fatigue often receive medication to alleviate their symptoms. However, no studies show any benefits of fatigue medication. Conversely, only an increased risk of side effects is noted[64]. Exercise is the only intervention proven to be successful in reducing fatigue in patients with MS[65-67].

Although numerous studies have attempted to discover the underlying pathophysiological mechanisms of MS-related fatigue, no single mechanism is clear. However, the theories are not lacking[68].

#### *2.3.5.1 Inflammation theory*

Inflammation is a central component in the pathophysiology of MS. The presence of inflammatory cytokines, such as TNFα, IFNγ, and IL-6, in the peripheral blood correlates with fatigue[69-72]. Moreover, the anti-inflammatory IL-10 is reduced in patients with MS with fatigue[73]. The data on inflammatory markers in CSF are more contradictory[74]. The presence of fatigue in other systemic inflammatory diseases

such as vasculitis, malignancies, and Sjogren's syndrome, further supports the notion of inflammation as a contributory factor for fatigue[75-77].

#### *2.3.5.2 Structural damage theory*

Researchers have thoroughly examined the potential association between white matter lesions and fatigue with highly conflicting results[78-82]. Therefore, white matter lesions alone cannot be responsible for the significant number of patients experiencing fatigue. Grey matter lesions, and atrophy of deep grey matter structures and the cerebral cortex, probably contribute to MS-related fatigue. Previous studies have linked MS-related fatigue to several different grey matter areas, including the cerebellar, parietal, and frontal cortices, and global grey matter atrophy including deep grey matter nuclei[79, 83-87]. Apart from the decreased number of grey matter neurons, malfunctioning neuronal networks, caused by grey matter lesions, could be another contributing mechanism in fatigue. Thalamic atrophy, and consequently dysfunction of the cortico-striato-thalamic network, is highly associated with the development of fatigue[68, 88, 89].

### 2.3.6 Cognitive impairment in MS

Cognitive impairment is a common complaint in patients with MS. The most affected cognitive ability is information processing speed (IPS), assessed using the Symbol Digit Modalities Test (SDMT) and Paced Auditory Serial Addition Test (PASAT)[90]. In addition, working and verbal memories and attention are other cognitive abilities frequently affected in patients with MS. Contrary to fatigue, structural damage and cognition are strongly correlated in patients with MS, particularly considering grey matter lesions and atrophy. Cerebral cortical damage and the deep cerebral nuclei are involved in the pathophysiology of cognitive impairment[83, 91-94]. In addition, NfL, a marker of neuronal damage, is strongly associated with both cognitive impairment and cerebral atrophy[95-99]. Moreover, white matter lesion burden contributes to cognitive deficits in patients with MS[81, 100-102]. Microglial infiltration and activation might contribute to the development of cognitive impairment; however, their exact roles need further clarification[103].

## 2.4 Treatment

### 2.4.1 Historical ("A long, long time ago…")

During the 19th century, Jean-Martin Charcot tested several different concoctions as treatments for MS, including phosphorized oils and the Calabar bean, which invariably proved more harmful than helpful[104]. His colleagues tested silver nitrate,

electrical therapy, mercury, and cod liver oil as treatments, without any beneficial effects on the disease course.

The disease was considered an inflammatory condition, and perhaps even an infectious condition. The concept of MS as an infectious disease led to disastrous experiments, with patients being placed in "fever boxes," administered typhoid vaccines to develop a fever, and even malaria-infected blood infusions to induce long-term episodic fevers. It was not beneficial for the patient. This was clear consequence of the doctor-patient relationship, where "the doctor wanted to do something, and the patient wanted something done," even if the treatment was experimental.

However, with the introduction of placebos and the concept of randomized controlled trials, the efficacy and safety of potential therapies could be evaluated in controlled settings. Kurtzke unsuccessfully attempted isoniazid therapy; however, neurologist Henry Miller found that adrenocorticotropic hormone (ACTH) was effective in treating relapses[105, 106]. Further studies demonstrated that high-dose steroids shortened relapses and decreased the risk of future relapses, albeit to a limited extent[107, 108]. However, long-term steroid treatment is associated with an extremely high risk of side effects, including iatrogenic hypocortisolism, type 2 diabetes mellitus, peptic ulcers, and osteoporosis[109].

Corticosteroids were the first drugs to have any actual, albeit limited, effect on MS. New immunomodulatory therapies began to be tested on patients with MS in the 1970s. Drugs such as cyclophosphamide, methotrexate, and azathioprine had demonstrated efficacy in patients with malignant or rheumatological conditions. However, the effects in MS were questionable[104].

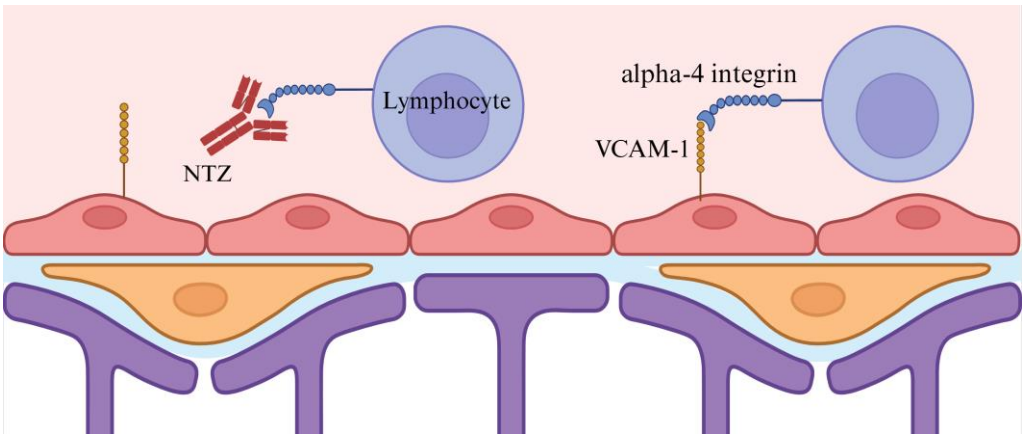
The 1990s heralded the first therapy proven to affect the relapse rate and MRI activity of patients with MS. In 1993, interferon beta-1b (Betaseron®) was approved for MS treatment[110]. Interferons inhibit antigen presentation and promote a shift from proinflammatory Th1 cells to regulatory, anti-inflammatory Th2 cells[111, 112]. Shortly thereafter, several therapies with similar mechanisms of action were introduced, with interferons and glatiramer acetate being the dominating therapies for MS into the 21st century.

### 2.4.2 21st century

Although interferons reduced the annual relapse rate (ARR) and the number of new lesions on MRI, patients continued to experience relapses, exhibit MRI activity, and converted to SPMS[112]. DMTs were yet to achieve clinical and radiological stability in patients, with no relapses, no new lesions on MRI, and no clinical worsening of disease, i.e., "no evidence of disease activity (NEDA)." In addition, interferons had disturbing side effects of flu-like sensations that considerably reduced quality of life.

Relapses occur as a consequence of lymphocytes infiltrating the CNS from the periphery, by crossing the BBB[113]. Targeting the passage of lymphocytes across the BBB garnered interest, and the antibody natalizumab (NTZ) was developed. NTZ (first licensed product, Tysabri®) targets alpha-4 integrin on the lymphocytes, preventing their adhesion to vascular cell adhesion molecule-1 (VCAM-1) on the endothelial wall, inhibiting attachment, and subsequent crossing across the BBB (Figure 1). NTZ, administered intravenously every 4 weeks, proved to be far more effective than previous therapies, leading to a significant reduction in ARR and number of new lesions on MRI. Initial studies revealed that NTZ was safe, with a beneficial overall risk profile[114-117].

**Figure 1: Mechanism of action - Natalizumab**




Natalizumab (NTZ) binds to alpha-4 integrin on lymphocytes, preventing adhesion between alpha-4 integrin and VCAM-1, thus preventing egress from the blood stream and into the central nervous system. Created by the author using BioRender.com.

However, in 2005, one year after its release onto the market, NTZ was withdrawn owing to reports of patients developing progressive multifocal leukoencephalopathy (PML)[118]. PML is an opportunistic disease, caused by the John Cunningham Virus (JCV). Approximately 50–60% of humans may be JCV carriers[119]. Under normal physiological conditions, the virus usually lies dormant in the kidneys, bone marrow, and lymphoid tissue. When patients carrying the JCV become immunocompromised, it can be reactivated, causing an active CNS infection and destroying the oligodendrocytes and astrocytes[120]. It remains unclear whether the virus migrates to the CNS when the patients become immunocompromised, or if it lies dormant in the CNS as well since the primary infection. PML is untreatable, with a high mortality rate, and survivors usually experience severe disabilities[118, 121].

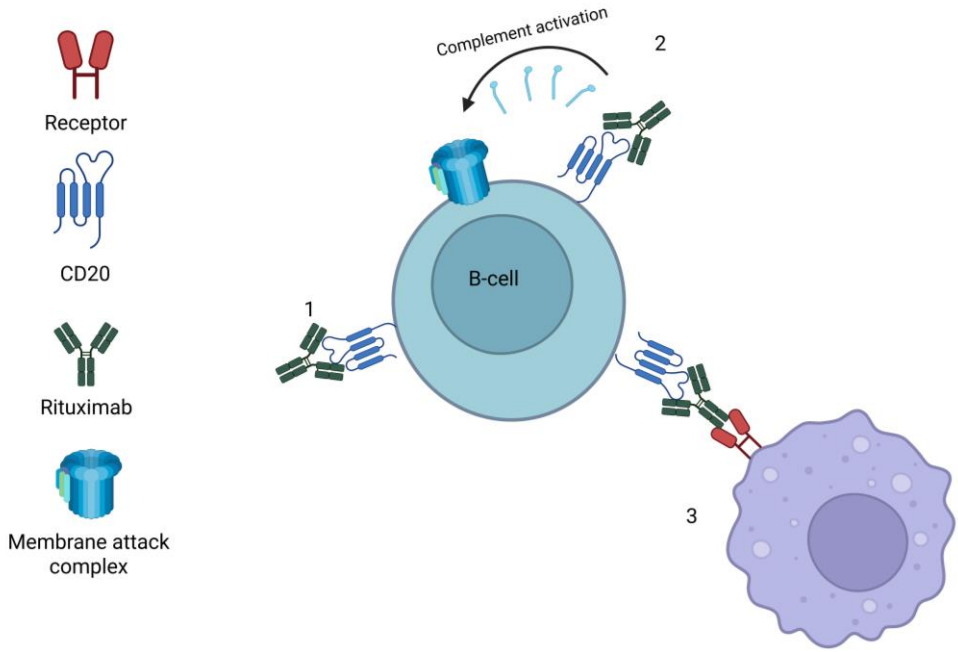
NTZ was re-introduced to the market in 2006. Currently, a few key factors are known for the development of PML in NTZ-treated patients. These are: 1) a long

treatment duration increases the risk of PML, 2) previous immunosuppressive therapy, and 3) JCV antibody levels in peripheral blood. The European Medical Authorities and Biogen have developed a risk stratification algorithm, based on JCV antibody index in blood, which is in use in Sweden. In patients with JCV antibody index >0.9, treatment must be terminated as the risk of PML is deemed too high[122]. Importantly, NTZ therapy must be terminated with caution and must overlap with another effective therapy. Autoreactive lymphocytes remain in the blood stream when terminating NTZ and might induce a severe rebound inflammatory relapse. These rebound relapses can result in severe disability and must be recognized as a serious risk when switching from or terminating NTZ therapy[123].

Subsequent studies have further supported the efficacy of NTZ, and it is considered a safe therapy, provided the risk of PML is closely monitored[124-127]. Currently, NTZ is considered a high-efficacy MS treatment, and it is the second most prescribed DMT in Sweden accounting for approximately 10% of all prescribed MS DMTs.

There have long been speculations on the role of B-cells in MS. The presence of OCBs and elevated k-FLC levels indicated that B-cell-derived plasma cells influenced the pathogenic process of MS[128, 129]. With increasing knowledge that B-cells were relevant in the disease process, the chimeric anti-CD20 monoclonal antibody, rituximab (RTX) (first licensed product, MabThera®) garnered interest among researchers. CD20 is found on the majority of B-cells, and RTX causes lysis of the CD20+ cells as follows: 1) direct antibody-induced apoptosis, 2) complement-mediated cytotoxicity, and 3) antibody-dependent cellular cytotoxicity mediated by NK-cells, granulocytes and macrophages (Figure 2). Two early trials demonstrated promising results of RTX in patients with RRMS and paved the way for researchers and physicians to further evaluate the effect of RTX in MS[130, 131]. Thereafter, numerous observational studies, non-randomized interventional studies, and one phase 3 trial, have supported the efficacy of RTX in RRMS[132-138].

**Figure 2: Mechanism of action - Rituximab**




Rituximab, a monoclonal antibody, has three different mechanisms of action: 1) Direct antibody-induced apoptosis, 2) complement-mediated cytotoxicity with complement factors forming the membrane attack complex causing lysis, and 3) antibody-mediated cellular cytotoxicity or phagocytosis by NK-cells or macrophages. Created by the author using BioRender.com.

In MS, RTX is administered intravenously every 6–12 months and is considered a highly effective and safe therapy. However, patients treated with RTX are at higher risk for developing bacterial infections requiring hospitalization than those treated with other commonly used DMTs[139]. This risk is partly mediated by potential hypogammaglobulinemia, which might pose a risk for serious bacterial infections[140, 141]. An extended dosing interval in RTX therapy and attempting to maintain the cumulative dose as much as possible have proven successful in minimizing the risk of hypogammaglobulinemia[142, 143]. Late-onset neutropenia (LON) is another potential serious side effect of anti-CD20 therapies. LON can lead to life-threatening infections, requiring hospitalization and intravenous broad-spectrum antibiotics. The frequency of LON is unknown[144]. RTX is used as an off-label drug in MS treatment. In Sweden, it is approved for rheumatoid arthritis, granulomatosis with polyangiitis, non-Hodgkin's lymphoma, and chronic lymphatic leukemia, albeit not MS[145]. Therefore, it's remarkable that RTX accounts for 61.1% of all currently active MS therapies.

Since 2008, several other DMTs have become available for RRMS treatment. In 2011, the first sphingosine-1 phosphate receptor (S1Pr) modulator, fingolimod (Gilenya®) was introduced in the market, and currently, three more S1Pr modulators (ponesimod, siponimod, and ozanimod) are available for RRMS treatment[146]. The anti-CD20 therapies approved for MS, ocrelizumab and ofatumumab, are likely comparable in efficacy and safety to RTX, with similar mechanisms of action. Ofatumumab might be less likely to cause hypogammaglobulinemia than rituximab and ocrelizumab, based on observed results from clinical trials. However, patients with low gamma globulin levels were excluded from participating in the ofatumumab trials, and if IgG or IgM levels plummeted 20 or 10% below lower normal limits, the treatment was discontinued[147]. As IgM levels decline before IgG levels, this might partly explain the lower frequency of hypogammaglobulinemia.

Immune reconstitution therapies comprise another principal therapy group. These include alemtuzumab, an anti-CD52 antibody; cladribine, a nucleoside analogue to deoxyadenosine; and autologous hematopoietic stem cell transplantation (AHSCT). Alemtuzumab causes near-total B- and T-cell depletion by inducing cell lysis by binding to the CD52 antigen on the cell surface. The broad elimination of autoreactive lymphocytes is hypothesized to lead to a reconstitution of the immune system with a highly decreased ARR, decreased to eliminated inflammatory activity on MRI, and the possible prevention of conversion to SPMS, as a consequence[148]. Cladribine selectively interrupts the DNA synthesis and breaks single-stranded DNA in B- and T-cells, leading to a selective depletion of activated lymphocytes with a long-lasting suppressive effect on the immune system[149]. AHSCT is the most effective therapy known to date. Patients are treated with high doses of cyclophosphamide and anti-thymocyte globulin, leading to near complete elimination of both lymphocytes and neutrophils in the peripheral blood and peripheral lymphoid organs. Thereafter, stem cells harvested from the patient before cytotoxic drug administration, are re-introduced, and the bone marrow regains its function, without autoreactive T- and B-cells, thus possibly curing the person from MS. However, these therapies have risks or side effects. Patients treated with alemtuzumab are at high risk of developing other autoimmune conditions, such as autoimmune thyroiditis, immune mediated thrombocytopenia, and glomerulonephritis[150, 151]. In AHSCT, the high dose cyclophosphamide therapy leads to an initial phase of pancytopenia, and thus, a high risk of serious infections [152]. Cladribine has not yet exhibited any serious side effects; however, it is considered less effective than alemtuzumab, AHSCT, anti-CD20 therapies, and NTZ.

### 2.4.3 Paradigm shift

The drugs introduced in the 21st century have led to a paradigm shift in MS treatment. Pre-2004, and pre-NTZ, when only interferons and glatiramer acetate were available, relapses and new lesions on MRI were a part of the disease course. Currently, clinical relapses or new lesions on MRI are no longer accepted, but is considered treatment failure.

In Sweden, >60% of patients with MS are treated with RTX because of the studies having revealed its safety and efficacy, along with the accumulated experience. Moreover, it is inexpensive. RTX costs approximately 2700 SEK per year, while ocrelizumab costs approximately 205 000 SEK per year. Therefore, seventy-six patients can be treated probably as effectively and safely using RTX compared with treating one patient with ocrelizumab.

NTZ is frequently used as a bridge from RRMS diagnosis until RTX therapy can be initiated, during which patients can be vaccinated, which is not possible during anti-CD20 therapies. However, the trend is to NTZ use less and less frequently, in favor of anti-CD20 antibodies.

# 3 Rationale

During the last two decades, RRMS treatment has undergone a paradigm shift: beginning with the approval of NTZ in 2004, followed by the approval of fingolimod in 2011, alemtuzumab in 2013, dimethyl fumarate in 2014, and cladribine in 2017. In 2018, the first anti-CD20 antibody, ocrelizumab, was approved for MS.

However, since 2008 neurologists worldwide, and particularly in Sweden, have used RTX to treat RRMS. Currently, >60% of Swedish patients with MS on DMTs are treated with RTX. NTZ, the second most common DMT, accounts for approximately 10% of prescribed DMTs. When natalizumab first was introduced in Sweden, only University Hospitals were allowed to prescribe the drug. This soon became unsustainable, and therapies began to be administrated at secondary referral hospitals. The secondary referral hospitals, such as Helsingborg General Hospital, were also early in implementing RTX into MS care, as it was considered a cheap and effective therapy. NTZ and RTX have both exhibited high-efficacy and safety, and patients treated with these drugs exhibiting NEDA. In 2019, to the researchers' knowledge, all RTX studies were based on data from university hospitals. The pilot study thus posed the following question: "Is RTX as safe and effective a drug in a general hospital setting as it is in a university hospital?"

Once the study was complete, another significant issue in patients with MS became a topic of interest. Nurses and physicians reported that NTZ-treated patients appeared to experience less fatigue than RTX-treated patients. The burden of fatigue is significant in patients with MS. Studies from the 1990s reveal that fatigue was reported as the worst symptom in these patients, particularly in an era when physicians were unable to successfully prevent clinical relapses and conversion to secondary progression. NTZ is an approved and highly efficacious drug for treating MS; however, it has some issues, such as the risk of PML when patients have a positive JCV-index, the risk of rebound inflammatory activity when terminating the therapy, and its expensiveness compared with RTX. Since most NTZ- or RTX-treated patients achieve NEDA, fatigue has once again gained interest. A pressing concern, as it involves anguish, sick leave, and disability pension. To the researchers' knowledge, no previous studies have compared NTZ with RTX with fatigue as primary outcome.

Therefore, the following questions were asked: “Are RTX-treated patients more fatigued than the NTZ-treated patients? If there are differences in fatigue levels, can plasma or CSF analyses explain these differences? Can biomarkers be found in plasma or in CSF that correlate to fatigue?”

# 4 Aims

This thesis primarily aims to provide comparative data on the most prominent clinical morbidities on high-efficacy therapy, fatigue, and IPS, in a patient population with RRMS treated with NTZ or RTX. Moreover, it aims to provide new data on the safety and efficacy of these DMTs by analyzing biomarkers in plasma and CSF samples from this population. Figure 3 illustrates the timeline of this PhD project.

Specific aims of the different studies:

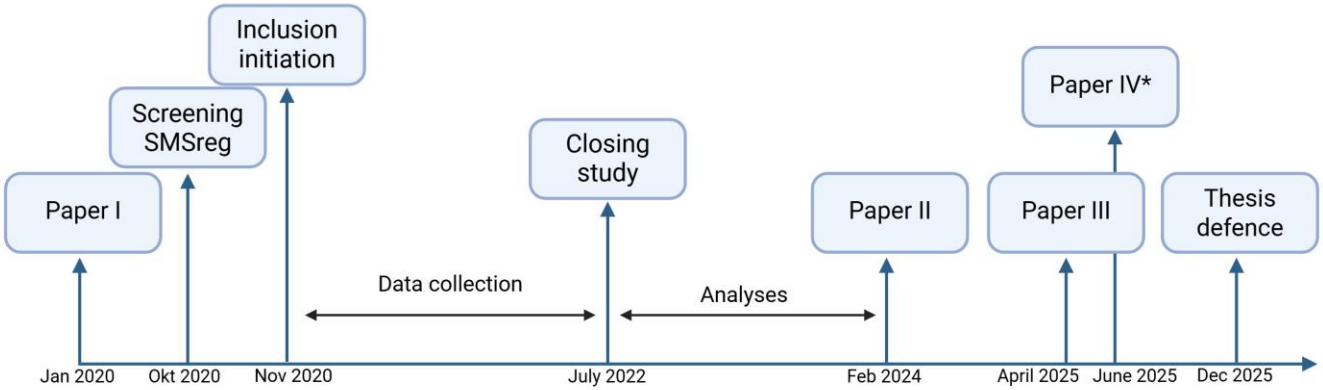
I. To evaluate the efficacy and safety of off-label RTX for MS at the Helsingborg General Hospital, Sweden

II. To investigate potential differences in patient-reported outcome measures (PROMs) on patients treated for at least 24 months with either NTZ or RTX, with fatigue as the primary outcome and to compare the IPS, measured by the SDMT as secondary outcome

III. To explore inflammatory and degenerative markers, i.e., GFAP, NfL, and IgG-biomarkers, in the CSF of NTZ- or RTX-treated patients, analyze potential between-group differences in biomarkers and to investigate potential associations between biomarkers, fatigue, and IPS

IV. To investigate potential associations between plasma GFAP, NfL, fatigue, and IPS, and to explore potential between-group differences in plasma GFAP and NfL levels

**Figure 3 – Overview of dissertation timeline**




*Paper IV is currently under review.

# 5 Rituximab at the general hospital level (Study I)

## 5.1 Methods

### 5.1.1 Study design and inclusion process

This retrospective observational study included patients with RRMS treated with RTX (MabThera®). Participants were identified using the Swedish MS Registry (SMSreg). All patients with MS (ICD G35.9) registered at the neurology outpatient clinic at the Helsingborg General Hospital were screened. Patients with 1) a definite diagnosis of MS according to the McDonald Criteria[58] registered at the neurology outpatient clinic at the Helsingborg General Hospital, 2) having received at least one dose of MabThera® until March 2019, and 3) having available follow-up data regarding safety and efficacy, were included. Patients treated with the then-recently introduced biosimilar, Ritemvia®, were excluded from the study, as it could not be guaranteed that its side effect profile was identical to MabThera®.

### 5.1.2 Data collection and outcomes

Data on demographical parameters, disease duration, treatment duration, DMTs before RTX, reasons for DMT discontinuation, ARR, potential adverse events, radiological disease activity on MRI (MRI activity), clinical worsening of disease, B-cell levels, and IgG-levels were collected by reviewing medical records and stored using Microsoft Excel 2013 (Microsoft Corp. Redmont, Washington, USA).

The outcomes were clinical worsening of disease (CWD), relapses during RTX therapy, MRI activity during therapy, and adverse events. CWD was defined as one or more of the following:

1) increasing weakness of the lower extremities affecting walking distance or requiring a change in mobility aids (crutches, walker, or wheelchair)
2) development of upper extremity coordination impairment or paralysis
3) loss of bladder and/or bowel control requiring intermittent catheterization and/or medication

This definition of CWD was preferred to the EDSS, owing to the lack of EDSS data in the SMSreg and medical records. A relapse was defined as new neurological symptoms or worsening of previously existing symptoms, persisting for at least 24 h in absence of fever or signs of infection. MRI data was collected from radiologists' reports. Follow-up scans were compared to the most recent previous scan. New lesions were considered a sign of disease activity.

Adverse events were classified as either infusion- or non-infusion-related events, and divided into mild, moderate, or severe events. Infusion-related events were divided into minor or major events, with minor events not requiring intervention other than a reduced RTX infusion pace, whereas major events required infusion discontinuation and diagnostic or therapeutic intervention from a physician. Non-infusion-related events were classified as events occurring at least 1 week after the latest RTX infusion and trichotomized as follows: 1) Mild, no treatment or treatment was administered without the need for inpatient care; 2) Moderate, with hospitalization albeit without persistent disability; or 3) Severe, potentially life-threatening.

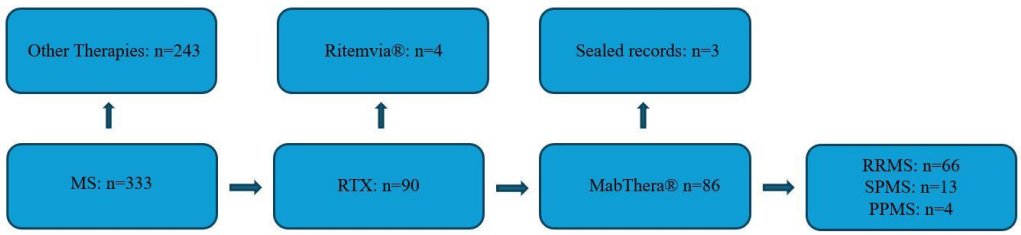
### 5.1.3 Statistics

All statistical analyses were performed using SPSS version 25.0 (IBM Corp., Armonk, NY, USA). Statistical significance was set at $p<0.05$. Wilcoxon signed rank test was used to analyze the potential difference in annual relapse rate before and after RTX initiation. McNemar's test was used to calculate the number of participants with new lesions on the most-recent brain MRI before RTX treatment compared with the those with new lesions on MRI during the entire follow-up period. Patients without baseline MRI or MRI data during the first year of follow-up were excluded from the MRI analysis. Kaplan–Meier survival curves were used to demonstrate time to, and the proportion of study participants free of, CWD.

## 5.2 Results

Among 333 patients screened using the SMSreg, 83 were finally included in the study (Figure 4).

**Figure 4: Study inclusion process**




Of the 90 RTX-treated participants, 4 received the biosimilar, Ritemvia®, and another 3 had sealed their medical records rendering data collection impossible. Thus, these 7 patients were excluded from the study

Abbreviations: MS, multiple sclerosis; RTX, rituximab; RRMS, relapsing-remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; PPMS, primary progressive multiple sclerosis

Baseline participant characteristics are presented in Table 1. Participants with progressive disease were older, and those with secondary progressive disease had a longer mean disease duration. Notably, 8/17 participants with progressive disease exhibited signs of disease activity in the year before RTX initiation, either as a clinical relapse or new lesions on MRI.

**Table 1: Baseline characteristics of study participants**

| | RRMS (n=66) | SPMS (n=13) | PPMS (n=4) | Total (n=83) |
|---|---|---|---|---|
| **Age, years, mean (range)** | 41.5 (17–69) | 51 (40–69) | 48.5 (45–58) | 44 (17–69) |
| **Female, n (%)** | 42 (63.6) | 11 (84.6) | 2 (50) | 55 (66.3) |
| **Disease duration, years, mean (range)** | 5.8 (0–25.7) | 17.1 (4.6–26.3) | 1.7 (0.3–2.1) | 6.4 (0–26.3) |
| **Treatment duration, months, mean (range)** | 23 (2–76) | 20 (1–75) | 17.5 (6–60) | 25.5 (1–76) |
| **Participants with new MRI lesions at RTX initiation, n (%)** | 35 (53) | 7 (53.8) | 1 (25) | 43 (51.8) |
| **ARR the year before RTX initiation, mean (SD)** | 0.46±0.6 | 0.38±0.5 | 0.75±0.74 | 0.46±0.6 |

Abbreviations: ARR, annual relapse rate; MRI, magnetic resonance imaging; RRMS, relapsing-remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; PPMS, primary progressive multiple sclerosis

### 5.2.1 Clinical outcomes

Patients with RRMS had a cumulative progression free survival of 86% over 3 years, compared with 30% in the PMS group. In participants with at least 12 months of follow-up (n=64; RRMS, n=50; PMS, n=14), ARR decreased from mean 0.38 (SD ±0.5) to 0.05 (SD ±0.19), $p<0.001$, with 75% of relapses after RTX initiation occurring within the first year of treatment.

MRI data at baseline and the first year of treatment, available for 76 participants, were analyzed. The number of participants with contrast-enhancing lesions (CELs) decreased from 39 at baseline to 5 during the first year of RTX treatment, with all cases except one occurring within 6 months from treatment initiation ($p<0.001$), with the number of CELs per MRI decreasing from 0.94 to 0.24 ($p<0.001$).

### 5.2.2 Safety

Regarding safety, 40 participants experienced at least one infusion-related adverse event, 72 events all-in-all. Of these, 94.4% were mild. Four participants experienced moderate infusion-related events, with three developing a chest rash, and one experiencing shortness of breath. Treatment, which recommenced after administration of a higher dose of corticosteroids and antihistamines, was well-tolerated. Conversely, 26 participants experienced at least one non-infusion-related adverse event, 59 events all-in-all, with infections being the most frequent (n=40). Four participants required hospitalization, all for infection-related adverse events, one of which was classified as severe as the patient developed pneumonia and concomitant LON. The patient was treated with broad-spectrum antibiotics and granulocyte colony stimulating factor (G-CSF) and recovered fully. Treatment was subsequently recommenced. Twelve participants discontinued treatment. Anti-drug antibodies (ADAs) were attributed for treatment discontinuation in one patient, and although the patient's CD20-positive B-cells were incompletely depleted, no signs of disease activity could be observed clinically or radiologically.

# 6 Natalizumab versus rituximab in multiple sclerosis (Studies II–IV)

## 6.1 Study design and inclusion process

The research with the working title "Biomarkers, cognition and PROMs in high-efficacy MS therapy – natalizumab versus rituximab" encompassing Studies II–IV, was a comparative multicenter cross-sectional study.

Inclusion criteria:

1) Age, 18–60 years
2) Definite diagnosis of RRMS according to the McDonald Criteria
3) Treatment with either NTZ or RTX for at least 24 months

Exclusion criteria:

1) Previous treatment with alemtuzumab or autologous hematopoietic stem cell therapy
2) Currently pregnant or breastfeeding
3) Comorbidities with other neurodegenerative diseases
4) Active malignancies
5) MRI findings suggestive of diagnosis other than MS

Initially, the upper age limit was set to 50 years. However, after an intra-team discussion, the age limit was extended to 60 years based on the rationale that participants <60 should not be cognitively impaired owing to reasons other than an MS diagnosis.

Eligible participants were identified using the SMSreg and subsequently recruited from three outpatient neurology clinics (the Skane University Hospital and Helsingborg General Hospital in Skåne county and the Danderyd Hospital in Stockholm County) between November 2020 and July 2022. At the Skåne County hospitals, participants were approached by a research nurse or the author. Eligible participants received information either in writing when they were at the clinic for treatment, or online via the "1177," a Swedish platform for medical information and

communication between patients and health care providers. At the Danderyd Hospital, eligible participants were approached by Prof. Svenningsson. Data, constituting the cross-sectional period as well, were collected between November 2020 and January 2024.

The full study protocol involved 7T MRI, with a protocol designed to identify white and grey matter lesions, as well as leptomeningeal enhancement. The results from this part of the protocol are yet to be analyzed, and hence, will not be presented in this thesis.

### 6.1.1 Data collection

#### *6.1.1.1 Demographics, patient-reported outcome measures, and clinical data*

Demographic data, patient-reported outcome measures, and SDMT results were extracted from the SMSreg. The following clinical data as well as cross-sectional and diagnostic lumbar puncture data were collected using both digital and paper medical records:

- Disease duration
- Treatment duration
- Annual relapse rate
- Total white matter lesion burden on MRI
- CSF mononuclear cells
- CSF polymorphonuclear cells
- CSF erythrocytes
- CSF-albumin
- Serum/plasma albumin
- Albumin quotient
- CSF IgG
- Serum/plasma IgG
- IgG-index
- IEF statement regarding presence of OCBs and interpretation of intrathecal IgG synthesis, and BBB damage

#### *6.1.1.2 Blood samples*

Between May 2021 and September 2022, blood samples from each participant were collected in two EDTA tubes and centrifuged (2000 g, 4 °C) for 10 min. Subsequently, 1 ml of plasma was aliquoted into four LoBind cryotubes and stored at −80 °C within 30–60 min.

#### *6.1.1.3 CSF samples*

Between June 2021 and July 2022, 7 mL CSF was collected from each study participant, immediately placed on ice, and later centrifuged (400 g, 4 °C) for 10 min. Next, 1 mL of CSF + CSF supernatants were aliquoted into LoBind cryotubes, and stored at -80 °C. Subsequently, the samples were defrosted, divided between five 200-uL LoBind tubes, with 400 uL from each participant being reserved for immunoglobulin analyses at the Clinical Chemistry Laboratory at the Skåne University Hospital.

#### *6.1.1.4 EDSS*

EDSS data were mainly collected using the SMSreg. Medical records were reviewed in cases of missing data from the registry, where the treating physician had assigned each patient an EDSS score based on clinical examination at the annual follow-up. In cases without assigned EDSS scores, it was assigned based on an intra-team consensus decision according to clinical examination findings and previous EDSS scores. If an EDSS score estimation was deemed impossible, the EDSS was administered telephonically by a research nurse or the author[153].

### 6.1.2 Patient-reported outcome measures

PROMs were used to assess fatigue, treatment satisfaction and IPS in the study participants.

#### *6.1.2.1 Fatigue Scale for Motor and Cognitive functions (FSMC)*

The 20-item FSMC questionnaire assesses fatigue in daily activities, using a 5-point Likert scale (1 [“completely disagree”] to 5 [“completely agree”]). Ten items, each presented as a statement, encompass cognitive and motor fatigue, respectively. Cognitive, motor, and total fatigue scores grade fatigue severity accordant with predetermined threshold values. High scores indicate worse fatigue.

#### *6.1.2.2 Treatment satisfaction questionnaire for medication (TSQM-10)*

The modified TSQM-10 assesses treatment satisfaction. It is based on TSQM-9, with an added question on side effects, as it is applied in the SMSreg. The 10 items, presented as questions, are graded on a Likert scale and address treatment effectiveness, drug administration and side effects (items 1–7, grades 1–7), belief in

benefits of treatment (items 8 and 9, grades 1–5), and overall satisfaction (item 10, grades 1–7). The score of items 1–9 is added, with item 10 presented separately. A high score indicates high treatment satisfaction.

#### *6.1.2.3 Symbol Digit Modalities Test (SDMT)*

The SDMT is used to assess IPS. The participant is presented with a test sheet comprising 110 symbols in a random sequence. Each symbol has a corresponding, pre-specified number between 1 and 9. The participant must pair as many symbols with the correct number as possible within 90 s, and subsequently, achieve a score based on the number of successful pairings within the time frame. A difference of ≥8 points is deemed clinically significant[154].

### 6.1.3 Plasma and CSF analyses

The cross-sectional CSF samples underwent routine analyses at the same hospital clinical laboratory as the LP (Helsingborg n=17, Lund n=5, Malmö n=6, and Danderyd n=2). IEF was analyzed at the Clinical Chemistry Laboratory, Skåne University Hospital, using a Sebia Hydrasys 2. Participants from Danderyd Hospital were excluded from the OCB and k-FLC analyses owing to missing data.

k-FLC was analyzed using a particle-enhanced nephelometric immunoassay on a Siemens BN-system. A k-FLC index >3.43 was considered pathologic[155]. Longitudinal comparison of the number of OCBs was performed by comparing the cross-sectional IEF plates with the diagnostic LP plates. The diagnostic IEF plates were re-evaluated by two of the researchers. A change of ≥5 bands, and at least 50% change in band numbers, was defined as a distinguishable change between time of diagnosis and cross-section. Samples with ≥2 CSF unique OCBs were considered OCB-positive.

GFAP and NfL concentrations were measured using the Simoa® NEUROLOGY 2-PLEX B Kit (product number: 103520, Quanterix, Billerica, MA, USA) at the Clinical Memory Research Unit, Lund University. A SR-X (Quanterix, Billerica, MA, USA) was used, according to the manufacturer's instructions, for the analyses. All samples were analyzed in duplicate. CSF and plasma samples from the same participant were analyzed in the same run.

Coefficients of variation (CV) indicates the variation in signal between duplicates and should be <20%. For CSF, the average GFAP intra- and inter-plate CV was 8.19% and 9.5 %, respectively, and the average NfL intra- and inter-plate CV was 4.25% and 7.7%, respectively. The CVs of duplicate measurements for all CSF samples were <18%.

For plasma, the average GFAP intra- and inter-plate CV was 9.58% and 9.49%, respectively, and the average NfL intra- and inter-plate CV was 8.85% and 7.66%, respectively. Serum samples with CV >20% were re-analyzed.

### 6.1.4 Statistics

Limited data availability regarding fatigue in patients with RRMS treated with NTZ and RTX rendered accurate power calculations impossible. A difference of 15% in fatigue levels was estimated to be clinically significant which, based on the results from the TYNERGY-trial, would be 9 points on the FSMC[156]. The study was estimated to require 50 participants/group to achieve 90% power, provided that SD was approximately to 1 and the p-value was set at 0.05.

Statistical analyses were performed in R version 4.3.3 (Studies II and IV) and IBM SPSS Advanced Statistics 29.0 for Windows (Study III). Statistical significance was set at $p<0.05$.

Linear regression models were used to compare continuous outcomes between participants treated with NTZ or RTX. These comparisons included FSMC, SDMT, CSF GFAP and NfL, and plasma GFAP and NfL. Potential confounders included EDSS, age, number of MRI-lesions at treatment initiation, disease duration, and sex. Time on RTX and NTZ was included in the FSMC and SDMT models, with educational level being included in the SDMT model. Owing to the small sample size in Study III, confounding factors in the CSF GFAP and NfL models were limited to sex and age, and a Bonferroni correction was performed to limit the risk of type 1 errors. Statistical significance was consequently adjusted to $p<0.0167$.

In Studies III and IV, the associations between the CSF and plasma biomarkers, FSMC, and SDMT were explored using scatterplots, and subsequently analyzed using Pearson correlation coefficient, and 95% confidence intervals (CI).

Wilcoxon signed-rank test was used for non-normally distributed continuous data (CSF IgG, IgG-index, mononuclear cells, CSF OCB number, k-FLC index, and CSF albumin). Fisher's exact test was used for the between-group comparison of a distinguishable change in the OCB numbers. Change over time was analyzed using paired t test and Wilcoxon Signed Rank Test for normally and non-normally distributed data, respectively.

#### *6.1.4.1 Sensitivity analyses*

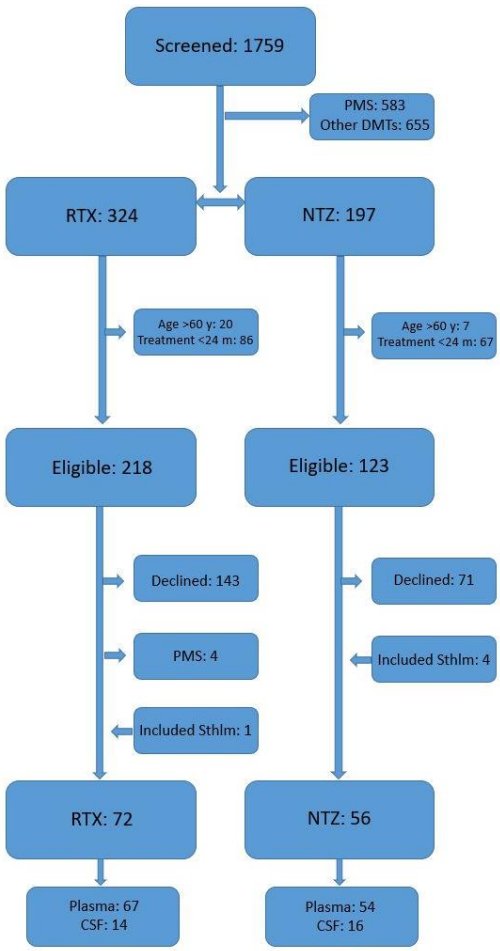
In Study II, a sensitivity analysis was conducted, excluding participants in the RTX group who were initially treated with NTZ and subsequently switched to RTX, to eliminate the possible negative bias introduced in the RTX group from an involuntary change of an effective medication. In Study IV, a sensitivity analysis was conducted after excluding observations with CV>20%.

## 6.2 Results

### 6.2.1 Study population

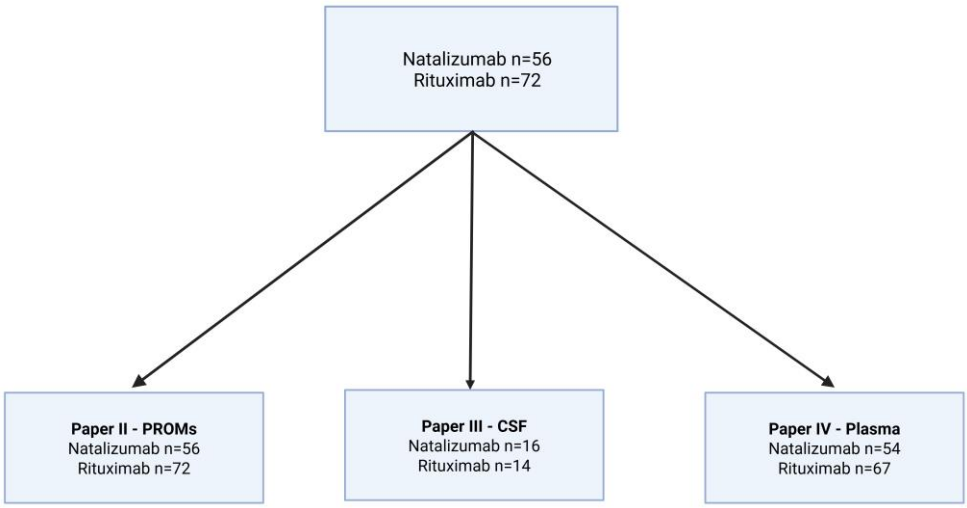
Among 1759 patients screened using the SMSreg, 128 were recruited as participants (NTZ, n=56; RTX, n=72). Of these, 121 provided blood samples and 30 underwent LP. Figures 5 and 6 illustrate the inclusion process and division into different study populations. Tables 2 and 3 provides an overview of Studies II–IV, including demographics and baseline clinical features. NTZ-treated participants, on average, younger, with longer treatment time, albeit shorter disease duration than the RTX-treated participants. EDSS and annual relapse rate were low in both treatment groups.

**Figure 5: Participant inclusion process**




Abbreviations: PMS, progressive MS; RTX, rituximab; NTZ, natalizumab; y, years; m, months; Sthlm, Stockholm; CSF, cerebrospinal fluid

**Figure 6: Participants included in the studies**




Abbreviations: PROMs, patient reported-outcome measures; CSF, cerebrospinal fluid

**Table 2: Overview of Studies II–IV**

| | **Study II** | **Study III** | **Study IV** |
|---|---|---|---|
| **Design** | Cross-sectional with a longitudinal component | Cross-sectional with a longitudinal component | Cross-sectional |
| **Participants (total)** | 128 | 30 | 121 |
| **NTZ** | 56 | 16 | 54 |
| **RTX** | 72 | 14 | 67 |
| **Data** | FSMC, SDMT, TSQM-10 | FSMC, SDMT, GFAP, NfL, OCBs, k-FLC | FSMC, SDMT, GFAP, NfL |
| **Materials** | PROMs | PROMs, CSF | PROMs, plasma, CSF |

Abbreviations: NTZ, natalizumab; RTX, rituximab; FSMC, Fatigue Scale for Motor and Cognitive Functions; SDMT, Symbol Digit Modalities Test; TSQM-10, Treatment Satisfaction Questionnaire for Medication-10 Items; GFAP, glial fibrillary acidic protein; NfL, neurofilament light; OCBs, oligoclonal bands; k-FLC, kappa free light chain; PROMs, patient reported-outcome measures; CSF, cerebrospinal fluid

**Table 3: Clinico-demographic features**

| | NTZ (n=56) | RTX (n=72) | Total (n=128) |
|---|---|---|---|
| **Age in years, mean (SD)** | 40 (8.8) | 45.6 (8.6) | 43.2 (9.1) |
| **Female, n (%)** | 49 (88) | 55 (76) | 104 (81) |
| **Disease duration, median years (range)** | 10.8 (2.8–42.1) | 12.6 (2.3–31.0) | 11.6 (2.3–42.1) |
| **Treatment time, median years (range)** | 6.4 (2.5–14.8) | 4.0 (2.2–9.4) | 4.5 (2.2–14.8) |
| **EDSS, median (range)** | 1.0 (0–6.5) | 1.5 (0–6.0) | 1.0 (0–6.5) |
| **Annual relapse rate** | 0.024 | 0.014 | 0.02 |
| **MRI lesions at treatment initiation, n (%)** | | | |
| **1–9** | 9 (16.1) | 20 (27.8) | 29 (22.6) |
| **10–20** | 16 (28.6) | 17 (23.6) | 33 (25.8) |
| **>20** | 31 (55.4) | 35 (48.6) | 66 (51.6) |

Abbreviations: NTZ, natalizumab; RTX, rituximab; EDSS, Expanded Disability Status Scale; MRI, magnetic resonance imaging; SD, standard deviation

### 6.2.2 Patient-reported outcome measures (PROMs)

Table 4 presents the results of the PROMs and SDMT. After adjusting for potential confounders, no significant between-group differences were found regarding the FSMC results, with age being the dominant confounding factor. Increasing age had a strong correlation with increasing fatigue, and although the initial crude analysis indicated less fatigue in the NTZ-treated participants, this difference could be completely explained by the higher age of the RTX-treated participants. Regarding the TSQM-10, most participants in both groups were "very" or "extremely satisfied," with 92% and 79% in the NTZ and RTX group, respectively, scoring either a 6 or 7 on the 10$^{th}$ item. No participant scored below "somewhat satisfied," that is, a 4.

The NTZ-treated participants performed significantly better on SDMT than the RTX-treated participants (Δ8.5 points). The NTZ-treated participants improved significantly from treatment initiation to cross-sectional SDMT (mean Δ8.9 points, 95% CI 6.0–11.8, p=0.002), whereas the RTX-treated participants had, on average, a mean decrease of 1 point on the SDMT from treatment initiation to cross-section. The sensitivity analysis revealed no significant difference (p=0.057). However, most NTZ-treated participants underwent the SDMT every 4–6 weeks, compared to the RTX-treated participants who underwent the SDMT every 6–12 months.

**Table 4: Results of PROMs and SDMT**

| | NTZ | RTX | p-value* |
|---|---|---|---|
| **FSMC** | **N=51** | **N=59** | |
| **Cognitive, mean (SD)** | 25.5 (11.6) | 29.2 (13.2) | 0.985 |
| **Motor, mean (SD)** | 23.6 (11.4) | 28.2 (13.3) | 0.857 |
| **Total, mean (SD)** | 49.1 (22.3) | 57.4 (26.3) | 0.936 |
| **SDMT** | **N=56** | **N=73** | |
| **Cross-section, mean (SD)** | 64.7 (12.8)** | 56.2 (12.0) | 0.003 |
| **Treatment initiation, mean (SD)** | 55.8 (12.1) | 56.6 (11.3) | N/A |
| **TSQM-10, median (range)** | **N=50** | **N=57** | |
| **Treatment satisfaction, median (range)** | 6 (4–7) | 6 (4–7) | N/A |

Abbreviations: NTZ, natalizumab; RTX, rituximab; FSMC, Fatigue Scale for Motor and Cognitive Functions; SDMT, Symbol Digit Modalities Test; TSQM-10, Treatment Satisfaction Questionnaire for Medication – 10 Items

*p-value represents the difference between the treatment groups at cross-section after adjustment for potential confounders

**SDMT score increased significantly from treatment initiation to cross-section in the NTZ group

### 6.2.3 Biomarkers and their associations to treatment and PROMs

#### *6.2.3.1 CSF and plasma biomarkers*

Table 5 presents the results of the CSF and plasma biomarkers. Among 30 participants who underwent an LP at cross-section, longitudinal comparisons of intrathecal IgG biomarkers were available for 14, with a mean time of 9.8 years between diagnostic and cross-sectional LP. IgG-index, and number of mononuclear cells decreased significantly compared with cross-sectional LP. No change was observed in OCB numbers. Only one NTZ-treated participant had become OCB-negative during treatment. OCB numbers and k-FLC levels were pathologically elevated in 97% and 89% of participants, respectively. No significant between-group differences were observed when comparing OCB numbers and the k-FLC index.

GFAP and NfL did not significantly differ between groups, with the exception that RTX-treated participants exhibited significantly lower CSF GFAP levels than the NTZ-treated participants (p=0.039). No significant between-group difference in CSF NfL was observed.

NfL levels correlated significantly in CSF and plasma (r=0.59; CI 95% 0.28–0.79). No association was found between CSF and plasma GFAP (r=0.33, CI 95% -0.04–0.62).

### *6.2.3.2 Biomarkers, fatigue, and IPS*

No significant associations were found between FSMC, SDMT, k-FLC, OCB numbers, CSF GFAP, CSF NfL, plasma GFAP, or plasma NfL.

**Table 5: Cerebrospinal fluid and plasma biomarker results**

| | All | NTZ | RTX | p-value |
|---|---|---|---|---|
| **CSF, median [IQR]** | **N=30** | **N=16** | **N=14** | |
| **OCB 1st LP** | 10 [7-20] | 10 [9-20] | 7 [4-16] | 0.14 |
| **OCB 2nd LP** | 8.5 [3-16] | 9 [5-16] | 8 [3-18] | >0.9 |
| **IgG index 1st LP** | 0.82 [0.66-1.23] | 1.01 [0.63-1.24] | 0.79 [0.68-1.28] | >0.9 |
| **IgG index 2nd LP** | 0.60 [0.52-0.73]* | 0.58 [0.51-0.73]* | 0.64 [0.56-0.73] | 0.4 |
| **Mono 1st LP** | 4 [2-10] | 6 [3-12] | 4 [0-6] | 0.3 |
| **Mono 2nd LP** | 0 [0-0]* | 0 [0-0]* | 0 [0-4]* | 0.035 |
| **k-FLC index** | 16.45 [9.28-51.52] | 15.40 [10.34-23.93] | 39.88 [7.40-66.80] | 0.2 |
| **NfL, pg/ml** | 307 [213-353] | 287 [204-347] | 326 [248-353] | 0.28† |
| **GFAP, pg/ml** | 11142 [9147-12287] | 11552 [9400-12372] | 9766 [9147-11994] | 0.039† |
| **Plasma, mean [SD]** | **N=121** | **N=54** | **N=67** | |
| **GFAP, pg/mL** | 85.1 [32.3) | 81.5 [30] | 88 [33.9] | N/A |
| **NfL, pg/mL** | 6.1 [2.5] | 5.7 [2.6] | 6.4 [2.4] | N/A |

* Statistically significant decrease from first to second LP, $p<0.05$.

† Adjusted for age and sex

Abbreviations: LP, lumbar puncture; IQR, interquartile range; CSF, cerebrospinal fluid; OCB, oligoclonal bands; RTX, rituximab; NTZ, Natalizumab; k-FLC, kappa free light chain; NfL, neurofilament light; GFAP, glial fibrillary acidic protein; Mono, mononuclear cells.

# 7 Ethical considerations

All studies included in this thesis were approved by the Swedish Ethical Review Authority. Four permits were approved: Studies I–IV (reference numbers 2019-03571, 2020-03608, 2020-06639, and 2023-06366-02). All participants provided written informed consent prior to enrolment.

Several ethical issues need to be addressed for studies such as these. First, a thorough review of medical files entails an intrusion into the patient's privacy. The data is strictly private. Therefore, it must be carefully and considerately handled. It was ensured that only information related to the study purpose and aim was collected. Nevertheless, the research team is in possession of sensitive information that potentially could harm the enrolled participants. Informed consent was obtained from each participant before the medical record review for the cross-sectional study, although not for the pilot study. The nature of the data collected from the medical records in the pilot study and the subsequent studies does not differ; however, the data of patients who were not informed of the intrusion needs more careful handling than those who had explicitly consented. These sensitive aspects have been treated with utmost respect, and it has been ensured that no study participant is harmed.

Second, patients may have been reminded of their illness, especially when they themselves prefer to disregard it as much as possible. Eligible patients were contacted several times, and through different means, to seek their participation in the comparative study of NTZ and RTX. In one instance, when the author was contacting eligible patients by phone, a patient mentioned that she was well and did not wish to do anything that reminded her that she had ever received the diagnosis. Currently, many patients with MS lead an ordinary life, and maintain contact with healthcare providers only on their annual follow-ups or when they receive treatment. Thus, contact can be limited to once or twice a year. Therefore, it is understandable that some patients might not choose to be reminded that they have an illness, and the author reminding them was not a pleasant experience for the patients.

Third, 30 participants in the comparative study of NTZ and RTX underwent an LP as part of the study protocol. Despite its safety, an LP has some risks. The most common complication is post-dural puncture headache (PDPH). It occurs in 6–36% of individuals, and its benignity notwithstanding, it can cause significant anguish to the patient[157, 158]. In some cases, PDPH might require interventions such as caffeine infusion or even an epidural blood patch. A few cases of PDPH were encountered

in the study cohort. Considering the results of the study, it is fair to catechize: “was it worth it?” Despite the inevitable materialization of such questions, they are irrelevant for this study. Intrinsically, research is “not knowing,” and risk and benefit must be balanced based on the pre-study data. Hence, the risk to benefit determination to perform a rather “harmful” procedure should be evaluated based on the viability of the hypothesis of the trial *before the research commences*, and not retrospectively, after the study concludes.

# 8 Discussion

## 8.1 Main findings

Currently, NTZ and RTX (off-label) are Sweden's most-prescribed DMTs for MS, accounting for approximately 10% and 61%, respectively, of all MS therapies.

This thesis provides further evidence of the clinical efficacy and safety of RTX in patients with MS. In RTX-treated patients, the ARR decreased from a relapse of every 2.6 years to once every 20 years, and MRI activity was almost completely eliminated. Furthermore, we report one case of ADA and one of RTX-induced LON as well.

In this limited study cohort, NTZ-treated participants do not experience less fatigue than the RTX-treated participants. Moreover, levels of intrathecal IgG-biomarkers, CSF NfL and plasma GFAP and NfL levels were not lower in the NTZ-treated participants than in the RTX-treated. Notably, RTX-treated participants exhibited lower CSF GFAP levels than the NTZ-treated participants. NTZ-treated participants performed significantly better on the SDMT than the RTX-treated participants, with those in the NTZ group improving their SDMT scores significantly from treatment initiation to cross-section.

## 8.2 Clinical efficacy

This thesis further supports the notion of RTX being a highly effective therapy for RRMS. In the retrospective observational study of RTX in patients with MS, the ARR and the number of participants exhibiting MRI activity decreased significantly.

Several clinical trials have evaluated the efficacy and safety of RTX in RRMS. Bar-Or et al. were the first to report on the clinical efficacy of RTX in RRMS[130]. In a phase 2 trial, Hauser et al. showed that RTX significantly lowered the ARR and number of CELs compared to placebo[131]. In 2016 a Swedish single arm open-label phase 2 study further evaluating the efficacy of RTX in RRMS[132], the number of CELs per patient decreased from 0.37 to 0.03. In the only phase 3 trial on RTX and MS performed today, the ARR was 0.015 in the RTX group, and 79% were free of

MRI activity at 24 months, which was an 80% reduction of disease activity compared with dimethyl fumarate, an approved first-line treatment of RRMS[138]. Moreover, several observational studies have evidenced the efficacy of RTX in RRMS, with the present study's results further supporting this notion[135, 159, 160].

Clinical worsening of disease, in the absence of relapse activity, was primarily observed in the PMS group in the present study cohort. However, a few participants in the RRMS group exhibited progression independent of relapse activity (PIRA). Notably, it is unclear whether these participants developed progressive disease over the years or if these were isolated events not leading to further progression or even reverting to a lower EDSS again. Data on PIRA in patients with MS treated with anti-CD20 therapies, particularly RTX, are limited. However, in post-hoc analyses from the OPERA I and II (ocrelizumab) trials, 15–20% of participants experienced PIRA, with the data being supported by subsequent studies[161-164].

Currently, PIRA is a widely discussed subject in MS research; however, it merits consideration. First, the definition of PIRA has varied between different trials. The most widely used definition is based on EDSS scores, which itself has limitations. EDSS is highly rater dependent, especially in patients with EDSS scores of 1–3.5[165]. A PIRA event can thus be owing to a mere change of treating physician. Hence, studies require that the change in EDSS score is sustained for at least 3 or 6 months, as in the OPERA trials. The OPERA investigators also had a central consistency check on expert review basis to ensure reliability of EDSS. This limits inter-rater variability, albeit not eliminating it. Retrospective observational studies with real-life data, such as that by Iaffaldano et al., revealed that patients may change treating physicians, possibly altering the EDSS scores confirmed by the previous physician 6 months earlier, thus confirming a PIRA event[164]. Moreover, EDSS can be affected by daily variations in each patient, constituting up to a 1.5-point difference in the EDSS[166]. This renders retrospective observational studies on EDSS-based PIRA unreliable as true measures of PIRA. The OPERA I and II trials used a composite PIRA definition, comprising the EDSS, 9-hole peg test, and the timed 25-foot walk test. The most frequent PIRA event was caused by a 20% increase in the timed 25-foot walk test and not a change in the EDSS, rendering PIRA an even more uncertain outcome measure, as the criteria differ greatly.

Furthermore, PIRA needs to be distinguished from secondary SPMS. The median time from diagnosis to confirmed SPMS in untreated patients is 20 years (range, 1–51 years), and following the natural course of MS, 60–80% of patients with RRMS convert to SPMS. PIRA only describes an event, such as an increase in EDSS of 1 point that is sustained over 6 months. SPMS is an irreversible process leading to continuous worsening of clinical symptoms gradually, distinguishing it from PIRA. Strijbis et al. also found in a retrospective analysis of the data from the NTZ trials AFFIRM and SENTINEL that a significant part of PIRA events be attributed to MRI activity. They also found that the number of events with improvement independent of relapse activity was greater than the number of PIRA events

documented. These findings further support that PIRA is not to be mistaken for progressive MS[167]. The clinical experience of physicians and nurses in specialized MS clinics in Sweden indicates that high-efficacy DMTs prevents, or at least delays, conversion to SPMS when prescribed to treatment-naïve patients with RRMS. However, to the researchers' knowledge, data on high-efficacy DMTs' effect on the conversion rate is insufficient. NTZ and RTX prescriptions to patients with early RRMS may affect the disease course, and in many cases, prevent the conversion to SPMS. However, when high-efficacy DMTs are prescribed later in the course of the disease, RRMS can nevertheless convert to SPMS[168]. Thus, a part of the pathological process remains unaffected by high-efficacy DMTs, especially when introduced later in the disease course.

The reason for conversion to SPMS is not fully understood. Theories include neurodegeneration independent of inflammatory activity, microglial and astrocyte activity, and compartmentalized inflammation from meningeal tertiary lymphoid-like structures seen as leptomeningeal enhancement on MRI[169-173]. In patients with SPMS, leptomeningeal enhancement is a frequent MRI finding, correlating with cortical atrophy and worse clinical outcome[174, 175]. Moreover, GFAP correlates well with clinical disease progression, indicating that reactive astrocytes play a role in the conversion to SPMS[174, 176]. In a recent phase III trial, the Bruton's tyrosine kinase (BTK) inhibitor, tolebrutinib, was demonstrated to slow disease progression in patients with SPMS, albeit modestly[177]. Moreover, BTK inhibitors penetrate the BBB and affect the CNS microglia and macrophages, possibly altering disease progression. The evolution of SPMS may be less affected by anti-CD20 therapies prescribed later in the disease process, as they fail to affect the meningeal tertiary lymphoid-like structures, as well as reactive astrocytes[178, 179].

## 8.3 Safety

In the retrospective study of RTX, four participants (4.8%) experienced moderate or severe adverse events. These findings are consistent with those in larger cohort studies on the infection rates in RTX-treated patients. Salzer et al. reported an exceptionally low rate of infections requiring hospitalization: 1.7%, in >800 RTX-treated patients[135]. The risk of serious infections was associated with age and hypogammaglobulinemia. For risk mitigation, many Swedish centers terminate treatment when the patient is approximately 55–60 years of age, and studies on the expansion of the dosing interval of RTX after 1–2 years of treatment are ongoing. Collectively, awareness of the increased risk of serious infections in patients with MS undergoing RTX therapy is vital, because more knowledge equates to better risk mitigation.

In the retrospective study on RTX, one patient had developed LON. Patients treated with anti-CD20 therapy can develop LON, albeit rarely. This condition is known within rheumatology and hematology, but the frequency in patients with MS is unclear. LON has been reported in RTX-, ocrelizumab-, and ofatumumab-treated patients[180]. LON is defined as an absolute neutrophil count <1.5/nL developing from week 4 up to 1 year after the latest anti-CD20 therapy infusion, placing patients at a potential risk for life-threatening infections[181]. Despite the several theories on the mechanisms underlying LON, evidence remains inconclusive[182]. No known LON predictors exist; however, Protopapa et al.'s study suggested laboratory assessment 4–6 weeks after initiation of B-cell depleting therapy[180]. Furthermore, they highlighted the possibility of increased risk of disease activity following G-CSF administration to increase the neutrophil count. Clinical relapses have been described in patients with neutropenia undergoing G-CSF stimulation, owing to its possible stimulating effect on T-helper cells, potentially causing disease exacerbations[182, 183]. However, considering the number of Swedish and global patients treated with anti-CD20 therapy for MS, the exceptionally few case-reports on the matter renders it likely that LON is an extremely rare side effect. Nevertheless, treating physicians need to be aware of this potentially life-threatening adverse effect of RTX therapy.

One patient developed ADA against RTX. It was not discovered by clinical or radiological activity, rather by insufficient B-cell depletion. Studies on ADA in rheumatological diseases have suggested a neutralizing effect of these antibodies[184, 185]. However, in MS, data is limited. In a 2018 cross-sectional study, serum samples from 339 RTX-treated patients were analyzed[186]. Approximately 40% of patients were positive for ADA, including 37% of those with RRMS. However, no association between clinical activity and ADA positivity was observed. Apart from the aforementioned study, data on the effect of ADA in MS remains limited. However, ADA might be assumed have a neutralizing effect on RTX, and further studies are needed to elucidate the clinical relevance of these ADA.

## 8.4 Fatigue

In this thesis, RTX-treated participants were found to be no more fatigued than the NTZ-treated participants after adjusting for confounders. Age was the primary factor affecting fatigue score, although the between-group difference was only 6 years.

Only a few previous studies have analyzed fatigue in patients treated with high-efficacy DMTs. Azoulai et al.'s study compared fatigue levels in 300 patients treated with either high-efficacy DMTs (ocrelizumab, NTZ, or RTX), or "other DMTs"[187]. No significant between-group difference was found. However, whether treatment

groups were comparable in age, EDSS, or ARR is unclear. Since the study was non-interventional, it is probable that participants in both groups were clinically stable and did not have an active disease. Otherwise, it is likely that the "other DMTs"-group patients would have switched to a high-efficacy therapy. Therefore, the "other DMTs"-group participants may have had low inflammatory activity at treatment initiation, thus explaining the lack of differences.

Svenningsson et al.'s TYNERGY trial found that NTZ-treated patients decreased their total FSMC score by 9 points on average during the first year of treatment, with a baseline mean total FSMC score of 71.2[156]. However, a comparison of the results of the TYNERGY trial and those of the present study yields some noteworthy similarities and differences. Mean total FSMC scores in the present study's treatment groups (Table 4) are well below the average 62.2 observed at the conclusion of the TYNERGY trial. Regarding the confounding factor of age, the TYNERGY trial participants are fully comparable to that of the present study's NTZ-treated participants (39.7 and 40 years, respectively). However, the TYNERGY trial participants had a longer disease duration and higher EDSS prior to NTZ initiation, both factors known to correlate with worse fatigue, than the present study participants[188-190]. Furthermore, the follow-up data in the TYNERGY trial only extends to 12 months from treatment initiation. Therefore, it cannot be discounted that FSMC scores continue to decline beyond 12 months. Iaffaldano et al. demonstrated a significant reduction in fatigue levels over 2 years in NTZ-treated patients, using the fatigue severity scale, further supporting that NTZ might have an effect on MS-related fatigue. To the researchers' knowledge, only one study has evaluated the effect of RTX on MS-related fatigue[191]. de Flon et al.'s study could not demonstrate any improvement in FSMC scores in clinically stable patients switching from platform DMTs (i.e., interferon-beta and glatiramer acetate) to RTX. Owing to the lack of data at treatment initiation, no longitudinal analyses of FSMC scores could be performed in the present study. Therefore, it is impossible to state whether or not the population in the present studies demonstrated the trends observed in Svenningsson et al. and Iaffaldano et al.'s studies. Notably, in contrast to those in Svenningsson et al. and Iaffaldano et al.'s studies, the study population in de Flon et al.'s study was clinically stable when initiating high-efficacy DMT and did not experience any clinical relapses. Evidence, however not conclusive, suggests that relapses are closely associated with fatigue. That is, cessation of aggressive inflammation follows a transition to highly effective therapy and lead to a reduction in fatigue levels at the group level[192-194].

Collectively, regardless of treatment strategy, differences in fatigue levels in clinically stable patients are likely negligible and require large materials for their discovery. The main factor affecting fatigue levels when initiating DMTs is most likely the reduction in disease activity; however, this needs further evaluation.

## 8.5 Information processing speed

IPS, as assessed by the SDMT, is the most commonly affected cognitive ability in patients with MS[90]. In the present study population, NTZ-treated participants performed significantly better on the SDMT than the RTX-treated participants, increasing their SDMT score from baseline to cross-section. A similar longitudinal improvement was not observed in the RTX group.

Previous studies on NTZ and SDMT have either demonstrated significant improvement or stable results over time[195-200]. Manouchehrinia et al. found that the probability of an increase in the SDMT score in NTZ-treated patients was higher than that for any other examined DMT (i.e., fingolimod, alemtuzumab, teriflunomide, dimethyl fumarate, peginterferon beta-1a, RTX, daclizumab, ocrelizumab, ofatumumab, and cladribine)[195]. However, in the aforementioned study, RTX was combined with the other monoclonal antibodies into one category, making it impossible to distinguish any distinct effects of RTX. To the researchers' knowledge, only de Flon et al. have conducted a longitudinal study on RTX and patient related outcome measures such as fatigue, treatment satisfaction and cognition measured by SDMT. They reported a significant improvement in SDMT score over time in RTX-treated patients. However, the improvement comprised only 3.2 points, and despite being statistically significant, it did not reach the estimated clinically significant cut off of 8 points[191, 201]. Two other studies, one on RRMS and another on PMS, had included RTX in their analyses on SDMT[202]. Longetti et al. reported that SDMT performance in patients with RRMS remained stable during the first few years of treatment, regardless of the DMT[203]. Regarding other anti-CD20 therapies, only studies evaluating the effect of ocrelizumab on SDMT are available. A post-hoc analysis from the OPERA trials reveals that 33% of ocrelizumab-treated patients improved by at least 8 points on SDMT over time, and in Schuckmann et al.'s study, ocrelizumab-treated patients remained stable in the SDMT scores over 24 months, albeit not exhibiting any significant improvement[204, 205]. When considering high-efficacy DMTs as one unit, their effect on SDMT does not appear to outperform that of platform DMTs[206].

Moreover, the thin line separating cognitive fatigue from cognitive impairment in MS merits consideration. Mills et al. defined reversible cognitive impairment as a distinct feature of cognitive fatigue, affecting attention and memory[207]. MS affects IPS, memory, language, executive function, and complex attention[90, 208]. Considering the sizable overlap in symptoms of cognitive impairment and cognitive fatigue, the observed differences in fatigue from health care professionals might actually represent less cognitive impairment in NTZ-treated patients.

The most relevant objection to the reliability of the present study's results is that most NTZ-treated participants performed the SDMT every 4–6 weeks for several years, suggesting that at least some of the observed effect is attributable to a practice

effect. Several studies have revealed that exchanging the SDMT form, as was the routine in the present study's centers, limits or even eliminates the learning effect. However, there are studies indicating a pronounced practice effect when SDMT is performed repeatedly with short intervals and participants may have developed techniques to perform better at each testing, thus warranting caution when interpreting the findings[209-214].

Regarding the data from the OPERA trials on ocrelizumab and the meta-analyses describing the effects of DMTs on SDMT, NTZ is unlikely to have unique features that render it superior to RTX or other anti-CD20 therapies on IPS. Overall, improvements in SDMT are probably a reflection of the reduction in inflammation and relapse activity, rather than a unique property of NTZ affecting cognition[215]. Studies with more data on the effects on RTX and other anti-CD20 therapies would likely have yielded similar results, i.e. initial improvement that subsequently plateaus.

Cognitive fatigue and cognitive impairment are closely linked[216]. Therefore, a true difference in fatigue between treatment groups cannot be discounted, considering the differences in the SDMT results.

## 8.6 Biomarkers, IPS, and fatigue

### 8.6.1 Information processing speed

No significant associations in plasma or CSF between IPS, GFAP, and NfL were found. These findings are consistent with those in previous studies on GFAP and IPS, which have yielded negative results both in CSF and plasma[217-220]. No significant associations between the SDMT and intrathecal IgG biomarkers were observed in the present study population. Although data on OCB, k-FLC index, and its relationship with the SDMT is limited, intrathecal IgG production may be associated with impaired cognition in general and IPS in particular[221, 222]. The relationship between the SDMT and NfL remains unclear. The results on NfL in both CSF and plasma are contradictory, with most studies reporting no associations, while others have found clear associations[205, 218, 220, 223-228].

The cause of impaired IPS in MS remains moot, with no conclusive evidence. Paramagnetic rim lesions have been reported to be associated with decreased SDMT scores, along with cortical and thalamic atrophy. EDSS severity and ambulatory function, progressive disease, depression, and anxiety are other contributory factors to impaired IPS[228-238], suggesting its complexity and multifacetedness, which are unmeasurable via a single plasma or CSF analysis. Impaired cognition in MS is likely a consequence of multifactorial impact from accumulated white matter damage, grey matter atrophy and inflammation on global cerebral networks.

The present study, comprising 30 participants undergoing an LP and 121 supplying a blood sample, may be too small to detect any associations. However, the termination of inflammation by the high-efficacy DMTs, NTZ and RTX, may have limited the extent of disability accrual and grey matter damage, thus preserving cognitive functions.

### 8.6.2 Fatigue

No associations were found between fatigue levels and biomarkers in CSF or plasma. Potential links between GFAP, NfL, and fatigue in patients with MS have been extensively investigated, without evidence of causation[74, 223, 239-241]. Moreover, there are, to our knowledge, no studies supporting a link between intrathecal IgG-production and MS-related fatigue.

Fatigue is not a unique feature for MS, rather it is a common symptom of chronic infections, systemic autoimmune illnesses, and malignancies[75, 77, 242]. The results of research on potential associations between MS-related fatigue, inflammatory biomarkers in CSF, grey and white matter lesions, are highly contradictory[68, 243, 244]. Although, some studies reveal that chronic active cerebral lesions, and thalamus and cerebral cortex grey matter lesions are associated with fatigue[68, 245].

Inflammation is the common denominator in all the aforementioned conditions. Data suggest that inflammatory markers in plasma, such as IL6, TNF-alpha, and IFN-gamma, in patients with MS are linked to fatigue, although the data is limited[72]. These cytokines are implicated to contribute to fatigue in several other inflammatory disorders[75, 246]. Cortical atrophy is associated with fatigue as well[68, 85]. It occurs in systemic autoimmune diseases such as Crohn's disease and rheumatoid arthritis, and is closely linked to systemic inflammation[247-249]. Moreover, data supporting astrocyte and microglial activation as contributing causes to fatigue in neuroinflammatory and autoimmune diseases are available[250].

Overall, the presence of fatigue in several inflammatory conditions supports the concept that MS-related fatigue does not have a solitary cause, rather it is a multifactorial entity with inflammation at its core. Therefore, any effect by DMTs in patients with MS-related fatigue may be due to reduced inflammation.

### 8.6.3 Comparison of biomarkers in NTZ- and RTX-treated participants

This thesis found that RTX-treated participants exhibited lower CSF GFAP levels than the NTZ-treated participants. No significant difference was observed for CSF NfL, k-FLC, OCBs, or IgG-index. No differences were found for plasma GFAP or NfL.

The significantly lower levels of CSF GFAP in the RTX-treated participants could potentially indicate a lower risk of conversion to SPMS. However, no significant difference was found for plasma GFAP. A few studies have found that NTZ reduces GFAP in patients with MS, while most suggest that high-efficacy DMTs, including anti-CD20 therapies, do not appear to affect neither plasma nor CSF GFAP[217, 251-256]. Moreover, recent studies indicate that the reliability of GFAP differs between plasma and CSF. CSF GFAP is more easily affected by repeated thawing and freezing than plasma GFAP[257]. Plasma GFAP exhibits a much better correlation with clinical parameters in Alzheimer's disease than CSF GFAP[258]. The present study's analyses on CSF were performed in 30 participant, a small sample. Thus, the difference in CSF GFAP may be consequent to a type 1 error.

NfL concentration is highly sensitive to relapses and focal inflammation in MS[259]. Elevated levels are indicative of clinical relapses or MRI activity[260]. In the present study, no significant differences in NfL levels were observed, neither in plasma nor CSF, which was expected. Previous studies demonstrated a significant decrease in both plasma and CSF NfL levels in NTZ- or RTX-treated patients. Moreover, plasma and CSF NfL levels correlate well, as observed in this thesis. NTZ and RTX significantly decrease NfL levels as a consequence of reduced inflammation, providing further evidence that these DMTs limit axonal damage and disease activity[132].

No between-group differences were observed regarding markers of intrathecal IgG production. No difference was observed in OCB numbers from diagnosis to cross-section, although IgG index was significantly decreased. Despite thorough research, the antigen targets of these IgG antibodies constituting the OCBs remain unknown. The antibodies may be a secondary immune response to cellular damage, thus having intracellular targets. In such a case, the absence of OCBs may indicate low to no degree of cellular injury[261]. This is evidenced by the presence of IgG OCBs at MS diagnosis, which indicates a more aggressive disease course, with more relapses and earlier conversion to progressive disease[262]. Studies examining the effect of high-efficacy DMTs on OCBs have yielded contradictory results. Some report that NTZ and cladribine reduce the number of, and even eliminate, OCBs. Conversely, other studies report no effect of NTZ, RTX, and cladribine on OCB numbers[263-267]. Notably, the IgG index decreased significantly in both treatment groups from diagnostic LP to cross-section, indicating that NTZ and RTX do affect IgG production.

The origin of OCBs remains unknown; however, plasma cells established in meningeal tertiary lymphoid follicular structures may be, at least partially, responsible for OCB production[268]. These structures can be observed on post-contrast MRI FLAIR sequences as leptomeningeal enhancements, and histological reports have revealed that they include long-lived plasma cells capable of producing immunoglobulins[173, 269]. NTZ affects only the lymphocytic passage across the BBB, RTX is too big a molecule to cross the BBB, and because long-lived plasma cells

are mainly CD20 negative, those established in lymphoid structures may not be affected by the investigated therapies[270, 271]. To date, convincing evidence of a lasting effect of DMTs on OCBs is lacking. Established plasma cell populations in tertiary follicular structures may be self-sustaining, thus remaining unaffected by immune reconstitution therapies such as alemtuzumab, cladribine, and autologous hematopoietic stem cell transplantation. However, in addition to long-lived plasma cells, short-lived plasma blasts can be found in the CNS of patients with MS, which contribute to the intrathecal IgG production[272]. Both NTZ and RTX decrease short-lived plasma blasts in the CNS, thus plausibly explaining the divergent results on IgG index and OCB numbers[266, 273, 274].

k-FLC index did not differ between treatment groups. This thesis found a strong association between k-FLC and OCB numbers, which may reflect a similar immunological process. Therefore, provided that the OCBs are unaffected, the k-FLC index remains unaffected as well. This thesis provides further support for the close link between OCBs and k-FLC.

## 8.7 Limitations and reconsiderations

### 8.7.1 Study I

This retrospective observational study has several limitations. The study population did not include a control group. The accuracy of medical records, and the prospectively collected data in the SMSreg, were relied upon for the study. The information in medical records and registries may not be entirely accurate. Moreover, it is possible that relevant information regarding serious adverse events and infections is missing from medical records, rendering the results less reliable. MRI data was collected using the original radiologist's reports, meaning that, the images were not re-evaluated by a neuroradiologist. For clinical worsening of disease, the research team's own definition was used, owing to the lack of EDSS reports. The effect of RTX in the whole MS population is discussed, not always clearly distinguishing between PMS and RRMS.

Retrospectively, a few things that could have been executed differently. Owing to the study's retrospective observational design, a formal control group was not possible. To improve comparability, a group treated with another DMT, such as dimethyl fumarate, could have been included. However, this was difficult because most patients with MS in Helsingborg are treated with RTX. The possibility of matching for age, disability, disease severity, and activity remains uncertain. The recruitment of a neuroradiologist would have improved the reliability of the MRI results and enabled a more in-depth analysis. Finally, PMS and RRMS must be clearly separated when analyzing the efficacy of a DMT. Most current DMTs have a clinically and statistically significant effect on relapses, MRI activity, and clinical

worsening of disease in RRMS. The effects on PMS without relapses or new lesions on MRI, are merely modest. Therefore, we could have made a more clear distinction between PMS and RRMS in the discussion and conclusion.

### 8.7.2 Studies II–IV

First, regarding the inclusion process and the power estimations, the studies aimed to compare the well-known issue of fatigue in patients with MS. This might incentivize patients experiencing discomforting fatigue to participate, considering the personal relevance of the issue. Conversely, it is possible that patients affected the most by fatigue are too extremely fatigued to participate. Therefore, the resultant study population may not reflect the population in general. The estimated sample size to demonstrate non-inferiority was 50 participants per treatment group, provided that the difference between groups was <15%. Insufficient data on estimated effect size from previous studies precluded the performance of formal power calculations. Data on the estimated effect size of DMTs on fatigue is unavailable, and power calculation with the currently available data remains an issue. However, estimating that treatment with RTX and NTZ lead to a 5% and 20% decrease in fatigue levels, respectively, a sample size of 101 patients per treatment group would be needed, providing 90% power. An underpowered study can never rule out a difference between groups, why type 2 errors cannot be discounted.

Returning to the inclusion process, patient selection was limited to the patients available at the selected outpatient neurology clinics in Lund, Malmö, Helsingborg and Danderyd undergoing treatment with either NTZ or RTX, which provided an upper limit of possible participants in each study group. More than 50% of eligible participants declined participation. Amongst those who declined participation, the fear of LP being cited as the main reason. Despite being offered the option to omit certain parts of the study protocol, many of these patients remained unwilling to participate. The limited sample size in the treatment groups, and especially the small number of participants who provided CSF, could potentially have caused type 2 errors throughout the thesis. Therefore, true differences in fatigue levels between NTZ- and RTX-treated participants are possible; however, the study was not large enough to detect them.

Second, body mass index was not included in the models of GFAP and NfL. Both GFAP and NfL are inversely correlated with body mass index and blood volume and could be both lower and higher than that revealed by the results[275]. Data on anxiety and depression, factors that affect both fatigue and IPS, were not included. It cannot be discounted that one treatment group experiences far more depression and anxiety than the other, thus obscuring a true difference in the case of fatigue, or causing a difference in the SDMT results. These clear limitations should have been included in the protocol during project planning.

Third, the lack of FSMC data at baseline is another major limitation of this study. Longitudinal data is needed to accurately evaluate the effects of DMTs on fatigue. FSMC is not routinely included in SMSreg as part of the annual follow-up. Consequently, potential changes in fatigue levels over time are impossible to evaluate, precluding the drawing of causative conclusions regarding the effects of the studied DMTs on fatigue. No fatigue-specific outcome measure is included in the Swedish MS Society's recommendations on annual follow-up. The SMSreg is a prospectively collected data set aimed at ensuring quality care for patients with MS across the country. Considering the impact of fatigue on patients with MS, it is notable that no fatigue-specific scale is included in the recommended annual follow-up. In the current era where NTZ, RTX, and other high-efficacy DMTs eliminate relapses and possibly progression, the problem of fatigue in MS care is amplified. Therefore, to enable further research on fatigue in MS, a validated fatigue scale, such as FSMC, should be included in the recommended outcome measures at the annual follow-up.

The repeated assessments of SDMT in Study II need addressing. "Practice makes perfect," and despite the forms being switched at every visit, limiting the practice effect, the observed results seen maybe partly attributed to a practice effect. This became apparent when examining the results, after participant inclusion was concluded. As an alternative to observe differences in IPS, participants should have performed the PASAT, as this test is not used in clinical practice in Sweden. To researchers' knowledge, participants in the study have not previously performed PASAT, and the inclusion of the test could eliminate potential practice effects in the study. The wide-spread use of PASAT in studies on cognitive abilities in MS renders comparisons to previous studies possible as well.

Fourth, distinguishing between fatigue and impaired IPS can prove difficult. Patients with cognitive impairment tend to be fatigued and fatigable. Conversely, fatigued patients tend to have more cognitive issues than those without fatigue. Therefore, a true difference in fatigue levels between treatment groups cannot be discounted, considering the difference in the SDMT results between the NTZ- and RTX-treated participants. Owing to the limited study population, all the non-significant results in this thesis may have been subjected to type 2 errors, and the positive results in the CSF study, to type 1 errors.

# 9 Conclusion and future perspectives

This thesis provides further evidence of the efficacy of RTX for MS. RTX remains an off-label therapy for RRMS, despite accumulating evidence of its safety and efficacy. The studies in this thesis found no significant differences in fatigue in NTZ- or RTX-treated participants. CSF GFAP was significantly higher in NTZ-treated participants than RTX-treated participants. However, no other between-group differences in biomarkers were found. Among the several studies published every day, these results are merit consideration for a few reasons.

RTX is inexpensive. In resource-limited countries, where the access to newer and expensive medications such as the anti-CD20, ofatumumab and ocrelizumab, medications might be constrained, RTX could be a feasible option for patients with RRMS. More data on the benefits of RTX in MS equates to its higher likelihood of prescription. The thesis provides further evidence of its efficacy on relapses and MRI activity; nevertheless, information on important safety concerns needs to be addressed.

This thesis is novel in that no previous studies have directly compared NTZ and RTX. A more seldom-used outcome, fatigue, was chosen for the project. Although no difference was found in fatigue levels between NTZ- and RTX-treated participants, the possibility cannot be discounted. However, NTZ-treated participants were found to have performed significantly better on the SDMT than the RTX-treated participants. Cognitive fatigue and impaired IPS are closely associated with each other, and it is possible that some of the differences observed in the SDMT scores represent the actual differences in well-being observed by health care professionals.

Moreover, based on the observed significant between-group differences in CSF GFAP levels, the results suggest the possible effectiveness of RTX in preventing disease progression. However, this is unlikely as the risk of type 1 errors is high, considering the small sample size. No significant between-group differences were observed in plasma GFAP, which is considered a more stable and reliable biomarker, supporting the attribution of the CSF finding to a type 1 error.

Collectively, the results suggest that off-label RTX therapy is no less effective than the approved NTZ therapy. However, owing to the limited sample size, a difference between the NTZ- and RTX-treated patients cannot be discounted.

The 2024 revised McDonald criteria enable a definite diagnosis of MS for patients previously diagnosed with RIS. This facilitates earlier therapeutic intervention, aiding the further exploration of fatigue and cognitive impairment in patients without clinical disabilities, undergoing treatment with NTZ and RTX. This will provide opportunities for controlled studies in patients who have neither experienced relapses nor exhibit any clinical signs of disability, to evaluate if patients with MS develop fatigue and cognitive impairment while undergoing treatment with high-efficacy DMTs.

Larger, controlled studies evaluating the effect of high-efficacy DMTs on fatigue and IPS should be conducted. It is probable that both symptoms are partly inflammation-induced, and controlled longitudinal studies on DMTs could potentially spotlight both the origin and potential effect of these DMTs on these symptoms.

An increased research focus on fatigue and cognition in general is strongly advocated. The Swedish MS Society could include a fatigue specific scale in their annual follow-up guidelines to advance the cause. This would facilitate research on fatigue and centralize it in discussions on national MS care. In the 1990s, before the DMT paradigm shift, when the only available therapy was corticosteroids, patients with MS reported fatigue as the most disabling symptom, and currently, patients continue to report fatigue and cognitive impairment as the main reasons for disability pension and sick leave. Better treatment options for fatigue and prevention of cognitive impairment will improve quality of life for patients with MS and be highly beneficial to society owing to reduced societal burden.

It is imperative that the MS research community remembers its purpose: to provide the best possible care for the patients. Increased focus on fatigue, the issue that causes them much anguish, is a good starting point.

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