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# **ANTI-CD20 THERAPY IN MULTIPLE SCLEROSIS - CLINICAL AND PARACLINICAL OUTCOMES**

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Cover illustration: Rituximab antibody by Wilhelm Juto

# Anti-CD20 therapy in multiple sclerosis - Clinical and paraclinical outcomes

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To Anna, Wilhelm and Hilma

*“The measure of a life, after all, is not its duration, but its donation.”*

Corrie ten Boom



## ABSTRACT

Multiple sclerosis is a chronic inflammatory disease of the central nervous system and the most common non-traumatic cause of neurological disability in young adults. Rituximab is a B-cell depleting drug targeting the CD20 epitope on B-cells, which drive inflammation in relapsing-remitting multiple sclerosis by acting as antigen-presenting cells to activate T-cell responses against central nervous system autoantigens. Despite not being formally approved for this disease, rituximab is Sweden's most common multiple sclerosis disease-modifying treatment.

The objective of this thesis was to through observational studies increase the knowledge base for the risk-benefit of rituximab in multiple sclerosis by determining clinical and paraclinical outcomes, in comparison with drugs approved for this disease, in real-world cohorts.

Study I is a multicentre retrospective study of 241 relapsing-remitting multiple sclerosis patients switching from interferon- $\beta$  or glatiramer acetate to rituximab, natalizumab or fingolimod due to breakthrough disease. Our results indicate a superior efficacy with rituximab and natalizumab compared to fingolimod, a similar tolerability profile between treatments, but a significantly higher overall drug persistence with rituximab.

Study II is a single-centre retrospective study of all 808 relapsing-remitting multiple sclerosis patients ever treated with rituximab at Karolinska. Data on reason for therapy stop, new therapies and clinical and radiological outcomes after rituximab termination were recorded. Rituximab was stopped in 92 (11%) cases, with 7 (< 1%) of all patients doing so due to lack of efficacy. Pregnancy plans and adverse events were the most frequent reasons for stopping therapy and disease activity remained low regardless if a new disease-modifying drug was started or not.

The cross-sectional Study III surveyed frequency of anti-drug antibodies and their potential impact on efficacy and safety outcomes in 339 rituximab treated multiple sclerosis patients. Presence of anti-drug antibodies was high; 37% and 26% in patients with relapsing-remitting and progressive disease, respectively. High anti-drug antibody titres were associated with incomplete B-cell depletion, but not with reduced clinical effectiveness or tolerability.

Study IV is a retrospective study comparing rates of regional atrophy and T1-weighted lesion volume accumulation on magnetic resonance imaging in relapsing-remitting multiple sclerosis patients starting rituximab ( $n = 15$ ) or interferon- $\beta$  ( $n = 67$ ) as initial treatment. The rituximab group had lower rate of T1-weighted lesion volume accumulation, but higher volume loss in some brain regions compared to interferon- $\beta$ , possibly due to pseudoatrophy.

The results add to a growing body of evidence suggesting equal or better effectiveness and tolerability of rituximab compared to commonly used drugs in relapsing-remitting multiple sclerosis. Furthermore, rituximab interruption is not associated with a rebound effect and anti-drug antibodies, while frequent, do not seemingly impact on therapeutic effect or tolerability. Lastly, rituximab is associated with slower brain lesion volume accumulation compared to a first-line treatment. The finding of a higher rate of regional brain volume loss with rituximab treatment, however, warrants further studies conducted over longer time periods.

# SAMMANFATTNING

Multipel skleros är en kronisk inflammatorisk sjukdom i centrala nervsystemet och utgör den vanligaste icke-traumatiska orsaken till neurologiskt handikapp hos unga vuxna. Rituximab är en monoklonal antikropp riktad mot ytstrukturen CD20 på B-celler, som misstänks vara involverad i patogenesen för multipel skleros genom att fungera som antigenpresenterande celler för sjukdomsdrivande T-celler. Trots att rituximab inte är formellt godkänt för multipel skleros har den kommit att bli den vanligaste sjukdomsmodifierande terapin för denna sjukdom i Sverige.

Målsättningen för denna avhandling har varit att förbättra kunskapsläget för rituximab som behandling för skovformad multipel skleros genom att belysa effekt och biverkningar i jämförelse med läkemedel som har formellt godkännande.

Studie I är en multicenter retrospektiv observationsstudie av 241 patienter med skovformad multipel skleros som på grund av bristande effekt växlat behandling från interferon- $\beta$  eller glatiramer acetat till rituximab, natalizumab eller fingolimod. Resultaten indikerar att rituximab och natalizumab har bättre effekt än fingolimod, att tolerabiliteten var likartad mellan behandlingarna, men att större andel behandlade med rituximab kvarstod på behandling över tid.

Studie II är en singelcenter retrospektiv observationsstudie av samtliga 808 patienter med skovformad multipel skleros som behandlats med rituximab vid Karolinska. Orsak till eventuella behandlingsavbrott, terapibytet samt kliniska och radiologiska utfall efter utsatt rituximabbehandling noterades. Behandlingen med rituximab avbröts hos 92 (11%), varav 7 (< 1%) på grund av bristande effekt. Planerad graviditet och biverkningar var de vanligaste skälen till uppehåll eller avbrott i behandlingen och sjukdomsaktiviteten kvarstod låg oberoende om en ny sjukdomsmodifierande terapi insattes eller inte.

Studie III är en tvärsnittsstudie där förekomst av antikroppar riktade mot rituximab hos 339 multipel skleros-patienter bestämdes. Antikroppar mot rituximab detekterades hos 37% och 26% av patienter med skovformad respektive progressiv multipel sklerossjukdom. En hög koncentration av anti-rituximabantikroppar var associerat till minskad B-cellselimination, men ej till sviktande klinisk/radiologisk behandlingseffekt eller förekomst av biverkningar.

Studie IV är en retrospektiv observationsstudie där förekomst av regional volymförlust och lesionsvolymökning på magnetkamerabilder jämfördes mellan patienter som startat rituximab ( $n = 15$ ) eller interferon- $\beta$  ( $n = 67$ ) som första behandling för skovformad multipel skleros. Patienter behandlade med rituximab uppvisade mindre lesionsvolymökning, men större volymförlust i flera hjärnregioner jämfört med interferonbehandlade patienter, möjligen på grund av pseudoatrofi.

Avhandlingens resultat bidrar till en växande kunskapsbas som tyder på likvärdig eller bättre effekt med rituximab vid multipel skleros jämfört med flera för sjukdomen godkända terapier. Dessutom är behandlingsavbrott inte förknippat med snabb återkomst av sjukdomsaktivitet och även om anti-läkemedelsantikroppar är vanligt förekommande, så saknar de betydelse för effekt eller tolerabilitet. Slutligen är rituximab kopplat till långsammare ökning av lesionsvolymen jämfört en traditionell första behandling för sjukdomen. Fyndet av en högre grad av regional hjärnvolymförlust vid rituximabbehandling motiverar längre uppföljningsstudier.



## LIST OF SCIENTIFIC PAPERS

This thesis is based on the following articles, which will be referred to in the text by their corresponding roman numerals.

- I. Boremalm M\*, **Juto A\***, Axelsson M, Novakova L, Frisell T, Svenningsson A, Lycke J, Piehl F, Salzer J. *Natalizumab, rituximab and fingolimod as escalation therapy in multiple sclerosis*. European Journal of Neurology 2019;26(8):1060-7.
- II. **Juto A**, Fink K, Al Nimer F, Piehl F. *Interrupting rituximab treatment in relapsing-remitting multiple sclerosis; no evidence of rebound disease activity*. Multiple Sclerosis and Related Disorders. 2020;37:101468.
- III. Dunn N\*, **Juto A\***, Ryner M, Manouchehrinia A, Piccoli L, Fink K, Piehl F, Fogdell-Hahn A. *Rituximab in multiple sclerosis: Frequency and clinical relevance of anti-drug antibodies*. Multiple Sclerosis Journal. 2018;24(9):1224-33.
- IV. **Juto A**, Ouellette R, Frisell T, Piehl F, Granberg T. *Greater T1 lesion volume accumulation rate with interferons compared to rituximab in treatment-naïve relapsing-remitting multiple sclerosis patients*. Manuscript.

\*These authors contributed equally.

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## LIST OF ABBREVIATIONS

3D	Three-dimensional
ADA	Anti-drug antibody
ADEM	Acute disseminated encephalomyelitis
AE	Adverse event
AHSCT	Autologous Haematopoietic Stem Cell Transplantation
APC	Antigen-presenting cell
ARMSS	Age-Related Multiple Sclerosis Severity
ARR	Annualised relapse rate
ATZ	Alemtuzumab
BBB	Blood-brain barrier
BTK	Bruton's tyrosine-protein kinase
CI	Confidence interval
CIS	Clinically isolated syndrome
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTCAE	Common Terminology Criteria for Adverse Events
CXCR4	C-X-C chemokine receptor type 4
DMF	Dimethyl fumarate
DMT	Disease modifying treatment
ECL	Electrochemiluminescence
EDSS	Expanded Disability Status Scale
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
FDA	U.S. Food and Drug Administration
FGL	Fingolimod
FLAIR	Fluid-attenuated inversion recovery

GA	Glatiramer acetate
GBCA	Gadolinium-based contrast agent
GM	Grey matter
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HLA	Human leukocyte antigen
HR	Hazard ratio
IC	Immune complex
IFN $\beta$	Interferon beta
IgG	Immunoglobulin G
IR	Infusion reaction
JCV	John Cunningham virus
MHC	Major histocompatibility complex
MOG	Myelin-oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MSD	Meso Scale Discovery
MSSS	Multiple Sclerosis Severity Score
NEDA	No evidence of disease activity
NMOSD	Neuromyelitis Optica spectrum disorder
NTZ	Natalizumab
OCR	Ocrelizumab
OFA	Ofatumumab
PML	Progressive multifocal leukoencephalopathy
PPMS	Primary progressive multiple sclerosis
RASGRP2	RAS guanyl-releasing protein 2
RCT	Randomised clinical trial
RIS	Radiologically isolated syndrome

RRMS	Relapsing-remitting multiple sclerosis
RTX	Rituximab
SMSreg	Swedish multiple sclerosis registry
SPMS	Secondary progressive multiple sclerosis
VLA	Very late antigen
WM	White matter



# 1. INTRODUCTION

I started working as a physician at the Neurology department at Karolinska University Hospital in 2016, where I rapidly became involved in the care of patients with multiple sclerosis (MS). Prior to that, my knowledge about MS was limited and had mostly been obtained during my medical studies, during which I had understood that the disease was highly heterogeneous in terms of symptoms with a handful of available treatments that were all relatively recently developed and expensive.

At Karolinska, it was soon obvious to me that many MS patients were quite young and that the degree of neurological disability was highly variable, ranging from none to patients needing a wheelchair. Further, it became clear to me that most of the patients who were neurologically intact were younger with shorter disease duration than those with more severe neurological impairment and more often on a disease-modifying treatment (DMT) compared to the latter patient category. I here thought that this might be one of those moments when access to novel and effective drugs can drastically change the traditional course of a disease.

Shortly thereafter, I had the opportunity to discuss my observations with senior colleagues working in the section of neuroinflammation. One of them had just recently received a major American grant to compare outcomes between different MS DMTs, with a special focus on patient-centred experiences. This also opened an opportunity for me to get involved in research and I was thrilled to be enrolled as a PhD student with a project aiming to describe outcomes with what then had become the most commonly prescribed MS DMT at our hospital, the monoclonal antibody rituximab (RTX). This was also highly relevant, since the use of RTX, not being formally approved for use in MS, was highly controversial at the time. In fact, the use of RTX was much higher at Karolinska Solna compared to the sister clinic Karolinska Huddinge. During the following years, when working clinically at the Neurology department, I noticed a steadily increasing number of patients with relapsing-remitting MS (RRMS) who received RTX while the frequency of MS patients in need of hospitalisation due to an MS relapse continuously decreased.

From my point of view, this PhD project has been highly clinically relevant and important for our patients, which has served as an important source of motivation for me throughout these years. As proof of this, I was proud that our study on slow return of disease activity after stopping RTX (Study II) received the “Best Poster Award” at the “European Committee for Treatment and Research in Multiple Sclerosis” (ECTRIMS) in September 2019 in Stockholm. No one could then imagine what would happen just a year and a half later with the Coronavirus disease (COVID-19) pandemic. Here, the knowledge that there is no rebound disease phenomenon after discontinuation of RTX became important, as we could postpone RTX re-infusions without fearing disease flares.

In conclusion, it has been a highly rewarding and privileged journey to conduct my PhD studies at Karolinska Institutet, which has developed me as a researcher in addition to on a personal level. I look forward to continuing doing clinical research in the years to come, hopefully, in collaboration with many of the researchers I have worked with during my doctoral studies.

## **2. BACKGROUND**

### **2.1 MULTIPLE SCLEROSIS**

MS is a chronic neuroinflammatory disease of the central nervous system (CNS), leading to myelin loss and axonal damage with resulting neuronal death. Focal lesions (plaques) are typically located around the post-capillary venules and these plaques have signs of breakdown of the blood-brain barrier (BBB).<sup>1</sup> MS lesions are developed in both grey and white matter (WM) and can be found anywhere in the CNS, including the optic nerve and spinal cord.<sup>2-4</sup> Among young adults, MS is the leading cause of non-traumatic disability and one of the costliest chronic health conditions.<sup>1,5</sup> The total mean annual cost per patient in Sweden has been estimated to 244.000 SEK (61% healthcare), 384.000 SEK (45% healthcare) and 888.000 SEK (13% healthcare) for mild, moderate and severe MS, respectively.<sup>6</sup>

MS was first described as a new neurological disease in 1868 by Jean Martin Charcot when he suggested that the observations of widespread neurological symptoms in patients noted by others were caused by the same disease, which he termed “Sclérose en plaques”.<sup>7</sup> This term was changed to MS in 1954 in English literature.

#### **2.1.1 Diagnosis**

MS is diagnosed when lesions disseminated in both time and space have been confirmed, according to the McDonald criteria which were most recently updated in 2017.<sup>8</sup> Neurological examinations can be sufficient to establish the diagnosis, but most patients are also examined with magnetic resonance imaging (MRI) and cerebrospinal fluid (CSF) analysis. The dissemination in space is fulfilled on MRI if T2 lesions are found on at least two out of four typical MS locals; that is juxtacortical/cortical, periventricular, infratentorial and/or spinal.<sup>8</sup> The dissemination in time is fulfilled when both enhancing and non-enhancing lesions are present, if new T2 lesion(s) are seen on follow-up MRI, if oligoclonal bands are found in CSF and/or in the event of a relapse due to a CNS lesion at a different location than the first relapse.<sup>8</sup>

Clinically Isolated Syndrome (CIS) is a term used when a patient has had only a single clinical symptom typical of MS. Radiologically Isolated Syndrome (RIS) is analogously the term for when there are MRI findings typical of MS without any typical MS symptoms. MS typically presents in people of 20-40 years of age as relapsing-remitting MS (RRMS), where intermittent relapses with complete or incomplete recovery are followed by clinically stable periods. Over time, a majority of patients with RRMS develop secondary progressive MS (SPMS), characterised by permanent neurological deficits and progression of clinical disability. Around 15% of MS patients have primary progressive MS (PPMS), characterised by a progressive course from its onset, which has slightly different diagnostic criteria than RRMS although the dissemination in space on MRI criteria still needs to be fulfilled.<sup>8</sup> Each MS phenotype can be classified as active or inactive based on neurological examination and MRI where relapses, contrast-enhancing lesions and/or new lesions on follow-up are all considered disease activity.<sup>9</sup> Further, patients with PPMS or SPMS can be classified according to whether disability has progressed over a given time.<sup>9</sup>



A clinical relapse is defined as an episode of new or worsened focal neurological deficits lasting more than 24 hours in the absence of fever or infection.<sup>8</sup> In addition, relapses can also manifest as an irritative phenomenon such as trigeminal neuralgia, epileptic seizures or Lhermitte's paraesthesia, the latter phenomenon first suggested as an early sign of MS by Jean Lhermitte.<sup>10</sup> A pseudo-relapse is recurrence or worsening of neurological deficits caused by one or more CNS lesions of older date and are usually accompanied by fever, infection or another stress factor.<sup>11</sup>

CSF analysis via lumbar puncture can help rule out conditions mimicking MS and verify the MS diagnosis. Typically, in MS a normal or slightly raised white blood cell count and protein levels are seen as well as an increased immunoglobulin G (IgG) index and presence of oligoclonal bands that are absent in serum.<sup>1</sup> Among the many differential diagnoses to MS are infectious diseases, metabolic disorders, vascular disorders, systemic autoimmune disease and non-MS demyelinating diseases. Some of the conditions belonging to the latter group are Neuromyelitis Optica spectrum disorder (NMOSD), Myelin-oligodendrocyte glycoprotein (MOG) encephalomyelitis and Acute disseminated encephalomyelitis (ADEM).

The differences between NMOSD and MS include higher patient age at onset in NMOSD, usually between 40-60 years and it is further distinguished from MS by a typical sparsity of brain lesions whereas spinal lesions are often long, covering 3 vertebral segments or more.<sup>12</sup> Bilateral optic neuritis is frequently seen in NMOSD and there can also be an involvement of the optic chiasm whereas this is rarely seen in MS. Moreover, in contrast to MS, CSF oligoclonal bands are typically absent whereas serum Aquaporin-4 autoantibodies are detected in a majority of patients.<sup>13</sup> There are only a few approved DMTs in NMOSD, all of which are restricted to the use in patients who are Aquaporin-4 IgG positive, although other treatments including RTX have displayed high efficacy in terms of reducing the annualised relapse rate (ARR) and disability progression.<sup>14</sup>

Patients with MOG-IgG-associated encephalomyelitis, as the name implies, generally display serum MOG antibodies in connection with acute attacks. The disorder differs compared to MS by higher risk of relapses after cessation of steroid treatment as well as lack of response to several MS DMTs, while RTX, intravenous immunoglobulins and other immunosuppressive drugs generally are efficient.<sup>15</sup>

ADEM usually debuts in early childhood, frequently after an infection, often displaying a monophasic disease course.<sup>16</sup> Symptoms include neurologic deficits in several locations in addition to encephalopathy. In contrast to in MS, MRI often reveals apparent cortical and deep grey matter (GM) lesions as well as bilateral diffuse lesions.<sup>16</sup> Typically, high-dose corticosteroids are administered in the acute phase of the disease and most patients with monophasic ADEM recover without neurological sequelae.<sup>16</sup>

### **2.1.2 Signs and symptoms**

MS symptoms can be of almost any kind, depending on what part of the CNS is affected.<sup>1,17,18</sup> The widespread distribution of MS CNS lesions was highlighted in a publication in 1916 by the Pathologist James Walker Dawson,<sup>19</sup> where he also described histopathologic changes around the ventricles and in the corpus callosum, which later was named "Dawson's fingers" by Charles Lumsden due to its characteristic appearance on MRI.<sup>20</sup>

In the majority of patients (85%), the disease debuts in the form of an initial clinical attack (i.e. CIS) manifested as optic neuritis, myelitis, brainstem or cerebellar syndromes or a cerebral hemispheric syndrome.<sup>1</sup> Optic neuritis is characterised by lesions to the optic nerve and causes loss of central vision, impaired colour vision and painful eye movements. About one in four MS patients has optic neuritis as their first MS symptom and the risk of developing MS within 15 years after an optic neuritis is up to 72%.<sup>21</sup> Approximately 30%-40% of patients have motor symptoms as first sign of MS and almost all patients develop motor disturbances during the disease course.<sup>1</sup> These symptoms are characterised by pyramidal signs such as the Babinski sign, increased reflexes and clonus as well as paresis and spasticity. In up to 40% of patients, sensory symptoms are the first clinical sign of the disease, mainly caused by myelitis and brainstem lesions.<sup>1</sup> These symptoms include reduced pain and light touch sensation, decreased vibration and joint positioning sensation, paraesthesia and Lhermitte's sign. Further, these symptoms can worsen with increased body temperature (Uhthoff's phenomenon).

Symptoms resulting from lesions in the brain stem and cerebellum are also frequent and seen in up to 70% of patients with MS.<sup>1</sup> These include impaired ocular movement, oscillopsia, diplopia, ataxia and gait imbalance, dysmetria, decomposition of complex movements, dysphagia and slurred speech. Signs of sphincter and sexual dysfunction occur in between 30%-99% of patients during the disease course.<sup>1</sup> Bladder dysfunction can manifest as urinary urgency, hesitancy, increased frequency and urge incontinence. Constipation and less frequently bowel incontinence is seen and in men, erectile dysfunction can occur. Approximately 40%-70% of MS patients have cognitive impairment that affects processing speed, episodic memory, attention and executive functions.<sup>1</sup> At later disease stages the frequency of cognitive impairment may be even higher.<sup>22</sup> Fatigue affects at least 70% of people with MS, also frequently occurring in between relapses and is characterised by a feeling of tiredness or lack of energy.<sup>23</sup> Sleep disorders are seen in up to 50% of patients and include insomnia, obstructive sleep apnoea and restless leg syndrome.<sup>1</sup> Up to two-thirds of MS patients have affective disorders where depression is most common.<sup>24</sup> Approximately 40% of MS patients experience pain which includes trigeminal neuralgia, dysesthetic pain, back pain, visceral pain and painful spasms.<sup>1</sup>

### **2.1.3 Epidemiology and aetiology**

Approximately 2.8 M people worldwide have MS,<sup>25</sup> of which around 20.000 patients are suffering from the disease in Sweden with an incidence rate of 10.2 per 100.000 person-years (6.2 for men and 14.0 for women).<sup>26</sup> Life expectancy has previously been reported to be reduced by 7-14 years.<sup>27</sup> However, this may be an overestimate for modern MS care since early treatment with even the first emerging MS treatments in the 1990s have shown a 47% reduction in all-cause mortality 21 years later.<sup>28</sup>

The aetiology of MS is incompletely understood, but environmental and genetic factors have been identified to play important roles. Smoking, Epstein-Barr virus (EBV) infection, low vitamin D levels and obesity during adolescence are the most well-established environmental risk factors.<sup>29</sup> The heritability in MS involves polymorphism in many genes, each of which contributes to a small risk increase. Of these, polymorphism in human leukocyte antigen (HLA) class I and II genes entail the highest risk of developing MS.<sup>30</sup> As to the risk of developing MS within families already

affected by the disease, this is highest (approximately 25%) in monozygotic twins, dropping to around 3 % in ordinary siblings.<sup>17,30</sup>

#### **2.1.4 Immune system pathophysiology**

The mechanism behind the BBB breakdown in MS is incompletely understood. However, this facilitates migration of proinflammatory cells such as macrophages, T-cells and B-cells into the CNS. Once inflammation is established in the CNS, this leads to demyelination, oligodendrocyte loss, reactive gliosis and neuro-axonal degeneration.<sup>31,32</sup> B-cells, T-cells and myeloid cells have bidirectional interactions and in MS patients with unregulated disease activity, the net effect of these are relapses.<sup>33</sup>

#### **T-cell involvement**

T-cell involvement in MS in part emerges from studies using the animal model Experimental Autoimmune Encephalitis (EAE), where relapses were found to be mediated by aberrantly activated pro-inflammatory CNS-specific effector T-cells, including CD4<sup>+</sup> T helper cells and CD8<sup>+</sup> cytotoxic T-cells.<sup>34,35</sup> These effector T-cells in part become overly active due to insufficient function of regulatory T-cells.<sup>36,37</sup> Antigen presentation to T-cells occurs in the periphery and CNS by antigen presenting cells (APCs), as B-cells (illustrated in Figure 1) and myeloid cells (macrophages, dendritic cells and microglia).<sup>38</sup> These APCs drive the responses that are thought to underlie relapses in MS, including the activation of myelin-reactive T helper cells which is regarded as a key step in MS pathophysiology.<sup>1,38</sup>

A study investigating the T helper cell signature in MS found that it is characterised by the expression of the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) and the C-X-C chemokine receptor type 4 (CXCR4).<sup>39</sup> Among functions of GM-CSF are activation of myeloid cells and migration of these cells to inflamed tissues.<sup>40</sup> CXCR4 has been shown to modulate the chemokine protein stromal cell-derived factor 1 alpha (SDF1 $\alpha$ ) at the BBB, thereby possibly contributing to recruitment of pathogenic cells into the CNS.<sup>39</sup> Interestingly, higher frequencies of T helper cells that displayed increased production of GM-CSF among other pro-inflammatory molecules, were found in the CSF as compared to peripheral blood in MS patients.<sup>39</sup> Further, the population of T helper cells expressing this signature decreased in peripheral blood after initiation of treatment with dimethyl fumarate (DMF). In addition, evaluation of the expression of GM-CSF and CXCR4 on T helper cells at CNS inflammatory sites in autopsy samples of untreated MS patients confirmed that this signature was present in lesions from all patients.<sup>39</sup> Together, these observations strongly suggest a central role in MS pathogenesis of T helper cells displaying this signature.

#### **B-cell involvement**

Peripheral B-cell tolerance seems to be disturbed in MS patients, possibly due to dysfunctional regulatory T-cells leading to presence of autoreactive B-cells in the circulation.<sup>41,42</sup> B-cells can differentiate into plasma cells and produce autoantibodies in the CNS, triggering cellular and complement-dependent cytotoxic effects.<sup>43</sup> However, the antibody-dependent functions of B-cells likely contribute less to MS pathophysiology than its antibody-independent functions, such as acting as APCs to activate myelin reactive CD4<sup>+</sup> T helper cells,<sup>33,38</sup> as depicted in Figure 1. In

addition, although relapse frequency is significantly reduced with B-cell depleting anti-CD20 therapies, the immunoglobulin profile in CSF in which MS patients have higher antibody titres than healthy controls, does not change in MS patients following this treatment.<sup>44,45</sup> Further, the CSF immunoglobulins in MS patients mainly recognise intracellular self-antigens, suggesting that these are produced as a secondary response to already occurred cell death rather than being the primary disease driving mechanism.<sup>46,47</sup> Moreover, the perivascular MS lesions characteristic of RRMS typically contain few B-cells and plasma cells.<sup>1</sup> In line with this, anti-CD20 therapies do not deplete plasma cells, which have a relatively long half-life, meaning they are present when clinical improvement is seen upon administration of these therapies.<sup>1</sup>

Of all B-cell subtypes in MS patients, memory B-cells are particularly prone to produce pro-inflammatory cytokines and hence it is logical that all approved MS therapies affect memory B-cell responses.<sup>33</sup> Further underscoring the inflammatory promoting properties of memory B-cells is that atacicept, a recombinant fusion protein that inhibits plasmablasts and plasma cells but not memory B-cells, exacerbated MS relapses in a clinical trial.<sup>48</sup> After discontinuation of anti-CD20 DMTs, mainly naïve B-cells re-emerge during the reconstitution phase. These naïve B-cells produce higher levels of regulatory cytokines and lower levels of pro-inflammatory cytokines, which has been associated with decreased T-cell and myeloid-lineage proinflammatory responses.<sup>33</sup> Hence, while memory B-cells probably contribute to inflammation in MS, the naïve B-cells in contrast could have a regulatory function thereby preventing new relapses.<sup>33</sup>

## **B-T cell interactions**

The study by Jelcic et al. reports several important observations regarding the B-T cell interplay,<sup>38</sup> which are summarised in this paragraph. Firstly, self-reactivity, defined as peripheral autoprolieration of T helper cells, is elevated in MS patients carrying the HLA-DR15 haplotype.<sup>38</sup> In fact, both autoprolieration of B and T-cells was higher in RRMS patients in remission as well as in healthy controls carrying the HLA-DR15 haplotype. In addition, after B-cell depletion T-cell autoprolieration was reduced. Conversely, transfer of autologous autoprolierating memory B-cells strongly activated and induced autoprolieration of T helper cells. These observations strongly suggest a central role for B-cells in triggering and/or sustaining T-cell autoprolieration. Notably, autoprolierating B-cells predominately showed a memory phenotype in addition to increased expression of major histocompatibility complex (MHC) class II cell surface receptors, as compared to non-prolierating B-cells. Further underscoring that autoprolieration of T-cells is primarily dependent on memory B-cells, is that the numbers of naïve B-cells and memory B-cells displayed a negative and positive correlation with the frequency of autoprolieration T-cells, respectively. The study also showed that RAS guanyl-releasing protein 2 (RASGRP2) is expressed in peripheral B-cells. Hence, RASGRP2 is possibly both an inducing antigen that activates autoreactive T helper cells as well as a target antigen in the CNS, since it is expressed also in the brain.

### **2.1.5 Lesion locations**

Lesions in MS patients are typically distributed around veins with periventricular, juxtacortical or infratentorial location in addition to in the spinal cord.<sup>49</sup>

## **White matter lesions**

CIS and RRMS are generally characterised by active demyelinating lesions whereas these are less frequent in patients with PPMS and SPMS. The progressive disease phenotypes are typically characterised by inactive lesions, although inflammatory mechanisms still play a role in these conditions, supported by that anti-CD20 therapy can have a disease-modifying effect in progressive MS.<sup>50</sup> Other types of plaques are chronic active lesions which are characterised by a low degree of inflammation in the lesion centre.<sup>1</sup> There is a reduction in white and GM volume with progression of MS disease paralleled by an increased lesion volume.<sup>22</sup> The total volume of WM lesions is moderately correlated with overall disability and cognitive impairment.<sup>51,52</sup>

## **Normal-appearing white matter**

Normal-appearing WM are areas that have normal appearance macroscopically but show diffuse inflammation and neuro-axonal damage upon microscopic examination when the tissue is stained. The term is also applied in imaging studies for tissue that appears to be normal on conventional MRI. These findings are seen in both RRMS and patients with progressive disease but are more severe in the latter phenotype.<sup>53</sup>

## **Grey matter lesions**

Cortical lesions are topographically related to inflammatory follicular infiltrates in the meninges and generally arise in the sulci and in deep invaginations of the brain surface.<sup>54,55</sup> A longitudinal in vivo study with 7 Tesla MRI has indeed also shown that cortical lesions preferentially accumulate in sulci.<sup>56</sup> Cortical lesions have less oedema and lower degree of inflammation compared to WM lesions.<sup>57</sup> Thus, the pathophysiology driving these lesions seems to be related to inflammation of the meninges, as well as to microglial activation.<sup>58</sup> These findings have also been corroborated in vivo using the combination of 7 Tesla MRI and positron emission tomography.<sup>59</sup> MS patients of all disease stages, including CIS, have cortical demyelination in the forebrain and cerebellum, being more pronounced in PPMS and SPMS.<sup>53,60-63</sup>

As to deep GM lesions, the study by Herranz et al. found signs of neuroinflammation in thalamus in both relapsing and progressive MS patients.<sup>59</sup> Consistent with these results, a post-mortem study of MS patients found that deep GM damage occurs in both relapsing and progressive disease and that inflammation in active lesions displays activated microglia/macrophages and signs of active demyelination, although to a lesser extent than is seen in WM lesions.<sup>64</sup> Specifically, active deep GM lesions had significantly more CD3<sup>+</sup> T-cells in the perivascular spaces and parenchyma than inactive deep GM lesions and controls. Deep GM demyelination was significantly more pronounced in progressive and relapsing MS patients as compared to controls, in many regions including the caudate nucleus, hypothalamus, thalamus and putamen.

## **Shadow plaques**

Remyelination in MS decreases with the disease duration and can be seen as “shadow” plaques. These display varying degrees of remyelination and occur in approximately 50% of all WM lesions and almost 90% of all GM lesions.<sup>1</sup> It has been proposed that remyelination is an important

mechanism behind clinical recovery after relapses and that stimulating this process could be a target for future DMTs.<sup>65</sup>

### **2.1.6 Clinical Scales**

#### **Expanded Disability Status Scale**

The Disability Status Scale was developed in 1955 and expanded in 1983, whereafter it has been known as the Expanded Disability Status Scale (EDSS), which is widely used in both research and the clinical setting.<sup>66</sup> Eight functional systems are evaluated and graded: Pyramidal, Cerebellar, Brain Stem, Sensory, Bowel and Bladder, Visual, Cerebral and Other. These grades are weighted together resulting in a final score ranging from 0 (normal neurological examination) to 10.0 (death due to MS) in steps of 0.5. EDSS is mainly useful for neurologists to gain an understanding of a patient's neurological function and the examiner should be certified for conducting this assessment. Limitations with EDSS include uncertainties regarding evaluator interindividual variability and that it is heavily weighted towards examining (particularly lower extremity) motor functions leaving other functions often affected in MS, such as cognition, unexamined.

#### **Multiple Sclerosis Severity Score**

The EDSS score and disease duration were determined for approximately 10.000 MS patients and compiled in a reference table as decile scores for disability, the Global Multiple Sclerosis Severity Score (MSSS).<sup>67</sup> With this tool, the EDSS score of an MS patient, measured at a single time point, can be compared with the EDSS score distribution in MS patients with similar disease duration. It has not been adopted to predict future disability on an individual patient level given its vulnerability to fluctuations in disease severity but can be used to compare predicted future disability between groups of patients. Disease duration was replaced by age in an alternative model, the Age-Related Multiple Sclerosis Severity (ARMSS) score, to reduce bias related to uncertainty regarding disease onset dates and this algorithm displays similar power to detect disability differences between groups of patients as the MSSS.<sup>68</sup> The MSSS and ARMSS are used in Study III.

#### **Symbol Digit Modalities Test**

The Symbol Digit Modalities Test (SDMT) is a symbol substitution test that assesses attention and processing speed.<sup>69</sup> The person undertaking the test is asked to decode symbols to numbers using a reference key. The number of correct substitutions gives the test score, which is then typically normalised into z-scores based on age and sex.

#### **Patient Reported Outcome Measures**

Among other scores used to assess MS patients is the Multiple Sclerosis Functional Composite (MSFC), which was introduced as an alternative to EDSS but has not been widely adopted. Furthermore, the Multiple Sclerosis Impact Scale (MSIS-29) examines the impact of MS on physical and physiological functioning from a patient perspective and is used primarily for clinical follow-up and to a lesser extent in clinical trials.<sup>70</sup> Moreover, the Fatigue Scale for Motor and Cognitive Functions (FSMC) is a questionnaire consisting of two scales focusing on physical and cognitive aspects of MS fatigue, respectively.<sup>71</sup> In addition, the Fatigue Severity Scale (FSS) is a 9-item fatigue questionnaire not specifically developed for MS that measures fatigue severity.<sup>72</sup>

## **2.2 THERAPIES IN MULTIPLE SCLEROSIS**

A broad range of DMTs are now available for RRMS, either as approved or off-label therapies.<sup>73</sup>

### **2.2.1 Interferons and glatiramer acetate**

Endogenous interferon beta (IFN $\beta$ ) is secreted in response to infection by mainly fibroblasts and it has anti-inflammatory properties.<sup>74</sup> Two different therapeutic formulations of IFN $\beta$  exist, IFN $\beta$ -1a and IFN $\beta$ -1b, which largely have similar biological effects.<sup>74</sup> The therapeutic effect seen in MS by injecting IFN $\beta$  subcutaneously or intramuscularly is thought to in part be due to reduction in T-cell activation and inhibition of BBB leakage.<sup>74</sup>

Glatiramer acetate (GA) is a synthetic mixture of polypeptides administered subcutaneously.<sup>75</sup> Its therapeutic mode of action is not known in detail. It has been suggested that GA by binding HLA class II molecules on the T – cell surface induces immunological tolerance through a process called anergy and that it activates anti-inflammatory Th2 cells.<sup>74</sup>

Although the ARR decreased by 30%-40% with both treatments, neither of them was shown to have an effect on the risk of developing permanent disability, risk of conversion to progressive MS or on progression rate in progressive MS.<sup>73</sup> The treatments appear safe according to long-term follow-up data with injection-related reactions being the most common adverse events (AEs).<sup>73,77</sup>

### **2.2.2 Fingolimod**

Fingolimod (FGL) is an oral therapy for RRMS.<sup>73</sup> By targeting the sphingosine-1-phosphate receptor which is expressed on different types of cells, including immune cells, it is thought to entrap lymphocytes in lymph nodes which is considered its main mechanism of action.<sup>73</sup> Two pivotal Phase III RRMS studies led to its approval as an RRMS DMT, the FREEDOMS study in which patients received 1.25 or 0.5 mg of FGL daily or placebo during two years<sup>78</sup> and the TRANSFORMS study where patients received either of the two FGL dose regimens used in the FREEDOMS trial or IFN $\beta$  weekly over one year.<sup>79</sup> These studies showed a ARR reduction of 38%-52% compared to IFN $\beta$  and of 53%-60% compared to placebo. FGL significantly reduced the number of gadolinium-enhancing lesions as compared to placebo treatment. Among side effects are bradycardia, heart block and increased risk of opportunistic infections.<sup>73</sup>

### **2.2.3 Natalizumab**

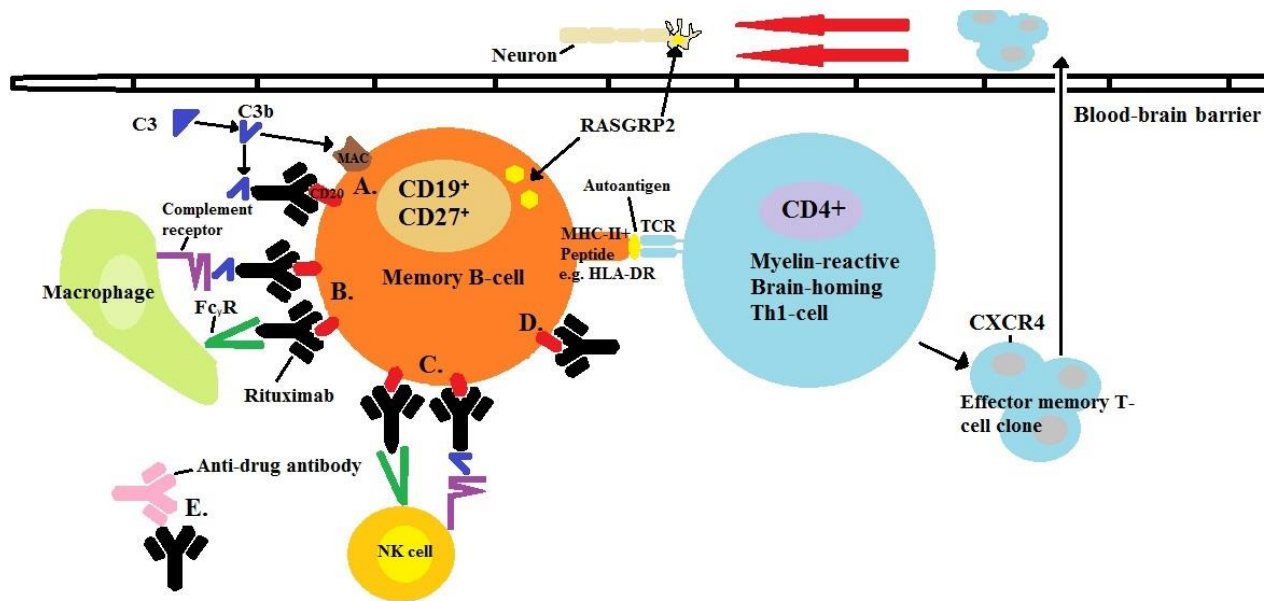
Natalizumab (NTZ) is a humanized monoclonal antibody, administered once monthly intravenously.<sup>73</sup> Present on the surfaces on most lymphocytes and monocytes are the cell adhesion molecule very late antigen 4 (VLA4). NTZ binds selectively to the  $\alpha$ 4 subunit on the VLA4 molecule, preventing the interaction between its ligand on the brain vascular endothelium. This blocks the transmigration of immune cells across the BBB, thereby counteracting MS inflammation.<sup>80</sup> NTZ was approved based on two pivotal phase III RRMS studies, the AFFIRM trial comparing NTZ in monotherapy with placebo<sup>81</sup> and the SENTINEL trial where NTZ or placebo was given weekly together with IFN $\beta$ .<sup>82</sup> The AFFIRM study reported a reduction in ARR by 67%, a reduction in the number of contrast-enhancing lesions by more than 90% at both one and two years and a decrease in the proportion of patients with disability progression after two years by 40%, as compared to placebo. In the SENTINEL trial, combination therapy reduced the

ARR by 55%, decreased the mean number of gadolinium-enhancing lesions at two years by 89% and lowered the risk of disability progression at two years by 24%, as compared to IFN $\beta$  alone. Long-term follow-up data indicate that NTZ is generally safe and well-tolerated.<sup>83,84</sup> Progressive multifocal leukoencephalopathy (PML), an often deadly demyelinating disease of the WM in the brain where no effective treatment is available, is rarely seen in NTZ treated patients and is caused by lytic infection of oligodendrocytes with the human polyomavirus John Cunningham virus (JCV).<sup>83</sup> Approximately 50%-60% of the population have a positive JCV serology, indicative of potential latent infection and NTZ likely impairs the immune system's ability to eradicate this viral infection in the brain.<sup>77</sup> Risk factors for PML include prior use of immunosuppressants, positive anti-JCV serology and long duration of NTZ therapy.<sup>85</sup> It is very unlikely that a JCV-negative patient develops PML, therefore this titre is monitored prior and at six months intervals during NTZ therapy in seronegative patients as the rate of seroconversion of anti-JCV antibodies is 1%-2% per year.<sup>86</sup> An updated algorithm was subsequently developed taking into account the concentrations of serum anti-JCV antibodies (anti-JCV antibody index), defined as  $\leq 0.9$ ,  $> 0.9$  to  $\leq 1.5$  and  $> 1.5$ , respectively.<sup>87</sup> The annual risk of developing PML ranges from 0.01% with index  $\leq 0.9$  during the first treatment year to 10.2% with index  $> 1.5$  during year 6, both risk estimates reflecting patients without previous DMT use.<sup>87</sup>

#### **2.2.4 Rituximab**

RTX is a chimeric monoclonal antibody binding to the CD20 protein present on naïve and memory B-cells, but not B-cell progenitors and plasma cells, resulting in systemic B-cell depletion.<sup>73</sup> Anti-CD20 DMTs also deplete the small subset of CD20 expressing T-cells, of which a pathogenic function in MS has not been established.<sup>33</sup> RTX is approved for B-cell lymphoma, rheumatoid arthritis and certain forms of systemic vasculitis.<sup>73</sup> Further development for other indications, such as RRMS, has been terminated by the manufacturer and hence it is used as an off-label treatment in RRMS. It is usually given to MS patients in Sweden as a 500 mg intravenous infusion every six months. The B-cell depleting mechanism of anti-CD20 therapies to varying degrees includes lysis of CD20-expressing B-cells by antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity and induction of apoptosis,<sup>88</sup> as illustrated in Figure 1. The highly consistent efficacy data with different anti-CD20 DMTs in RRMS suggest that these results represent a class effect rather than drug-specific mechanisms of action related to their structural differences.<sup>89</sup>





**Figure 1.** The B-cell depleting mechanisms of rituximab in peripheral blood are depicted in the left part of the figure, denoted as A-D. A) Complement-mediated cytotoxicity; B) Phagocytosis and antibody-dependent cell-mediated cytotoxicity; C) Antibody-dependent cell-mediated cytotoxicity; D) Direct apoptosis. E) Illustration of the binding of a neutralizing anti-drug antibody to the rituximab antibody. Middle and right part of figure illustrating the peripheral B-T cell interaction that activates myelin reactive brain homing  $CD4^+$  cells with subsequent formation of effector memory T-cell clones invading the central nervous system, contributing to inflammation in active brain lesions (upper part of image). Abbreviations: C3, complement component 3; CXCR4, C-X-C chemokine receptor type 4;  $FC\gamma R$ , Fc-gamma receptor; HLA, human leukocyte antigen; MAC, membrane attack complex; MHC, major histocompatibility complex; NK, natural killer cell; RASGRP2, RAS guanyl releasing protein 2; TCR, T-cell receptor.

Two phase II studies on RTX have been done with MS patients.<sup>90,91</sup> The first of these was a double-blind, placebo-controlled trial on 104 RRMS patients lasting 48 weeks showing that two doses of 1000 mg RTX given two weeks apart reduced the relapse frequency as well as the contrast-enhancing lesion count, as compared to placebo.<sup>90</sup> The group receiving RTX had more AEs (78% of patients) compared to the placebo group (40%) within 24 hours of the infusion, mostly being mild to moderate. Notably, after the second infusion, both groups displayed a similar number of AEs, including infections and serious AEs. The second study was a double-blind, placebo-controlled trial during 96 weeks which included 349 patients with PPMS.<sup>91</sup> In this study, patients were given 1000 mg RTX or placebo every six months. The study did not meet its primary endpoint, defined as time to confirmed disease progression measured as an increase in EDSS. However, a subgroup analysis of patients under 51 years of age and patients with increased inflammatory activity showed that these groups had a delay in time to confirmed disease progression, as compared to placebo. Type and frequency of AEs were similar between groups apart from serious infections that were more common in the RTX group (4.5%) as compared to the

placebo group (< 1%). The group receiving RTX had a higher frequency of mild to moderate infusion reactions (IRs) in connection with the first infusion only, as compared to placebo.

Of note, in a small open-label proof of concept study, 12 RRMS patients received 100 mg RTX twice yearly, leading to a profound reduction in radiological and clinical disease activity as compared to baseline.<sup>92</sup> Although other anti-CD20 DMTs are now increasingly used in RRMS globally, RTX remains the by far most commonly used anti-CD20 DMT in RRMS patients in Sweden with annual RTX treatment starts in Sweden growing from approximately 50 patients in 2011 to 1159 patients in 2016.<sup>93</sup> Experience from clinical practice and observational studies performed by our group suggest the discontinuation rate for RTX in RRMS is low, also in comparison with approved drugs.<sup>89,94,95</sup> Further, Study II, an observational retrospective study on all RRMS patients ever treated with RTX at our centre confirmed its high efficacy and low rate of discontinuation.

### **2.2.5 Ocrelizumab**

Ocrelizumab (OCR) is a humanised monoclonal antibody belonging to the anti-CD20 group of DMTs and given as an intravenous infusion every six months.<sup>96</sup> OCR was developed to reduce the immunogenicity and frequency of infusion-related reactions seen with RTX, and the drugs differ from each other in the variable regions of the heavy and light chains.<sup>96</sup> Compared to RTX, the OCR mechanism of action is to a higher extent associated with antibody-dependent cell-mediated cytotoxic effects and to a lesser extent complement-dependent cytotoxic effects in vitro.<sup>96</sup>

One phase II study<sup>96</sup> and three phase III studies<sup>50,97</sup> have been conducted and led to the approval of OCR for both RRMS and progressive MS. The phase II study was a double-blind, randomised clinical trial (RCT) enrolling 218 RRMS patients receiving either 600 mg or 2000 mg of OCR, weekly IFN $\beta$  or placebo at baseline. At week 24, the patients in the placebo, IFN $\beta$  and OCR 600 mg groups received 600 mg of OCR whereas the high dose (2000 mg) group received 1000 mg of OCR.<sup>96</sup> At week 24, patients receiving 600 mg or 2000 mg of OCR had an 89% and 96% reduction, respectively, in number of contrast-enhancing lesions as compared to placebo. The ARR at week 24 were 80% vs. 73% lower with 600 mg and 2000 mg OFA, respectively, as compared to placebo. The estimated mean ARR from week 24 to 48 was lower in the 600mg group compared to the 2000 mg group. One patient in the 2000 mg OCR group died due to development of a systemic inflammatory response syndrome. Disregarding this event, serious AEs frequencies were similar between groups, but infusion-related reactions were more common with OCR (35%-44%) as compared to placebo (9%) in connection with the first infusion. At the subsequent infusion, this difference disappeared.

Of the phase III studies, the ORATORIO study was a double-blind, placebo-controlled trial on 732 PPMS patients comparing OCR 600 mg every six months to placebo.<sup>50</sup> At week 24, confirmed disability progression was lower for OCR than for placebo (hazard ratio, HR, 0.75; 95% confidence interval, CI, 0.58-0.98). By week 120, patients receiving OCR had less worsened performance on the 25-foot walk test and had less global brain-volume loss compared to placebo (0.90% vs. 1.09%,  $p = 0.02$ ). Neoplasms were more common in the OCR group (2.3% compared to 0.8%) in addition to oral herpes infections, upper respiratory tract infections and infusion-related reactions that were more frequently occurring with OCR than with placebo.

The other two phase III studies were the OPERA I and II studies, two double-blind trials involving 821 and 835 RRMS patients, respectively. In both studies patients either received OCR 600 mg every six months or IFN $\beta$  three times weekly.<sup>97</sup> Compared to placebo, the proportion of patients with confirmed disability progression at week 24 was lower with OCR treatment (HR 0.60, 95% CI 0.43-0.84). Patients receiving OCR had lower ARR (46% and 47% lower in OPERA I and II, respectively) as well as lower mean number of contrast-enhancing lesions (94% and 95% lower in OPERA I and II, respectively) as compared to placebo. IRs occurred in 34% of patients receiving OCR and the frequency of neoplasms were similar between groups.

OCR has been approved for CIS, RRMS and PPMS by the U.S. Food and Drug Administration (FDA) and for RRMS and PPMS by the European Medicines Agency (EMA).

### **2.2.6 Ofatumumab**

Ofatumumab (OFA) is a fully human anti-CD20 B-cell depleting monoclonal antibody.<sup>98</sup> It was first approved for chronic lymphatic leukaemia in the US and was originally evaluated as MS treatment in studies where it was administered intravenously. In a phase II double-blind, placebo-controlled study 38 RRMS patients received two infusions of 100 mg, 300 mg or 700 mg OFA or placebo two weeks apart.<sup>98</sup> Treatments were crossed over at week 24 between OFA and placebo groups. There was a > 99 % reduction of the total number of contrast-enhancing lesions during weeks 8-24 in the OFA treated patients, which was greater than with placebo treatment ( $p < 0.001$ ). Relapses were noted through weeks 0-24 and the proportion of patients who experienced this was similar between OFA and placebo arms (19% vs. 25%, respectively). There were no relapses in patients treated with 700 mg OFA. Only grade  $\leq 3$  AEs occurred.

The MIRROR study was a 24-week phase II double-blind, placebo-controlled study in which 232 RRMS patients received 3, 30 or 60 mg OFA every 12 weeks, 60 mg OFA every 4 weeks or placebo, administered subcutaneously.<sup>99</sup> At week 12, the patients in the placebo group was given a single dose of 3 mg OFA whereas the remaining groups had unchanged treatment throughout the study. For all OFA treatment groups, a statistically significant (65%) reduction in the mean cumulative number of new contrast-enhancing lesions was seen compared to placebo at week 12. Post hoc analysis (excluding weeks 1-4) showed a  $\geq 90\%$  suppression of new lesions at week 12 for all cumulative OFA doses  $\geq 30$  mg, compared to placebo. The proportion of patients who had relapses during the 24-week follow-up was 25% compared with 9-22% in placebo and OFA treated groups, respectively.

However, the drug was withdrawn for use in chronic lymphatic leukaemia from the European Union as requested by the manufacturing company in 2019. Thereafter, two double-blind, phase III RCTs have been published comparing subcutaneously administrated OFA vs. oral teriflunomide in RRMS patients (ASCLEPIOS I and ASCLEPIOS II). These studies were identical and conducted in parallel with patients receiving 20 mg of OFA every 4 weeks after initial 20 mg doses at days 1, 7 and 14 or 14 mg of teriflunomide daily for up to 30 months.<sup>100</sup> In total, 946 and 936 patients received OFA and teriflunomide, respectively and the median follow-up was 1.6 years. In trial I, the ARR was 0.11 in OFA treated patients vs. 0.22 in the teriflunomide group (difference, -0.11; 95% CI -0.16 to -0.06). The ARR in trial II was 0.10 vs. 0.25 in favour of OFA treatment (difference, -0.15; 95% CI -0.20 to -0.09).

The difference between treatment groups in proportions of patients with contrast-enhancing lesions and new or enlarging T2 lesions were expressed as rate ratios. In trial I, the rate ratio for OFA vs. teriflunomide treatment was 0.03 (95% CI 0.01 to 0.05) for contrast-enhancing lesions and 0.18 (95% CI 0.15 to 0.22) for new or enlarging T2 lesions. In trial II, the rate ratio for OFA vs. teriflunomide treatment was 0.06 (95% CI 0.04 to 0.10) for contrast-enhancing lesions and 0.15 (95% CI 0.13 to 0.19) for new or enlarging T2 lesions. For brain volume change, there were no significant differences between treatment groups.

In the pooled data from the trials, the proportion of patients with confirmed disability progression was lower in the patients receiving OFA compared to those treated with teriflunomide at 3 months (10.9% vs. 15.0%, HR, 0.66; 95% CI, 0.50-0.86) and at 6 months (8.1% vs. 12.0%, HR, 0.68; 95% CI, 0.50-0.92).

OFA has been approved for CIS and relapsing forms of MS by the FDA and for RRMS by EMA.

### **2.2.7 Hematopoietic stem cell transplantation**

Autologous Haematopoietic Stem Cell Transplantation (AHSCT) is not an approved MS therapy in Europe and less than 1% of patients with RRMS are candidates for this treatment.<sup>1</sup> To date, only one RCT in RRMS patients comparing AHSCT with approved MS treatments has been published.<sup>101</sup> The study had a 1:1 treatment allocation and reported that only 3 AHSCT treated patients had confirmed disease progression during a 2-year follow-up compared to 34 among those receiving approved MS DMTs, among which NTZ was the most common treatment.<sup>101</sup>

AEs associated with AHSCT range from secondary malignancies, reduced fertility, acute toxicity from chemotherapy and secondary autoimmunity.<sup>102</sup> In the study by Burt et al., grade 3 toxicities were the most common AE in the AHSCT group with 72 recorded events in 52 patients whereas infection was equally common in both treatment groups (0.19 vs. 0.23 per patient-year in the AHSCT vs. approved MS DMT groups, respectively).<sup>101</sup> Further, thyroid disease only occurred in the HSCT group. These results are partly consistent with an observational study by Alping et al. comparing AE frequencies in MS patients treated with either alemtuzumab (ATZ), AHSCT or non-induction therapies.<sup>103</sup> As to AHSCT, they found a higher incidence of thyroid disease as well as infections in patients receiving this treatment as compared to those receiving non-induction DMTs. Interestingly, MS patients treated with AHSCT seem to normalize their brain atrophy rate, after an initial period of possible pseudoatrophy.<sup>104</sup>

### **2.2.8 Other DMTs used in MS**

Mitoxantrone is a chemotherapeutic agent, which was developed to treat malignancies<sup>105</sup> and is approved for treatment of progressive MS and highly active RRMS if no alternative therapies are available.<sup>73</sup> AEs as increased risk of acute leukaemia and colon cancer as well as cardiotoxicity has been reported and it is seldom used in MS.<sup>106,107</sup>

Teriflunomide is an oral immunomodulatory drug taken daily.<sup>108</sup> Three phase III studies have shown statistically significant effects in patients with RRMS and CIS with a reduction in disability progression by 26%-31%, a decrease in ARR by 31%-36% and a reduced risk of relapse or a new

MRI lesion compared to placebo.<sup>109-111</sup> Side effects include increased liver enzymes, respiratory and urinary tract infections.<sup>73</sup>

DMF is taken orally several times daily.<sup>73</sup> The mechanism of action in MS is poorly understood but might include altered expression of several antioxidant genes.<sup>73</sup> Two phase III studies with different dosing regimens have shown that DMF reduces the ARR by 44%-53% as well as the relative risk of disability progression by 34%-38 % compared to placebo.<sup>112,113</sup> Side effects are usually mild.<sup>73</sup>

ATZ is a humanised monoclonal antibody binding to the CD52 epitope found on a broad range of immune cells. It induces depletion and reconstitution of B and T lymphocytes leading to long-standing changes in adaptive immunity.<sup>114</sup> It is approved as an induction therapy in RRMS and is administered intravenously over five days at therapy initiation and again over three days 12 months later. In two phase III studies which included 667 and 581 RRMS patients, respectively, ATZ was compared to IFN $\beta$ .<sup>114,115</sup> In these studies, the ATZ treated group displayed a 49%-55% relative risk reduction for relapses compared to IFN $\beta$  treated patients.<sup>114,115</sup> Side effects of ATZ includes IRs, infections as well as thyroid cancer,<sup>115</sup> limiting its use in MS. However, in the study by Alping et al., only thyroid disease, but no other AEs, occurred with an increased frequency in ATZ treated patients as compared to patients on non-induction DMTs.<sup>103</sup>

Cladribine is an oral induction therapy approved for RRMS causing a decrease in T and B-cells levels lasting months to years.<sup>116</sup> Two phase III studies have been conducted on 1326 RRMS patients and 616 CIS patients, respectively.<sup>117,118</sup> In both these studies, patients received either cladribine cumulative doses of 3.5 mg/kg, 5.25mg/kg or placebo. In the RRMS study cladribine groups had reduced brain lesion count, lower risk of 3-month sustained progression of disability and lower ARR compared to placebo.<sup>117</sup> A post hoc subgroup analysis showed that the proportion of patients with absence of disease activity over 96 weeks was higher with cladribine compared to placebo.<sup>119</sup> In the CIS study, the risk of conversion to clinically definite MS was lower for cladribine compared to placebo (HR 0.38 [95% CI 0.25-0.58] for the higher dose and 0.33 [95% CI 0.21-0.51] for the lower dose).<sup>118</sup> Lymphopenia is a known side effect, however, a meta-analysis of cladribine RCTs found no increased cancer incidence associated with its use compared to other MS DMTs.<sup>120</sup>

Daclizumab is a humanised monoclonal antibody binding to the CD25 protein on the interleukin-2 receptor. Among its mechanisms of action is reduction of early T-cell activation.<sup>121</sup> It was approved as an RRMS treatment following two phase II trials<sup>122,123</sup> and one phase III trial<sup>124</sup> but was withdrawn in 2018 in light of the occurrence of severe and sometimes lethal inflammatory brain disorders.<sup>125</sup>

Among emerging therapies are inhibitors of Bruton's tyrosine-protein kinase (BTK), which are involved in intracellular signalling in both lymphocytes and cells of the innate immune system, hence possibly facilitating modulation of immune reactions in the brain as well as in the periphery.<sup>126</sup> In a placebo-controlled trial in RRMS evaluating the selective oral BTK-inhibitor evobrutinib, patients in the active treatment arm had significantly fewer contrast-enhancing lesions during weeks 12 and 24 compared to the placebo arm.<sup>127</sup>

## **2.3 TREATMENT STRATEGIES**

Axonal damage is the main determinant of irreversible disability and occurs already in CIS and RIS.<sup>128</sup> In addition, short intervals between the first and second relapse and the number of MRI lesions at clinical onset have a negative prognostic value, meaning that disease activity during the early RRMS phases predicts long-term disability.<sup>129</sup> Inflammatory activity due to dysfunctional adaptive peripheral immunity dominates in the early phases and this can be regarded as the therapeutic window in MS, whereas at later disease stages the pathogenic mechanisms are mostly restricted to within CNS and related to innate immunity.<sup>129</sup> In line with this, the response to DMTs has been shown to be larger in CIS than in RRMS.<sup>129</sup>

MS DMTs can be subdivided into first, second and third-line therapies depending on their therapeutical efficacy and safety profile.<sup>73</sup> As a general principle, first-line therapies are the least effective DMTs displaying the most favourable safety profile while second and third-line therapies are increasingly effective but display a less favourable AEs profile.<sup>73</sup>

### **2.3.1 Escalation and induction therapy**

European and American RRMS guidelines recommend the escalation therapy strategy, the approach that is currently dominant in the clinical setting.<sup>1</sup> This means treatment is initiated in patients diagnosed with CIS or RRMS with any of the first-line therapies IFN $\beta$ , GA, teriflunomide or DMF. In the event of intolerable AEs, the treatment can be replaced by a different first-line agent whereas in patients having new relapses or MRI lesions a more potent (second or third-line therapy) should replace the first-line DMT.<sup>1</sup> The escalation therapy approach should be considered in patients with a favourable prognostic profile.<sup>129</sup>

As opposed to this, the induction therapy strategy means treatment-naïve patients receive a drug with long-lasting immunological effects as first treatment.<sup>1</sup> To date, only alemtuzumab and mitoxantrone are classified as induction therapies<sup>129</sup> although OCR has been suggested to also belong in this category.<sup>1</sup> The strategy is mainly applied in patients recently diagnosed with MS with negative prognostic factors such as severe and frequent relapses, high number of MRI lesions and accumulation of disability.<sup>1</sup> By using induction therapies, rapid suppression of inflammation is achieved, thereby possibly inducing a tolerogenic state as a result of a reset of the immune system.<sup>1</sup> It is not known what is the most appropriate strategy after successful induction, but the use of a less-potent drug as maintenance therapy could be considered.<sup>129</sup>

### **2.3.2 Escalation and induction therapy in relation to risk of conversion to progressive MS**

As to the comparison of patient outcomes between those remaining on the same DMT vs. those escalating therapy, Cree et al. performed a prospective observational ten-year follow-up study comparing MS patients who either started a first-line DMT or changed from first to second-line treatment, with those remaining untreated or remaining on a first-line DMT. They found that long-term disability did not differ between patients whose treatments were escalated compared to those who remained on the same treatment, suggesting that the value of treatment escalation is limited.<sup>130</sup> However, that study had several limitations, one of which that CIS and RRMS patients were grouped together suggesting that some of these patients did not have MS, which may affect the cohort so that it to a high extent contained patients with a benign disease course. Further, the study

did not apply the escalation therapy approach by definition, since they defined therapeutic escalation as change either from no treatment to first-line therapy or from first to second-line therapy. Consequently, it is difficult to interpret the results of the study.

However, a more clinically relevant comparison of the risk of developing progressive MS is between patients where either escalation or early intensive therapy has been practiced, since these are the most common strategies. Importantly, escalation therapy seems to increase the risk of conversion to SPMS compared to early intensive therapy.<sup>131,132</sup>

## **2.4 IMMUNOGENICITY**

### **2.4.1 Biological treatments and immunogenicity**

Immunogenicity in the context of treatment with biological medical products is an immune response where anti-drug antibodies (ADAs) against the drug are produced.<sup>133</sup> A vast amount of research since the introduction of biopharmaceuticals for treatment of inflammatory disorders has confirmed that immunogenicity frequently is associated with the use of these drugs, including DMTs used in MS. For IFN $\beta$ , immunogenicity has been associated with increased relapse rate.<sup>134</sup> Persistent ADA positivity has been associated with loss of efficacy and risk of infusion-related AEs for NTZ.<sup>135</sup> Immunogenicity is being increasingly recognised by governmental authorities and a risk assessment of immunogenicity with approved methods is required to get market approval for biologic drugs.<sup>136</sup>

### **2.4.2 Production of anti-drug antibodies**

The response by the immune system towards therapeutic proteins is considered as belonging to either of two general categories. In the first category, involving the classic immune pathway, the production of ADA is the result of an either thymus-dependent (also called T-cell dependent) or T-cell independent response. In comparison, the second category involves a breach of B and/or T-cell tolerance although the exact mechanisms underlying this are less well defined.<sup>137,138</sup>

In the T-cell-independent response, B-cells are activated as the therapeutic protein cross-links specific B-cell receptors and induces ADA production.<sup>137,138</sup> These ADAs are, in contrast to them produced by T-cell-dependent activation, limited in both isotype and affinity and if memory B-cells are generated, these are short-lived.<sup>137</sup>

In the T-cell-dependent response, the therapeutic protein is taken up by a dendritic cell (DC) and processed to small peptides while the DCs migrates towards the T-cell zone of the lymph node.<sup>139</sup> During the movement of the DCs, some of these peptides bind to the HLA/MHC class II molecules with ensuing presentation on the dendritic cell surface, which hereby acts as an APC.<sup>137</sup> When CD4<sup>+</sup> T-cells recognise a specific antigen-MHC class II complex and interact with its T-cell receptor (TCR), with simultaneous co-stimulation by signals from the APC, naïve T-cell activation occurs.<sup>137</sup> The activated CD4<sup>+</sup> T-cells move towards the boundary between the T-cell zones and the B-cell follicle, where interaction with B-cells that have been activated by the same antigen occurs.<sup>139</sup> The CD4<sup>+</sup> T-cell and B-cell then form a secondary follicle in which the latter undergo somatic hypermutation and class switch recombination to produce high-affinity ADA.<sup>139</sup> The

activated B-cells can also differentiate into either short-lived plasmablasts or, after leaving the germinal centre, to memory B-cells or long-lived plasma cells.<sup>140</sup>

### **2.4.3 Consequences of immunogenicity**

The binding between an ADA and a therapeutic protein forms a so-called immune complex (IC). ICs may cause development of AEs, decreased drug efficacy, and altered pharmacokinetic profile.<sup>141-143</sup> The size of the IC may differ between patients treated with the same drug.<sup>144</sup> Large ICs are cleared from the circulation more rapidly than smaller ICs.<sup>141,144</sup>

### **2.4.4 Immunogenicity in rituximab-treated MS**

RTX and other anti-CD20 therapies have been associated with development of ADA<sup>90,145</sup> but until recently the consequences of this in terms of risk of inadequate therapeutic response and AEs in RRMS have been unknown. However, in Study III, we showed that RTX ADA occurs in MS patients in decreasing frequency with infusion numbers and that RTX ADA was associated with incomplete B-cell depletion, but not with infusions/AEs or clinical outcomes on group level.<sup>146</sup> Our results are consistent with the finding in the MIRROR study on OFA in RRMS patients in which complete B-cell depletion was not necessary for a profound treatment effect.<sup>99</sup>

### **2.4.5 Immunoassays for detecting ADA**

There are several immunoassays available for detection of RTX ADA. Among these are the ADA bridging Enzyme-linked immunosorbent assay (ELISA) and the Electrochemiluminescence (ECL) method using the Meso Scale Discovery (MSD) platform.

When performing the bridging ELISA, RTX is first added to a microplate. Patient and control samples are then added to separate microplates, facilitating binding between any present RTX ADA and RTX. Unbound sample is subsequently washed off, whereafter RTX labelled with horseradish peroxidase is added which binds RTX ADA. After a second washing step, a chromogenic substrate is added after which the amount of RTX ADA on the plate is determined based on its absorbance by spectrophotometry.<sup>147</sup>

In the ECL-MSD technique, biotinylated and ruthenylated RTX antibodies are incubated with patient serum samples. If there is RTX ADA in the samples, these will form a bridge between biotinylated and ruthenylated RTX. This complex is then captured on an MSD Streptavidin plate and the light intensity by spectrophotometry is used to determine the amount of RTX ADA in the sample.<sup>148</sup>

The bridging ELISA is widely used as a screening method for detection of ADA and is available commercially, although this method has its limitations with a higher limit of detection because of its extensive washing steps which remove ADA with low affinity. Consequently, bridging ELISA has lower sensitivity for detecting low-affinity ADA, which are the predominately produced types of IgG and IgM ADA early in the immune response. Low-affinity ADA can have the ability to neutralise therapeutic monoclonal antibodies. Other disadvantages with bridging ELISA are lower serum and drug tolerance, also contributing to its inferior sensitivity compared to the ECL-MSD assay.<sup>149</sup>



## **2.5 MRI IN MS PATIENTS**

### **2.5.1 The role of MRI in MS from a historical perspective**

The first study applying MRI in MS was published in 1981, where higher sensitivity to MS brain lesions was demonstrated compared to computed tomography.<sup>150</sup> To fully elaborate on the important subsequent milestones in the history of MRI in MS is beyond the scope of this thesis, but some should be mentioned. The first RCT that implemented MRI outcomes was a multicentre study published in 1993 comparing IFN $\beta$ , which subsequently became the first-ever approved MS DMT, vs. placebo.<sup>151</sup> They reported that RRMS patients receiving active treatment reduced their disease activity more than those receiving placebo, as measured by both the number of active and new lesions on MRI. In 2001, MRI was included in the first version of the McDonald diagnostic criteria for MS.<sup>152</sup> By the 2005 update of these criteria, MRI could provide evidence of both dissemination in space and time.<sup>153</sup> It has also become clear that MRI is highly useful to rule out differential diagnoses,<sup>154</sup> as well as to shorten the time until diagnosis, thereby decreasing the time from onset to initiation of a DMT.<sup>155</sup> Further, MRI is crucial to monitor efficacy in clinical trials as well as in the clinical setting. Presence of contrast-enhancing lesions and new or enlarged MS lesions is now accepted as primary outcomes in phase II trials for RRMS and remains important secondary outcomes in phase III trials. This is in addition to being regarded as valid biomarkers of disease activity in the clinical setting.<sup>1</sup> In fact, MRI is now considered as the only examining tool that reliably assesses disease activity in MS.<sup>156</sup> Thus, the use of MRI in MS is steadily increasing and (as discussed under section 2.5.4) it is now widely accepted that commonly used MRI measures are strongly correlated to clinical outcomes and that these can to some extent predict the disease course.

### **2.5.2 MRI in follow-up of multiple sclerosis patients**

According to the Swedish National Board of Health and Welfare guidelines, RRMS patients of less than 60 years of age should perform annual MRIs, except for patients who have not displayed new lesions for 3-5 years while not taking a DMT.<sup>157</sup> These recommendations do not include RRMS patients aged 60 years and above nor patients with PPMS or SPMS. These guidelines are largely consistent with the recommendation from the Swedish Multiple Sclerosis Association and the Swedish Neuroradiological Society, stating that MRI should be performed frequently in patients with inflammatory active disease whereas patients with stable disease can be examined with MRI less frequently.<sup>158</sup>

In addition to MRI, the follow-up of MS patients consists of neurological examination where the EDSS score is often assessed, regularly as well as whenever patients report new neurological symptoms possibly reflecting a relapse. It has been suggested that these measures could be combined in a composite measure denoted as “No evidence of disease activity” (NEDA), referring to patients with no relapses, no disability progression and no new T2 hyperintense or contrast-enhancing lesions.<sup>159</sup> A disadvantage with NEDA is that it mainly focuses on inflammatory activity and thus ignores other aspects of the disease as neurodegeneration and cognition.<sup>1</sup>

The same cohort of CIS and RRMS patients as discussed in section 2.3.2 was reassessed in 2019 where it was found that whole-brain atrophy rate was associated with increased long-term

disability<sup>160</sup> and brain volume loss is included in NEDA-4.<sup>161</sup> Importantly, the absence of clinical signs of disease activity does not preclude ongoing neuroinflammation, as patients clinically stable on injectable first-line DMTs who switched to RTX therapy subsequently displayed lower frequencies of inflammatory signs on MRI, as well as lowered their NFL levels in CSF.<sup>162</sup> Correspondingly, in a yet later version, the NEDA-5, CSF NFL is included.<sup>163</sup>

### 2.5.3 MRI protocols

The principle of MRI is based on the use of strong magnetic fields and radio waves that generate a signal which is reconstructed, resulting in a three-dimensional description of the body. The major steps in an MRI examination can be very simply described as that a patient is placed in a magnet, the MRI scanner, after which radio waves are applied. The radio waves are then turned off and the signal the patient emits at that stage is received by the scanner's receiving coils and used for reconstruction, after which the volume being examined can be depicted as an image.

MRI protocols in MS vary depending on the purpose of the scan. Succinctly, two different MRI protocols are recommended in Sweden; one longer investigational protocol and a shorter routine follow-up protocol.<sup>164</sup> T1- and T2-weighted images are acquired along with fluid-attenuated inversion recovery (FLAIR) images in both protocols to allow adequate lesion detection. In the more extensive investigational protocol, additional sequences (diffusion-weighted imaging and susceptibility-weighted imaging) are acquired to rule out differential diagnoses but also to detect treatment-related AEs. Scans aiming to detect ongoing inflammation in the form of BBB disruption are performed with the administration of gadolinium-based contrast agents (GBCAs). GBCAs are per default applied in the investigational cohort while it is only optional in the routine follow-up protocol.

Tissues with long T2-relaxation times are brighter than those with short T2-relaxation times on T2-weighted images, hence fluids appear bright while more complex tissues with lower water content such as WM are relatively dark.<sup>165</sup> T2-weighted images are therefore sensitive to pathological fluid accumulation (i.e. oedema) as in MS lesions, which appear hyperintense. The T2-weighted FLAIR image is also sensitive to fluid accumulations but removes the CSF-signal, which is especially useful for identification of periventricular lesions and cortical/juxtacortical lesions.<sup>165</sup> On T1-weighted images, tissues with the longest T1-relaxation time have the darkest signal, hence CSF is dark whereas GM is grey and fat is bright.<sup>166</sup> Lesions with high signal on T2-weighted images that have low signal on spin-echo T1-weighted images compared to normal-appearing WM either disappear or persist, representing reversible oedema/demyelination or permanent axonal loss, respectively.<sup>165</sup>

Gadolinium is a metallic element that, when used as an intravenous contrast agent, leaks into the brain wherever the BBB is impaired.<sup>165</sup> Gadolinium shortens the T1-relaxation time of the surrounding tissue where it accumulates and hence brightens inflammatory active lesions and is an established radiological biomarker of ongoing MS inflammation.<sup>1</sup> Previously, mainly linear GBCAs were used in MRIs of MS patients, which are less kinetically stable compared to the macrocyclic GBCAs that are mostly being used now.<sup>167</sup> The study by Forslin et. al found, apart from that signal intensity in the dentate nucleus and globus pallidus increases with the number of linear GBCA infusions, that higher signal intensity in the dentate nucleus is associated with lower

verbal fluency scores.<sup>167</sup> However, the use of contrast agents in routine follow-up of MS patients is steadily decreasing. This is at least partly explained by a lower diagnostic value because of the reduced disease activity in the MS population in general, as a large proportion of patients today receive very effective treatment.

#### **2.5.4 MRI biomarkers in relation to MS disease activity**

There is a compelling amount of evidence of a close association between MRI biomarkers and clinical outcomes. For example, Sormani et al. performed a meta-analysis of 31 MS DMT RCTs where a high correlation ( $R^2 = 0.71$ ) between treatment response on MRI lesions (measured by number of new or active T2 lesions and gadolinium-enhancing lesions) and relapses was found.<sup>168</sup> Moreover, there has been demonstrated a correlation between baseline T2 lesion volume and disability after 2 years in newly diagnosed MS patients,<sup>169</sup> whereas another study found that increased T1 lesion volume at baseline predicts worsening of EDSS at 5-year follow-up.<sup>170</sup> Of note, there seems to be a difference in the correlation between lesion burden and disability progression in MS patients between T1-weighted and T2-weighted MRI images, with the predictive associations being numerically higher for T1 lesions.<sup>170</sup> Moreover, whole-brain atrophy on T1-weighted imaging has been shown to significantly correlate with disability progression in trials with NTZ and FGL.<sup>171,172</sup>

Regarding cognitive deficits, a common problem in RRMS patients and as previously discussed poorly assessed by EDSS, lesion volume measurements have been shown to predict cognitive performance 8.5 years later.<sup>22</sup> This exemplifies how the currently available conventional MRI data from clinical routine assessments can be used in alternative ways to increase sensitivity to identify patients at increased risk of a more aggressive disease course. In addition, there is extensive ongoing research within the field of MRI in MS to find new methods to monitor the disease, both with conventional MRI data as well as non-conventional MRI techniques. A selection of these techniques will be briefly described in the following sections with emphasis on brain volume quantification, i.e. atrophy measurements since this was used in Study IV. To the best of our knowledge, brain atrophy rate had prior to Study IV not yet been studied in RTX treated RRMS patients, except in a study comparing several DMTs with only two RTX treated patients.<sup>173</sup>

#### **2.5.5 Brain atrophy as biomarker of MS disease activity**

Brain atrophy occurs already in early stages of MS and accelerates with disease duration.<sup>174,175</sup> Monitoring the atrophy rate is, however, associated with several challenges, including the pseudoatrophy phenomenon seen during the first year after the initiation of effective therapy, likely due to the resolution of oedema and brain swelling.<sup>156</sup>

It has been suggested that specific monitoring of atrophy rates in structures of importance for cognition, such as the thalamus and corpus callosum, is superior to global GM atrophy measurements in explaining the decrease in cognitive capacity in MS patients.<sup>176</sup> Indeed, one of the findings in the study by Ouellette et al. was that corpus callosal atrophy is associated with cognitive disability in MS,<sup>22</sup> supporting the hypothesis of disconnection in this structure as the underlying mechanism behind cognitive impairment in MS patients.

## 2.5.6 Methodological aspects of MRI atrophy quantification

There are several aspects that affect atrophy quantification, which have been described in a study by Rocca et al.<sup>177</sup> and the key points are elaborated upon in this section.

### Validation process

Accuracy and precision assessments are essential parts of the validation process when a new monitoring method is introduced.<sup>178</sup> Accuracy is the degree of bias associated with any measurement and precision reflects the repeatability and reproducibility of measurements. Precision can be assessed by performing several MRI measurements over a relatively short period of time where little real change in volume can have occurred, something that is often lacking in patients due to the logistical challenges in performing multiple MRI scans on the same day. However, such validations have been done specifically in MS with the tools that are used for Study IV.<sup>179</sup> Accuracy is more difficult to test, unless objects with known structural dimensions are used, which naturally is not possible of the brain *in vivo*. And brain MRI scans *ex vivo* come with even more challenges related to temperature, fixation, etc. Validation involves measurements under conditions made under different signal-to-noise ratio, image contrast, image geometry, optimisation of acquisition and processing parameters, to test method robustness and to characterise the measurement error. Further, for a method to be accepted for atrophy quantification, validation in both healthy controls and patients is required, being studied both cross-sectionally and longitudinally.

### Image contrast

The radiofrequency pulse flip angles are important to create the signal and hence affect the image contrast-to-noise ratios. Tissue compartments (CSF, GM and WM) are estimated based on their relative brightness in the image and therefore the radiofrequency pulse flip angles indirectly affect atrophy measurements. It is therefore important that the same scanner with the same hardware is used in longitudinal studies and that also the software remains stable across the study. Among other parameters influencing image contrast is the echo-time (the time from the middle of the radiofrequency pulse to the middle of the echo) and the repetition time (the time between two corresponding consecutive points in a sequence of pulses and echoes). Lastly, if inversion pulses are applied, the time between the inversion pulse and the subsequent pulse affects image contrast.

### Image sequence

Brain atrophy measurements are ideally made on T1-weighted images or a combination of multiple weightings including T1-weighted images. Most importantly, atrophy studies should be based on high-resolution three-dimensional (3D) acquisitions with voxel sizes around 1 mm<sup>3</sup> or less, in order to reduce partial volume effects causing measurement errors.

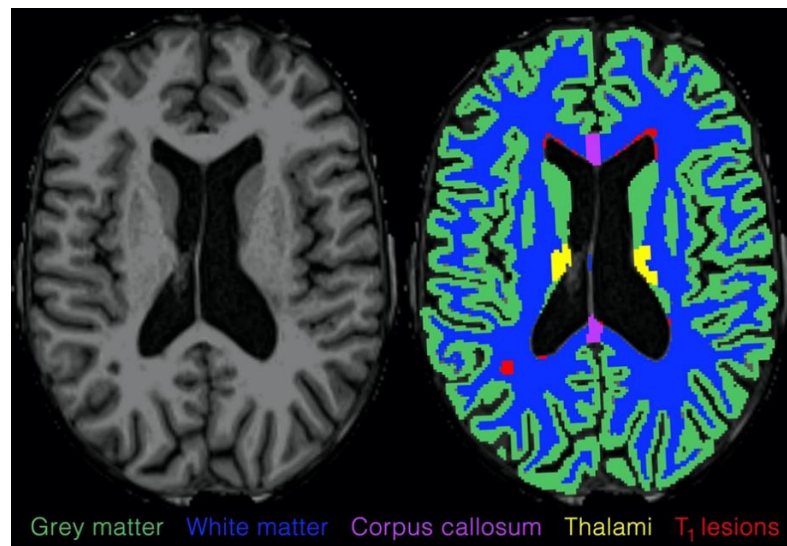
### True atrophy rate on patient level

The atrophy rate in untreated MS patients has been estimated to be 0.7-1% per year, with a median absolute error of 0.15% for longitudinal changes in brain atrophy, while in healthy adults the atrophy rate is 0.1-0.3% per year. The measurement of atrophy in a single patient depends on the

precision of the method used relative to the true change in atrophy over the follow-up, but is also affected by hydration status, clinical events and treatment status between scans.<sup>180</sup>

### FreeSurfer

There are several freely available and commercial software providing automatic methods for image analysis and brain segmentation for volumetrics. These programs often use *a priori* anatomical information to perform segmentation which generally requires registration to an atlas. FreeSurfer is commonly used and can provide comprehensive characterisation of brain anatomy, for the cortex as well as subcortical structures.<sup>181,182</sup> FreeSurfer uses image intensity normalisation, a process aiming to adjust for fluctuations in image intensity, which otherwise would make intensity-based segmentation more difficult. It also uses information about shape variation and intensity distributions in a set of images that are representative of a healthy population. Brain volumes obtained through FreeSurfer can be expressed as fractions, meaning they are normalised to the estimated total intracranial volume, which corrects for variation in head size between individuals and potential scaling effects between scans. FreeSurfer was used in Study IV and Figure 2 illustrates how this software measures subcortical structures using segmentations.



**Figure 2** shows a T1 weighted MRI of an RRMS patient (left) in Study IV including the regions of interest (right) as depicted in FreeSurfer. Each region is represented by a colour as follows: grey matter (green), white matter (blue), corpus callosum (purple), thalami (yellow) and T1 lesions (red) Abbreviations: MRI, magnetic resonance imaging; RRMS, relapsing-remitting multiple sclerosis.

### Sources of error in image acquisition and processing

There are several potential sources of error in image acquisition and processing, including artefacts caused by head motion, imperfect patient repositioning in longitudinal studies and errors in image segmentation. Furthermore, lifestyle factors as well as comorbidities may affect brain volumes.<sup>183</sup> In addition, brain volume fluctuates throughout the day, with higher volumes in the morning.<sup>183</sup>

## **Global versus regional atrophy quantification**

Global volumetric measures average over a larger volume and hence have greater precision and statistical power, whereas regional measures describe anatomical changes in more detail and are not diluted by areas where there is little or no change. GM atrophy rates increase with the disease duration, being between 8.1 and 14 times greater in MS patients of relapsing and progressive stages, respectively, compared to healthy controls.<sup>175</sup> In contrast, WM volumes are relatively constant during all MS disease stages.<sup>175</sup>

### **2.5.7 Myelin quantification in MS**

Although currently used DMTs in MS mainly prevent active inflammation, there is a growing interest in remyelination promoting therapies. Interestingly, in RRMS patients, there is early axonal pathology in the normal-appearing WM in areas outside focal inflammatory demyelinating lesions.<sup>63</sup> Whereas conventional MRI techniques are not sensitive to demyelination in normal-appearing tissue, MRI myelin quantification techniques have successfully been developed to address this problem, where diffuse demyelination has been found to be associated with cognitive and clinical disability.<sup>184</sup> Furthermore, myelin has equal or higher correlation with disability compared to periventricular lesion fraction and monitoring DMT effects on myelination could therefore be a possible biomarker of therapeutic response.<sup>184</sup>

### **3. AIMS**

#### **3.1 GENERAL AIMS**

The overall aim of this thesis was to determine the efficacy, safety and tolerability of RTX as DMT in RRMS in a real-world setting by studying patients followed at our MS centre receiving this drug.

#### **3.2 SPECIFIC AIMS**

The specific aims were to study:

- Compare outcomes in MS patients switching to either RTX or approved MS drugs due to lack of effect or AEs on their initial therapy.
- Determine the frequency of interrupted RTX treatment in all RRMS patients treated at our centre, the reasons for drug discontinuation and frequency of clinical and radiological signs of disease activity after stopping RTX.
- Determine frequency and clinical relevance of ADAs against RTX among RRMS patients receiving this treatment.
- Compare rates of brain lesion accumulation and atrophy between RRMS patients receiving either IFN $\beta$  or RTX as their first DMT.

## **4. MATERIALS AND METHODS**

### **4.1 THE SWEDISH MS REGISTRY**

The Swedish MS Registry (SMSreg) is a validated database with close integration to the clinic where treatment and clinical course are noted for approximately 80% of the patients with an MS diagnosis in Sweden.<sup>185</sup>

### **4.2 STUDY SUBJECTS**

The patient cohorts in Studies I, II and IV were initially identified through the SMSreg, with additional steps in the selection processes subsequently performed, described in the sections below.

#### **4.2.1 Study I**

Included were all patients who had switched treatment between January 1<sup>st</sup> 2011 and December 31<sup>st</sup> 2015 from IFN $\beta$  or GA to NTZ, RTX or FGL due to either a relapse, contrast-enhancing lesion on MRI or both. The study population was restricted to patients followed up at Umeå University Hospital, Karolinska University Hospital, Danderyd Hospital and Sahlgrenska University Hospital. Patients were excluded from the study if having either missing or incomplete patient data, were deemed non-compliant to therapy, were included in a clinical trial with unknown treatment allocation or if they had high titres of ADAs prior to changing treatment. A total of 241 patients were included in the study of which 105 received NTZ, 48 RTX and 88 FGL. In the group receiving RTX, this was administered as infusions of 500-100 mg every six months in the majority of patients.

#### **4.2.2 Study II**

This cohort consisted of all RRMS patients who had received at least one infusion of RTX between 2009 to 2018 at Karolinska University Hospital/Centre for Neurology and who had either interrupted their treatment or had more than one year since last RTX infusion. RTX had been administered to 808 RRMS patients in total. Of these, 92 patients who had interrupted RTX treatment were included in the study after the exclusion of patients who only had received this drug at another centre, whose MS diagnoses had been changed to other diseases prior to data extraction, who developed SPMS prior to RTX initiation or where RTX had been initiated after the development of PML. Additional clinical information on the 92 patients terminating RTX treatment was retrieved from medical charts until April 11<sup>th</sup>, 2019. The most common treatment regimen among the included patients was a 500 mg RTX infusion every six months with a minority of patients initially receiving 1000 mg at six-month intervals with subsequent dose reduction to 500 mg per infusion occasion.

#### **4.2.3 Study III**

This study included 339 relapsing-remitting and progressive MS patients receiving RTX at the Karolinska University Hospital from which at least one serum sample for ADA quantification had been obtained immediately prior to a scheduled RTX infusion from November 2015 to November 2016. Pre-treatment samples and samples from patients treated at other hospitals, patients with other diagnosis than MS, non-Swedish residents and ADA samples taken after RTX infusion were



excluded from the study. In total, 387 samples from the 339 patients were included in the study, which consisted of 238 RRMS patients and 101 patients with SPMS. The majority of patients (> 95%) had received either 500 mg (n = 322) or 1000 mg (n = 33) of RTX every six months.

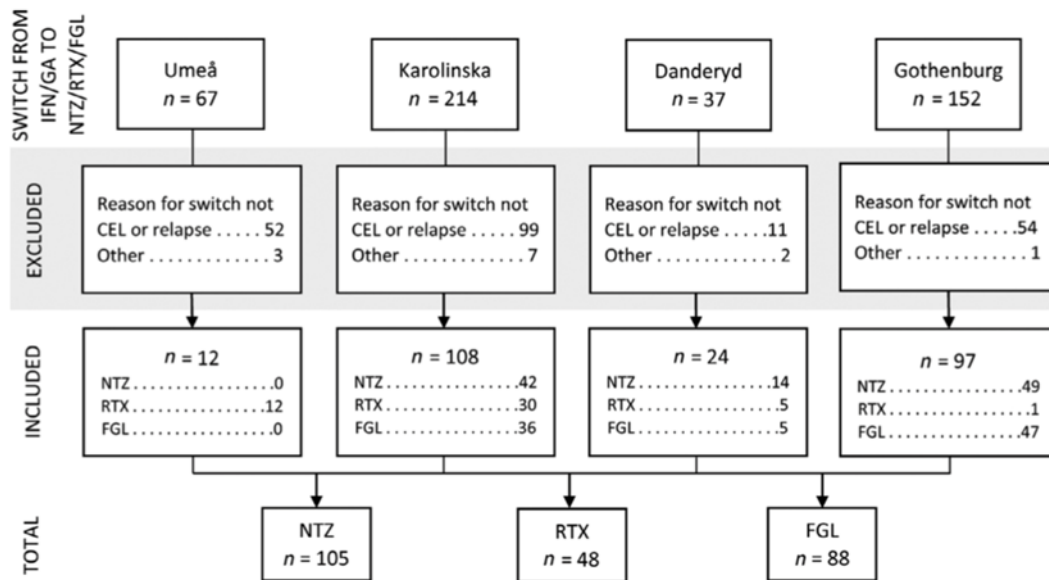
#### **4.2.4 Study IV**

The study was based on all RRMS patients starting either IFN $\beta$  or RTX as first ever DMT between 2005 and 2020 at Karolinska University Hospital. Additional inclusion criteria were  $\geq 2$  comparable MRI 3D T1-weighted MRI scans performed > 1 year apart, the baseline scan performed > 3 months after DMT initiation and follow-up scan obtained within one year of the most recent RTX infusion or within 3 months after the most recent IFN $\beta$  injection. Exclusion criteria were > 10 years from disease onset to diagnosis, > 5 years from diagnosis to DMT start, non-compliant to therapy or any comorbidity that would likely affect MRI outcomes. Of 107 RTX and 172 IFN $\beta$  treated patients screened for inclusion in the study, 15 and 67 patients fulfilled the study criteria and were included, respectively. In the RTX treated group, this was administered as 500 mg infusions every six months in the majority of patients (n = 9) and in the remaining patients as 1000 mg at initial infusion followed by 500 mg every six months in (n = 3), as 1000 mg at initial and six-month infusion followed by 500 mg every half year (n = 1), or 1000-2000 mg at initial infusion followed by 1000 mg every six months (n = 2), until follow-up MRI. The vast majority of included IFN $\beta$  treated patients had been prescribed subcutaneous injections of 44 microgram of IFN $\beta$ -1a three times a week.

### **4.3 STUDY DESIGN AND OUTCOME VARIABLES**

#### **4.3.1 Study I**

This was a retrospective observational study. Eligible patients were identified through the SMSreg where information regarding age, sex, hospital, date of disease onset, EDSS score and type of latest DMT before and after therapy switch was collected. Further, it was noted the reason for discontinuation of treatments, the number of contrast-enhancing lesions on final MRI before therapy switch and on MRI performed  $\geq 3$  months after therapy change in addition to the number of relapses on pre-switch treatments and on post-switch treatments occurring  $\geq 3$  months after treatment start. Moreover, type and severity of AEs according to the Common Terminology Criteria for Adverse Events (CTCAE) Version 4.0 on post-switch treatment was noted from date of treatment initiation to its discontinuation plus 1, 3 and 6 months for NTZ, FGL and RTX, respectively or until data censure or if follow-up was lost, whichever came first. Persistence to post-switch medication was measured from date of administration of its first dose to date of last administration if discontinued or until data censure or loss of follow-up. All collected data were subsequently validated against medical records at each study site. A flow chart depicting the selection process of the study cohort is shown in Figure 3. In the study, the majority (81%) had switched from IFN $\beta$ , where among replacement therapies NTZ (43.6%) was the most drug followed by FGL (36.5%) and RTX (19.9%). Treatment allocation differed substantially between MS centres, with patients mainly recruited from the Karolinska University Hospital and Umeå University Hospital constituted the whole RTX treatment group.



**Figure 3** depicting the selection process of the 241 patients included in Study I. Abbreviations: CEL, contrast-enhancing lesion; FGL, fingolimod; GA, glatiramer acetate; IFN, interferon beta; NTZ, natalizumab; RTX, rituximab. © 2019 European Journal of Neurology. Reproduced with permission from John Wiley & Sons, Inc.

#### 4.3.2 Study II

Study II was a retrospective observational study. Reasons for termination of RTX was noted for all patients and grouped in decreasing priority order as due to lack of effect, AEs, stable disease, confirmed or planned pregnancy and other reasons. In patients with  $\geq 2$  reasons for stopping RTX treatment only the most important one as defined in the priority order stated above was noted. Lack of efficacy was defined as a clinical relapse or contrast-enhancing lesion on brain MRI  $\geq 3$  months after first RTX infusion or a new brain T2 lesion compared to a reference scan obtained  $\geq 3$  months after RTX initiation. AEs leading to RTX interruption were graded according to the CTCAE, version 4.0. IRs on RTX were noted from date of infusion until three days later and the remaining types of AEs regarded as caused by RTX were noted from three months after initial RTX infusion until 1 year after the last infusion. Stable disease leading to RTX discontinuation was denoted as such, referring to patients where the disease course was considered to be mild and not requiring a DMT by the treating neurologist. Women stopping RTX treatment due to planned or confirmed pregnancy were categorised as such. Interruption due to other reasons included patients who wished to terminate the treatment without fulfilling categorisation into any of the other discontinuation subgroups or patients that were lost to follow-up. A period of  $\geq 1$  year between RTX infusions was considered as terminated treatment in all discontinuation categories, regardless of whether the drug later was reintroduced.

In the subgroup of patients interrupting RTX treatment due to lack of efficacy, B-cell counts during and after this treatment was noted as well as the presence of ADA when available. A B-CD19<sup>+</sup> cell count of  $< 0.01 \times 10^9$  cells/L was regarded as complete B-cell depletion,  $0.01$  to  $< 0.09 \times 10^9$  cells/L as incomplete B-cell depletion and  $\geq$  as normalised B-cell levels, equivalent to the laboratory

reference range for B-CD19<sup>+</sup> cell quantification. Moreover, changes in EDSS between treatment with RTX and its replacement therapy was noted for this subgroup.

After RTX discontinuation, information regarding type of replacement therapies was noted for all patients in addition to new T2 lesions from date of last infusion until the most recent MRI, compared to a reference scan performed  $\geq 3$  months after initial infusion was noted, irrespective of subsequent therapies. Further, clinical relapses and contrast-enhancing lesions on MRI were noted from last RTX administration until most recent MRI, regardless of RTX substitute treatments. A standardised protocol on 1.5 or 3 Tesla MRI scanners was used for all MRI examinations, with evaluation of at least one trained neuroradiologist. All patient data gathered from the SMSreg was validated against medical records. Figure 5 under section 5.2.1 illustrates the process of selecting the study cohort.

### 4.3.3 Study III

Study III was a cross-sectional study on serum samples from RTX treated patients that had been collected in a non-active selection manner during a time period and using a selection process described in section 4.2.3. Review of medical records and the SMSreg was performed to collect clinical information at baseline and during follow-up. These variables included contrast-enhancing lesions, EDSS scores and relapses. Using EDSS, a Global MSSS and ARMSS score were calculated to further assess disease activity throughout the study.<sup>68</sup> The follow-up period for the clinical outcomes was from RTX treatment initiation to completion of data collection (November 2016). Information on IRs in connection with previous infusions and in conjugation with sampling for ADA screening was noted and graded according to the CTCAE version 4.0. At least one trained neuroradiologist interpreted the MR images that typically were obtained annually using protocols that all included administration of GBCAs on T1-weighted sequences.

B-cell counts were determined using a Flow cytometry method at a certified in-house clinical laboratory from samples taken within a 30-day period prior to serum sampling and administration of RTX, where complete B-cell depletion was defined as a B-cell count  $< 0.01 \times 10^9$  cells/L.

Samples from a subgroup of MS patients (n = 321) were screened for presence of RTX ADA with an in-house validated ECL-MSD assay and the ELISA. This effort was made to compare RTX ADA detection sensitivity between assays. The differences between these assays are described in section 2.4.5. Using three human anti-RTX clones with different affinity as well as a rat anti-RTX clone, a higher sensitivity of the ECL-MSD vs. the ELISA assay was noted, with the ECL-MSD assay displaying higher detection frequencies of RTX ADA. These results led to the selection of the ECL-MSD assay for detection and quantification of RTX ADA in the whole patient cohort, which was done using a three-step approach. Samples detected as positive in the screening assay, i.e. the first step, were confirmed positive in a competitive assay, i.e. the second step, to confirm that the ADAs were specifically targeting RTX. In the third step, the sample was titrated and the titre, expressed as arbitrary units per millilitre (AU/mL), was determined as the last dilution step in which the sample still gave a positive signal in the assay.

#### **4.3.4 Study IV**

Study IV was a retrospective observational study where study candidates were assessed regarding clinical inclusion and exclusion criteria through review of medical records. The final study cohort was determined after assessment of the MRI-specific inclusion and exclusion criteria. All brain MRI scans were conducted with 1.5 Tesla Siemens Aero/Avanto scanners at Karolinska University Hospital in Huddinge using 3D T1-weighted magnetisation-prepared rapid gradient echo without GBCA. The 3D T1-weighted volumes were quality assessed by a radiologist prior their post-processing in FreeSurfer v. 7.1.1. (Harvard University, Boston, MA, USA).<sup>186</sup> Except for cortical thickness, all volumetric outputs were estimated by dividing by the cortical intracranial volume to take account for variation in head size between subjects and to mitigate any potential scaling effects between the scans. By subtracting the follow-up value by the baseline value, divided by the mean value of the two scans, symmetrised annual percent change for volumes of T1 lesions and brain were retrieved. Annualised rates of lesion accumulation and volume change were obtained by dividing by the number of years between scans.

#### **4.4. STATISTICAL ANALYSIS**

In all studies, a p-value of  $< 0.05$  was considered statistically significant. In Studies I, II and IV IBM SPSS Statistics Versions 24, 22 and 25 were used to perform statistical analyses, respectively. In Study III, Stata (StataCorp, 2009, Stata Statistical Software: Release 14; StataCorp LP, College Station, TX, USA) was used to perform statistical calculations.

##### **4.4.1 Study I**

Annualised relapse and drug discontinuation rates were calculated in addition to the incidence of AEs. To compare the HRs for relapse, AEs and medication persistence of RTX and FGL with NTZ treatment, Kaplan-Meier curves and Cox proportional hazard models with 95% CIs were used. NTZ was used as reference drug in these analyses since this was the most frequent medication in the study. To adjust for potential confounding factors in baseline characteristics, i.e. centre, sex, age at inclusion, disease duration, EDSS score at baseline, duration of last treatment before therapy switch and time from disease activity to therapy switch, multivariate Cox proportional hazard models were used.

##### **4.4.2 Study II**

The statistical analyses in this study were descriptive. For baseline demographic data comparing patients with ongoing vs. terminated RTX treatment, standard deviations were used for means and range for median to illustrate the distribution of data within these subgroups. To establish the distribution of reasons for terminating RTX, the number of patients within a category was divided by the total number of patients interrupting the treatment. Mean MRI follow-up time for each RTX discontinuation subgroup was calculated as the sum of the time difference from date of last RTX infusion to most recent MRI for all patients in the subgroup, divided by the number of patients in the subgroup.

### 4.4.3 Study III

Patients were categorised as either highly RTX ADA positive ( $> 5$  AU/ml), RTX ADA positive or RTX ADA negative and the number of patients in each of these categories were calculated by MS subtype. The risk of RTX ADA positivity in RRMS patients compared to progressive MS patients was expressed as odds ratios with 95% CIs. For patients with more than one treated serum sample, the most recent one was used for the analyses, except for the analyses of IRs and AEs where all samples were treated as individual events to determine if variability in RTX ADA status correlated with these two outcomes. A treatment index was calculated by dividing treatment duration by number of previous RTX infusions at the time of ADA sampling, thereby taking into account the variation in time between the infusions.

To compare the proportion of patients experiencing IRs or AE between RTX ADA categories, Fisher's exact test was used. Baseline was defined as start of RTX treatment. To compare the proportion of patients displaying contrast-enhancing lesions on MRI between RTX ADA categories at baseline and follow-up, Wilcoxon signed-rank test was used. Absolute change, i.e. delta change, in number of contrast-enhancing lesions on MRI, global MSSS and ARMSS score from baseline to most recent measurement, by RTX ADA category, was also calculated.

Logistic regression models were used when assessing the association between RTX ADA category and B-cell count, this outcome variable here treated as a binary variable, with adjustment for the potential confounders age, sex, disease duration, treatment index and MS type. To assess if change on global MSSS and ARMSS score differed by RTX ADA status, linear regression models were used, adjusted for the same variables as in the logistic regression models.

### 4.4.4 Study IV

Mann-Whitney U test was used for comparison between treatment groups regarding demographic data and clinical characteristics at baseline with the exception for sex, where the Chi-Square test was used. To compare brain volume fractions at baseline MRI per treatment group, Mann-Whitney U test was used for WM lesions, whole-brain and cortical thickness, whereas for GM, thalamus, WM and corpus callosum this was done using the independent t-test. To compare differences in lesion accumulation and atrophy rates between treatment groups multiple and simple linear regression models were used, as well as the independent t-test.

In the multiple regression model, the outcome or dependent variable was either the annualised atrophy rates or the T1 lesion accumulation rate. As predictors or independent variables, we included sex, age at diagnosis, treatment type, corticosteroid treated relapse  $< 6$  months prior DMT start, number of relapses before DMT start, time from onset to diagnosis, time from diagnosis to DMT initiation and time from DMT start to baseline MRI.

Since the T1 lesion volume at baseline scan could be affected by ongoing treatment, including this variable in the model could potentially bias the results. To address this, all analyses were run with and without T1 lesion volume at baseline MRI as predictor.

To test if assumptions for multiple regression were met, histograms and Q-Q plots were used to assess if distribution of data was normal with logarithmic transformation of outcome variable if

data were non-normally distributed. Scatter plots were used to assess linearity, independence of error and constant error variance. Outliers were defined as patients with > 3 times the cohort mean value on Cook's distance in each regression model. Outliers were excluded one at the time in a stepwise manner, starting with the patient with the highest value on Cook's d, until all patients indicated as outliers had been removed. For each outcome, the regression model with the lowest p-value for the overall F-test was retained. Bootstrapping with 2000 samples was performed to obtain CIs for regression coefficients.

To compare unadjusted crude means and differences in lesion accumulation and atrophy rates between groups, simple linear regression and independent t-test were used unless assumptions for these tests were not met, where Mann-Whitney U test instead was used. Normality was assessed using Q-Q plots, histograms and Shapiro-Wilk's test whereas Levene's test was used to test for homogeneity of variance. The same outlier patients were removed in the independent t-test and simple regression analyses for each outcome, where exclusion was performed in the step-wise manner described for the multiple regression models.

#### **4.5 ETHICAL CONSIDERATIONS**

There are several laws, regulations and guidelines that regulate research on humans. Among these, the Declaration of Helsinki regarding medical research written by the World Medical Association in 1964 and updated in 2013, is perhaps one of the most important. It states that obtaining a patient's informed consent is necessary, that a risk-benefit assessment of a planned research project should be conducted together with a risk vs. benefit analysis for the individual and/or society. Further, it stresses the right to anonymity for the individual and confidentiality of personal information.

Moreover, the Swedish Ethical Review of Research Involving Humans (Etikprövningslagen)<sup>187</sup> and the Biobanks in Medical Care Act<sup>188,189</sup> and the Act containing supplementary provisions to the EU General Data Protection Regulation Personal Data Act<sup>190</sup>, all apply to research on humans and handling of research data in Sweden. The risk-benefit assessments performed before initiation of the STOP-MS and COMBAT MS projects, that generated the data used in the studies included in this thesis, clearly indicated that the potential benefits of these studies outweighed the possible disadvantages for the patients. To prevent that any unauthorised individual gained access to the research data used in any of the studies constituting this thesis, all extracted data in this project have been stored with encryption in restricted spaces with replacement of patients' social security numbers with the anonymised SMSreg IDs.

Only patients who have given written (biosamples collected) or oral (consent to registration SMSreg) consent to sharing their data in MS research are included in the studies of this thesis and all participants have received information about the possibility to withdraw their consent whenever they wish to do so.

All studies included in this thesis were approved by the Regional Ethical Review Boards in Stockholm (diary numbers 02-548, 2009/2107-31/2, 2020-03471) and Umeå (diary number 2013/445-31).

## 5. RESULTS

### 5.1 Study I

Of the 241 patients included in the study, the majority (81%) had switched from IFN $\beta$ , where among replacement therapies NTZ (43.6%) was the most common drug followed by FGL (36.5%) and RTX (19.9%). Treatment allocation differed substantially between MS centres, with patients mainly recruited from the Karolinska University Hospital and Umeå University Hospital constituted the whole RTX treatment group.

#### 5.1.1 Efficacy with escalation therapies

A total of 13 patients had  $\geq 1$  MRI examination with at least one contrast-enhancing lesion, one in each group treated with NTZ (1.0%) and RTX (2.1%) and in 11 (12.5%) patients treated with FGL. A total of 26 patients experienced  $\geq 1$  relapse, distributed as 6 (5.7%) in the NTZ group, 4 (8.3%) in the RTX group and 16 (18.2%) with FGL. This yielded ARR of 0.02, 0.03 and 0.07 for the NTZ, RTX and FGL groups, respectively.

There was no statistically significant difference in time to first relapse in the RTX group compared to the NTZ group, where crude and adjusted HRs for this outcome were 1.6 (95% CI, 0.5-5.8) and 1.0 (95% CI, 0.2-5.6), respectively. There was a significantly shorter time to first relapse comparing the FGL vs. the NTZ group, illustrated by crude and adjusted HRs of 3.8 (95% CI, 1.5-9.8) and 3.4 (95% CI 1.3-9.2), respectively.

#### 5.1.2 Adverse events with escalation treatments

In total, 19 patients had at least one recorded AE, of which 8 (7.6%) in the NTZ group, 5 (10.4%) in the RTX group and 6 (6.8%) among the FGL treated patients. This was translated into an incidence of AEs of 0.03, 0.04 and 0.03 for NTZ, RTX and FGL groups, respectively. The HRs comparing the proportion of patients experiencing an AE in the RTX vs. the NTZ group and in the FGL vs. the NTZ group showed no significant differences between the groups: 1.4 (95% CI, 0.5-4.5) and 1.0 (95% CI, 0.3-2.9), respectively.

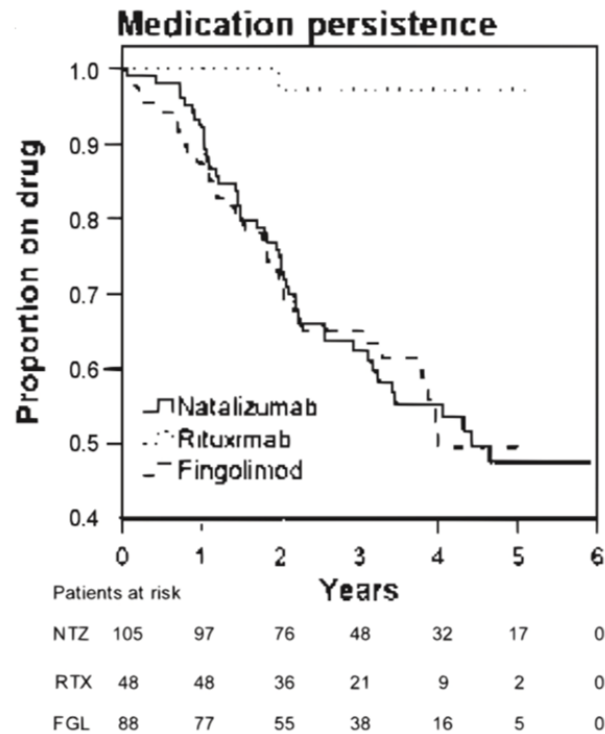
During follow-up, the NTZ group had four grade 2, three grade 3 and one grade 4 event caused by depression. Among the RTX treated patients, there were five grade 2 and one grade 5 event, a suicide in a patient with psychiatric co-morbidity, as previously reported.<sup>89</sup> In the FGL cohort there were four grade 2 and three grade 3 events. There was no recorded grade  $\geq 3$  IR, as defined by the CTCAE scale, in any of the NTZ and RTX treated patients.

#### 5.1.3 Drug persistence with escalation drugs

A total of 83 patients interrupted therapy, with 47 (44.8%) being in the NTZ group, 1 (2.1%) in the RTX group and 35 (39.8%) in the FGL group. This corresponded to an annualised drug discontinuation rate of 0.15, 0.01 and 0.15 in the NTZ, RTX and FGL groups, respectively.

The drug survival was significantly higher in RTX vs. NTZ treated patients in both unadjusted and adjusted analyses: HR 0.05 (95% 0.01-0.34) and 0.05 (95% CI, 0.01-0.38), respectively. Adherence to therapy was similar in FGL vs. NTZ groups in both crude and adjusted analyses: HR 1.0 (95%

CI, 0.6-1.6) and 1.0 (95% CI 0.6-1.7), respectively. Figure 4 displays the drug survival in the treatment groups.



**Figure 4.** Medication persistence per treatment group and year during follow-up. Abbreviations: FGL, fingolimod; NTZ, natalizumab; RTX, rituximab. © 2019 European Journal of Neurology. Reproduced with permission from John Wiley & Sons, Inc.

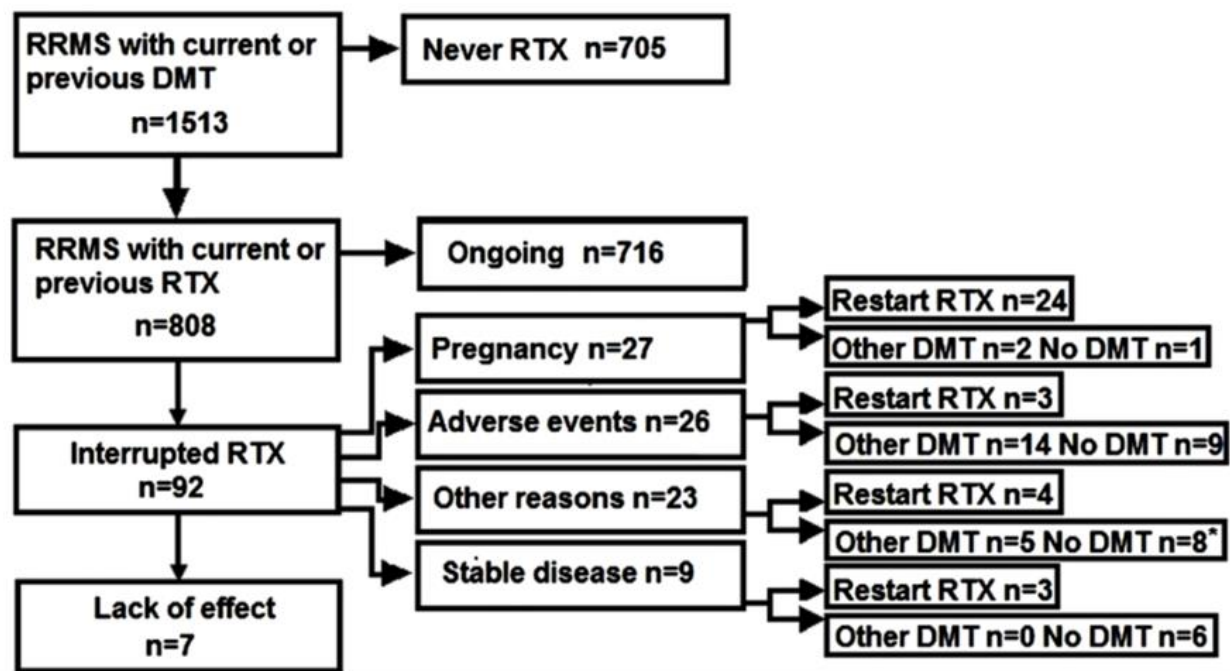
## 5.2 STUDY II

Of the 808 RRMS patients ever treated with RTX at Karolinska University Hospital, 92 (11.4%) patients had interrupted this treatment. Patients terminating RTX were similar to those continuing this therapy regarding age, disease duration, EDSS and number of previous therapies.

### 5.2.1 Reasons for interrupting rituximab treatment

Figure 5 shows the distribution of patients per reason for RTX discontinuation among the 92 patients who had ever terminated this therapy, and whether RTX treatment was subsequently restarted within each category.





**Figure 5** illustrating the identification of the cohort terminating RTX, with sub-classification into reasons for RTX discontinuation and initial subsequent DMTs. Abbreviations: DMT, disease-modifying treatment; RRMS, relapsing-remitting multiple sclerosis; RTX, rituximab. © 2019 Elsevier B.V. Reproduced with permission from Elsevier B.V.

The most common reason for interrupting RTX treatment was planned or confirmed pregnancy (n = 27; 29.4%), followed by AEs (n = 26; 28%), other reasons (n = 23; 25%), stable disease (n = 9; 9.8%) and lack of effect (n = 7; 7.6%). In the group terminating RTX due to AEs, four had a grade 3 AE, eleven had a grade 2 AE and four had a grade 1 AE as defined by the CTCAE scale. There was no grade  $\geq 4$  AE recorded. It was not possible to grade the remaining patients (n = 7) in this group on the CTCAE scale due to insufficient information in medical records, all of which described increased susceptibility for infections on RTX treatment and of whom three patients had serum immunoglobulin G levels below the lower threshold of 6.7 gram/litre on the reference value interval.

### 5.2.2 Patients terminating rituximab due to lack of effect

Four of the seven patients terminating RTX due to objective signs of lack of efficacy had contrast-enhancing lesions on MRI scans of which two also experienced relapses. Two of the remaining patients reported diffuse worsening of MS symptoms on RTX treatment, without distinct relapses or neuroradiological signs of disease activity. The seventh patient reported general malaise including fatigue, without objective signs of active disease.

### 5.2.3 Outcomes after RTX treatment discontinuation

Thirty-four of the patients stopping RTX treatment had re-started RTX as initial subsequent therapy, mainly in the group of patients interrupting treatment due to planned or confirmed

pregnancy. At data censoring, twenty-seven patients had switched to a different DMT, most frequently to NTZ or OFA (n = 7 for both). Twenty-four patients were free of DMT use and seven were lost to follow-up.

Of notice, excluding the subgroup of patients terminating RTX due to lack of effect, only three patients experienced relapses and four displayed new T2 lesions, one of which had both, after RTX discontinuation. Seventy-seven patients (84%) performed  $\geq 1$  MRI scan after terminating RTX where gadolinium was administered in 78% of the patients with no enhancing lesions found, with a mean MRI follow-up time from RTX discontinuation of 29 months (range 7-92 months).

Of the patients discontinuing RTX due to family planning or confirmed pregnancy, 25 (92.6%) had restarted this treatment at data censor, whereas in the AEs group this number was 4 (15.4%) and in the group terminating RTX due to other reasons 4 (17.4%). Notably, 4 (44.4%) of the patients in the groups interrupting RTX due to stable disease later restarted this treatment.

Among the patients discontinuing RTX due to lack of effect, two underwent AHSCT, subsequently displaying absence of disease progression. One patient was lost to follow-up soon after terminating RTX treatment. The remaining four patients displayed incomplete B-cell depletion with RTX treatment, three of which in the context of serum RTX ADA, and switched therapy to OFA. Three of these patients responded well to OFA displaying complete B-cell depletion and no signs of disease activity. The fourth patient had a CTCAE grade 3 IR immediately after the first OFA infusion, which was then discontinued and instead initiated FGL treatment.

### **5.3 STUDY III**

A total of 387 blood samples from 339 patients with relapsing-remitting and progressive forms of MS were included after application of the exclusion criteria, as detailed in section 4.2.3.

#### **5.3.1 Frequency of anti-rituximab antibodies**

In the whole study cohort, 34% of all MS patients displayed RTX ADA of which 18% had a positive titre  $> 5$  AU/mL. Patients with RRMS had higher frequency of RTX ADA compared to patients with progressive disease (37% vs. 26%, respectively) and consistent with this finding, adjusting for age, sex, disease duration and treatment index showed an almost twice as high risk (odds ratio: 1.9, 95% CI: 1.04-3.5,  $p = 0.038$ ) of becoming ADA positive in RRMS patients compared to patients with progressive MS. The frequency of RTX ADA decreased significantly with increasing number of infusions in both MS subtype groups ( $p < 0.001$ ).

#### **5.3.2 Assay comparison**

In the subgroup of samples (n = 321) that were analysed with both ECL and ELISA the proportion of patients detected as RTX ADA positive was 32.7% vs. 5.6%, respectively. All samples detected as RTX ADA positive on the ELISA were also positive on the ECL. The ECL method yielded positive results at lower concentrations of RTX ADA compared to the ELISA.

#### **5.3.3 Anti-rituximab antibodies and B-cell counts**

The association between RTX ADA and B-cell count was assessed in the 236 patients in whom a B-cell count had been obtained within one month prior to ADA sampling. A significantly higher

proportion of all ADA positive patients displayed incomplete B-cell depletion compared to ADA negative patients (45% vs. 26%, respectively; odds ratio: 3.8,  $p < 0.001$ ). Patients with low-positive ADA titres were significantly more likely to have complete B-cell depletion compared to patients with high-positive ADA titres ( $p < 0.008$ ) and had similar probability of adequate B-cell suppression as the ADA negative cohort ( $p = 0.128$ ). The risk of having incomplete B-cell depletion in patients with  $> 5$  AU/mL RTX ADA was significantly greater than in ADA negative patients (odds ratio: 5.8,  $p < 0.001$ ).

#### **5.3.4 Anti-rituximab antibodies and clinical outcomes**

In 19% of the patients a grade 1 or 2 IR was registered whereas 7% of the patients experienced a grade 1 to 3 AE. The number of IRs and AEs did not differ between ADA positive and ADA negative patients ( $p = 0.6$  and  $p = 0.28$ , respectively) and no difference in the severity of these events was found between these patient categories. Moreover, no association was found between IRs or AEs and the ADA titres, MS subtype or RTX dose.

Both ADA positive and negative patients displayed a strong suppression of disease activity after commencing RTX treatment, with an average reduction of the number of contrast-enhancing lesions of  $-0.92$  (95% CI:  $-0.33$  to  $1.5$ ) vs.  $-1.2$  (95% CI:  $-0.61$  to  $1.8$ ), respectively. When adjusting for the potential confounding factors detailed in section 4.4.3, there was no difference in the clinical outcomes between ADA categories. Among the five (1.5%) patients that discontinued RTX treatment during the study period, four were RTX ADA positive and all four interrupted RTX treatment due to lack of efficacy.

### **5.4 STUDY IV**

The RTX and IFN $\beta$  groups were similar regarding sex distribution, age at diagnosis, accumulated number of relapses at treatment initiation, time from onset to diagnosis, time from diagnosis to DMT start and had a similar proportion of patients with at least one contrast-enhancing lesion at baseline MRI. There was a trend for greater T1 lesion volume at baseline MRI in the RTX vs. IFN $\beta$  group (4.2 vs. 2.3 mL;  $p = 0.066$ ). Further, a higher proportion of RTX treated patients had received pulsed corticosteroid treatment for an MS relapse within six months prior DMT start (60 vs. 27%;  $p = 0.014$ ) and the interval between initiating a DMT and baseline MRI was shorter in the RTX group (8.2 vs. 29.3 months;  $p < 0.001$ ). Conversely, the IFN $\beta$  treated patients had a longer interval between the baseline and follow-up scans (58.0 vs. 29.8 months;  $p = 0.001$ ).

B-cell counts had been obtained from all RTX treated patients at least once  $\geq 3$  months after initial RTX infusion until date of follow-up MRI, where incomplete B-cell depletion, i.e.  $\geq 0.01 \times 10^9$  cells/L, was found in 10 of 15 patients at  $\geq 1$  time point.

The treatment groups were similar at baseline MRI regarding GM fraction, thalamic fraction and corpus callosum fraction. Patients treated with RTX had a higher T1 lesion fraction at baseline MRI compared to the IFN $\beta$  group (0.27% vs. 0.14%, respectively;  $p = 0.019$ ). The RTX treated patients had compared to the IFN $\beta$  group a greater whole-brain fraction (77.6% vs. 72.8%, respectively;  $p = 0.007$ ) and higher WM fraction (34.1% vs. 31.6%, respectively;  $p < 0.001$ ) at baseline MRI. Cortical thickness was higher in the IFN $\beta$  group compared to the RTX treated patients (2.45 millimetres vs. 2.37 millimetres, respectively;  $p = 0.008$ ) at baseline MRI.

### 5.4.1. Interferons versus rituximab and T1 lesion accumulation rates

In both the unadjusted and adjusted analyses, T1 lesion annual accumulation rate was greater with IFN $\beta$  compared to RTX therapy. In the crude analysis, this was 0.44% compared 1.57%, in favour of RTX treatment compared to IFN $\beta$  use ( $p = 0.008$ ). In the adjusted analyses, the annualised rate of T1 lesion volume accumulation was 1.74 percentage points ( $p = 0.006$ ) and 1.48 percentage points ( $p = 0.024$ ) lower with RTX compared to IFN $\beta$  treatment in the models excluding and including T1 lesion volume at baseline MRI, respectively.

### 5.4.2 Atrophy rates with interferon and rituximab treatments

The crude mean atrophy rates were higher with RTX than IFN $\beta$  treatment for whole-brain ( $-0.56\%$  vs.  $-0.12\%$ , respectively;  $p = 0.002$ ) and GM ( $-0.59\%$  vs.  $-0.11\%$ , respectively;  $p = 0.016$ ). A trend for greater unadjusted mean atrophy rate was seen with RTX vs. IFN $\beta$  treatment for cortical thickness ( $-0.35\%$  vs.  $-0.08\%$ , respectively;  $p = 0.052$ ), thalamus ( $-0.66\%$  vs.  $-0.35\%$ , respectively;  $p = 0.060$ ) and WM ( $-0.53\%$  vs.  $-0.30\%$ , respectively;  $p = 0.075$ ), but not in corpus callosum ( $-0.55\%$  vs.  $-0.32\%$ , respectively;  $p = 0.68$ ).

The trends between the treatment groups in crude atrophy rates were largely corroborated in the adjusted analysis where the RTX group had higher atrophy rate for whole-brain ( $-0.22$  percentage points;  $p = 0.048$ ), GM ( $-0.51$  percentage points;  $p = 0.010$ ) and for cortical thickness ( $-0.36$  percentage points;  $p = 0.009$ ). This pattern was also seen for thalamus and WM in the adjusted model where the RTX group tended to have a higher atrophy rate compared with IFN $\beta$  treatment ( $-0.24$  percentage points;  $p = 0.099$  and  $-0.46$  percentage points;  $p = 0.082$ , respectively). The multiple regression analysis for corpus callosum yielded uncertain results due to data not being normally distributed, but indicated no difference in atrophy rates between treatments, consistent with the result in the unadjusted analysis. Table 1 depicts the regression coefficients for RTX as predictor in the simple and multiple regression analyses for T1 lesion accumulation and atrophy rates.

	RTX as predictor in unadjusted model				RTX as predictor in multiple linear regression			
	95% confidence interval				95% confidence interval			
	Beta	Lower	Upper	P-value	Beta	Lower	Upper	P-value
T1 lesion accumulation	-1.13	-2.49	0.47	0.197	-1.74	-2.86	-0.46	<b>0.006</b> <sup>‡,§</sup>
Whole brain	-0.29	-0.48	-0.13	<b>&lt;0.001</b> *	-0.22	-0.42	-0.01	<b>0.048</b> #
Grey matter	-0.48	-0.87	-0.12	<b>0.023</b> <sup>†</sup>	-0.51	-0.90	-0.12	<b>0.010</b> **
Cortex	-0.27	-0.52	-0.04	0.051 <sup>†</sup>	-0.36	-0.61	-0.10	<b>0.009</b> <sup>††</sup>
Thalamus	-0.30	-0.62	-0.03	0.062 <sup>†</sup>	-0.24	-0.54	0.05	0.099 <sup>‡‡</sup>
White matter	-0.23	-0.77	0.26	0.453	-0.46	-0.94	-0.04	0.082 <sup>§§</sup>
Corpus callosum	-0.22	-1.03	0.64	0.609	-0.08	-0.92	0.61	0.888 <sup>##</sup>

**Table 1** RTX as predictor of MRI outcomes in simple and multiple linear regression. See supplementary Table 5 in Study IV for explanation of footnotes. Abbreviation: RTX, rituximab.

## 6. DISCUSSION

The successive addition of new DMT options for RRMS, all of which vary in efficacy, safety, tolerability, duration of effect and mode of administration, in theory should give better opportunities for individualised treatment. In practice, however, a multitude of choices may not necessarily lead to better outcomes, unless there is sufficient know-how for how to channel patients to the right DMT depending on specific patient characteristics and reliable methods to monitor treatment outcomes. Evidence-based medicine relies on generation of efficacy data from RCTs, being the accepted standard for establishing efficacy and overall safety of drugs. However, RRMS studies generally running over two years have limited sensitivity for predicting long-term outcomes of a disease that evolves over years and decades. Furthermore, generalisability is restricted by strict inclusion criteria, excluding patients with certain comorbidity and prior treatment history, thus not representing the full spectrum of the RRMS population actually treated. Nevertheless, acknowledged international guidelines generally divide current DMTs into first-line and escalation agents largely depending on existing RCT trial data. From this point of view, the extensive use of RTX, a DMT not formally approved for RRMS, in Sweden represents a highly contested practice. The increasing off-label use has been driven by a series of retrospective real-world studies demonstrating an apparent benefit of RTX compared to different comparators in different settings. Still, there are several issues that have not been addressed. The main objective for my thesis has been to fill four important knowledge gaps regarding the use of RTX in RRMS; comparative effectiveness in patients switching from first-line DMTs due to lack of effect, immunogenicity of RTX and its potential impact on effect and tolerability, the risk of rebound with extended dosing intervals and comparative effect on MRI metrics. In the following paragraphs, the implications of the generated data for each study are discussed.

### 6.1 Main findings and relation to previous research

#### 6.1.1 Study I

The results of this study illustrate that NTZ and RTX seem to have superior effects compared to FGL in terms of suppressing clinical and radiological disease activity, consistent with our experience from clinical practice and previous observational studies. Also in line with our experience from clinical practice, patients on RTX had the highest drug persistence, where positive JCV serology was the leading cause of terminated treatment among NTZ treated patients whereas lack of effect was the dominant reason for stopping therapy in the FGL group. Importantly, frequency and severity of AEs were similar between treatment groups.

As to comparative efficacy between the drugs evaluated in Study I, this has been evaluated in other observational studies where Granqvist et al.<sup>94</sup> compared initial DMTs in RRMS in a study cohort to some extent overlapping with Study I, and found an identical ARR for RTX of 0.03. Consistent with these results are the finding of an ARR of 0.04 for RTX treated RRMS patients in a non-comparative observational study by Salzer et al.<sup>89</sup> Granqvist et al. reported an ARR for NTZ of 0.14 and for FGL 0.16, which was higher than the ARR for NTZ in our study (0.02) as well for FGL (0.07). This relatively large difference for NTZ is somehow surprising, since the NTZ-treated patients in both studies displayed signs of a more inflammatory active disease prior starting NTZ

therapy, in comparison with the other treatment arms in each study. The difference in ARR for FGL between the studies should be interpreted with caution due to a small number of patients (n = 17) in the study by Granqvist et al.<sup>94</sup>

Further supporting the finding in Study I of a superior efficacy with RTX vs. FGL is the study by Alping et al.<sup>191</sup> on patients switching from NTZ to RTX or FGL due to JCV-positivity. The relapse frequency was almost equal in the studies for FGL (17.6% in their study vs. 18.2% in Study I), while the relapse frequency for RTX was 1.8% in their study compared to 8.3% in Study I. However, Study I had an almost twice as long follow-up to detect such events.

Interestingly, the ARR with RTX treatment in these observational studies is lower than in the ASCLEPIOS I and II trials where ARR for OFA was 0.11 and 0.10, respectively.<sup>100</sup> This difference is likely due to a higher sensitivity to detect relapses with the more robust follow-up protocol applied in RCTs compared to in retrospective observational studies, since it is unlikely that two structurally highly similar anti-CD20 DMTs would have highly different efficacy.

As to frequency of AEs, we replicate the finding by Granqvist et al.<sup>94</sup> of no difference in frequency of AEs between NTZ, RTX and FGL, although that study recorded grade 1-5 CTCAE AEs, whereas in Study I this was noted for grade 2-5 AEs. The findings in these studies are in contrast to the study by Alping et al., where RTX-treated patients had significantly lower risk of experiencing AEs compared to FGL.<sup>191</sup>

Notably, the low rate of discontinuation of RTX therapy in Study I is supported by our findings in Study II, where frequency and reason for interrupted RTX treatment among all RRMS patients ever receiving this drug at our centre were described, as well as by our findings in Study III, the RTX ADA study. Further, the annualised drug discontinuation rate for RTX was 0.03 in the study by Granqvist et al.,<sup>94</sup> which is almost the same as in Study I (0.01). Importantly, we here reproduce the finding by Granqvist et al.<sup>94</sup> of a superior drug survival with RTX compared to both NTZ as well as FGL and both studies found that AEs were the most frequent reason to stop FGL treatment whereas JCV-positivity was the most common reason for terminating NTZ.

### **6.1.2 Study II**

We here highlighted that interruption of RTX treatment in RRMS is an infrequent event, that <1% of all patients ever receiving RTX terminated this treatment due to lack of effect and that the disease activity after RTX discontinuation is low, regardless of reason for RTX discontinuation and whether a new DMT is started or not.

Interestingly, in the subgroup stopping RTX due to pregnancy, there were no subsequent relapses during follow-up, a finding consistent with a study by Razaz et al.,<sup>192</sup> although study cohorts were partly the same. In the study by Razaz, the relapse rate among women suspending RTX within 1 year prior to conception remained low beyond childbirth and this was lower compared to both patients who suspended NTZ and untreated patients. These results indicate that the treatment effects of RTX in RRMS are long-lasting.

Seven of the twenty-six patients terminating RTX treatment described increased susceptibility to infections since starting RTX, with a minority of these patients (3 of 7) displaying serum IgG levels

below 6.7 gram/litre. This illustrates that hypogammaglobulinemia can occur during RTX treatment, which is being increasingly recognised and recently described as the most common laboratory abnormality in RTX treated MS patients.<sup>193</sup>

In the subgroup of patients terminating RTX due to lack of effect, the majority displayed incomplete B-cell depletion as well as RTX ADAs with subsequent initiation of treatment with OFA infusions, with adequate treatment response in three of four of these patients. However, since there seems to be no association between incomplete B-cell depletion and clinical outcomes, as illustrated in Study III, we can only speculate why these patients experienced lack of efficacy with RTX with subsequent adequate treatment response on OFA. Notably, given the small number of patients, the observational study design and relative short follow-up time after switching to OFA, we cannot rule out that the lack of relapses after initiation of OFA was simply due to chance or reflecting a stable period in the disease course. Nevertheless, that initiation of OFA was successful in most of these patients suggests that a therapy switch within the same class of anti-CD20 drugs could be meaningful in RTX treated patients who have incomplete B cell-depletion and signs of disease activity.

The CTCAE grade 3 IR during the first OFA infusion in one of these patients is notable, although the subcutaneous route of administration for the approved version of this drug is probably associated with a lower risk of such side effects.

### **6.1.3 Study III**

We here determined the frequency of RTX ADAs in relapsing and progressive MS patients using two different assays and interestingly, found that RTX treatment in MS is more immunogenic than previously reported,<sup>90,91,194</sup> also compared to other diseases where it is used.<sup>195,196</sup> In addition, we here highlight that the frequency of ADAs is dependent on which assay is used, where the ECL-MSD method was more sensitive than the ELISA. That ADA frequency decreased with length of treatment suggests that the drug is more immunogenic during early treatment and possibly also early during the disease course, the latter being supported by the finding that the frequency of ADA was higher in the relapsing than in the progressive MS group, consistent with results from previous studies.<sup>90,91</sup> The finding of no association of ADA status and clinical outcomes is consistent with previous studies of RTX in RRMS<sup>90,91,194</sup> and in other diseases.<sup>197,198</sup>

### **6.1.4 Study IV**

We demonstrated that treatment with RTX is associated with a lower rate of T1 lesion volume accumulation than with IFN $\beta$ . In contrast, most regional brain volumes seemed to decrease more in RTX vs. IFN $\beta$  treated patients, in both crude and adjusted analyses. When controlling for the time from treatment initiation to baseline MRI in the adjusted model, the whole-brain atrophy rate for RTX was lower compared to that in the uncontrolled model for this outcome, suggesting presence of pseudoatrophy in this group.

Of note, RTX treated patients displayed a more severe disease activity prior DMT start, as illustrated by a trend of higher T1 lesion volume at the baseline MRI ( $p = 0.066$ ) and a higher proportion of patients receiving high-dose cortisone prior DMT start ( $p = 0.014$ ), as compared to the IFN $\beta$  treated patients. Adjusting for these differences did not markedly change the outcomes,

supporting the conclusion that RTX is more efficient than IFN $\beta$  in terms of slowing rate of T1 lesion volume increase.

Our finding of a lower rate of T1 lesion volume accumulation is interesting given the previously mentioned correlation between T1 lesion volume and progression of disability in MS.<sup>169</sup> Further, Ouellette et al. identified T1 lesion volume fraction as an independent predictor of cognitive performance 8.5 years later in a study on RRMS and progressive MS patients.<sup>22</sup> This result in Study IV could therefore indicate that IFN $\beta$  treated patients are at greater risk of disability progression as well of developing cognitive decline.

However, the interpretation of higher brain atrophy rates with RTX compared to IFN $\beta$  treatment is challenging. Of note, in the OPERA I study comparing OCR vs. IFN $\beta$  the result was the opposite of Study IV for whole-brain atrophy, in favour of the anti-CD20 treatment. One explanation could be that this discrepancy is due to the pseudoatrophy phenomenon, given the short time from RTX initiation to baseline MRI in Study IV. This is supported by the finding of a greater atrophy rate with NTZ compared to placebo during the first treatment year in the AFFIRM study, which was reversed during the following year in favour of NTZ.<sup>199</sup>

Importantly, the results in Study IV could be interpreted as such that slowing focal lesion accumulation does not affect brain atrophy, suggesting that these represent different disease mechanisms, highlighting the need for more sensitive imaging techniques to be able to identify the underlying disease mechanism in neurodegeneration to attempt to develop new DMTs that target this.

Our findings suggest that T1 lesion volume could serve as biomarker of therapeutic response and hence possibly could be used as a tool to identify treatment non-responders at an early disease stage, who possibly could benefit from changing to a different DMT. However, future studies are warranted with a robust MRI protocol in larger cohorts to facilitate non-biased comparisons of atrophy rates between treatment groups, as well as have EDSS performed in all patients to enable correlation of MRI outcomes to development of neurological disability. Such studies would preferably be conducted as RCTs.

## **6.2 Methodological considerations and limitations**

The studies included in this thesis are retrospective cohort studies providing level 2b evidence (Studies I, II and IV) whereas Study III provides level 4 evidence due to its cross-sectional study design.<sup>200,201</sup>

### **6.2.1 Remarks on terminology**

The terms relapse and clinical relapse are used interchangeably in this thesis, including in its associated studies and refers to a clinical relapse as defined in section 2.1.1.

The term AE includes IRs in Studies I-II, whereas in Study III these terms are used separately.

The classification of comorbidities in Study IV as minor or major comorbidity was an arbitrary categorisation method, with the sole purpose of identifying large differences in the distribution of other diseases among included patients between treatment groups.



### 6.2.2 Internal and external validity

Precision is to what degree a study is free from random error and by increasing the study population size, precision generally increases.<sup>202</sup> The internal validity of a study is determined by to what degree alternative explanations to the result, due to systematic error i.e. bias, can be ruled out.<sup>203</sup>

Systematic error can be classified into three groups: selection bias, information bias and confounding.<sup>203</sup> Selection bias is when the association between the exposure and outcome is different among participants and non-participants in a study, where the latter group was failed to be included despite being eligible.<sup>203</sup> Hence, selection bias in a study can occur at any point from inclusion of patients to loss-to-follow-up.<sup>204</sup> Information bias, also called misclassification, is when the information collected about or from study subjects is erroneous, i.e. there is measurement error, An example of this is when a patient is categorised in the wrong group for a categorical variable.<sup>203</sup>

Misclassification can be non-differential, when it is unrelated to other study variables, whereas in differential misclassification the misclassification differs depending on the value of the other study variables.<sup>203</sup> Confounding can occur when a confounding factor is associated with the exposure of interest and also influences the disease outcome, biasing the results if not adjusted for in the analysis.<sup>205</sup> An example applicable to MS research would be a study comparing the efficacy with which two different DMTs (i.e. the exposure) slows disease progression (i.e. the outcome). Smoking, which negatively affects the disease course and is not a part of the causal chain leading from the exposure to the outcome, is then a potential confounder if unevenly distributed between treatment groups. Another, perhaps simpler, way of defining confounding is a confusion of effects, i.e. the effect of one exposure is mixed with the effect of a different exposure, leading to bias.<sup>203</sup> One way of preventing confounding is through randomisation, as applied in RCTs.<sup>202</sup>

A stratified analysis refers to dividing the study cohort into different strata for a confounding factor, where more strata with narrower boundaries usually control confounding more effectively than fewer strata with broader boundaries. A stratified analysis only controls for between-stratum confounding, not within-stratum confounding. The disadvantage with stratifying too extensively is that this produces small frequencies of events within cells leading to imprecise results.<sup>203</sup>

Residual confounding refers to within stratum-confounding and is also the term used to describe confounding factors that are not controlled at all in a study.<sup>203</sup> External validity is to what extent the results are representative of the target population, i.e. a measure of its generalisability to other populations than the study cohort.<sup>202</sup>

### 6.2.3 Strengths and limitations of Study I

The strengths of Study I include similar frequencies of MRI between centres and treatment groups and that these served population-based catchment areas, implying that study populations did not systematically differ between regions.

That geographical location strongly correlated with treatment allocation suggests that individual preferences among treating neurologists regarding DMT choice outweighed patient-specific disease characteristics, since it is unlikely that the between-region difference in DMT choice would

be a consequence of a systematic difference in patients' disease severity across regions. This decreases the risk of confounding.

There are several limitations in the study. The results suggest that there was a systematic difference in disease activity between the NTZ, RTX and FGL groups, where the former group higher EDSS score and shorter time to disease breakthrough on IFN $\beta$ /GA compared to the other groups, respectively. Further supporting the idea of between-group imbalance regarding disease activity prior treatment switch is the lower proportion of patients with contrast-enhancing lesions at baseline MRI in the RTX group compared to the other groups. This could be regarded as confounding reducing the internal validity of the study.

The frequency of contrast-enhancing lesions with NTZ and RTX was too low to allow for adjustment of potential confounders in the model for this outcome, hence only raw frequencies were calculated, implying a greater risk of residual confounding in this analysis compared to the adjusted models for the other study outcomes.

The investigators at each study site were not blinded to treatment allocation, which could have affected the process of extracting data so that the exposure and outcome variables registered were affected. In addition, that there were different researchers extracting data at each site, infers a risk of inter-individual differences in terms of interpretation of data in clinical records. This could lead to information bias reducing the internal validity of the study.

Together, these limitations negatively affect the possibility to estimate the true difference in the outcomes of interest between treatments and hence decrease the interpretability of the study results. To overcome these limitations, additional larger observation studies with longer follow-up where treatment groups are similar regarding disease severity at baseline are needed to assess if these differences in outcomes between these treatments persist over time. However, ideally, an RCT comparing these drugs would be conducted that would automatically overcome many of the methodological issues associated with Study I.

#### **6.2.4 Strengths and limitations of Study II**

A strength is that the study design facilitated detection of all RTX treatment interruptions that had occurred during a relatively long period of time, extending beyond what is typically seen in most RCTs.

Among study limitations are that it is unknown with what frequency similar events (i.e. those causing treatment interruption in the 92 included patients) occurred among the RTX treated RRMS remaining on their therapy (89% or  $n = 716$ ), limiting our conclusions on frequency of these events only to the subgroup stopping RTX therapy. This aspect is important not only in relation to efficacy outcomes, but also regarding frequency of AEs where a larger and a more recently published study has described a higher incidence of both severe and mild infections in RTX treated MS patients compared to both approved MS drugs and the general population.<sup>206</sup>

As in Study I, the researcher extracting data was not blinded to exposure status which might have affected the interpretation of data in medical records, a possible source of information bias and reduced internal validity in Study II.

### **6.2.5 Strengths and limitations of Study III**

A strength of this study was that the person extracting clinical data from the SMSreg and clinical charts was blinded with regard to ADA status, increasing the internal validity of the study.

A study limitation was the random ADA sampling, where exclusion of patients based on timing of sampling of B-cell count in relation to ADA sampling resulted in that only a subgroup of all RTX treated MS patients at the hospital were included. Hence, the study cohort is not representative of the total cohort of RTX-treated MS patients at the hospital at the time. This could introduce selection bias since the ADA status and its relation to the study outcomes is unknown for these non-included patients.

Further, the method for counting B-cells did not discriminate between memory B-cells and naïve B-cells. Mainly naïve B-cells re-emerge during the reconstitution phase after discontinuation of anti-CD20 therapies, exerting anti-inflammatory effects resulting in decreased T-cell proinflammatory responses whereas memory B-cells are particularly prone to produce pro-inflammatory cytokines.<sup>33</sup> This combined with that, due to the cross-sectional study design, it was unknown if patients with incomplete B-cell depletion had displayed complete depletion earlier during the treatment course, makes it difficult to draw conclusions on the association between incomplete B-cell depletion and clinical outcomes in Study III.

### **6.2.6 Strengths and limitations of Study IV**

Among the strengths of this study were that only treatment-naïve patients were included, avoiding any carry-over effect from previous treatments. Further, that all patients were scanned using the same scanner and that all volumetric data were tissue fractions, meaning it was normalised to the total intracranial volume, are additional strengths of the study and increases its internal validity.

There are several limitations of the study. One of the most important is the non-randomised design, making it impossible to completely control for confounders, such as differences in diseased severity between treatment groups, which may have affected the study outcomes. These include a higher proportion of RTX treated patients who had received high-dose cortisone relapse treatment during the six months prior DMT initiation and an almost twice as high T1 lesion volume at baseline MRI in the RTX group compared to the IFN $\beta$  group, although some of these differences between treatment groups were not statistically significant. While adjusting for these differences did not considerably change the outcomes, considering that RTX treated patients had more inflammatory active disease, we cannot rule out that the increased atrophy rate in RTX-treated patients in part is due to confounding by indication. That is, we cannot tell if the increased atrophy rate in the RTX group was a consequence of the DMT or simply reflecting a more severe disease course in this group. This decreases the internal validity of Study IV.

In addition, the shorter interval between DMT initiation and baseline MRI in the RTX group, which probably introduced pseudoatrophy in this subgroup, can be regarded as a form of measurement error, leading to misclassification or information bias which further reduces the study's internal validity. Moreover, since clinical visits were not synchronised with MRI, we could not include EDSS in the analysis, which would have been of value to correlate our MRI findings to clinical signs of disease progression. In addition, since information on presence of serum ADA was lacking,

we could not assess ADA status in relation to B-cell count, which would have been interesting since the majority of RTX treated patients had incomplete B-cell depletion during this treatment and the finding in Study III of an increased risk of having this in patients with high-positive ADA titres. Another important weakness is that many patients were excluded since they had not been scanned in the same MRI scanner, although fulfilling the clinical criteria for study inclusion, hence we cannot rule out that this has caused selection bias, further decreasing the validity of the study.

## **General Discussion**

### **The main goals of our research**

RTX has been increasingly used as a DMT in MS patients in Sweden since more than a decade ago although this use is off-label. As of today, more than 5000 patients have RTX as their MS treatment in Sweden. The experience from clinical practice is that RTX is highly efficient in depressing disease activity and with a significantly better drug survival than previously approved MS therapies. In order to assure both the treating neurologists, authorities and patients that it is on par with the best approved MS drugs it is warranted that its efficacy, tolerance and AEs are adequately assessed.

Importantly, there is substantial regional differences in Sweden regarding choice of DMT. This problem is highlighted in Study I, where some participating centres consequently used only one or two of the three studied DMTs. This suggests that local tradition or preferences outweigh patient disease characteristics when choosing DMT, which is problematic from an ethical point of view, i.e. patients are potentially not offered an as efficient or safe treatment across regions, as well as from a healthcare economic perspective, since treatment costs substantially differ between drugs.

Fortunately, the Patient-Centered Outcomes Research Institute (PCORI) in the United States launched a funding initiative in 2015 for treatment of MS. One of the projects granted funding was the study entitled “COMparison Between All immunotherapies for Multiple Sclerosis” abbreviated COMBAT-MS which is conducted in Sweden and California, US. The COMBAT-MS study is a prospective non-intervention observational cohort study assessing the long-term safety and efficacy of RTX treatment in MS compared with other common MS DMTs regarding both clinical and radiological parameters in a real-life population of patients with MS. The current project has been partly financed with this grant.

Importantly, despite the approval of the anti-CD20 drugs OCR and OFA for different types of MS, RTX has continued to be the anti-CD20 drug of choice for the vast majority of MS patients in Sweden. So far, our experience from clinical practice is that RTX is as efficient and well-tolerated as the approved anti-CD20 MS drugs based on the information from the pivotal studies for these drugs.

No patients treated with RTX in Studies I and II reported any malignancy during follow-up. In contrast, the phase III ORATORIO study on OCR in PPMS reported a higher frequency of neoplasms with this anti-CD20 drug compared to placebo.<sup>50</sup> The ASCLEPIOS trials,<sup>100</sup> that led to the approval of OFA, have not reported a higher incidence of malignancies with this drug as compared to placebo. However, one weakness with Studies I and II as well as the ORATORIO and ASCLEPIOS trials is the relatively short follow-up time, limiting the possibility to detect cancers

developing during these studies. However, in a recently published observational study with over 4000 RTX treatment initiations with a mean follow-up of 2.3 years, no increased invasive cancer incidence was found with RTX treatment compared to the general population.<sup>207</sup>

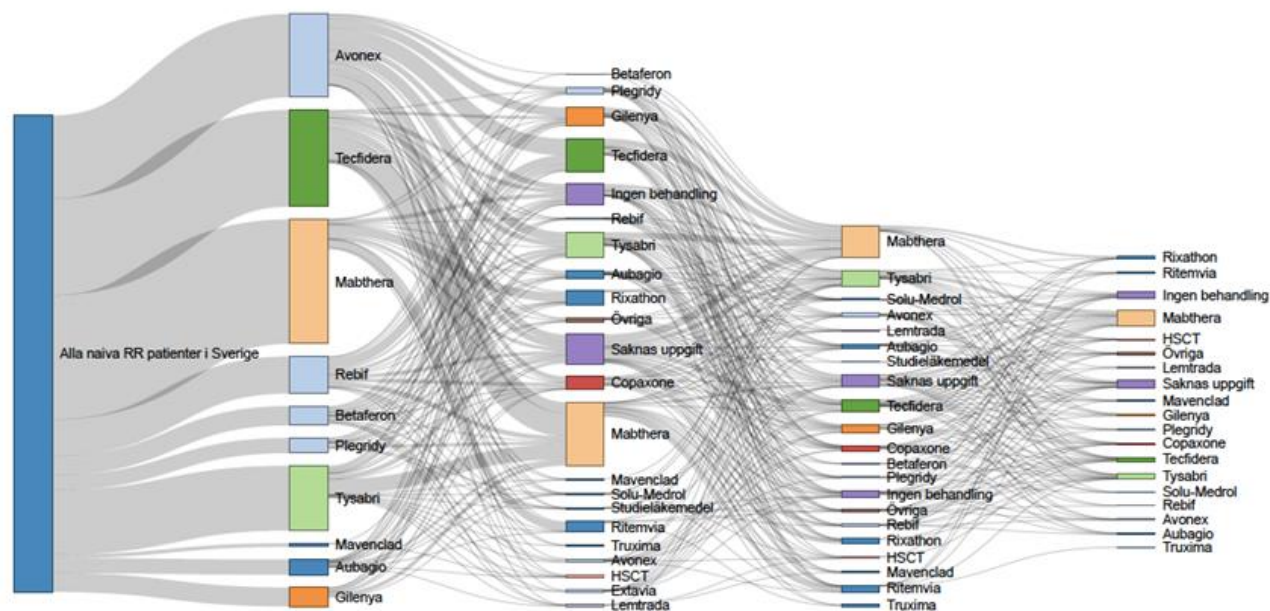
Naturally, it would be interesting to compare RTX vs. the approved anti-CD20 MS drugs in terms of clinical efficacy and safety profile, however, given the small number of patients receiving these newer antibody drugs it is currently not possible to conduct such studies at our centre or in Sweden.

It is worth noting that the annual cost for these newer anti-CD20 drugs is substantially higher than for RTX. In addition, there are now several RTX biosimilars available in Sweden, reducing the annual treatment cost for this treatment substantially. Therefore, from a cost-benefit perspective for the health care system, it is eligible to choose RTX or any of its generic equivalents as these are as efficient and safe as the approved anti-CD20 therapies.

### **Future role of rituximab in RRMS treatment**

The Swedish Medical Products Agency published in 2019 a systematic risk/benefit assessment report on RTX treatment in MS where it was concluded that no new safety concern had been identified in relation to the off-label use of RTX in patients with active MS, why there was no need for regulatory action. However, the report stressed that the magnitude of efficacy in relation to a well-defined population and dosing had not been reliably confirmed from a regulatory view and called for further studies focusing on efficacy and safety of RTX in MS.<sup>208</sup>

One important aspect of MS treatment is that a substantial number of patients change between three or more treatments during their disease course. One possible interpretation of this is that we, as treating neurologists, are not that successful in choosing the right drug for the right patient. As illustrated Figure 6, a large proportion of patients change therapy within 5 years from treatment initiation. For example, among first-line treatments such as IFN $\beta$ , this is true for 4 out of 5 patients. Hence, selecting a treatment strategy that works over time is a challenge. The experience from clinical practice in Sweden is that patients stay on RTX treatment for longer periods compared to approved MS DMTs, which is illustrated in Figure 6. Consistent with this observation, in Study II there was a low proportion of RRMS patients who terminated treatment (11%) among those who had ever received this drug our centre, as well in Study I where drug persistence was significantly greater for RTX compared to NTZ and FGL.



**Figure 6** illustrating all RRMS patients starting a first DMT in 2012 or later and changing therapy within 5 years of start of first DMT where lines illustrate subsequent therapies per DMT switch. Given brand names for drugs included in Studies I–IV correspond to the following generic names: Avonex (interferon beta), Betaferon (interferon beta), Copaxone (glatiramer acetate), Gilenya (fingolimod), Mabthera (rituximab), Plegridy (peginterferon beta), Rebif (interferon beta), Rixathon (rituximab), Ritemvia (rituximab), Tysabri (natalizumab). Abbreviations: DMT, disease-modifying treatment; HSCT, Haematopoietic Stem Cell Transplantation; RRMS, relapsing-remitting multiple sclerosis. Permission not required.

To date, the comparison between RTX and other MS therapies has largely been confined to real-world observational studies. One of the most awaited studies is the RIFUND study, an ongoing Swedish academic-driven phase III RCT comparing RTX and DMF in patients with CIS and early RRMS in terms of clinical, radiological and cerebrospinal fluid marker outcomes. The results from this study, if in favour of RTX treatment, likely would increase use of RTX as MS treatment also in countries with restrictions on use of drugs used off-label unless there is evidence fulfilling more strict regulatory requirements.

In summary, the work conducted during this PhD project has hopefully, together with other ongoing and finished projects conducted under the COMBAT-MS umbrella, contributed to a better understanding of the efficacy and AE profile associated with the off-label RTX use in MS. However, the effort to validate RTX as a proper MS treatment must continue and it is up to the academic society to continue this strive.

## 7. CONCLUSIONS

RTX is a highly efficient therapy in RRMS diminishing disease activity to the same extent as the most efficient approved MS treatments. The drug is well tolerated in the vast majority of patients. The AEs associated with RTX treatment are mostly related to IRs and there is no increased incidence of PML or malignancies with the treatment. The presence of anti-RTX antibodies correlates with incomplete B-cell depletion but not with clinical outcomes. The lesion volume accumulation rate on T1-weighted images is lower with RTX than IFN $\beta$  treatment and potentially a surrogate marker for disease progression but needs to be validated as such in future studies with more robust study designs. RTX treatment is possibly equal to or less efficacious compared to IFN $\beta$  in terms of slowing brain atrophy, depending on brain region. However, the multiple technical limitations in our material combined with differences in disease characteristics between treatment groups undermine the interpretability of atrophy rates in Study IV, advocating for new studies with a more structured study protocol and longer follow-up to confirm these findings.

## 8. FUTURE PERSPECTIVES

Studies with longer follow-up are warranted to assess comparative efficacy and frequencies of AEs of RTX and other MS treatments over many years of drug exposure. The SMSreg is a great platform to conduct such studies, with over 90% of all MS patients in Sweden included in it. Thus, it will be possible to substantially increase the follow-up time for all relevant outcomes for all currently used DMTs by conducting additional retrospective observational studies in the future. Further, the Swedish MS Society is a network of neurologists and other healthcare professionals with a special interest in MS, of whom many are very prominent world-leading researchers, who work in close collaboration and have published guidelines for diagnosis, management and follow-up of MS patients, contributing to standardised high-quality care of MS patients throughout Sweden.

Although RTX is not an induction therapy for MS *per se*, the disease seems to display few if any signs of inflammatory activity years after treatment discontinuation, as indicated in Study II. As a consequence of this finding, combined with a similar experience from our clinical practice, additional studies on MS patients are ongoing where infusion frequency and dose of RTX are lowered, aiming to determine the lower threshold of its efficacy. Of notice, the doses and frequencies of RTX administration used today in MS patients are much lower than in other diseases, for example rheumatic disorders. Since it is likely that the risk of AEs decreases with lower doses, it is motivated to perform such studies, not only from an ethical point of view but also because a decreased AE frequency most certainly correlates to higher drug persistence and, consequently, increases the likelihood that the patients can maintain their RTX MS treatment. In addition, by reducing the risk of AEs such as infections, healthcare costs are reduced as patients' working capacity is maintained to a greater extent and the need for hospitalisation due to infections is reduced. Lower and less frequent RTX dosing would also decrease the treatment cost per patient.

Further, as MS is a disease that often debuts at young adulthood, more often in women than in men, studies on RTX in relation to pregnancy are needed. Hence, studies are ongoing that more closely assess the outcomes with RTX treatment in women of childbearing age.

And finally, there is room for improvement in the currently used MRI techniques in the follow-up of MS patients. New methods that can contribute to earlier detection of patients at greater risk of a more aggressive disease course or those not responding adequately to treatment would be of great value in optimising treatment choices for each patient.



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