

Circulating lymphocyte subsets as prognostic markers in multiple sclerosis

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CIRCULATING LYMPHOCYTE SUBSETS AS PROGNOSTIC MARKERS IN MULTIPLE SCLEROSIS

A first step towards systems immunology

Max Cornelius Mimpfen

Circulating lymphocyte subsets as prognostic markers in multiple sclerosis
A first step towards systems immunology

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof.dr. P. Habibović,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
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"If the brain were so simple we could understand it, we would be so simple we couldn't."

— Lyall Watson

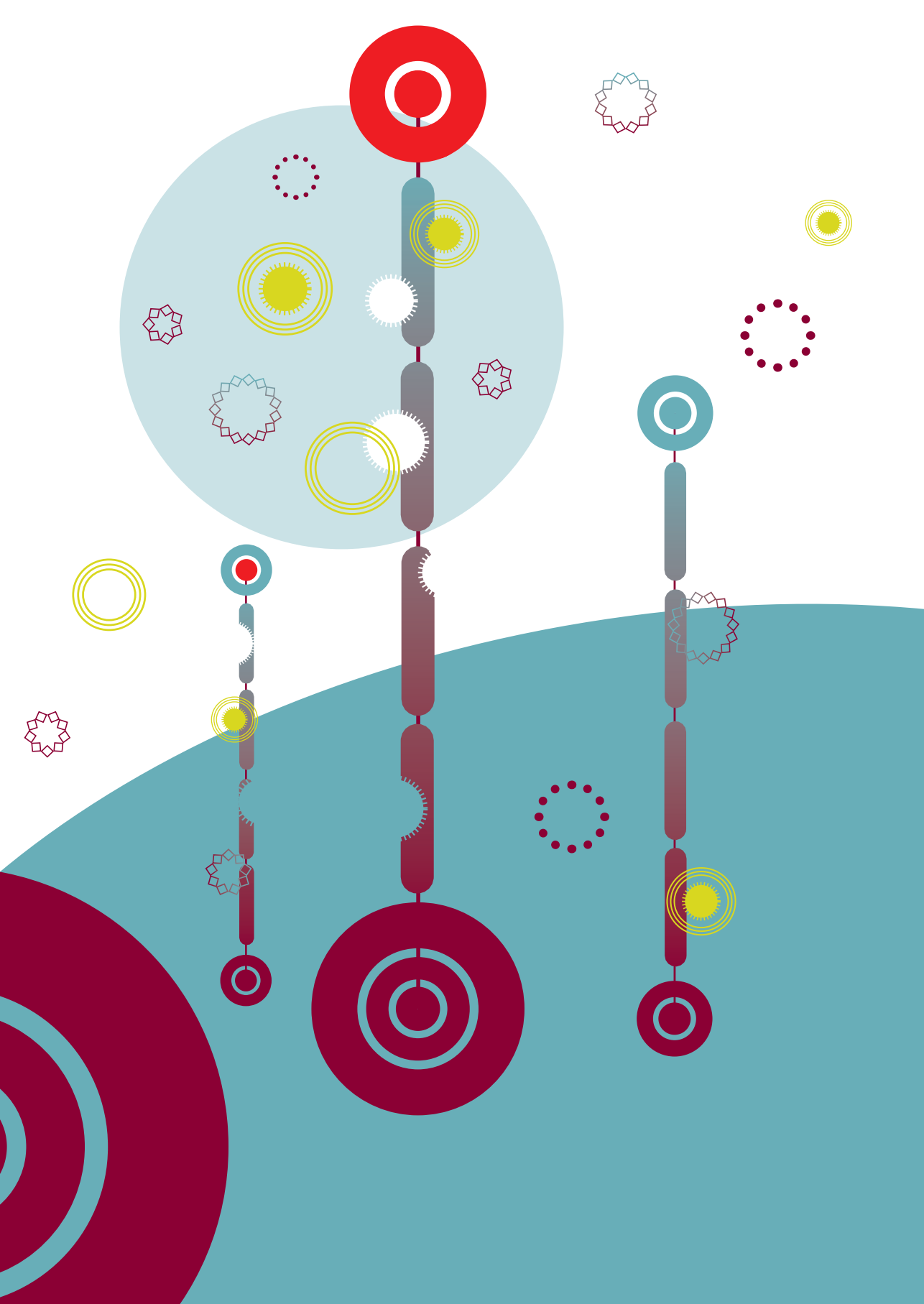
TABLE OF CONTENTS

Abbreviations		8
Chapter 1	Introduction	11
Chapter 2	Vitamin D3 supplementation and neurofilament light chain	33
Chapter 3	Vitamin D3 supplementation and genotypes	43
Chapter 4	Natural killer cells in multiple sclerosis	57
Chapter 5	Prognostic value of NK/T cell ratios	89
Chapter 6	The IL-2 – IL-2R pathway influences NK – T cell interaction	107
Chapter 7	Prognostic value of transitional B cells: a step towards systems immunology	119
Chapter 8	General discussion	141
	Impact paragraph	175
	Nederlandse samenvatting	183
	Curriculum Vitae	193
	Dankwoord	197

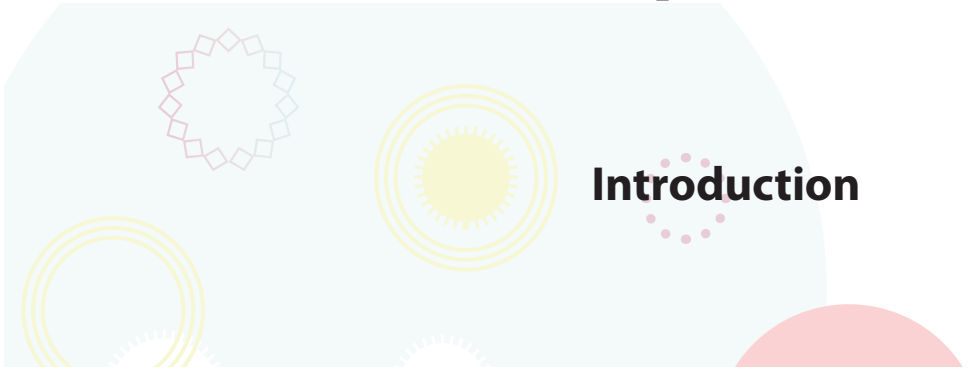
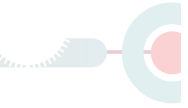
ABBREVIATIONS AND GLOSSARY

1,25(OH)₂D₃	1,25-dihydroxyvitamin D; calcitriol
25(OH)D	25-hydroxyvitamin D; calcidiol
ADCC	Antibody dependent cellular cytotoxicity
APC	Antigen presenting cell
B cell	Bone marrow derived lymphocyte
BBB	Blood-brain barrier
BMI	Body mass index
CD	Cluster of differentiation
CD4⁺ T cell	T helper cell
CD8⁺ T cell	Cytotoxic T cell
CD25	Interleukin-2 receptor alpha chain
CI	Confidence interval
CIS	Clinically isolated syndrome
CMV	Cytomegalovirus
CNS	Central nervous system
CSF	Cerebrospinal fluid
CUA	Combined unique active lesions
CYP	Cytochrome p450
CV	Coefficient of variation
DBP	D binding protein
DIS	Dissemination in space
DIT	Dissemination in time
DMT	Disease modifying treatment
EAE	Experimental autoimmune encephalitis
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
FACS	Fluorescence-activated cell sorting
GM-CSF	Granulocyte macrophage colony-stimulating factor
GWAS	Genome wide association study
HLA	Human leukocyte antigen
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IQR	Interquartile range
IM	Infectious mononucleosis
IU	International units
JC virus	John Cunningham virus

LC-MS/MS	Liquor chromatography-tandem mass spectrometry
METC	Medical ethical committee
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
N	Number
NEDA	No evidence of disease activity
NfL	Neurofilament light chain
NK cell	Natural killer cell
NKG2	Natural killer group 2
OR	Odds ratio
PBMC	Peripheral blood mononuclear cells
PPMS	Primary progressive multiple sclerosis
RRMS	Relapsing remitting multiple sclerosis
SNP	Single nucleotide polymorphism
SOLAR	Supplementation of Vigantol Oil versus placebo as add-on therapy in Rebif (Interferon Beta) treated patients with relapsing remitting multiple sclerosis
SOLARIUM	SOLAR immune modulating effects
SPMS	Secondary progressive multiple sclerosis
T cell	Thymus derived lymphocyte
Th	T helper cell
Treg	Regulatory T cell
VCA	Viral capsid antigen
VDR	Vitamin D receptor



Chapter 1



Introduction

Multiple sclerosis (MS) is an immune-mediated disease of the central nervous system (CNS), characterised by demyelination and axonal loss.[1] Currently, no cure for this disease exists, although therapies are becoming increasingly potent at reducing or even halting the disease progression.[2] Although the exact mechanism of disease has not yet been fully elucidated, we know of many factors that are involved in determining the risk of developing MS, as well as risk factors for a more severe disease progression. These factors can roughly be divided in environmental factors like vitamin D status,[3] infection with Epstein-Barr virus[4] and smoking,[5] and genetic factors, of which there are many, conferring a heritable risk of MS based on the degree of family relation.[6, 7] Research has pointed out a prominent role for lymphocytes as both aggravators and protectors in MS. Much research has gone into investigating the altered functions of these lymphocyte subsets, yet there is a paucity of evidence regarding the use of circulating lymphocyte composition to predict disease prognosis or chance of therapy success. In other words, although we understand how certain lymphocyte subsets are altered in MS, we do not understand what a certain composition of lymphocytes in the bloodstream means for the patient. As such, we investigated the clinical value of certain lymphocyte subsets in MS patients, exploring if they have potential to predict whether patients are at an increased risk for MS disease activity.

MULTIPLE SCLEROSIS

MS affects roughly 2.8 million people worldwide, with the highest prevalence rates in Western Europe and North America, e.g. the prevalence rate of MS in The Netherlands is 150 per 100,000 people.[8] MS disproportionately affects women more than men and this imbalance seems to be slowly increasing, now approaching a 3:1 ratio.[9] It can develop at any age, although it typically presents between the ages of 20 and 40 years old.[10] MS is the most common inflammatory disease of the CNS and also the most common cause of non-traumatic neurological disability in young adults.[11] Roughly, symptoms can be divided into prodromal symptoms and symptoms associated with clinical events. In the prodromal phase, *i.e.* before the first clinical event, patients show an increased risk of gastric symptoms (e.g. nausea, vomiting), urinary dysfunction, anorectal dysfunction, headaches and pain in the back, neck or limb.[12] Symptoms of clinical episodes may include sensory disturbances (like pain or numbness), muscle weakness, fatigue, vertigo, balance problems, gait disturbances, tremors, blurred vision, or any combination of these symptoms.[13] These symptoms, since they can persist, may influence a patient's employability, make it difficult to create and uphold social relationships, and even interfere with core social developments in adolescents, like sexuality and romantic relationships. As a result of the disability caused by MS, patients are more likely to suffer

from mental health problems such as depression and anxiety, as well as physical comorbidities like obesity.[14] Taken together, MS affects patients not only on a personal level, but also on a social and economic scale. The average cost related to MS in Europe is €22,800 per patient per year for mild cases, and up to €57,500 per patient per year in severe cases.[11]

MS can be categorised into several subtypes according to their clinical presentation.[15] The most common form of MS is relapsing-remitting (RR)MS, with roughly 85% of all MS patients adhering to this subtype. As the name implicates, this subtype is characterised by periods of clinical disability, called relapses, which are then followed by a remission phase. Recovery from the clinical disability may be complete, but often some level of disability remains permanently. The disease course of this subtype is illustrated in **Figure 1**, combined with the proposed underlying mechanisms of disease. As this is by far the largest group of MS patients, this subtype is the most investigated and best understood. Consequently, most therapies for MS are targeted towards RRMS.

Between 15 and 30% of all RRMS patients will enter a phase where relapses stop and are replaced by a gradual deterioration.[16] This secondary progressive (SP)MS subtype usually starts around the 5th or 6th decade of life[17] and is less well investigated and understood than the RRMS subtype.

A minority of MS patients (roughly 15%) never suffers any relapses, but instead shows a gradual deterioration pattern from the onset of disease. This primary progressive (PP) MS variant usually starts later than the mean onset of MS, typically around the age of 50.

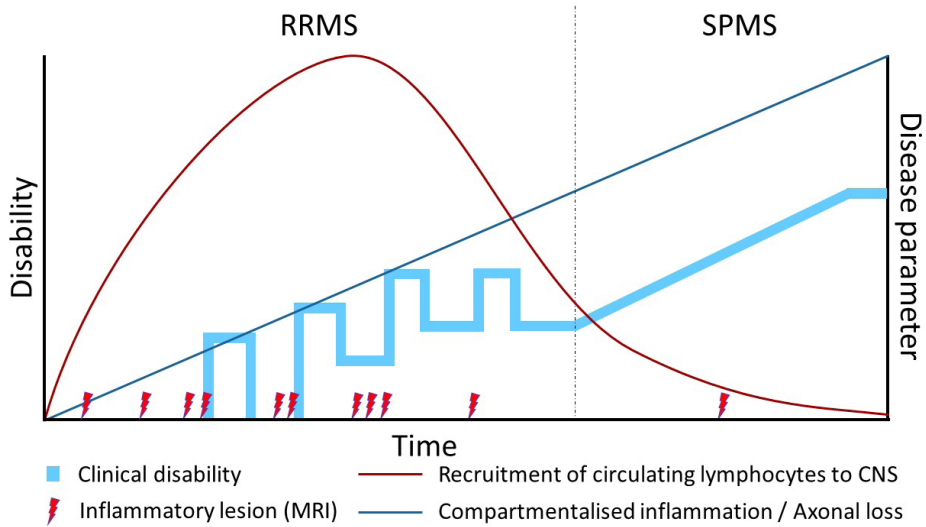


Figure 1. Overview of inflammatory processes during the lifetime of a MS patient. Recruitment of circulating lymphocytes and inflammatory lesions in the CNS may occur before any clinical disability is found, often named the 'prodromal phase'. Although the recruitment of circulating lymphocytes eventually diminishes and some patients enter a new phase of gradual deterioration (SPMS), inflammation and axonal loss keep increasing over time, with signs of active inflammation being found in post-mortem CNS samples of MS patients.

DIAGNOSIS AND FOLLOW-UP

With its extensive and diverse scale of symptoms, MS is challenging to diagnose based on clinical manifestations alone. As such, additional instruments are necessary to specify the diagnosis of MS and to monitor disease activity in MS patients. The diagnosis of MS relies on the McDonald criteria 2017, which is heavily based on magnetic resonance imaging (MRI) findings. This technique utilises a powerful magnetic field to visualise the CNS. MRI is especially potent in visualising the brain at a high resolution, which makes it an excellent tool for finding relatively small anomalies. MS shows a distinct pattern of damage and localisation, which greatly supports the diagnosis of MS.[18] As such, MRI is currently the gold standard for MS diagnosis and monitoring.[19, 20] To provide supportive evidence of an MS diagnosis, cerebrospinal fluid (CSF) analysis may be performed by determining the white blood cell count, protein concentration and IgG oligoclonal bands.[21]

The most important concepts in the diagnosis of MS are 'dissemination in space' (DIS) and 'dissemination in time' (DIT). DIS refers to multiple regions of the CNS being affected, based either on different symptoms (affecting different regions of the brain) or multiple lesions on MRI scans in different areas of the CNS. DIT implies that there must

be a temporal component to the anomalies, *i.e.* that symptoms or lesions must have appeared at different points in time. In an attempt to highlight recent or newer lesions on an MRI scan, it is common to administer contrast material intravenously during the scan. Active focal inflammation in the CNS will cause contrast leakage, thus confirming the recentness of the lesion, whereas old lesions do not show contrast enhancement. Finding both a contrast enhancing lesion (recent) and a non-contrast enhancing lesion (old) is therefore proof of DIT. Alternatively, a patient suffering from two separate clinical relapses, or repeating an MRI scan revealing new lesions, are both proof of DIT.

MRI is also used for the follow-up of diagnosed MS patients to assess whether new (contrast enhancing) lesions have appeared, which is an indicator of active disease. If multiple new lesions appear, this may prompt the treating neurologist to switch to a new treatment in an attempt to attenuate focal inflammatory activity in the CNS.

As useful and informative as MRI scans are, they come with a few downsides. For one, MRI scans are both expensive and time-consuming to obtain. Additionally, they require patients to lie still in an enclosed space for a prolonged amount of time. Patients, especially those suffering from claustrophobia, may find this process uncomfortable. As such, efforts are being made to develop follow-up tools which are less time-consuming for the patient, as well as less expensive to obtain. One of the results of these efforts is the discovery of the neurofilament light chain (NfL) biomarker in serum.[22] This marker is one of the degradation products of neurons in the CNS and can be used to measure the level of neuro-axonal damage in patients.[23-25] This marker is not specific to MS, but MS patients do indeed show higher baseline levels of NfL in serum, as well as a sharp increase during a relapse. On a patient level, the role for NfL in monitoring MS has yet to be determined, although early results appear promising.[23]

Currently, no curative treatment for MS exists, although much progress has been made in the last few decades regarding disease-halting therapies. Currently, every disease modifying therapy (DMT) used in MS is an immune-modulatory drug with varying degrees of efficacy and side-effects. As a general rule of thumb, therapies with higher efficacy tend to carry a higher risk of side effects.[2] Therefore, a clinician is tasked with the careful monitoring of clinical and radiological progression of the disease and, if the patient shows signs of progression, to carefully balance the suppression of disease activity and the tolerability of side effects. One difficulty in this process is the lack of prospective markers in MS, which means the clinician is unable to pre-emptively adjust for new disease activity. This means that intensifying treatment happens only after the patients has suffered new clinical disability.

DISEASE MECHANISM

MS is a notoriously complex disease with many interplaying factors. Despite its complexity, however, studies in the last decades have gradually uncovered several contributing factors which seem to be involved in the disease process. The current leading theory is that MS is the result of an unlucky combination of common environmental factors on the one hand, and common genetic factors on the other. These factors then cause anomalies in the immune system, leading to a CNS specific vulnerability to inflammation by enhancing pro-inflammatory factors and suppressing regulatory factors.[26]

Migration studies can be used to see how environmental factors and genetic factors relate to each other. Adult individuals that migrate from a low-risk area to a high-risk area do not seem to suffer an increased risk for MS. However, children born to these migrants in high-risk areas show similar risks of developing MS as native children from these high-risk areas. As such, environmental factors seem to take precedence over genetic factors for the risk of developing MS, which also implies that some fraction MS cases may be preventable if these environmental factors are addressed. These results also imply that the window of risk for developing MS lies in childhood years. Indeed, it has been suggested that individuals may be vulnerable up until the age of roughly 15 years old.[27]

Genetic factors

Genome wide association studies (GWAS) have identified a multitude of genetic variations which influence the risk of developing MS. Virtually all of these genetic variations are on genes involving immune cells or cell signalling pathways commonly used by immune cells.[7, 28] Although many factors contribute, the odds ratio (OR) associated with these variations are usually around 1.1-1.2. The genetic variant with the strongest association with MS is HLA-DRB1*15:01, a variant on the major histocompatibility complex type 2 (MHC class II), showing an odds ratio of >3 for heterozygotes and >6 for homozygotes.[29] Although its association has been shown, the mechanism by which the risk of MS is increased remains unknown. It is hypothesised that its role is through antigen presentation, although this theory remains controversial.[30]

Environmental factors

The most established environmental factors in MS are vitamin D deficiency, infection with Epstein-Barr virus (EBV), smoking, and obesity.[5]

Epidemiological studies showed a latitude gradient in the incidence of MS, with increasing latitudes being associated with a higher incidence of MS.[31] This phenomenon

was then linked with reduced sun exposure in higher latitudes, causing lower levels of UVB exposure and consequently, a reduced vitamin D generation. Although recent studies suggested that the latitudinal gradient has slowly been disappearing, vitamin D deficiency has since then been established as a risk factor for the development and disease course of MS.[3] Although its relevance has been shown epidemiologically,[32] genetically,[7] and clinically,[33, 34] the mechanisms by which vitamin D influences the immune system remain unknown. Currently, vitamin D is thought to play a homeostatic role in the immune system, tempering the pro-inflammatory milieu which is detrimental in MS. Recent efforts to treat MS patients with vitamin D supplements have shown mixed results, but may reduce MRI lesions when supplemented in high doses.[33, 35-37]

In order to further investigate the effect of vitamin D in MS, our group previously designed the SOLARIUM study to broadly explore phenotype and function of the adaptive immune response and compare it between patients receiving high-dose vitamin D₃ supplementation and patients receiving placebo. The study focussed on circulating T cells, which are thought to be the key players in the MS disease process (see also: 'Immune cells in multiple sclerosis'). SOLARIUM was a sub study of the international SOLAR study, which investigated the effect of high-dose vitamin D₃ supplementation on clinical outcome measures.[33] The SOLARIUM study approached Dutch participants of the SOLAR study for additional measurements, evaluating the composition of the T cell compartment before and after vitamin D₃ supplementation. The SOLARIUM study showed no difference in relative presence of T cell subsets between patients receiving high-dose vitamin D₃ supplementation and patients receiving placebo.[38] There was also no difference in pro- or anti-inflammatory cytokines produced by T cells. In conclusion, although vitamin D is heavily implied to reduce inflammatory activity in MS, it does not do so by altering the composition of the circulating T cell compartment or its cytokine profile.

Epstein-Barr virus (EBV) is a virus from the Herpes family and is most well-known for its associated disease, *mononucleosis infectiosa*, or Pfeiffer's disease. The virus primarily infects epithelial cells and B cells in the oropharyngeal region, after which it causes a latent infection by immortalising (memory) B cells.[39, 40] As such, EBV infections are lifelong and EBV may show signs of resurgence in immune-compromised individuals. Roughly 90% of the human population show signs of infection with EBV.[41]

The associations between EBV infection and MS are numerous. First, individuals with a history of *mononucleosis infectiosa* show a two-fold risk increase for developing MS.[42] Furthermore, EBV-specific antibodies are significantly higher in MS patients compared to the general population[43] and some studies have proposed anti-Epstein-Barr nuclear

antigen-1 (anti-EBNA-1) as a prognostic marker for disease activity,[44] although this remains controversial.[45] Finally, although roughly 90% of the general population is latently infected with EBV, data suggests that every single MS patient is infected with EBV, and that the EBV-seronegative MS patient does not exist.[46-48] These findings have led to some groups indicating EBV infection as a prerequisite for the development of MS.[40]

Recently, a large cohort study indeed revealed infection with EBV to be the leading cause of MS (OR 32).[4] As the leading role for EBV infection in MS becomes more established, interesting hypotheses regarding treatment and even prevention are starting to be formulated, although these require further understanding of the exact mechanism with which Epstein-Barr virus is necessary for development of MS.

Smoking and obesity are associated with many auto-immune diseases and are considered to be general promoters of inflammation in the body. As such, in susceptible individuals, these factors may tip the scales towards development of disease or increase the risk of relapses in MS patients.[49, 50] Indeed, smokers appear to be roughly 1.5 times more likely to develop MS compared to non-smokers.[51, 52] Similarly, one standard deviation increase in BMI was associated with a 41% increase in odds of MS (OR 1.4).[53]

An unlucky combination/interplay between these factors may lead to an inflammatory process, eventually causing MS. It is not completely understood how these factors alter the immune compartment to develop MS, or how these altered immune cells then cause MS exactly, but much is known about the involved cell types in multiple sclerosis and how they are altered in MS patients.

Immune cells in multiple sclerosis

The immune system can be roughly divided into a 'native' and an 'adaptive' component. The native component refers to cells that can immediately respond to a pathogen without requiring prior activation or sensitisation. The adaptive component of the immune system is slower to respond, as it requires other cells to activate it, but is generally more effective in eliminating specific pathogens. During an immune response there is a constant interplay between the innate and adaptive components of the immune system. The distinction between innate and adaptive immunity, suggesting the existence of two separate immune systems, therefore, is rather arbitrary. Since the adaptive immune system plays a prominent role in MS, the disease is classified as an auto-immune disorder, which entails that the immune system mistakenly attacks normal body cells.

Like most auto-immune disorders, the immunological disturbance causing MS cannot be attributed to a single cell subset. Instead, many immune cells seem to be involved in causing the inflammatory lesions that ultimately lead to clinical disability. A simplified overview of the involved cells is shown in **Figure 2**. Of these cells, lymphocytes (T cells, B cells and NK cells) appear to be the major relevant population, both as aggravators of inflammation and as protectors and regulators of the disease.[54]

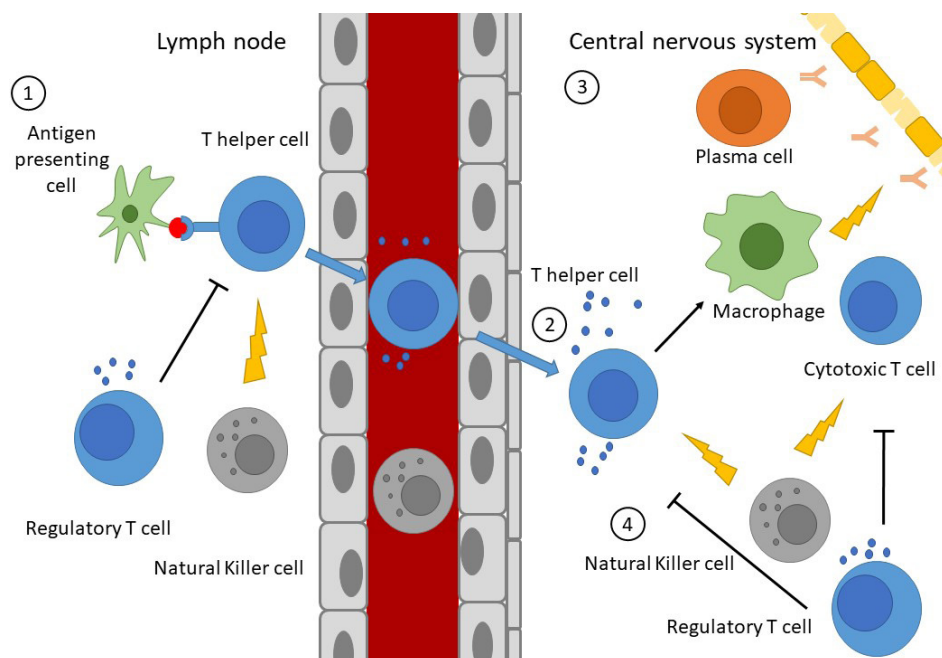


Figure 2. Simplified overview of involved cell types in MS. Antigen presenting cells (APCs) present a CNS-specific antigen to T helper cells in the cervical lymph nodes (1), thereby sensitising them to the host-protein and initiating the disease process. T helper cells migrate into the circulation and eventually past the blood-brain-barrier, where they produce pro-inflammatory cytokines (2), thus activating and sensitising effector cells for this CNS-specific antigen. In response to the cytokines produced by the T helper cell, B cells, T effector cells and macrophages enter the CNS area and are directed to attack the CNS (3), leading to myelin sheath damage of the neurons. Regulatory T cells at the lymph nodes and in the CNS inhibit the inflammatory process, while NK cells kill autologous activated T helper cells (4), although in MS these anti-inflammatory efforts appear flawed and insufficient to fully halt the inflammation process.

Historically, MS has been regarded for a long time as a disease mediated by $CD4^+$ T cells. [55] $CD4^+$ T cells producing interferon-gamma (IFN- γ), known as T helper 1 or Th1 cells, and later IL-17 producing $CD4^+$ T (Th17) cells and IFN- γ and GM-CSF producing Th17.1 cells have been regarded as the main effectors of inflammation.[55-57] These faulty Th1 and Th17 cells would be wrongly primed in the cervical lymph nodes (draining the CNS) and then later be reactivated in the CNS, where they would produce pro-inflammatory cytokines, thereby inducing local inflammation and breakdown of the blood-brain barrier. This pro-inflammatory environment and the breakdown of the blood-brain barrier

also leads to recruitment of more immune cells, including T helper cells, thus sustaining the inflammatory milieu.

Another subset of T cells, the regulatory T cell (Treg), is also associated with MS disease activity. Tregs naturally suppress inflammatory activity, for example by releasing anti-inflammatory cytokines, as well as suppressing the differentiation of Th1 and Th17 cells. In MS patients, Tregs seem to be less potent at limiting Th1 and Th17 cells, thus increasing and prolonging the inflammatory capacity of Th1 and Th17 cells.[58, 59]

T cells have been extensively investigated, among others in the context of prognosis. Indeed, some studies hint towards a prognostic value of certain T cell subsets. An increased presence of CD4⁺ cytotoxic T lymphocytes was associated with an increase in subsequent disability,[60] and an increase in the production of certain cytokines by CD8⁺ T cells was associated with a higher risk of MRI activity after several years.[61] However, these associations explained only parts of the disease risk, implying other factors influenced the risk of disease activity as well.

B cells are most well-known for their capacity to produce antibodies against antigens. Early research into the composition of cerebrospinal fluid (CSF) of MS patients showed that oligoclonal intrathecal IgG production is a sensitive hallmark of MS.[62] This revealed B cells to be involved in MS, although classic models suggest these as a rather passive effector population, which is activated through T helper cells and only then starts producing antibodies.[63]

This view was changed when more antibody independent functions of B cells were revealed. B cells are not only capable of producing antibodies, but can also secrete a multitude of pro- and anti-inflammatory cytokines, and are able to present antigens to other lymphocytes.[64] Furthermore, B-cell activating factor, which is associated with increased proliferation of B cells, is increased in MS lesions in the CNS.[65] Finally, B cells are the main host of latent EBV infection, which has been established as a key player in the development of MS.[40] As such, when the relevance of EBV infection in MS became more clear, B cells became increasingly interesting to investigate. Because of these growing appreciations, tests were conducted with B cell depleting therapies, particularly rituximab (chimeric anti-CD20), which is most commonly known for its use in rheumatic autoimmune diseases. Rituximab, and later ocrelizumab (humanised anti-CD20) showed good clinical benefit on both relapses and MRI activity.[66] Interestingly, anti-CD20 therapy depletes all B cells except for antibody producing B cells (plasma cells), which both showed B cells to be major players in the MS disease process, as well as their mechanism of action to be mainly antibody independent rather than their

antibody production for which they were originally known. Ever since, new MS disease models tend to place more emphasis on B cell involvement and tend to move away from the classic T cell centric view of MS.[54, 67, 68]

More recently, NK cells have been put forward as important players in the MS disease process. This growing appreciation is due to reports of NK cells, particularly the CD56^{bright} subset (known for its regulatory properties), being capable of killing (autologous) activated T cells.[69-71] This interaction highlighted an interesting and crucial interplay between NK cells and T cells, where NK cells hypothetically could limit MS disease activity by suppressing Th1 and Th17 cells.

The NK cell – T cell interaction was highlighted by the findings from clinical trials involving daclizumab, an anti-CD25 monoclonal antibody which targets the alpha chain of the IL-2 receptor (IL-2R).[72, 73] In short, IL-2 has a large variety of functions in the immune system. Most notably, it is essential for Treg survival, as well as for proliferation of activated T cells. The IL-2 receptor has varying affinities towards IL-2, based on its composition. The IL-2R may consist of an alpha chain (IL-2R α , CD25), a beta chain (IL-2R β , CD122) and a common gamma chain (γ_c , CD132).[74] A receptor composed of only IL-2R α shows a low affinity towards IL-2, whereas a receptor composed of IL-2R β and γ_c shows a medium affinity. A combination of all three subunits begets the highest affinity receptor. Although many cells express the medium-affinity IL-2R, the high affinity composition is mainly expressed by Tregs and activated T cells.[74]

This distribution of the high-affinity IL-2R caused some to hypothesise that blocking IL-2R α would cause activated T cells to stop proliferating, thereby reducing inflammation in MS. It was also thought that this reduction of activated T cell activity would be more potent than the reduction in regulatory capacity by blocking Tregs. Indeed, the resulting drug (daclizumab) showed clinical benefits in MS patient, reducing their relapses and MRI lesions. However, later studies revealed that blocking IL-2R α actually enhanced activated T cells *in vitro*, rather than suppressing them.[75] As such, an alternative explanation had to be found to explain the clinical benefits of blocking IL-2R α . Further research revealed a third population to be altered by daclizumab, namely NK cells.[76] By blocking the high-affinity IL-2R, less IL-2 was being used by T cells and thus, more IL-2 was available for cells with a medium-affinity IL-2R. NK cells seemed to benefit the most from this increased IL-2 pool and expanded up to 400%.[77] Although daclizumab was later discontinued due to side effects, the trials have shown us the importance of NK cells in the suppression of activated T cells.

The introduction of B cells and NK cells as central players in the MS disease process is relatively new. Still, much effort has gone into elucidating the interactions between these cell types and how they influence one another. These interactions are summarised in **Figure 3**. Noteworthy in this overview is that T cells, B cells and NK cells show both activating and suppressing capabilities, and both anti- and pro-inflammatory properties. This further highlights the intricate interplay between these cells and the difficulties in understanding the MS disease process. As more (seemingly contradictory) pathways are discovered and the interplay between lymphocytes becomes increasingly complex, some groups are suggesting a different approach to the MS disease model. A systems biology approach, whereby the entire system is analysed to find relative imbalances or disturbances, is becoming increasingly popular in several medical fields[78] and has been used to investigate different genetic influences in MS.[79, 80] However, studies using a systems biology approach for circulating white blood cells are at present lacking.

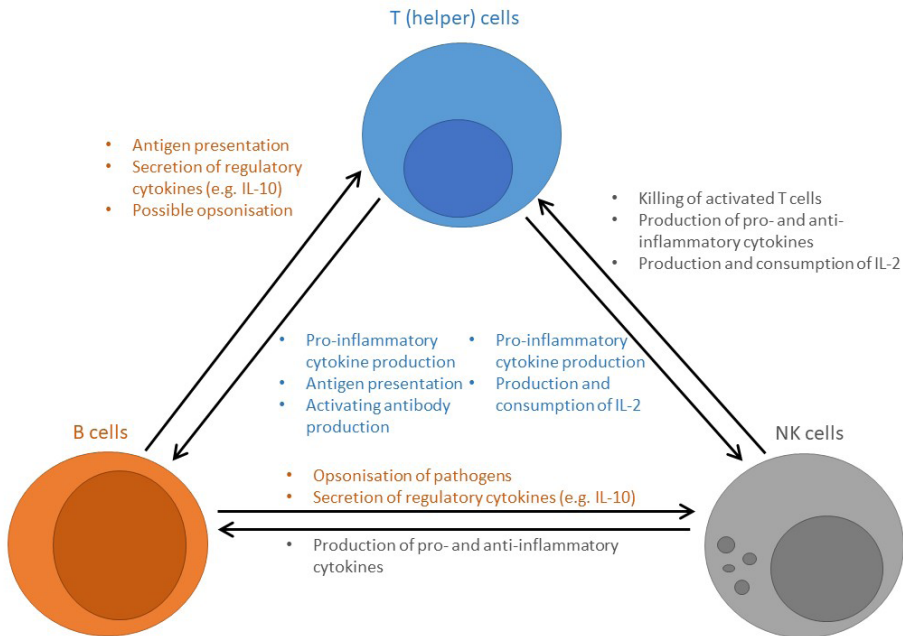


Figure 3. Simplified overview of the interaction between T (helper) cells, NK cells and B cells

The second question arising from this new information is more focused on the patient: now that we know the importance of these lymphocytes, how do they affect the MS disease process and can they be used to reveal patient specific information about their disease. For example: does a higher level of NK cells directly translate to a milder disease course, considering they should suppress activated T cells? Some studies, especially those

related to pharmaceutical treatments, give us an insight in the opportunities that may lie here. One example has already been mentioned, namely the studies involving daclizumab.[75] Indeed, these studies reported CD56^{bright} NK cells to suppress the inflammatory activity of MS and, as such, be beneficial in MS. One Australian group expanded on this by reporting that patients with a higher relative presence of CD56^{bright} NK cells are less likely to show signs of MRI activity after 2.5 years follow-up.[81] This hints towards the prognostic potential of NK cells, although the results are yet to be replicated. In any case, an increase in CD56^{bright} NK cells appears to be favourable for the patient.

In a similar vein, studies involving therapies like fingolimod and interferon- β -1a have attributed at least some of their therapeutic effects to the increase in a specific B cell subtype: the transitional B cell.[82-85] This immature B cell subset shows anti-inflammatory properties, such as the production of interleukin-10.[86] This makes it increasingly likely that transitional B cells have a positive effect on MS and that an increase in transitional B cells is beneficial for the patient. However, transitional B cells have not directly been investigated for their prognostic potential.

THIS THESIS

The involvement of lymphocytes in MS has been investigated extensively, and many of the new disease modifying therapies are increasingly focused on single lymphocyte subsets or functions. As such, MS research is mainly focused on further understanding the changes and aberrations of lymphocyte composition and function in MS patients. However, very little is known about what these changes in lymphocytes mean for MS patients. In other words, the translation of these changes and aberrations of lymphocytes to clinical effects and consequences is relatively underappreciated. Some of the current challenges in MS treatment are prediction of therapy success and prediction of disease activity. One could hypothesize that the composition of the immune system may show clues as to which lymphocytes are most affected and, in turn, which cell-based therapies would have the highest chance of success. Similarly, if lymphocytes show signs of aberration before the moment this disease activity becomes clinically manifest, one could use regular blood tests to evaluate the chances of developing disease activity and then altering current therapy accordingly, thereby possibly preventing disease activity altogether.

Aim of the thesis

We aim to investigate the clinical effect of altered proportions of lymphocyte subsets in the blood, looking to explore their potential as prognostic/predictive markers for

disease activity in order to create a better understanding of the MS disease process, as well as first steps towards tools to help clinical decision making, e.g. which DMT to use or when to intensify treatment, ultimately aiming to reduce clinical disability in RRMS patients.

Outline of this thesis

For this thesis, we studied the data of the SOLARIUM study. Using the data gathered regarding the immune composition of the participants, combined with the outcome markers measured after 48 weeks in the SOLAR study, we can explore the relation between the composition of the lymphocyte compartment and disease activity. Considering the SOLARIUM study originally investigated the effects of vitamin D₃ supplementation on the immune system, it is prudent to first expand our understanding of the interaction between vitamin D and our outcomes of interest.

As such, in **Chapter 2** we evaluate the relation between vitamin D levels and NfL levels in serum, in order to find out whether NfL related findings should be corrected for vitamin D₃ levels. Additionally, we explore whether certain patient characteristics, like BMI or age, affect vitamin D₃ levels in our patient cohort. Then, in **Chapter 3**, the influence of several vitamin D-related genetic variants that are associated either with a poor vitamin D status or with a higher risk of MS are investigated to explore their potential relationship with vitamin D metabolism and supplementation.

After investigating the effects of vitamin D on our measurements of outcome, we then aim to explore the relation between the lymphocyte compartment and disease activity. Given that the relation between T cells and disease activity has been investigated extensively, we mainly focused on NK cells and B cells in our exploratory research.

We continue in **Chapter 4**, where we review the role of NK cells in MS, as well as their relation with environmental factors and their involvement in the therapeutic effect of currently used treatments, in order to establish and underline their supposed involvement in the MS disease course. Following the potential outlined in this narrative review, we evaluate the clinical application of NK cells in **Chapter 5**, where we evaluate the prognostic value of NK/T cell ratios for disease activity in MS, as measured by relapses, MRI activity and NfL levels. Then, in **Chapter 6**, we take a closer look at the mechanisms behind the NK cell – T cell interaction by investigating the influence of the IL-2/IL-2R pathway on NK/T cell ratios. Here, we use serum measurements, expression patterns on T cells and genetic variants to confirm the IL-2/IL-2R pathway as the main controlling agent of the NK cell – T cell interaction, as suggested by the daclizumab trials.

In **Chapter 7**, another lymphocyte compartment, the B cell compartment, is investigated in a similar manner to the NK/T ratio. We explore their prognostic value, but also their relation with EBV related serology, as B cells are the main host for the MS associated virus. Afterwards, all prognostic factors which have been reported in this thesis were put together in a model in order to address the collective contribution to the prognosis of MS disease activity. Finally, we summarize and discuss our findings in **Chapter 8**.

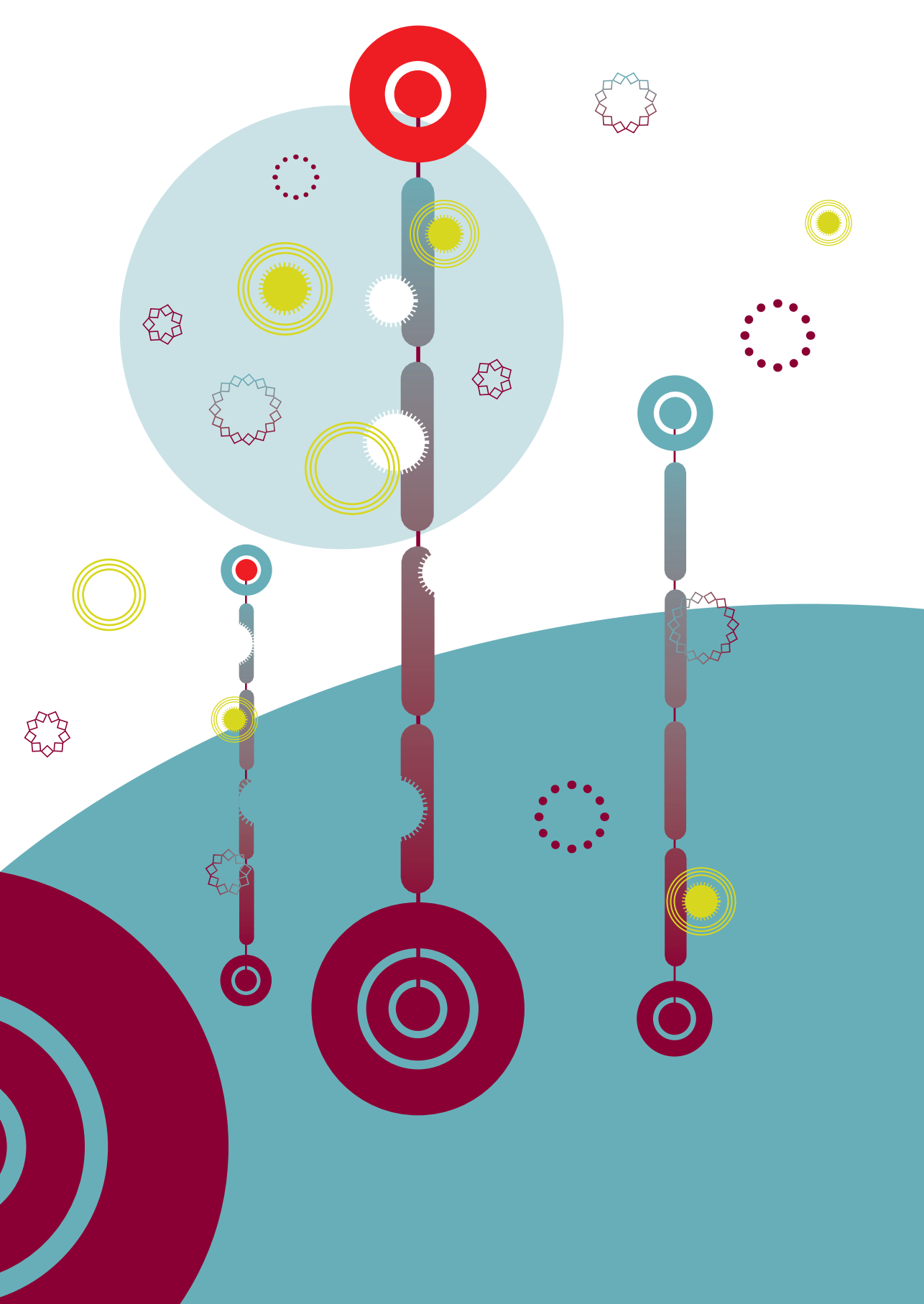
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Chapter 2



Vitamin D₃ supplementation and neurofilament light chain

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ABSTRACT

Objectives: Low circulating vitamin D levels are associated with an increased risk of active MRI lesions and relapses in several cohorts with relapsing remitting multiple sclerosis (RRMS). Randomized controlled supplementation trials are, however, negative on their primary endpoints, while secondary MRI endpoints suggest anti-inflammatory effects. Circulating levels of neurofilament light chain (NfL) are a biomarker of disease activity in RRMS. We explored whether 48-week high-dose vitamin D₃ supplements were associated with lower circulating NfL levels.

Materials & Methods: Of N = 40 Dutch interferon beta-treated participants with RRMS of the SOLAR trial, plasma samples at baseline and 48-week follow-up were available. Of these participants, N = 24 were supplemented with 14 000 IU/d vitamin D₃ and N = 16 with placebo. Twenty-five hydroxyvitamin D₃ (25(OH)D₃) levels were measured with LC-MS/MS, and NfL levels were measured in duplicate with Simoa.

Results: Serum 25(OH)D₃ levels at 48 weeks were increased in the vitamin D₃ when compared to placebo group (median level 281 [IQR 205-330] vs 72 [39-88] nmol/L; P < .01). NfL levels at 48 weeks did not differ between the treatment groups (median level 25.4 [IQR 19.6-32.2] vs 25.3 [17.9-30.1] pg/mL; P = .74). Higher week 48 NfL level showed a trend toward association with a higher risk of combined unique active lesions on the week 48 MRI scan (OR 2.39 [95% CI 0.93-6.12] for each 10 pg/mL increase; P = .07).

Conclusions: Supplementation of high-dose vitamin D₃ for 48 weeks was not associated with lower NfL levels. This study does not support an effect of vitamin D₃ on this biomarker of neuro-axonal injury.

BACKGROUND

Low circulating levels of 25-hydroxyvitamin D (25(OH)D) are associated with a higher risk of MRI lesions and relapses in several cohorts with relapsing remitting multiple sclerosis (RRMS).[1] Randomized controlled trials on high-dose vitamin D₃ supplements thus far missed their primary endpoints,[2-8] yet some suggested benefits on secondary MRI endpoints.[2, 4, 7] In the SOLAR trial, we did not detect an increased proportion of interferon β 1 α -treated participants with RRMS reaching no evidence of disease activity (NEDA-3) in the high-dose vitamin D₃ supplements vs placebo arm, yet observed a lower number of combined unique active lesions (CUA) and a reduced T2 volume increase on the 48-week MRI scan.[4] In a sub-study among Dutch SOLAR participants (SOLARIUM), we observed no effect of vitamin D₃ supplements on general markers of lymphocyte homeostasis,[9] but did observe a reduction in circulating anti-Epstein-Barr virus Nuclear Antigen-1 (EBNA-1) IgG antibodies.[10] Anti-EBNA-1 IgG levels have been identified as a biomarker for disease activity of RRMS in several but not all cohorts.[11] Similarly, a Norwegian trial on average-dose vitamin D₃ supplements vs placebo in RRMS did not show an effect on clinical disease parameters,[5] but showed a reduction of anti-EBNA-1 IgG.[12] In the latter study, blood levels of neurofilament light chain (NfL) were measured, but did not show a significant variation.[13] Blood NfL levels have been identified as a biomarker of adverse disease outcomes in RRMS.[14-16] To further explore a possible benefit in taking vitamin D₃ supplements for people with MS, we measured NfL levels in participants of the SOLARIUM cohort. Since serum levels of 25(OH)D were measured with a radioimmunoassay in the SOLAR trial and exceeded the detection limit of the assay, we measured in addition plasma 25(OH)D₃ levels with liquid chromatography-mass spectrometry.

METHODS

Study design

Regrettably, no biomaterials were available from the SOLAR trial to study NfL levels. Therefore, the effect of vitamin D₃ supplements on blood NfL levels was explored as post hoc measurements in the Dutch SOLARIUM study,[9, 10, 17] a sub-study of the SOLAR trial (NCT01285401).[4] In short, participants were interferon β 1 α -treated RRMS patients, with a first clinical event within the previous 5 years, but no relapse 30 days before inclusion. Patients were randomized to a placebo or vitamin D₃ group following procedures described elsewhere.[4] Patients in the vitamin D₃ group received cholecalciferol drops (Vigantol Oil, Merck) 7000 IU/d in the first 4 weeks, followed by 14 000 IU/d up to week 48. As part of the SOLAR study protocol, presence of CUA at the week 48

MRI scan (new or enlarging T2 lesions when compared to the baseline MRI scan and/or gadolinium-enhancing MRI lesions, Y/N) was registered.[4] Written informed consent was acquired, and the SOLARIUM study was approved by the Ethical Committee METC-Z (Heerlen, the Netherlands).

Vitamin D and NfL measurements

Plasma samples were collected at baseline and week 48 and stored at -80°C . Plasma levels of 25(OH) D_3 were measured using liquid chromatography-mass spectrometry/ mass spectrometry (LC-MS/ MS) following earlier published procedures.[18, 19] Coefficients of variation (CV) were 7.4% at 36 nmol/L, 4.0% at 88 nmol/L, and 3.1% at 124 nmol/L, respectively. Lower limit of quantification was 1 nmol/L. From the SOLAR dataset, serum 25(OH) D levels were available as determined using the DiaSorin immunoassay method. [4] Plasma levels of NfL were determined with a single molecule array (Simoa) analysis in duplicates following earlier published procedures.[16] Median intra-assay CV was 5.1% (interquartile range 3.0%-7.1%; min-max range 1%-18%).

Statistical analysis

Statistical analyses were conducted with GraphPad Prism (GraphPad Software) and SPSS (SPSS Inc, version 20.0). Descriptive statistics are provided for continuous variables as median and corresponding interquartile range (IQR), for proportions as number with corresponding percentage. Non-parametric paired (Wilcoxon signed ranks) or non-paired (Mann-Whitney U) statistics were applied, and correlations were tested with the Spearman correlation coefficient. Prediction of 25(OH) D_3 levels reached in the vitamin D_3 arm by BMI corrected for age was analysed in a linear regression model, and prediction of week 48 CUA by NfL levels corrected for age was analysed in a logistic regression model. A p-value $<.05$ was considered significant.

RESULTS

Of the SOLARIUM sub-study, material could be retrieved of $N = 40/53$ participants (75%), of which $N = 24/30$ (80%) were allocated to the vitamin D_3 arm, and $N = 16/23$ (70%) to the placebo arm. These two groups were balanced for most variables, except a larger proportion of placebo-treated patients experiencing 2 or more relapses the previous 2 years (**Table 1**). The characteristics of this subgroup were not different from those of the total SOLARIUM cohort (data not shown).

Plasma 25(OH) D_3 levels were significantly elevated in the vitamin D_3 group, with a median level of 81.80 nmol/L (IQR 58.56-123.17) at baseline and 281 nmol/L (IQR 205.29-

330.40) at week 48 ($P < .01$). The serum levels in the placebo group remained stable, 76.7 nmol/L (IQR 53.58-91.42) vs 71.9 nmol/L (38.95-87.87), respectively ($P = .42$). Plasma 25(OH)D₃ levels as measured with LC-MS/MS were overall higher than serum 25(OH)D levels reported with the radioimmunoassay (median 91.55 nmol/L [IQR 62.5-161.78] vs 70 nmol/L [IQR 46-119.75], resp., $P < .01$), but showed a strong positive correlation within the measurable range (<250 nmol/L; $R = .917$, $P < .01$). Participants in the vitamin D₃ arm with a higher BMI had numerically lower 25(OH)D₃ levels, BMI < 25 median 304.1 nmol/L (IQR 276.61-334.33) vs BMI ≥ 25 median 237.9 nmol/L (IQR 158.62-287.42; $P = .143$). In these participants, 25(OH)D₃ levels reached were best predicted in a linear model (overall model fit $R^2 = .330$; $P = .02$) containing BMI category ($\beta -96.37$ [95% CI -162.73 to -30.00]; $P < .01$) and age ($\beta 4.54$ [95%CI -0.431 to 9.503]; $P = .07$).

Table 1. Cohort characteristics

	Placebo (N=16)	Vitamin D ₃ (N=24)
Female sex (N [%])	N=11 (69%)	N=17 (71%)
Age (years; Med [IQR])	40 (33-47)	37 (31-42)
Duration (months; Med [IQR])	5.7 (4.1-8.2)	6.5 (4.5-11.1)
≤ 1 relapse previous 2 years (N [%])	N=2 (13%)	N=9 (37%)
Time since last relapse (months, Med [IQR])	8.0 (6.6-9.3)	6.5 (4.0-9.9)
BMI ≥ 25 (N [%])	N=9 (56%)	N=12 (50%)

Average plasma NfL levels were at baseline and week 48 equally low in placebo (median level 32.8 pg/mL [IQR 24.58-34.4] vs 25.3 pg/mL [IQR 17.85-30.05], resp.) and vitamin D₃ arm (median levels 23.7 pg/mL [IQR 19.8-33.65] vs 25.35 pg/mL [19.55-32.13], resp.) without any significant or relevant differences between timepoints or treatment arms (Figure 1A). Baseline NfL levels correlated positively with age (Spearman $R = .356$; $P = .03$), but not with other baseline variables. Participants with CUA ($n = 8$) on their 48-week MRI scan had numerically slightly higher week 48 NfL levels when compared to participants without CUA ($n = 29$, median 28.2 [IQR 22.7-38.4] vs 22.6 [18.3-28], resp., $P = .12$) (Figure 1B). Corrected for age, higher NfL levels corresponded with a trend toward an increased risk of CUA (OR 2.39 [95% CI 0.93-6.12] for each 10 pg/mL increase; $P = .07$). Of the eight participants with CUA, six participants had $N = 1$ new T2 lesion on their 48-week MRI scan, one participant had $N = 2$ new T2 lesions (NfL level 26.5 pg/mL), and one participant had $N = 3$ new T2 lesions (NfL level 40.8 pg/mL). No participants had gadolinium-enhancing MRI lesions on the week 48 MRI scan.

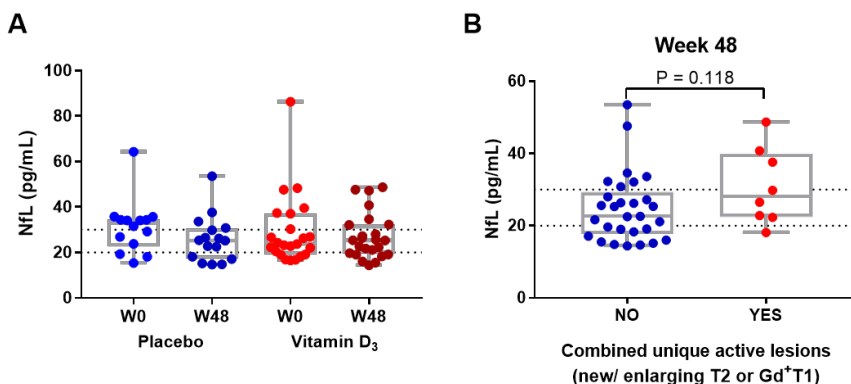


Figure 1. A) NfL levels stratified for treatment arm and timepoint (baseline and week 48). Man Whitney U test showed no significant differences between any of the timepoints. B) Week 48 NfL levels stratified for presence of combined unique active lesions (Y/N). The P value shows the Mann Whitney U test. The dashed lines indicate the cut-off levels for 20 and 30 pg/mL.

DISCUSSION

High-dosed vitamin D₃ supplements in interferon beta 1a–treated RRMS did not result in lower plasma NfL levels. This adds to the negative findings of this intervention on clinical endpoints in this study.[4] It is also in line with another study, where moderate doses of vitamin D₃ supplements did not affect both clinical and NfL endpoints.[5, 13] These negative results are paralleled by a positive effect of supplements on MRI endpoints. [4, 7] Therefore, despite these data, vitamin D₃ supplementation in RRMS remains a controversial issue.

Median blood NfL levels in our sample were in the same range as reported for instance in the FREEDOMS (27.1 pg/mL) and TRANSFORMS (24.1 pg/mL) studies by the same laboratory.[16] However, our data set showed only two samples with NfL levels exceeding 60 pg/mL (baseline 86.3 and 64.3 pg/mL, 2.53 and 3.42 months, respectively, after a clinical attack), where the range of NfL levels in these previous studies was up to 372.7-589.5 pg/mL.[16] These data support the general notion of inclusion of patients without severe inflammatory activity in the SOLAR trial, which may be attributable to the introduction of many new disease-modifying therapies at the time of the SOLAR study.[4] Treatment with interferon-β-1α did result in a drop of circulating NfL in an earlier study.[20] Since the association between MRI activity and 25(OH)D levels was also abrogated by treatment with interferon-β in this same cohort,[21] an effect of vitamin D₃ supplements on plasma NfL levels may be masked by this treatment. However, the trend toward an association with the presence of combined unique active lesions on week 48 MRI suggests that NfL may also reflect relevant inflammatory disease activity in

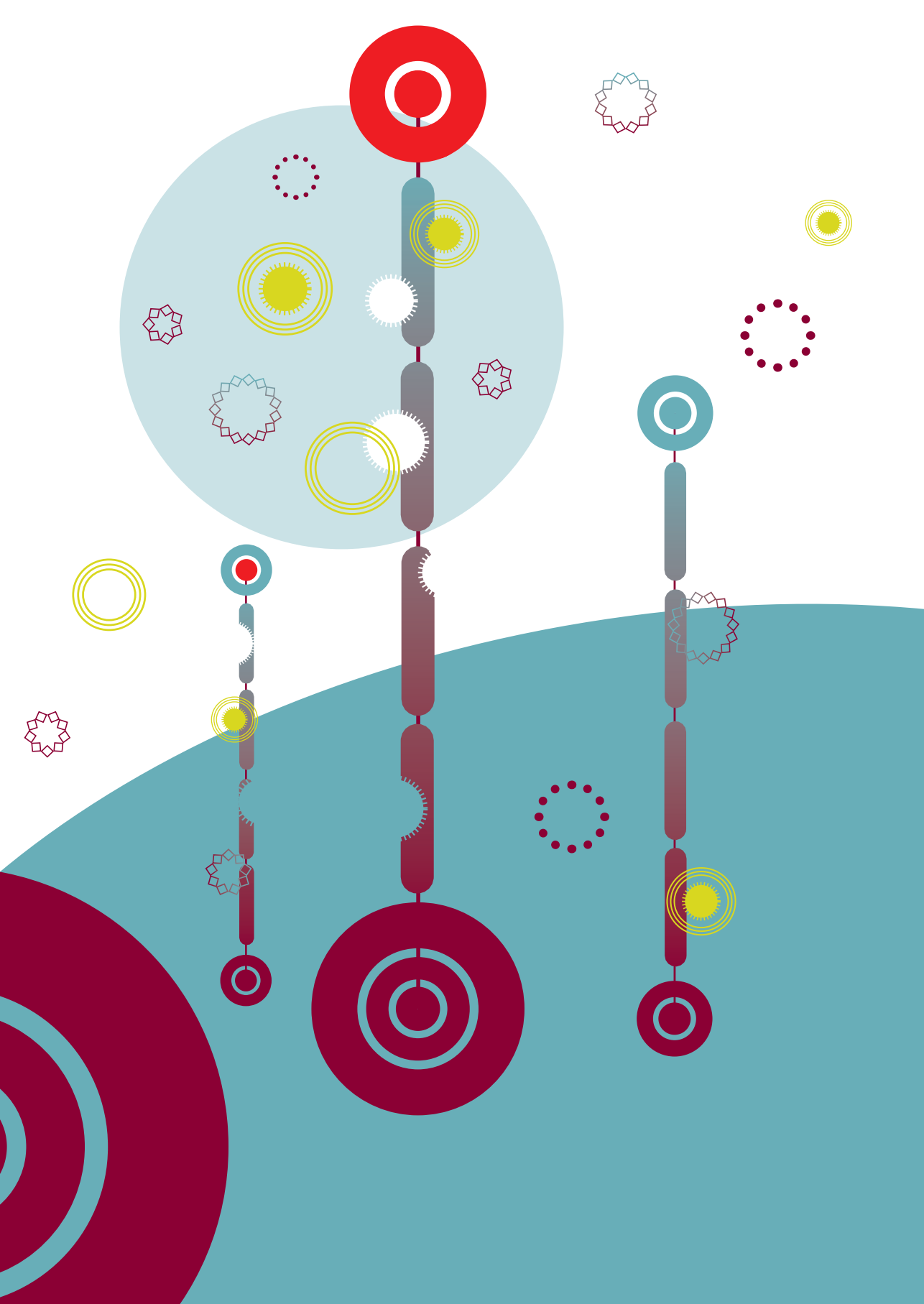
these low ranges. There are some limitations of the data presented. A relevant effect of vitamin D3 supplements on blood NfL levels in cohorts with a more pronounced disease activity cannot be excluded. Most importantly, the small sample size and exploratory nature of analyses performed carry a high risk of false-negative results. Regrettably, no biomaterials of the parental SOLAR trial are available for analysis. The samples of the SOLARIUM cohort were therefore the best to explore any effect of high-dosed vitamin D₃ supplements in the context of this trial. The Dutch MS cohort is likely to show a small seasonal fluctuation of 25(OH)D₃ levels.[22] This fluctuation was not taken into account in this study, but is unlikely to have substantially confounded the large variation in 25(OH)D₃ levels induced by highly dosed vitamin D₃ supplements. The strengths of our data include the double-blind, placebo-controlled, and randomized design, and the extensive monitoring of participants during follow-up in a clinical trial.

Ongoing studies on vitamin D₃ supplements in early MS and clinically isolated syndrome (CIS)[1] may be able to retrieve samples from a larger cohort of participants with more active disease for NfL measurement. Biomarkers as NfL and MRI are important to reveal biologically relevant effects of vitamin D₃ supplements. These may be insufficiently captured in clinical endpoints in relatively small trial cohorts. Upcoming randomized controlled trials and subsequent meta-analyses of published trials may bring an end to the controversy surrounding vitamin D₃ supplementation in MS.

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Chapter 3



Vitamin D₃ supplementation and genotypes

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ABSTRACT

Introduction: A poor 25-hydroxyvitamin D (25(OH)D) status is a much replicated risk factor for developing multiple sclerosis (MS), and several vitamin D-associated single nucleotide polymorphisms (SNPs) have been associated with a higher risk of MS. However, studies on the benefit of vitamin D supplementation in MS show inconclusive results. Here, we explore whether vitamin D-associated SNPs and MS risk alleles confound serological response to vitamin D supplementation.

Methods: 34 participants from the SOLARIUM study consented to genotyping, of which 26 had vitamin D data available. The SOLARIUM study randomised relapsing-remitting MS patients to placebo or 14,000 IU vitamin D₃ for 48 weeks. Participants were categorised as either 'carriers' or 'non-carriers' of the risk allele for 4 SNPs: two related to D binding protein (DBP) and associated with lower 25(OH)D levels (rs4588 and rs7041), and two related to vitamin D metabolism enzymes CYP27B1 and CYP24A1 and associated with a higher risk of MS (rs12368653; rs2248359, respectively). 25(OH)D levels were determined at baseline and after 48 weeks.

Results: The DBP-related SNPs showed no difference in 25(OH)D status at baseline, but carriers of the rs7041 risk allele showed lower 25(OH)D-levels compared to non-carriers after 48 weeks of supplementation (median 224.2 vs. 332.0 nmol/L, $p=0.013$). For CYP related SNPs, neither showed a difference at baseline, but carriers of the rs12368653 risk allele showed higher 25(OH)D-levels compared to non-carriers after 48 weeks of supplementation (median 304.1 vs. 152.0 nmol/L, $p=0.014$).

Discussion: Vitamin D-related SNPs affect the serological response to high-dose vitamin D supplementation. The effects on more common doses of vitamin D, as well as the clinical consequence of this altered response, need to be investigated further.

INTRODUCTION

Although the exact aetiology of multiple sclerosis (MS) remains unknown, many factors, both genetic and environmental, have been identified as contributors to the disease. Low levels of circulating 25-hydroxyvitamin D (25(OH)D) are a much replicated risk factor for both the pathogenesis[1-3] and disease course of MS.[4-6] Genome wide association studies show that some MS risk alleles are situated in or near genes coding for enzymes related to vitamin D metabolism.[7] Indeed, MS patients tend to have a genetic background that is associated with lower 25(OH)D levels,[8] and low 25(OH)D levels are in turn associated with a higher risk of MS disease activity.[4-6]. Many studies have investigated the supplementation of vitamin D in order to correct this lower level of 25(OH)D. However, these studies show mixed results, with some showing no clinical benefit,[9, 10] while others report a protective effect of high-dose vitamin D supplementation.[11-14]

A study by Bhargava et al. showed a attenuated elevation of circulating 25(OH)D levels after vitamin D supplementation in MS patients, compared to healthy controls.[15] Recently, Graves et al. reported that vitamin D related single nucleotide polymorphisms (SNPs) associated with lower 25(OH)D levels, associate with an increased relapse risk in paediatric MS patients.[16] However, it remains unclear whether these genetic variations are confounding factors in clinical supplementation studies. In this respect, elucidating the effects of vitamin D-related genetic background on supplementation studies is important for the interpretation of the results from these studies. Therefore, we have evaluated the influence of several vitamin D metabolism-related polymorphisms on the serological response to high dose vitamin D₃ supplementation, in a cohort of interferon-beta treated relapsing-remitting MS patients from the SOLARIUM study.[17]

METHODS AND MATERIALS

Patients

This study is a post-hoc extended analysis of the SOLARIUM study, which was a sub-study of the SOLAR study. The SOLAR study evaluated disease activity in interferon beta-treated RRMS patients using high dose vitamin D₃ supplements compared to placebo. The SOLARIUM study investigated the effect of high dose vitamin D₃ supplementations on the immune system composition. After randomisation, participants received either interferon-beta and a placebo or interferon-beta and vitamin D₃ supplements (cholecalciferol, Vigantol®Oil, Merck KGaA, Darmstadt, Germany) of 7,000 IU daily for 4 weeks, followed by 14,000 IU daily up to week 48. In- and exclusion criteria for the SOLAR and SOLARIUM studies are described elsewhere.[13, 17]

Originally, genetic markers regarding vitamin D status were planned to be gathered of all participants in the SOLAR study.[18] Ultimately, however, this analysis was not performed. Therefore, we acquired ethical approval to approach Dutch participants of SOLAR to acquire written informed consent for genetic analysis (Ethical Committee METC-Z, 11-T-03; Heerlen, the Netherlands). This resulted in 34 of 53 participants of the SOLARIUM study to provide consent for genetic analysis.

Vitamin D measurements

Plasma samples were collected at baseline and week 48 and stored at -80°C . Plasma levels of 25(OH)D were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) following earlier published procedures.[19, 20] Coefficients of variation (CV) were 7.4% at 36 nmol/L, 4.0% at 88 nmol/L, and 3.1% at 124 nmol/L, respectively. Lower limit of quantification was 1 nmol/L.[21] From the SOLAR dataset, serum 25(OH)D and 1,25(OH)D levels were available as determined using the DiaSorin immunoassay method, with an upper limit of 250 nmol/L.[13]

Of all participant providing consent for genetic analyses, $N=20/34$ were allocated to the high-dose vitamin D₃ supplementation arm (**Figure 1**). Of these 20 participants, three had no biomaterial available for 25(OH)D analysis by the LC-MS/MS method. For these three participants, 25(OH)D levels were available as determined with the immunoassay as used in the SOLAR trial.[13] Since these 3 individuals had week 48 levels of 25(OH)D below the assay upper detection limit (<250 nmol/L), and we earlier reported a good correlation between the immunoassay and LC-MS/MS within this range ($R=0.917$, [21]), we converted for these individuals their 25(OH)D status using a linear model.

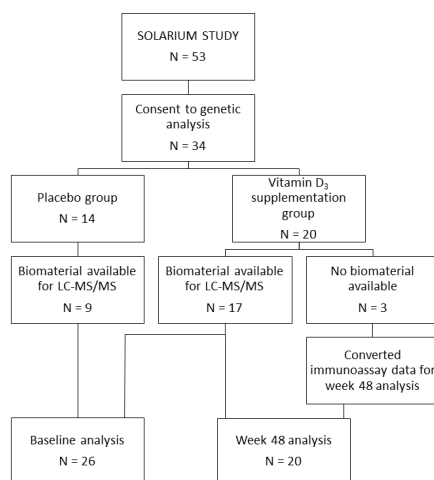


Figure 1. Flowchart depicting availability of data for our analyses. LC-MS/MS: liquid chromatography-tandem mass spectrometry.

Defining SNPs of interest

We selected for our study two SNPs that are missense variants in the gene encoding vitamin D-binding protein (DBP, or Group Component) (rs4588 and rs7041), as well as one SNP near the CYP27B1 gene (rs12368653) and one SNP near the CYP24A1 gene (rs2248359), which are all related to vitamin D metabolism. SNPs rs12368653 and rs2248359 have been identified as MS risk-alleles in a recent large GWAS.[7] The DBP-associated SNPs rs4588 and rs7041 have been reported as top-hits in several GWAS studies on determinants of 25(OH)D status in healthy and diseased cohorts.[22-24] Although not all GWAS studies support these specific DBP SNPs, DBP has been identified in all GWAS as a locus influencing 25(OH)D levels.[25, 26] Since the rs7041 and rs4588 SNPs were also associated with response to low-dose vitamin D supplementation in non-MS cohorts,[27-29] we included these SNPs in our analysis. We genotyped rs2248359, the others were imputed using the RICOPILI pipeline and the Michigan server (HRC-reference panel). Genotyping was performed using extracted DNA from buffy coats. This DNA was analysed on the Infinium PsychArray-24v1.3_A1 BeadChip (Infinium array technology) and the polymorphisms of interest were defined through standard protocols.

Statistical analysis

SPSS software (IBM SPSS, version 25.0. Chicago, IL) was used to evaluate the differences in vitamin D status between carriers of risk alleles versus non-carriers. Normality of data was assessed by visual inspection of normality plots, as well as the Shapiro-Wilk test. To compare baseline characteristics, Mann-Whitney U tests were used to compare continuous data, while chi square tests were used to compare dichotomous data. Because of sample sizes, a Mann-Whitney U test was used to compare 25(OH)D levels between carriers and non-carriers. Previously, we have shown BMI to be a confounder in vitamin D levels in MS patients. Therefore, we have marked all patients with a BMI ≥ 25 kg/m² in our analyses. A p-value of <0.05 was considered statistically significant.

RESULTS

Baseline characteristics

In the original SOLARIUM trial, 53 participants completed the 48 week follow-up. Of these participants, 34 participants consented to genetic analysis; 14 participants received placebo and 20 participants received high-dose vitamin D₃ supplementation. Regarding vitamin D assays, 26 of the 34 participants had material available for the LC-MS/MS method at baseline and at week 48. Of these 26 participants, 9 received placebo and 17 received high-dose vitamin D₃ supplementation. For the 3 remaining participants that received high-dose vitamin D₃ supplementation, immunoassay data were available and

these were converted as described in section 2.2 (**Figure 2**). After a 48 week follow-up, 6 of the 26 participants showed signs of MRI activity, 19 showed no signs of MRI activity and 1 was unavailable for MRI analysis. For all baseline characteristics, as well as treatment arm and MRI outcome, no statistically significant difference was observed between the SOLARIUM group and this subcohort (**Table 1**).

Table 1. Baseline characteristics of the studied participants

	Participants with available genetic material (N=26*)	Total SOLARIUM population (N=53*)	p-value
Sex (N[%])			
Female	18 [69]	35 [66]	0.777
Male	8 [31]	18 [34]	
Age (years: median [interquartile range])	39.9 [32.6 – 45.1]	36.2 [31.4 – 43.9]	0.591
Body Mass Index (BMI) (N[%])			
< 25 kg/m ²	11 [42]	24 [45]	0.802
≥ 25 kg/m ²	15 [58]	29 [55]	
Disease duration (months: median [interquartile range])	7.3 [5.2 – 11.7]	7.3 [4.4 – 11.8]	0.900
Attacks during past 2 years at baseline (N[%])			
≤ 1	17 [65]	37 [70]	0.691
> 1	9 [35]	16 [30]	
Time since last attack at baseline (months: median [interquartile range])	7.4 [4.6 – 11.3]	7.5 [5.0 – 10.4]	0.983
Treatment (N[%])			
Placebo	9 [35]	23 [43]	0.455
Vitamin D3	17 [65]	30 [57]	
MRI activity after 48 weeks follow-up (N[%])			
No MRI activity	19 [76]	37 [74]	0.851
MRI activity	6 [24]	13 [26]	

P-value is based on Mann-Whitney U test for continuous data and Chi square test for dichotomous data.

* MRI data after 48 weeks follow-up were available for N=25 participants from the genetic material group and N=50 participants from the total SOLARIUM group.

D binding protein SNP rs7041 shows increased serological response to supplementation

DBP is a known important determinant of 25(OH)D status.[30] Two missense SNPs in the DBP gene, rs4588 and rs7041, were imputed and 25(OH)D levels were compared between carriers and non-carriers of the allele associated with lower 25(OH)D levels. Baseline 25(OH)D levels did not significantly differ between the two groups (**Figure 2A**). However, after 48 weeks of high-dose vitamin D₃ supplementation, carriers of the rs7041 risk allele showed a lower serological response compared to non-carriers [median(IQR):

224.2 nmol/L (150.1-308.9) and 332.0 nmol/L (329.9-357.7), respectively, $p=0.013$] (**Figure 2B**). Accordingly, carriers of the rs7041 risk allele showed a reduced absolute increase of 25(OH)D compared to non-carriers [median(IQR): 148.9 nmol/L (44.6-208.2) and 245.3 nmol/L (226.5-265.3), respectively, $p=0.020$].

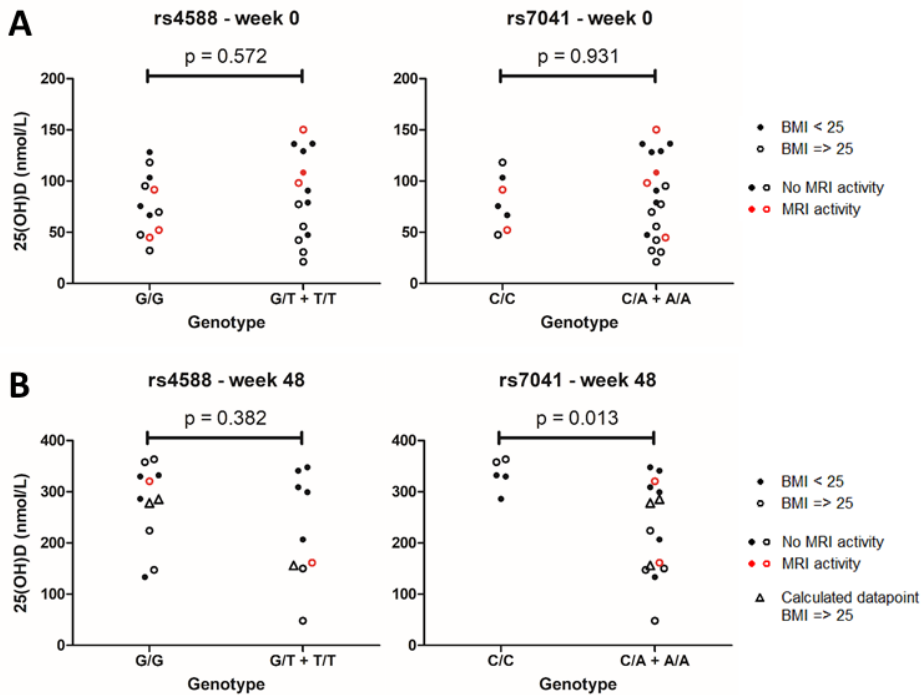


Figure 2. Differences in 25(OH)D levels between carriers and non-carriers of DBP related risk alleles. 25(OH)D levels at baseline (N=26) (**A**) and after 48 weeks of supplementation (N=20) (**B**), compared between non-carriers (left group) and carriers (right group) of the rs4588 and rs7041 risk alleles. P-value shown is calculated using a Mann-Whitney U test. Triangles indicate calculated data, derived from immunoassay values to replace missing values from the LC-MS/MS method.

CYP27B1-related SNP rs12368653 associated with higher vitamin D level after supplementation

Then, two known MS risk alleles that are related to vitamin D metabolism, rs12368653 (near *CYP27B1*) and rs2248359 (near *CYP24A1*), [7] were analysed for differences in 25(OH)D levels. Again, baseline 25(OH)D values did not significantly differ between carriers and non-carriers of risk alleles (**Figure 3A**). After 48 weeks of supplementation, carriers of the rs12368653 risk allele showed higher levels of 25(OH)D compared to non-carriers [median(IQR): 304.1 nmol/L (251.2-336.7) and 152.0 nmol/L (140.6-158.9), respectively, $p=0.014$] (**Figure 3B**). Additionally, carriers showed a higher absolute increase in 25(OH)D levels over 48 weeks compared to non-carriers [median(IQR): 211.0 nmol/L (170.0-

261.5) and 11.2 nmol/L (5.5-77.9), respectively, $p=0.023$]. 25(OH)D levels did not significantly differ between carriers and non-carriers of the rs2248359 risk allele.

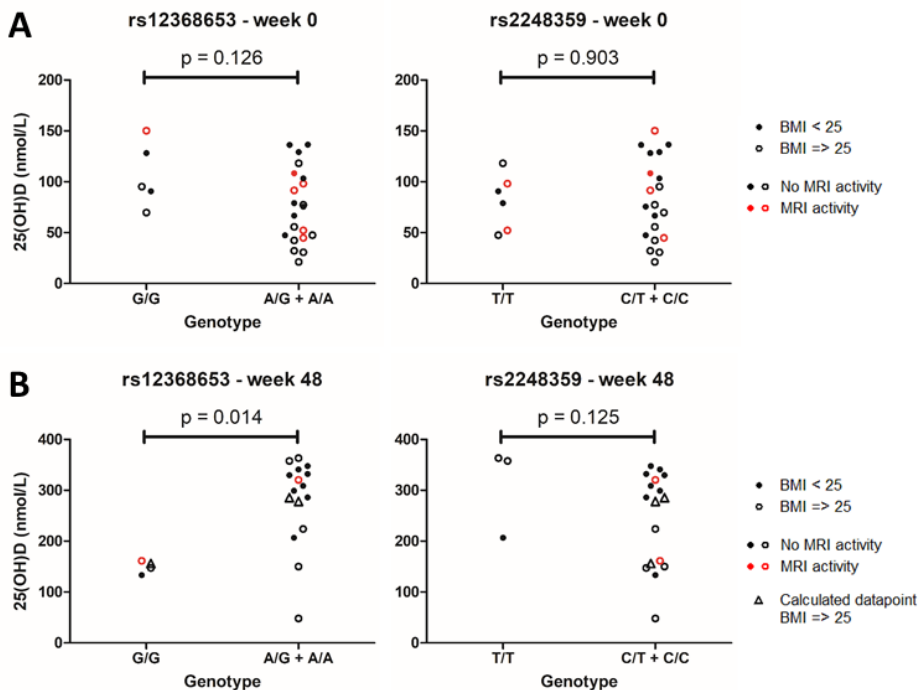


Figure 3. Differences in 25(OH)D levels between carriers and non-carriers of CYP related MS risk alleles. 25(OH)D levels at baseline (N=26) (A) and after 48 weeks vitamin D₃ supplementation (N=20) (B), compared between non-carriers (left group) and carriers (right group) of the rs12368653 and rs2248359 risk alleles. P-value shown is calculated using a Mann-Whitney U test. Triangles indicate calculated data, derived from immunoassay values to replace missing values from the LC-MS/MS method.

DISCUSSION

We explored the effect of vitamin D-related genetics on serological response to high-dose vitamin D₃ supplementation in a cohort of relapsing-remitting MS patients treated with interferon-β-1a. We report an association between two genetic markers and serological response to high-dose vitamin D₃ supplementation, where the *DBP*-linked rs7041 risk allele shows a decreased serological response, and the *CYP27B1*-linked rs12368653 risk allele shows an increased response in circulating 25(OH)D levels.

Studies investigating the *DBP* rs7041 risk allele and 25(OH)D status show varying results. A study by Sollid et al.[30] showed a difference in 25(OH)D at baseline, but not after supplementation. By contrast, a recent study by Al-Daghri et al.[27] showed no differ-

ence in 25(OH)D levels at baseline between rs7041 genotypes, but did show a decreased serological response to 6 months of vitamin D₃ supplementation for carriers of the risk allele. Our findings add to the latter observation, showing a relevant effect of genetic background on serological response to vitamin D supplements.

CYP27B1 (1 α -hydroxylase) catalyses the hydroxylation of the inactive 25(OH)D to the active 1,25(OH)₂D. The identification of the rs12368653 risk-SNP for MS further supports the role of vitamin D in developing MS. Our findings showed an increased serological response to vitamin D₃ supplementation in carriers of the risk allele. We speculate that this finding may reflect a more strict regulation of vitamin D metabolism in carriers of the *CYP27B1*-linked risk-allele. At present, no experimental data addressing the functional effects of this SNP on 1- α hydroxylation of vitamin D have been published. These findings appear contradictory with the findings from Bhargava et al. that MS is related to a decreased response to supplementation. One explanation for this may lie in the fact that the rs12368653 risk allele shows an odds ratio for developing MS of 1.1.[7] As such, the contribution of rs12368653 to the development of MS is very limited. Additionally, our findings are found in supra-physiological conditions and thus differ from the conditions in which MS develops. Nevertheless, differing alleles in rs12368653 appear to influence vitamin D metabolism in a biologically relevant manner, reducing the serological response to supplementation in the SOLARIUM study. As such, rs12368653 may be a relevant confounder in supplementation studies.

In our study, we find no difference in 25(OH)D levels before and after supplementation between carriers and non-carriers of both the rs4588 and rs2248359 risk alleles. However, both have been associated with 25(OH)D levels in other studies, with the rs4588 risk allele showing a decreased serological response to vitamin D₃ supplementation,[27] and the rs2248359 risk allele showing a decreased baseline level of 25(OH)D.[31] This discrepancy may be explained by our relatively small sample size, which increases the risk of type 2 errors.

Our findings suggest that genetic components have influenced the serological response to vitamin D₃ supplementation in the SOLARIUM study and may be a relevant confounder in general supplementation studies. However, it is currently unknown how this translates to clinical outcome in MS patients. As lower 25(OH)D levels have been linked to an increased risk of disease activity,[6] a relation between the serological effect of genetics and clinical outcome could be hypothesised. Additionally, findings by Graves et al.[16] show that children with a unfavourable genetic composition regarding vitamin D metabolism show an increased risk for relapses, which points towards a relevant relation between vitamin D genetics and clinical outcome in MS. Alternatively,

genetic background could also influence the occurrence of adverse events of high-dose vitamin D₃ supplementation, such as hypercalcaemia. Although hypercalcaemia could negatively affect the disease course of MS, [32] hypercalcaemia has not been observed in high-dose vitamin D₃ supplementation studies thus far.[33] Taken together, the influence of genetics on the clinical response to vitamin D₃ supplementation should be investigated further. It is also important to mention that our findings show an effect on supra-physiological levels of 25(OH)D after high-dose vitamin D₃ supplementation. It remains to be determined whether the reported influences of rs7041 and rs12368653 remain relevant with more physiological levels of vitamin D₃ supplementation.

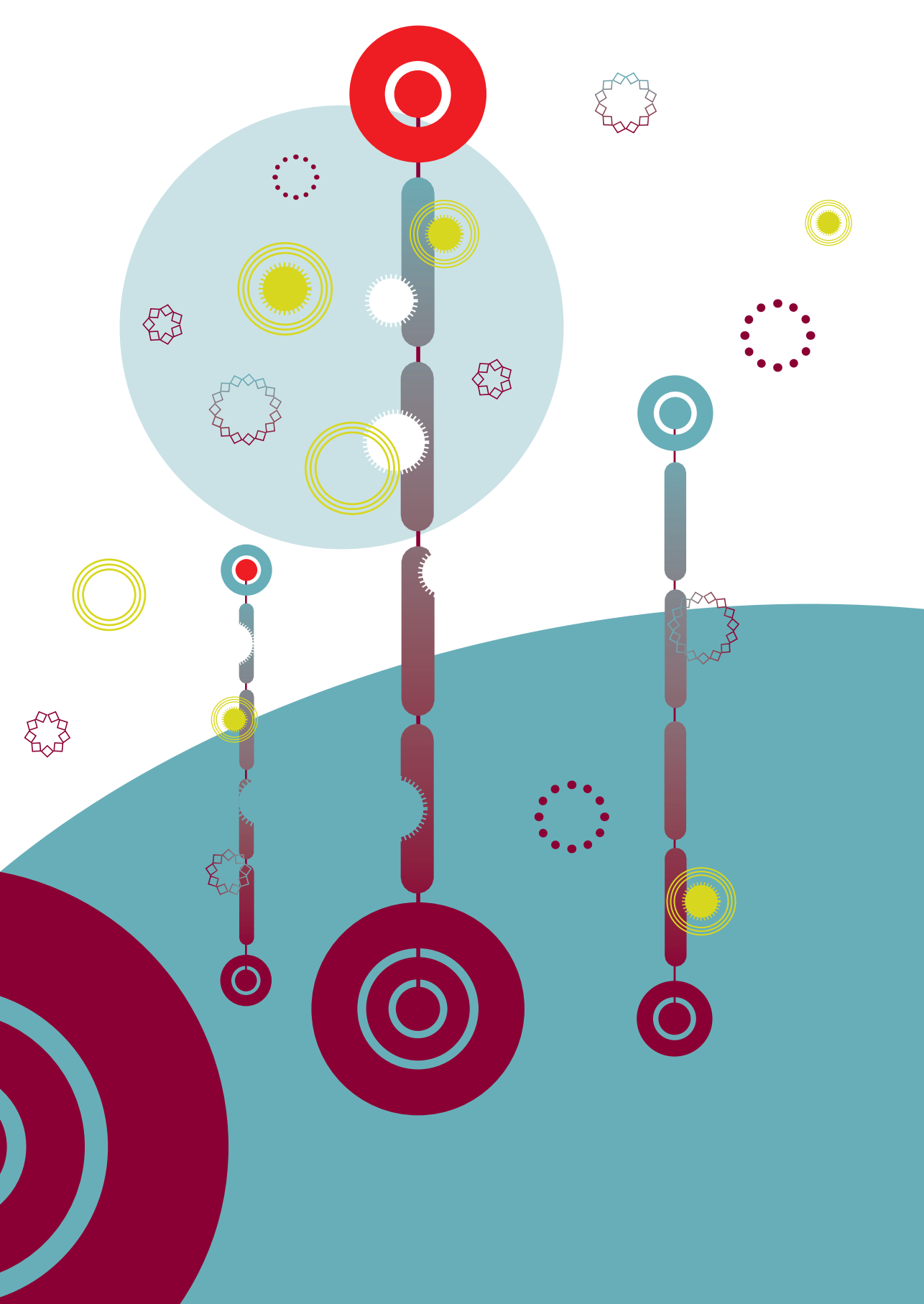
Strengths of our study include its double blind nature.[18] This study is limited by its relatively small sample size, which also made it impossible to analyse each SNP separately, and instead having to use a dichotomous carrier/non-carrier designation for risk alleles.

In conclusion, the vitamin D-related genetic background influences the serological response to high-dose vitamin D₃ supplementation and, as such, may need to be corrected for in later supplementation studies. The clinical consequence of this altered serological response should be investigated further.

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Chapter 4



Natural killer cells in multiple sclerosis

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ABSTRACT

As the most common non-traumatic disabling disease among adolescents, multiple sclerosis (MS) is a devastating neurological inflammatory disease of the central nervous system. Research has not yet fully elucidated its pathogenesis, but it has shown MS to be a complex, multifactorial disease with many interplaying factors. One of these factors, natural killer (NK) cells, lymphocytes of the innate immune system, have recently gained attention due to the effects of daclizumab therapy, causing an expansion of the immunoregulatory subset of NK cells. Since then, NK cells and their relation to MS have been the focus of research, with many new findings being published in the last decade. In this review, NK cells are pictured as potent cytotoxic killers, as well as unique immune-regulators. Additionally, an overview of our current knowledge regarding NK cells in MS is given. The role of NK cells in MS is reviewed in the context of well-established environmental factors and current disease modifying therapies to gain further understanding of the pathogenesis and treatment options in MS.

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disorder resulting in demyelination and destruction of neurons in the central nervous system (CNS).[1] In its most common phenotype, the relapsing-remitting (RR) variant (~85%),[2] patients experience periods of neurological disability, like sensory or motor loss of a limb, followed by (sometimes only partial) recovery.[3] Although the exact mechanism of the development and the progression of MS is still unknown, many factors which contribute to its pathophysiology have been identified.[4]

The view of MS on an immunological level has expanded and changed greatly over the years. Whilst classically viewed as a T helper-1 (Th-1) cell mediated disease,[5] many cells have since been identified as contributors to the disease. Notably, Th-17,[5, 6] CD8⁺ T-cells[7, 8] and B-cells[7, 9] are now generally considered to be involved in the inflammatory mechanisms of MS and treatments focussed on B-cells have shown positive results.[10] More recently, the natural killer (NK) cell has emerged as a contributor to the disease. NK cells are lymphocytes of the innate immune system that play a pivotal role in the defence against malignancies as well as viral infections. The identification of this new player in the field was mainly due to the treatment with daclizumab, an IL2 receptor alpha chain (IL2R α ; CD25) blocking monoclonal antibody that showed positive results in MS, potentially due to its effects on NK cells.[11-13]

The dysfunctions in the immune system of MS patients are the result of the interplay between genetic and environmental risk factors. Genome wide association studies have identified a wide array of genetic polymorphisms linked to the immune system as risk-alleles for MS.[14] Environmental factors include vitamin D,[15, 16] infection viral agents like Epstein-Barr virus (EBV)[17] and cytomegalovirus (CMV),[18] smoking[19] and adolescent obesity.[19, 20] The full effects of vitamin D on the immune system are not yet fully understood. Increasing evidence points towards a role in maintaining and restoring immune homeostasis and thereby a protective effect in MS.[21, 22] This is considered to be important both in the onset of disease as well as in severity and progression.[23] The EBV hypothesis, postulating a dysfunctional or disproportional reaction to EBV infection as the cause for MS, is currently one of the best fitting models for the pathogenesis of MS. The theory classically claims a 'molecular mimicry' model as explanation for the auto-immune reaction. Recently, some other studies have postulated a model where immortalized B-cells, infected with EBV, play a role in priming and activating lymphocytes in tertiary lymphoid follicles in the meninges.[24] Levels of anti-EBV nuclear antigen 1 (anti-EBNA1) and anti-EBV viral capsid antigen (anti-VCA), which are an indication of EBV activity, are also linked to higher MS disease activity.[17] Smoking and obesity are

regarded as general inducers of an inflammatory state, thereby potentially contributing to many auto-immune diseases, including MS.[19, 20]

In this review, the associations between NK cells and MS and its relation with environmental factors are explored. Additionally, the effects of known MS therapies on NK cells are listed.

NATURAL KILLER CELLS

Subsets and functions

Human NK cells in peripheral blood are phenotypically defined as lymphocytes that lack the expression of CD3, but differentially express CD16 and CD56. Figure 1 shows a main gating strategy for NK cells from PBMCs. Circulating NK cells can be divided into a CD56^{bright} and CD56^{dim} subset. Some, but not all patients also show a CD56^{dim}CD16⁺ phenotype, but generally this subset is pooled with the CD56^{dim} subset as they seem to fulfil the same role in immunity.[25] **Figure 1** shows 4 different distributions of the NK cell compartment in order to showcase the interpersonal variations of NK cells between patients. A further division in NK cell subsets can be made, based on several effector properties of these NK cells. Cichocki et al. divided the NK cell population into 4 subsets, based on their phenotype, i.e. CD56 and CD16, migratory function and memory-like function.[26] The discriminating characteristics of these subsets are further described below.

The circulating CD56^{bright} NK cells are immunoregulatory in nature through their cytokine production in response to chemical signalling.[27] Additionally, CD56^{bright} NK cells produce granzyme K used to kill activated CD4⁺ T cells.[28] As CD56^{bright} NK cells do not express receptors of the killer-cell immunoglobulin-like receptor (KIR) family, they are less prone to activation by cell-to-cell contact, but more sensitive to cytokine signalling by receptors like the IL-2R or IL-15R. In fact, CD56^{bright} NK cells express high levels of the IL-2R, both as medium affinity (IL-2Rβγ) and high affinity (IL-2Rαβγ) receptors,[29] ensuring its activity and survival in an environment with relatively low IL-2 levels. The cytokines produced by NK cells depend on the manner of activation of the cell. Co-stimulation of IL-12 and IL-18 efficiently induces production of IFN-γ, but not IL-10. On the other hand, IL-10 is produced upon stimulation of IL-12 together with IL-15 or IL-2. [30] This specific reaction to different cytokine mixtures, combined with a sensitivity to many chemokines, makes the CD56^{bright} NK cell a potent immunoregulatory cell which could play a protective role in auto-immunity.

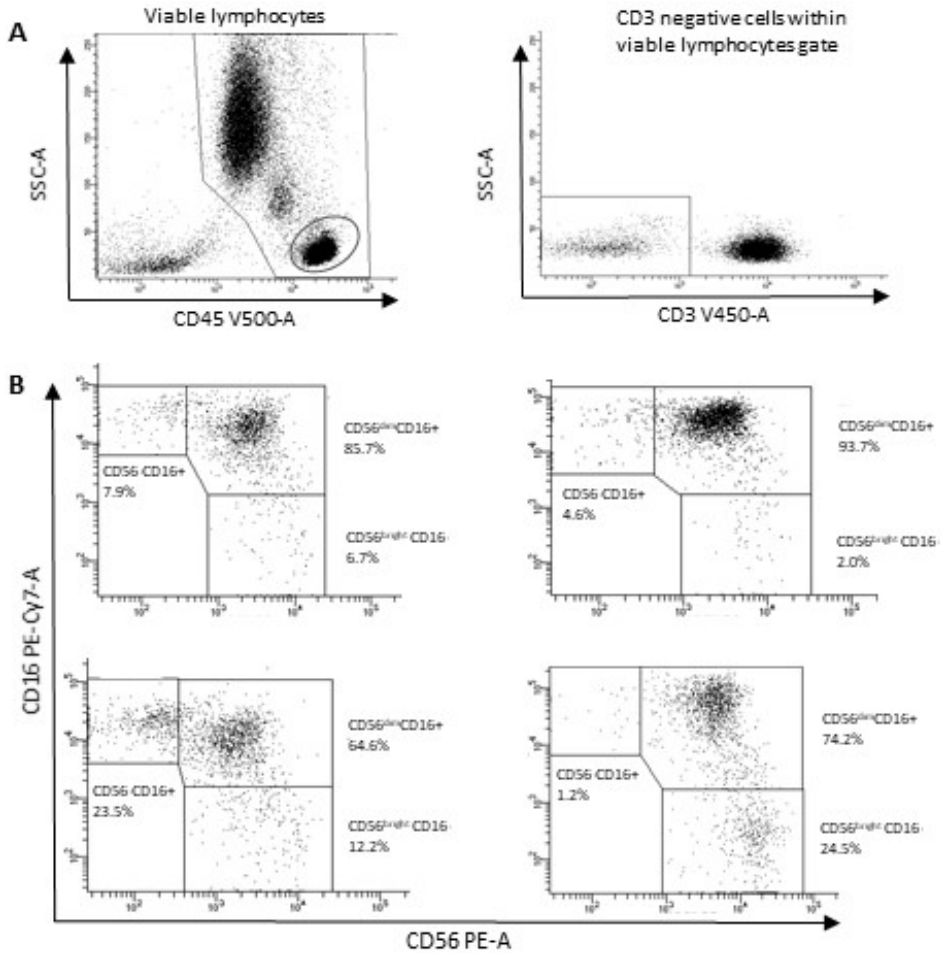


Figure 1. Flowcytometry imaging of NK cells. Pictured is a step-by-step process to find NK cells and their subsets. The first gate (lifegate) aims to find only living cells, after which doublets are removed (A). Then the remaining cells are used to find the lymphocyte population. Using CD3, we can differentiate T cells from B cells and NK cells. Using the CD3 negative population, CD56-CD16- (B cells) are removed (B). The result is a population of only NK cells. NK cells can be sorted in 3 subtypes based on their expression of CD56 and CD16. Although CD56^{dim}CD16⁺ and CD56-CD16⁺ are two different populations, they are functionally considered the same subtype. Also depicted are relative proportions of subtypes within the NK population. Four different NK cell populations are shown to illustrate the interpersonal variance in circulating NK cell phenotypes. Cy: cyanin; PE: phycoerythrin; SSC: side scatter.

Canonical CD56^{dim} NK cells are the most abundant in peripheral blood and the most well-known subtype. These cells fulfil a role of immune-surveillance, in that they ‘scan’ cells for infection or malignant activity. Once a CD56^{dim} NK cell is activated by a relative abundance of activating receptor stimulation, it utilises granzyme B and perforin to induce apoptosis in the target cell. The CD56^{dim} NK cell also releases IFN- γ in response to ‘finding’ an unhealthy cell, thereby creating a pro-inflammatory environment to further combat the infectious or malignant tumour.[31] This pro-inflammatory environment

could contribute to the deterioration of auto-immune diseases, like exacerbations in MS, by triggering a re-activation of the (auto-)immune response. CD56^{dim} NK cells also express CD16 (FcyRIII) which allows the NK cell to sense antibody coated cells and eliminate them through antibody dependent cellular cytotoxicity (ADCC), giving the CD56^{dim} NK cells another potent mechanism of eliminating potential threats. It is important to note that, while CD56^{bright} cells are primarily considered as cytokine producers and CD56^{dim} cells as cytotoxic cells, both subtypes are capable of utilizing both mechanisms. The main difference between the subsets is not their mechanisms of action, but rather the means of activation, as CD56^{bright} cells respond to soluble signalling, while CD56^{dim} cells respond to cell-to-cell signalling.

The concept of a 'memory' CD56^{dim} NK cell has been controversial, as memory is considered to be a defining and exclusive feature of the adaptive immune system. However, research in murine models from O'Leary et al.[32] demonstrated that NK cells can, in absence of T and B-cells, mediate a contact hypersensitivity response, typical of a memory-mediated delayed reaction. Later, Cooper et al.[33] demonstrated that previously activated murine NK cells responded more robustly to stimulation by cytokines. Additionally, when transferring these activated NK cells to naïve donors, they started proliferating whilst maintaining their sensitivity to stimulation. This finding was supported by Sun et al.[34], who demonstrated a rapid expansion of murine NK cells bearing the Ly49H receptor, which specifically recognizes murine cytomegalovirus (CMV), after infection with MCMV. Again, transferring these NK cells to naïve mice and introducing a viral challenge resulted in a far more robust NK cell response and protective immunity. This memory-like phenomenon can also be found in human NK cells. Within the innate immune system, this memory-like process is called 'trained immunity'.[35] These memory-like NK cells are typically found in people infected with human CMV[36] and have several characterising properties. For one, an upregulation of the inhibitory NKG2C receptor was found, together with a downregulation of the FcεRI gamma chain adaptor, which is coupled to CD16.[36, 37] The subsequent replacement of the γ chain with the ζ chain is thought to enhance antibody-dependent NK cell activation.[38, 39] As such, this subset seems to be less potent in its surveillance role, but far more potent in its antibody dependant role, particularly in cytokine production, compared to regular NK cells.[40] Another characteristic of memory-like NK cells includes the epigenetic silencing of promyelocytic leukemia zinc finger (PLZF) transcription factor, with a currently unknown consequence for the NK cell function.[36, 37] The relation between MS and these memory-like NK cells has been investigated,[41] but more research is necessary to gain a full understanding of the role memory-like NK cells may have in the inflammatory process of MS.

Tissue-resident NK cells are characterised by a lack of migratory receptors and thus a limited capacity to enter the blood stream. Tissue-resident NK cells have been found in the liver, spleen and uterus, each with different phenotypes.[42] To the best of our knowledge, no study investigating a CNS specific NK cell subset has been performed. However, since tissue-resident NK cells are thought to play a role in immune homeostasis,[43] a disturbance in this subset, even if not localized within the CNS, may alter the intrathecal immune response, thus affecting the course of MS. Obviously, since this remains mere speculation, research into this NK cell subset is warranted to elucidate its potential role in MS.

Activation and inhibition

As stated earlier, both CD56^{bright} and CD56^{dim} NK cells rely on a complex system of activation versus inhibition in order to fulfil their effector function. Many activating receptors and inhibitory receptors and their ligands have been identified, as listed in **Table 1**. [44-56] Table 1 also lists if the gene coding for the receptor/ligand has come up in GWAS studies, as well as their known up-/downregulation in MS.

Table 1. Activating and inhibitory human natural killer cell receptors

Receptor	Risk allele	Ligand	Risk allele	Receptor role	Comments	
KIR-family						
KIR2DL1		HLA-C2		Inhibiting	During CNS inflammation, HLA class I (-related) molecules are more abundant due to damaged cells. The subtype of HLA class I (-related) molecule is dependent on genetic predisposition.	
KIR2DL2/3		HLA-C1		Inhibiting		
KIR2DL4		HLA-G		Activating		
KIR2DL5		?		Inhibiting		
KIR3DL1		HLA-Bw4		Inhibiting		
KIR3DL2		HLA-A3, -A11	HLA-A	Inhibiting		
KIR2DS1		HLA-C2		Activating		
KIR2DS2		HLA-C1		Activating		
KIR2DS3		?		Activating		
KIR2DS4		?		Activating		
KIR2DS5		?		Activating		
KIR3DS1		HLA-Bw4	HLA-B	Activating		
CD94-NKG2						
NKG2A		HLA-E		Inhibiting		
NKG2C		HLA-E		Activating		
NKG2E		HLA-E		Activating		
NKG2D		MIC-A/-B, ULBP1/2/3/4	MIC-A	Activating		

NCRs

Table 1. Activating and inhibitory human natural killer cell receptors (continued)

Receptor	Risk allele	Ligand	Risk allele	Receptor role	Comments
NKp30		BAT-3, HSPG, B7-H6		Activating	BAT-3 is upregulated in PPMS[54]
NKp44		Viral HA		Activating	
NKp46		Viral HA, HSPG		Activating	
NKp80		AICL		Activating	
2B4		CD48	CD48	Activating	CD48 is upregulated in EAE[49]
DNAM-1	CD226	PVR, CD112	PVR	Activating	
TIGIT		PVR, CD112	PVR	Inhibiting	
TACTILE		PVR, CD112	PVR	Inhibiting	
LILR		HLA-I, UL18		Inhibiting	UL18 is related to CMV infection[52]
KLRG1		Cadherins		Inhibiting	Certain cadherins are downregulated during exacerbations[50]
FcγRIII (CD16)		Antibodies		Activating	
IL-receptors		IL			
IL-1R		IL-1		Inhibitory	IL-1β is found in the blood, CSF and CNS lesions of MS patients[44]
IL-2R	IL2RA	IL-2		Activating	
IL-7R	IL7R	IL-7		Activating	In MS, IL-7 levels are reduced and IL-7Ra is increased[56]
IL-10R	IL10RB	IL-10		Inhibitory	IL-10 protects against TNF-induced relapses in EAE[46]
IL-12R	IL12RB1	IL-12	IL12A/IL12B	Activating	Levels of IL-12 are elevated in progressive MS patients serum[45]
IL-15R	IL15RA	IL-15		Activating	IL-15 is elevated in serum and CSF of MS patients[53]
IL-17R		IL-17		Activating	IL-17 is significantly higher in MS patients[55]
IL-18R		IL-18		Activating	IL-18 is increased in serum of MS patients;[51] IL-18R is upregulated in CSF of MS patients[48]
IL-21R		IL-21		Activating	IL-21 is correlated with more severe MS disease course[47]

Overview of activating and inhibitory receptors on NK cells with their corresponding ligands and function. AICL: activation-induced C-type lectin; BAT-3: HLA-B-associated transcript 3; DNAM-1: DNAX accessory molecule-1; HA: hemagglutinin; HLA: human leukocyte antigen; HSPG: heparan sulphate proteoglycan; IL: interleukin; IL-R: interleukin-receptor; KIR: killer-cell immunoglobulin-like receptor; KLR: killer cell lectin-like receptor; LILR: leukocyte immunoglobulin-like receptor; MHC: major histocompatibility complex; MIC: MHC class I polypeptide-related sequence; NCR: natural cytotoxicity receptor; PVR: polio virus receptor; RAE-1: retinoic acid early transcript-1; TACTILE: T-cell activation, increased late expression, CD96; TIGIT: T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain; ULBP: UL16 binding protein. Adapted from: Pegram, H.J. et al., Activating and inhibitory receptors of natural killer cells. *Immunol Cell Biol*, 2011. 89(2): p.216-24

For activating receptors, several have been extensively studied and their mechanism of action is relatively well known. Prime examples are natural killer gene 2D (NKG2D, CD314), 2B4 (CD244), the natural cytotoxicity receptors (NCR) NKp30, NKp44 and

NKp46, and DNAM-1 (DNAX accessory molecule-1). While these receptors can be potent in activating NK cells, a combination of activating signals, like NKp46 with NKG2D, is always required in order to initiate activation.[57] This most likely constitutes a fail-safe mechanism to ensure NK cells do not kill healthy cells. Only signalling through CD16 can independently activate an NK cell. In this case, the specificity of the response is determined through B-cell activity, i.e. antibody production, thus providing a reason why no co-stimulation is needed when circulating NK cells are activated through CD16.

Inhibitory receptors are less abundant than their activating counterparts, but play a more dominant role in the triggering of the NK cell.[57] Inhibitory receptors include members of the KIR family, as well as NKG2A, CD96 (TACTILE) and T-cell immunoreceptor with Ig and ITIM domains (TIGIT). The latter two are paired receptors with the activating DNAM-1 receptor. Interestingly, although many variations of KIR have been identified, a single NK cell expresses only a few.[58] This random selection of inhibiting receptors seems a way for NK cells to better detect different phenotypes of infected/tumour cells.

KIR recognise self HLA-A, -B and -C molecules, while NKG2A recognises the less variable HLA-E molecules.[59] TIGIT, like DNAM-1, binds polio virus receptor (PVR) and nectin-2. When one of these inhibitory receptors is engaged, it seems that the activating signalling pathway is blocked, thus preventing the NK cell from initiating its effector function.[60] Even here, it seems that some signals are stronger than others, as it has been shown that NKG2D dependent signalling is easier to inhibit than ADCC signalling via CD16.[61] Inhibitory receptors also seem to be involved in the 'licensing' of NK cells. This detailed mechanism is reviewed elsewhere[57] and is beyond the scope of this review.

MULTIPLE SCLEROSIS

Disease characteristics

MS is the most common non-traumatic disabling disease to affect young adults.[62] Its prevalence varies greatly, between 2 in 100.000 in Japan and greater than 200 in 100.000 in Northern Europe and North America,[2] with its prevalence increasing with latitude.[63] There is also a gender bias, since women are more often affected compared to men (3:1).[64, 65] MS is most frequently diagnosed between the ages of 25 to 40, where a patient can present with a clinically isolated syndrome (CIS). This constitutes a mono- or poly-symptomatic event (depending on its corresponding lesion in the CNS) which develops acutely or subacutely and remains for at least 24 hours.[66] Afterwards there may be a period of recovery, although this is not guaranteed. Typical first presentations include a unilateral optic neuritis (ON), partial myelopathy, focal supratentorial

syndrome or focal brainstem or cerebellar syndrome.[67] Frequent symptoms in definite MS include fatigue, reduced walking range, paraesthesia, hypaesthesia, muscle weakness and imbalance.[68]

An important diagnostic tool in MS is magnetic resonance imaging (MRI) of the CNS, where typical T2-hyperintense areas may be found around the ventricles (paraventricular), directly adjacent to the cortex (juxtacortical), in the cortex (cortical), infra-tentorial (brainstem/cerebellum) and in the spinal cord. The addition of gadolinium contrast may show contrast enhancement, indicating leakiness of the blood brain barrier due to lymphocyte trafficking. In order to provide a definite MS diagnosis, a dissemination in space (DIS) and dissemination in time (DIT) is required.[66] With a better use of MRI techniques, DIS and DIT are found much earlier and it is no longer required to wait for a second clinical relapse before starting treatment.[67] Additionally, the most recent revisions of the McDonald criteria allow presence of cerebrospinal unique oligoclonal bands to replace the DIT criterion.[66]

RRMS is the most prevalent subtype of MS and the focus of almost all disease modifying therapies (DMTs).[2] For most of these patients (65% of all RRMS patients [69]), the disease later evolves into a steadily progressing variant, where relapses are replaced by gradual deterioration of the clinical condition. In more contemporary cohorts treated early with disease modifying therapies, a lower estimate of 15-30% secondary progression has been reported.[70] This secondary progressive MS (SPMS) variant has a less diverse repertoire of treatment options, with currently only interferon-beta being approved for active SPMS treatment.[71] Additionally, an upcoming drug named siponimod is showing promising results in treatment of SPMS patients, reducing their rate of deterioration significantly.[72] Patients may also present with gradually progressive neurological deterioration, without ever experiencing relapses. These patients suffer from a primary progressive MS (PPMS) and currently, only ocrelizumab (anti-CD20) is approved for treatment of PPMS.[73] This division into different subtypes is becoming increasingly controversial, as research shows little genetic and clinical distinguishing properties between the subtypes.[74] A more recent classification categorises MS as either relapsing-remitting or progressive. Progressive MS could then be characterized by both 'activity' and 'progression'.[75] Activity is described as the presence of clinical exacerbations, together with MRI findings. These are also the most common endpoints used in intervention trials for RRMS and are frequently reported as a measure of effectiveness of the drug. 'Progression' is monitored through yearly measurement of the expanded disability status scale[76] and implies a loss of function, regardless of new MRI lesions or exacerbations.

Natural killer cells in multiple sclerosis

The role of NK cells in MS has been a controversial topic, with studies reporting both a protective and a damaging role in MS and experimental auto-immune encephalomyelitis (EAE, the induced variant of MS in animals, usually mice).[77-79] Much of the exploratory research into NK cells is based on EAE models. Unfortunately, as murine NK cells do not express CD56 and express different receptors on NK cells, the results of EAE studies are not easily extrapolated to human patients. There are, however, some similarities between murine and human NK cells. The murine NK cell population can be subdivided into CD27^{high} and CD27^{low/-}, considered to be the equivalent of the human CD56^{bright} and CD56^{dim} NK population, respectively.[80] EAE is also different from MS in some aspects. For example, EAE is induced by immunisation with myelin peptides like myelin basic protein,[81] even though the exact target or mechanism of auto-inflammation is unknown in MS. These differences can cause discrepancies between murine and human studies.

Considering the difficulty of finding patients with MS before clinical manifestation and subsequent lack of understanding of MS pathogenesis, EAE is an excellent model to study the early, pre-clinical stage of MS. For example, one EAE study performed in mice found that in the pre-clinical stage of EAE, the murine NK cell compartment undergoes several changes. For one, The proportion CD27^{low/-} NK cells increased. Additionally, the receptors of the (immunoregulatory) CD27^{high} NK cell population shifted towards a more inhibitory phenotype, including downregulation of the activating Ly49D, Ly49H and NKG2D receptors.[82] Increasing our knowledge of immunological changes leading to clinical disease could increase our insight in MS pathogenesis and provide new therapeutic targets.

Enhancing the regulatory features of NK cells ameliorates the course of EAE. When blocking NKG2A and Qa-1 (the murine equivalent of HLA-E), NK cells inhibited CNS inflammation by killing T cells and microglial cells.[83, 84] On the other hand, recent research shows that NK cells in MS may also contribute to CNS damage. According to Liu et al., in MS and EAE, NK cells are in contact with neural stem cells (NSCs) and, in EAE, NSCs release IL-15 upon contact with NK cells.[85] IL-15 in turn supports proliferation and survival of NK cells, thus contributing to a stronger NK cell response. However, particularly during the later stages of EAE, NK cells kill NSCs, mainly due to reduced expression of Qa-1. Removing NK cells during the later phases of EAE indeed ameliorated the disease severity.[85] Liu et al. did not determine which subset of NK cells was responsible for these observations. Other studies show that the NK cell population in the CNS and CSF consists mainly of CD56^{bright} NK cells.[86, 87] This is true for MS patients, but also healthy controls and patients with other neurological disorders, which points towards a loca-

tion specific enrichment of CD56^{bright} NK cells rather than a MS specific phenomenon. [88] This might be related to the higher migratory capacity past the blood brain barrier (BBB) of CD56^{bright} NK cells compared to CD56^{dim} NK cells. [89] Even though CD56^{bright} NK cells are abundant in the CNS and CSF, this does not mean that they are responsible for the destructive findings as described by Liu et al. A post-mortem study shows that NK cells most likely damage the myelin in an antibody dependent mechanism, i.e. ADCC, suggesting that CD56^{dim} NK cells are responsible for the damage to the CNS. [90] More research is necessary to fully elucidate the mechanisms of action of both NK cell subsets in MS.

Given their immunoregulatory properties, CD56^{bright} NK cells have been monitored in untreated MS patients, patients with clinically isolated syndrome (CIS) suggestive of MS, and healthy controls. [91] Interestingly, the number of CD56^{bright} NK cells in peripheral blood is similar between MS patients and healthy controls. However, when stimulated with cytokines, the CD56^{bright} population in MS patients was less efficient in killing activated T-cells than in healthy controls. This difference in efficiency was attributed to an increased expression of HLA-E on T-cells, thus providing more ligand for NKG2A, which acts as a inhibitory receptor on NK cells. [91, 92] HLA-E is also found to be upregulated in MS patients in white matter lesions, endothelial cells and astrocytes. [93] Immune cells and neural cells in MS plaques also express higher levels of HLA-E. [92] This would imply that the reason for the weakened response is not a dysfunctional NK cell population, but rather a resistance of target cells towards NK cells due to HLA-E upregulation in MS.

The largest breakthrough for NK cells in MS came with the introduction of daclizumab. [94] This anti-CD25 antibody effectively blocks the α -component of the IL-2R, which is needed to turn a medium affinity IL-2R into a high-affinity IL-2R. [95] The intended mechanism of action of daclizumab was to inhibit T-cell activation, since these T cells express the high-affinity IL-2R and require IL-2 for activation/survival. [96] An MS risk-allele can be found in the IL2RA gene (coding for CD25), thus supporting the theory of its involvement in MS. [97] Additionally, this IL2RA gene seems to be influenced by vitamin D in T-cells, thus providing another connection with known MS risk factors. [98, 99] Early results showed positive outcomes using daclizumab in MS, [89, 100, 101] but paradoxically later studies revealed that blocking IL-2R actually enhanced the T-cell response *in vitro*, instead of dampening it. [102, 103] Additionally, blocking IL-2R meant that regulatory T-cells (Tregs), which normally suppress the immune response, were also inhibited. As such, blocking IL-2R seemed to be more pro- than anti-inflammatory by inducing T-cell immunity and dampening immune regulation by Tregs. Another mechanism of action was sought to explain the beneficial effect of daclizumab, which was found in the expansion of the CD56^{bright} NK cell population, expressing the medium-affinity IL-2R.

Because of the relative abundance of IL-2 due to blocking of the high affinity IL-2R, these cells could expand around 400-500%.[89, 102] Continuing this line of research, it was later found that CD56^{bright} NK cells can kill (autologous) activated T-cells, thereby severely limiting the activity of the T-cell population.[28, 102] The expansion of CD56^{bright} NK cells apparently outweighed the reduction in Treg activity. This finding provided solid support for the immunoregulatory and protective role of CD56^{bright} NK cells in MS. Indeed, one Australian study found an inverse correlation between MRI lesions and CD56^{bright} NK cells.[104]

Interaction with risk factors

Despite many genetic loci being associated with MS, genetic aberrations do not fully account for its pathogenesis and course. Only a fraction of the pathogenesis is explained by genetics (a maximum of 25% concordance in twin studies[105]), of which nearly 50% has been accounted for by recent work by the international MS genetic consortium. [97] Epidemiological data points towards a key role for environmental factors in MS. Of these factors, the ones that are currently the most impactful and best understood are vitamin D, infection with EBV and CMV, smoking and adolescent obesity. Full details on how these factors impact MS are beyond the scope of this review. However, looking at how these environmental factors interact with NK cells and vice versa could provide new insights into MS. As the interplay between the different immunological players and environmental factors is becoming increasingly complex, this review focuses mainly on the direct effects of environmental factors on NK cells.

Vitamin D

When looking at the distribution of MS patients worldwide, an increase in prevalence is found in increasing latitudes.[63, 106] Much of this effect is attributed to diminished sun-exposure in countries with higher latitudes, causing less vitamin D to be synthesised in the skin. Genetic pre-disposition did not seem related, as studies with adolescent migrants show a higher risk of developing MS for migrants who move from lower to higher latitudes. Conversely, adolescent migrants from higher latitudes have a lower chance of developing MS when moving towards lower latitudes.[107]

Vitamin D is gained through sun-exposure and nutrition, but requires some activating steps in the body before its active variant can be created.[108] Most studies regarding MS, NK cells and vitamin D have used vitamin D₃ (cholecalciferol) supplementation. Usually, vitamin D levels are measured in 25-hydroxyvitamin D (calcifediol) which is the precursor for active vitamin D (1,25-dihydroxyvitamin D or calcitriol).

Vitamin D is considered to support immune homeostasis,[109, 110] mainly through the suppression of pro-inflammatory cytokine production by effector T-cells.[111] Since NK cells can show gene expression for VDR [112], a direct effect of vitamin D is to be expected on NK cells as well. Indeed, treating NK cells with 1,25-dihydroxyvitamin D *in vitro* shows an increase in cytotoxicity without altering the proliferation of NK cells.[113, 114] Several findings support the notion that vitamin D influences NK cell cytotoxicity. For example, studies including elderly patients show that those with low 1,25-dihydroxyvitamin D levels have higher circulating NK cell numbers, likely as a compensation for a reduced cytotoxic potential per individual NK cell.[115, 116] Also, patients with chronic renal failure (who cannot produce sufficient 1,25-dihydroxyvitamin D) and vitamin D resistant rickets have an impaired immune response, including a decreased NK cell functionality, which can be restored by cholecalciferol or 1,25-dihydroxyvitamin D supplementation. [117, 118] It is important to note, however, that adding 1,25-dihydroxyvitamin D in a PBMC culture reduces NK cell cytotoxicity. This has been attributed to the production of prostaglandins by monocytes, which express relatively more VDR and thus respond more strongly to vitamin D. Prostaglandins in turn reduce the effectiveness of NK cells. [119, 120] As such, it seems more likely that a lack of vitamin D reduces the NK cell cytotoxicity, but an abundance of vitamin D does not cause the NK cell to become 'overactive'.

Besides the influence on cytotoxicity, vitamin D seems to also influence other facets of the NK cell. Indeed, Weeres et al. point towards a role for 1,25-dihydroxyvitamin D in the developmental process of NK cells, showing a reduced population and reduced cytotoxicity *in vitro*, using HSC cultures treated with physiological 1,25-dihydroxyvitamin D levels.[121] They suggest an immunoregulatory role of 1,25-dihydroxyvitamin D by impacting the early development of NK cells at the level of HSCs, seemingly favouring a development of HSCs into monocytes instead, although they do not report on the effect of 1,25-dihydroxyvitamin D on the differentiation into different NK cell subsets. Another influence of vitamin D is reported by Olson et al, who focussed their research on large granular lymphocyte leukaemia. This rare cancer of the T-cell or NK-cell lineage[122] is associated with EBV infection and characterised by hyperactivation of the 'signalling transcription and transduction' (STAT)-1, STAT-3 and STAT-5 pathway, resulting in overproduction of cytokines.[123, 124] The group studied the effect of 1,25-dihydroxyvitamin D *in vitro* on the malignant, hyperactive NK cell functionality and cytokine production and found a decrease in STAT-activation and IFN- γ production under stimulation of the VDR, pointing towards a regulatory role for 1,25-dihydroxyvitamin D in NK cells.

Despite the many promising results regarding the effects of 1,25-dihydroxyvitamin D, it remains unclear whether the supplementation of 25-hydroxyvitamin D actually alters

the NK cell population at any level. As such, future research on this subject should be focussed on the effect of supplementing cholecalciferol *in vivo* and monitoring possible effects on the NK cell population, NK cell subsets and their cytokine panel in MS patients. Nevertheless, these studies do provide evidence of 1,25-dihydroxyvitamin D interacting with NK cells and may offer new insights into the link between vitamin D and the pathogenesis and course of MS.

Viral infections

EBV is a virus of the Herpes family, which mainly transmits through contact with saliva of infected individuals.[125] EBV infects epithelial cells and B cells, after which it persists in B cells for the rest of the individual's life. EBV usually persists asymptotically in healthy and immune-competent individuals and around 90% of the worldwide population shows signs of EBV exposure. Generally, EBV infection happens during early childhood and is asymptomatic. However, EBV infection in adolescence/adulthood may manifest as infectious mononucleosis (IM) in 30-40% of patients, associated with chronic fatigue and lymphadenopathy.[126] Its association with MS is evident when looking at epidemiological studies. Of all MS patients, over 99% show signs of EBV exposure and a medical history of IM increases the risk of developing MS about two-fold compared to overall EBV exposed individuals.[127-129] Moreover, more recent evidence leads some authors to question if a truly EBV-seronegative MS patient even exists, further implying EBV's crucial role in MS pathogenesis.[130, 131] The exact mechanism through which EBV contributes to the pathogenesis of MS remains unclear, although a few hypotheses have been formulated, including molecular mimicry and B-cell immortalisation. In almost every case, a role is reserved for the immortalised EBV infected-B cell, which would normally be kept under strict control by the healthy immune system.[132] Markers for active EBV infection, such as anti-early antigen (anti-EA) IgM and IgA, were found in patients with relapses, but not in clinically stable patients.[133] Due to these findings, the authors suggested that disease activity in MS may be related to re-activation of EBV. Interestingly, a role for vitamin D can also be found in the response against EBV, as evidence shows that anti-EBNA-1 antibodies are reduced in patients receiving vitamin D₃ supplementation.[134]

NK cells may be involved in EBV in multiple ways. Obviously, as major players in the innate viral immunity, NK cells play a role in the early defence against EBV infection. Indeed, an expansion of NK cells is seen in infectious mononucleosis, which is correlated to lower viral load levels in some cases.[135] Likewise, depleting NK cells in mice results in failure to control EBV infection.[136] EBV infection even seems to elicit phenotype changes in NK cells, with an upregulation of NKG2A,[137, 138] but do not promote a memory-like NK cell phenotype.[139]

Besides EBV infection, CMV infection also seems to induce a wide array of changes in the immune system, specifically targeting NK and T cells.[140, 141] For example, as mentioned earlier, CMV is of key importance in the formation of NKG2C+ memory-like NK cells, which are more potent in their antibody-dependent mechanisms, but are more limited in their surveillance-dependent potency.

In MS, CMV seems to play a protective role in its pathogenesis, as CMV seropositivity was associated with a decreased MS risk (OR=0.73).[18] The mechanism of protection is not yet fully understood, however currently, the protective effect of CMV is attributed to its immune configurative properties. For example, one theory states that the memory-like NK cells induced by CMV infection are especially potent in fighting viruses of the herpes family, including EBV. [41, 142]

Lifestyle

Lifestyle factors seem to play a role in the pathogenesis and disease progression of MS. Of these factors, the most prominent ones are smoking[143, 144] and adolescent obesity.[145-147] So far, no specific component of tobacco smoke or adipose tissue has been linked to MS. Indeed, oral tobacco (snuff) actually seems protective against MS.[148]

The effect of smoking on NK cells has not been researched in the context of MS, but general studies regarding smoking and NK cells may provide clues how smoking affects MS specifically. Evidence is mainly found in COPD and lung cancer studies and is somewhat contradicting. Some studies point towards an enhanced NK cell response and promoted expression of IFN- γ due to smoking in murine NK cells,[149] as well as increased activated circulating NK cells.[150] However, other studies report a lower circulating volume of NK cells due to smoking,[151, 152] as well as a lower IFN- γ and TNF- α expression and reduced cytotoxicity.[153, 154] Some of these differences may be explained by presentation of data. Studies reporting an increase in NK cells mostly report a higher percentage of NK cells in smokers, which does not equal an absolute increase in NK cells. It could be possible that smoking reduces all lymphocytes, but it reduces other cells more than NK cells, thus increasing the percentage of NK cells in peripheral blood. However, although smoking clearly affects NK cells in multiple ways, no specific connection between alterations in the NK cell population and MS can currently be made.

Obesity has been proven to negatively influence the immune response[155, 156] and increase the risk of many disorders, including viral infections.[157, 158] There is also evidence of alterations in the NK cell population as a result of obesity, like reduced

cytotoxicity and cytokine production.[159-161] Additionally, the fact that vitamin D is fat soluble means that obesity directly impacts circulating vitamin D levels and could lead to a vitamin D deficiency. Again, no specific link can be established between MS and the effect of obesity on NK cells. While it is evident that obesity impacts NK cells and MS, a specific mechanistic relation is currently not feasible. However, both smoking and obesity are general inducers of a pro-inflammatory state. As such, seeing as there currently seems to be no mechanistic connection between smoking or obesity and MS, it seems that this shift towards a pro-inflammatory state caused by lifestyle factors simply lowers the threshold for auto-immunity. A combined theory could be formed with the EBV hypothesis, formulating that EBV infection in a pro-inflammatory environment due to smoking and obesity has a higher risk of causing auto-immunity.

NATURAL KILLER CELLS IN THERAPIES FOR MULTIPLE SCLEROSIS

As mentioned earlier, much of our knowledge about the CD56^{bright} NK cell in MS comes from studies regarding daclizumab. Studies regarding the efficacy of daclizumab showed a significant reduction in MRI lesions and clinical progression, with improvements up to 50% compared to interferon beta-1a treatment.[11-13, 100] Although the therapeutic effects were evident, serious concerns were raised regarding its safety profile. Hepatotoxicity was noted in the safety trials, causing authorities to restrict the prescription of daclizumab to only patients who did not respond to other treatments. After its approval, several cases of serious inflammatory brain disorders emerged, causing the suspension and recall of the drug.[162] As such, although CD56^{bright} NK cells seem to have a promising protective and therapeutic potential, no DMTs currently focus on the NK cell population. However, although other drugs were not developed with the intent of influencing the NK cell population, they might have an effect there. Several therapies and their effect on NK cells are listed below.

Interferon- β -1b

Interferon- β -1b is one of the earliest therapies for MS. It has a plethora of effector mechanisms, including a reduction of MHC class II molecule expression, reduction of T-cell proliferation, lowered IFN- β production and reduced expression of adhesion molecules. [163] Interestingly, interferon- β -1b also seems to upregulate MHC class I expression in murine neuron models. As MHC class I is the main inhibitory ligand for CD56^{dim} NK cells, it could be postulated that part of interferon- β -1b's immunoregulatory effect is due to reduction of cytotoxicity of the CD56^{dim} NK cell population.

Additionally, interferon- β -1b seems to alter the CD56^{bright}/CD56^{dim} NK cell ratio by expanding the CD56^{bright} population in peripheral blood,[164] as well as altering the phenotype of the NK cell.[165] This phenotype change consisted of a downregulation of the inhibitory LILRB1 receptor (which binds MHC-I class molecules) and an upregulation of the inhibitory NKG2A receptor (which binds MHC class-I antigen E). Thus, it does not seem to alter the overall inhibition or activation of the NK cell, but rather the method of inhibition. The exact mechanism by which interferon- β -1b facilitates the expansion of CD56^{bright} NK cells is unclear, but theories include a mobilisation from the lymph nodes to the peripheral blood. Although interferon- β -1b is proven to be effective in slowing disease progression and reducing clinical events, newer drugs are far more potent and have a better prognosis, resulting in a decline of popularity for interferon- β -1b.

Natalizumab

Natalizumab blocks very late antigen-4 (VLA-4), also known as α 4 β 1-integrin, a receptor which is crucial in the migration of immune cells between tissues. VLA-4 binds with its counter-receptor, vascular cell adhesion molecule (VCAM)-1 to facilitate cell migration into the CNS. Natalizumab's intended mechanism of action was to block VLA-4 on T-cells and monocytes so they would be unable to migrate into the CNS and thus be unable to exert their inflammatory effects there. Interestingly, circulating NK cells are increased with natalizumab use,[166, 167] possibly reflecting a reduced capacity to migrate towards tissues, including the CNS. Indeed, NK cells also express VLA-4 and seem to prefer using a VLA-4 dependent mechanism to migrate into the CNS.[89] As mentioned earlier in this review, NK cells in the CSF are mainly of the CD56^{bright} phenotype. Therefore, as CD56^{bright} NK cells are generally considered as immunoprotective in MS, it would seem that natalizumab exerts both positive and negative effects on the immunological composition of the CNS. Supported by natalizumab's positive outcomes,[168-170] the lack of T-cells in the CNS seems to outweigh the reduction of CD56^{bright} NK cells.

Glatiramer acetate

Glatiramer acetate (GA) is a drug derived from four amino acids common in MBP (Glu, Ala, Lys, Tyr). It was originally designed as a synthetic antigen capable of inducing EAE. However an opposite effect was found where glatiramer acetate actually protected against EAE instead of inducing it.[171] The main mechanism of action seemed to be based on a shift from Th-1 cells to Th-2 cells and activation of Tregs.[172, 173] It was demonstrated that monocytes under GA are less responsive to pro-inflammatory stimuli, secrete higher amounts of anti-inflammatory cytokine IL-10 and secrete lower amounts of the pro-inflammatory IL-12.[174] There also seems to be a role for NK cells in this shift towards Th-2 cells. *In vitro* experiments show that GA enhances the cytotoxicity of NK cells against both immature and mature DCs, which are implicated in the activation of

Th-1 cells.[175, 176] Killing immature DCs is part of the physiological repertoire of the NK cell and GA seems to amplify this by enhancing the interaction between NK cytotoxicity receptors and immature DCs.[176] Killing mature DCs is not a physiological mechanism of the NK cell, but seems to be caused by GA's ability to reduce MHC class I expression on DCs.[176] Thus, less inhibitory signals are received by the NK cells which causes it to kill the DCs. Furthermore, GA decreases the IFN- γ production of NK cells, but slightly increases the TNF- α production.[176] In stimulating cytotoxicity against both immature and mature DCs, GA ensures that Th-1 cells are not activated by antigen presentation. As such, it seems that NK cells are not directly enhanced or altered by GA, but they may be key players in clearing GA-altered DCs.

Dimethyl fumarate

Dimethyl fumarate (DMF) is a drug which alters many different immune cell populations, although its exact mechanisms are not fully elucidated. DMF's active metabolite, monomethyl fumarate (MMF), is proven to downregulate T- and B-cell responses through various mechanisms, such as induction of apoptosis, stimulation of Tregs and inhibition of migration to injured tissues.[177] Although the main focus of research has been the adaptive immune system, more recent evidence points towards an effect on the innate immune system as well. More specifically, a marked expansion of the CD56^{bright} population is noted in patients treated with DMF.[178-180] Additionally, an increase in NK cell degranulation is reported.[180] Seeing as DMF seems to influence nearly every part of the immune system,[177] it seems unlikely that the CD56^{bright} NK cell expansion is the sole cause of the immunoprotective effect of DMF. However, it seems likely that the CD56^{bright} population plays a role in the beneficial effects of DMF.

Fingolimod

Fingolimod (FTY720) is a sphingosine-1-phosphate (S1P) antagonist, with an intended effect of retaining autoreactive lymphocytes within the lymph nodes.[181, 182] Its phosphorylated form binds to four of the five S1P receptors (S1PR_{1,3,4,5}), used in the egress of lymphocytes from the lymph nodes, with S1PR₁ being the most crucial for most lymphocytes.[183] Fingolimod causes a lymphopenia due to an accumulation of lymphocytes in the lymph nodes, although NK cells seem less affected than T and B-cells. This may be due to the expression of S1PR₅ by NK cells, which is less affected by fingolimod than S1PR₁, thus giving NK cells an alternative method of egressing from lymph nodes. It should be noted that CD56^{dim} NK cells have a relative overexpression of S1PR₅, while the CD56^{bright} NK population expresses relatively more S1PR₁.

Some studies suggest that absolute circulating NK cell numbers do not change under fingolimod treatment.[184, 185] Nevertheless, the NK cell population does seem to be

affected by fingolimod, as a marked reduction of circulating CD56^{bright} NK cells without a loss of IFN- γ and TNF- α production is reported.[186] In the CSF, an expansion of the CD56^{bright} subset is noted.[187] As such, it can be postulated that at least some of fingolimod's effect is by enriching the CD56^{bright} population in the CSF, thus creating a more immunoprotective environment.

Ocrelizumab

One of the more recent additions to the arsenal of DMTs is ocrelizumab, an anti-CD20 monoclonal antibody developed to deplete the B-cell population. It is quite similar to the more well-known rituximab, another anti-CD20 monoclonal antibody which is used in a variety of auto-immune diseases including neuromyelitis optica, a differential diagnosis of MS.[188] The fact that B-cells are involved in MS is supported by several findings. For one, B-cells are found in MS plaques[189] and in meningeal follicles.[190] Also, one of the diagnostic hallmarks of MS, oligoclonal bands in the CSF, are produced by B-cells.[191] Additionally, as noted earlier, nearly all hypotheses regarding EBV's mechanism of inducing MS involve the infected, immortalized B-cell. As such, there are many ways for the B-cell to potentially influence MS and B-cell depletion seems like a viable method of limiting MS activity. Indeed, its effectiveness is evident from its clinical trials, showing reduced relapse rates and fewer MRI lesions.[192, 193] Also, it is currently the only drug to be approved for the treatment of primary progressive MS (PPMS).[194] Ocrelizumab's intended mechanism is to block a specific epitope of CD20 (which is different than those bound by rituximab). CD20 is expressed in the majority of B-cell lines, but not stem cells, pro-B cells and plasma cells.[195] Since plasma cells are the main producers of antibodies, antibody levels are not affected by ocrelizumab treatment.[196] Also, a small subset of T-cells (~6%) seems to express CD20.[197] By binding to CD20, ocrelizumab causes the depletion of B-cells mainly by mediating ADCC against the target cell and for a small part by mediating complement dependent cytotoxicity (CDC) and apoptosis.[10] Although CD20 is not expressed on NK cells, they are still involved in ocrelizumab's mechanism since they play a role in ADCC. As such, through the depletion of B-cells, no antibodies are produced to coat target cells, which in turn renders the ADCC mechanism of NK cells ineffective.

CONCLUSIONS

Multiple sclerosis is a remarkably complex disease with a multitude of interacting factors contributing to its pathogenesis and course. The relatively recent interest in innate lymphoid cells has revealed new key players in the disease, with NK cells being especially interesting due to their therapeutic potential, as shown in the clinical effect of

daclizumab. We reviewed not only the direct relation of NK cells with MS, but also their involvement in the well-known environmental risk factors associated with MS. Additionally, we reviewed the current therapies for RRMS and their effects on NK cells.

First, the more specific division of NK cells into four subsets gives rise to new perspectives in how NK cells might influence MS. The characterisation of memory-like NK cells is particularly exciting, as it shows a way for NK cells to easily create a pro-inflammatory environment. As such, this finding might reveal an additional target for therapy. As the concept is relatively new, the role of these memory-like cells must be investigated further to determine their exact role in the pathogenesis of MS and in exacerbations. Another characteristic of NK cells that warrants further investigation is the expression of NK cell receptors. Particularly the changes a NK cell undergoes in response to different infections (e.g. EBV or CMV infection) may play a role in the pathogenesis of MS, suggested by the protective effect of CMV infection. If a change in receptor expression is cause for a faulty immune response causing MS or its exacerbations, therapy blocking or altering the expression of these receptors may prove beneficial. Although NK cells are heavily implicated in MS exacerbations, as shown by Caruana et al., much less clinical evidence exists for a role in the pathogenesis of MS. To explore the early diagnostic potential of NK cells, a study correlating NK cell counts and conversion from CIS to MS is necessary.

With MS being a complex, multifactorial disorder, it is imperative to place the NK cell within the context of known environmental risk factors. As NK cells express a VDR, a direct link between NK cells and vitamin D levels can be established. It seems that vitamin D has a immunoregulatory effect on NK cells, thereby contributing to protection against MS. On the other hand, EBV is a known risk factor for MS and might even be a requirement for developing the disease. NK cells are the first line of defence against viruses and EBV seems to alter the NK cell phenotype. Smoking and obesity also contribute to the development of MS, but seem to do so in a non-specific way by stimulating a pro-inflammatory environment.

Although no current DMTs specifically target NK cells, some therapies do influence the NK cell population. Mostly, this seems to consist of an increase in CD56^{bright} NK cells, although none increase the CD56^{bright} population as much as daclizumab did. Considering the undeniable therapeutic effects of daclizumab, the line of CD56^{bright} NK cell enhancement as a therapy should be explored further, despite the initial setback of daclizumab's hazard profile.

In conclusion, NK cells are established as key players in MS. The more we learn about the way they influence the disease, the more we can look towards using NK cells as a diagnostic or safe therapeutic tool in combating MS.

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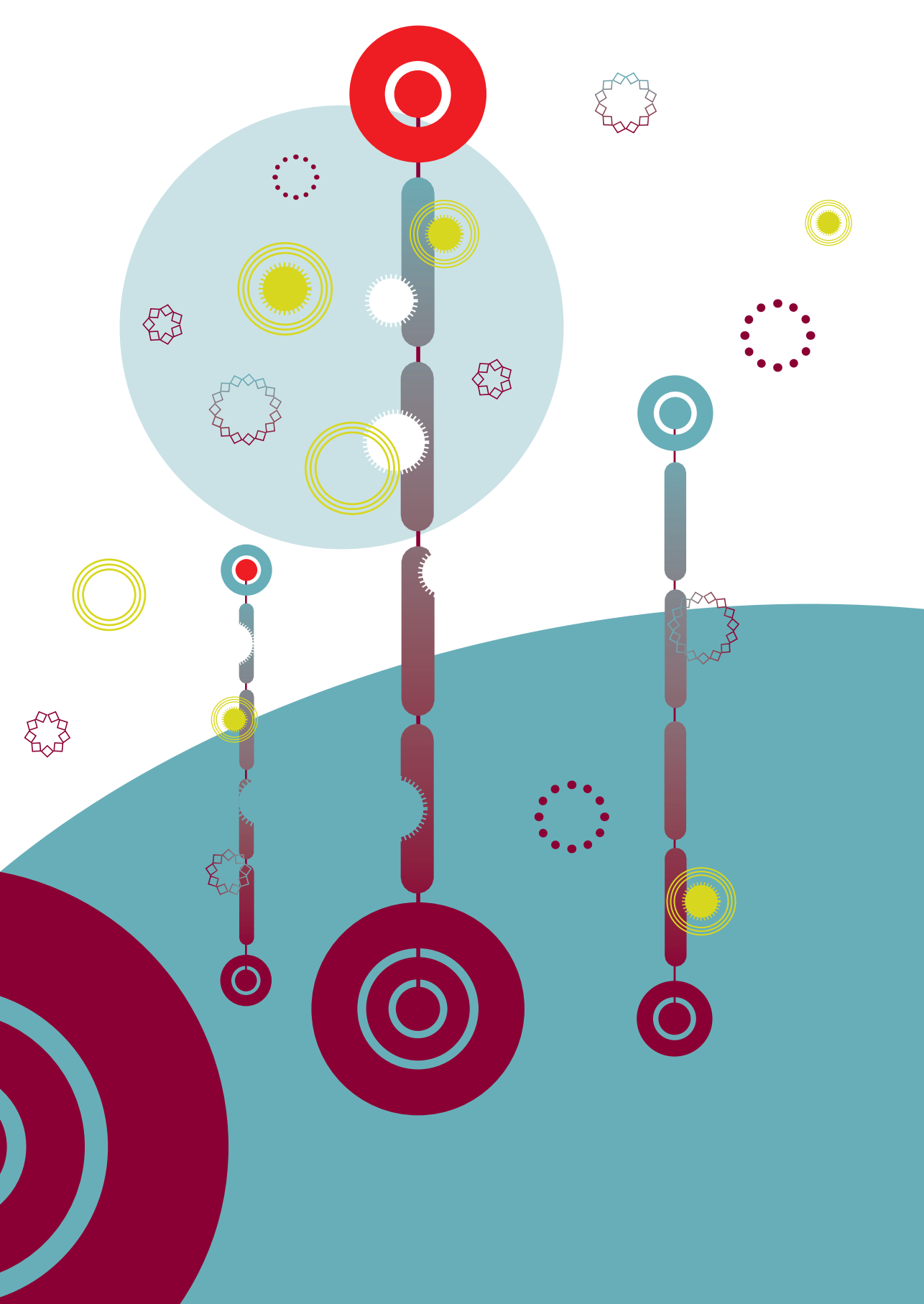
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Chapter 5



Prognostic value of NK/T cell ratios

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ABSTRACT

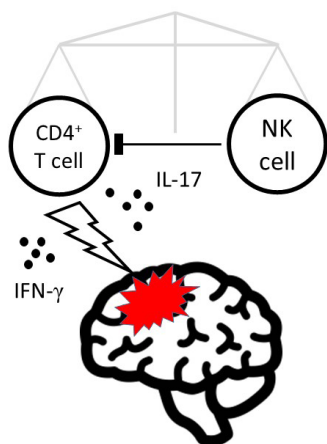
Background: Natural killer (NK) cells may play a role in multiple sclerosis (MS). Ratios of NK cells/CD4+ T cells have been proposed as a biomarker for the therapeutic effect of stem-cell transplantation in MS.

Objectives: To explore the relevance of this ratio in MS patients by analysing NK and T cell subsets, as well as their prognostic value for disease activity.

Methods: Baseline peripheral blood mononuclear cells of 50 relapsing-remitting MS patients, participating in our vitamin D supplementation study (SOLARIUM), were analysed with flow cytometry. Disease activity was measured as new MRI-lesions, relapses, and mean plasma neurofilament light chain (NfL) levels after 48 weeks of follow-up.

Results: The proportion of NK cells correlated negatively with CD4+ T cells [$R=-0.335$ $p=0.001$] and IL17-A+CD4+ T cells [$R=-0.203$ $p=0.043$]. Participants with MRI activity or relapses displayed lower NK/IL-17A+CD4+ T cell ratios [$p=0.025$ and $p=0.006$, respectively]. The NK/IL-17A+CD4+ T cell ratio correlated negatively with NfL levels [$R=-0.320$ $p=0.050$]. Vitamin D supplementation did not affect these ratios.

Conclusions: Our data suggests a protective role of an expanded NK cell compartment compared to the CD4+ T cell subset fractions in RRMS patients. NK/CD4+ T cell ratios may be a prognostic biomarker for disease activity in MS.



Graphical abstract. CD4+ T cells are considered pathogenic in multiple sclerosis (MS), contributing to lesions in the central nervous system via the production of pro-inflammatory cytokines. Recently, NK cells have been shown to be able to suppress (autologous) activated T cells, revealing an interesting NK cell / T cell interplay which may be relevant for disease activity in MS. When illustrating this interplay as an NK/T cell ratio, using different subsets of NK cells and CD4+ T cells, a prognostic value is found between NK/T ratios at baseline and disease activity at week 48.

INTRODUCTION

Multiple sclerosis (MS) is an immune-mediated, disabling disease which is most common in young adults.[1] The pathogenesis and mechanism of action of MS have been partially elucidated, although many facets still remain unclear. Classically, CD4+ T cells and interferon gamma (IFN- γ)⁺ CD4+ T (T helper type 1, Th1) cells in particular have been named as the main perpetrator in causing MS lesions in the CNS.[2] However, other immunological subsets, like IL-17+ CD4+ T cells,[3, 4] CD8+ T cells[5, 6] and B cells,[5, 7] have also been linked to MS.

More recently, natural killer (NK) cells are under investigation in MS.[8] NK cells show a strong enrichment for expression of MS susceptibility genes as reported in a recent genome wide association study.[9] In the experimental autoimmune encephalomyelitis (EAE) model of neuro-inflammation, both direct neurotoxic effects of infiltrating NK cells,[10] as well as a regulatory role of NK cells in controlling autoreactive T cell activation[11, 12] have been postulated. During the treatment of people with MS with the anti-CD25 antibody daclizumab, a drug with notable effects in reducing relapses and MRI activity, an expansion of the CD56bright subset of NK cells was observed.[13] This subset is generally considered to fulfil an immuno-regulatory role, with one of its functions being the killing of (auto-reactive) activated T cells.[13, 14] As such, an inverse correlation between NK cells and pathogenic T cells is hypothesized and supported by the findings of daclizumab trials. Interestingly, Darlington et al.,[15] investigated the re-emergence of Th17 in relapsing-remitting (RR) MS patients after autologous hematopoietic stem cell transplantation (aHSCT). Although the absolute number of NK cells did not predict IL-17A+ Th cell levels, the ratio between NK cells and CD4+ T cells did correlate with lower IL-17A+ Th cell levels after 3 weeks, 3 months, 12 months and 21 months. As IL-17A⁺ T cells are considered to contribute to the pathogenic T cell response in MS,[3, 4] the group hypothesized that a high NK/CD4+ T cell-ratio is a biomarker of a protective constitution of the lymphocyte compartment in MS.[15] However, no subsets of NK cells or CD4+ T cells were analysed, so little can be said about the role of, for example, CD56bright NK cells and IL-17A+ CD4+ T cells, in this hypothesis.

We aim to expand upon this hypothesis by exploring the correlation between NK cells (and their subsets) and CD4+ T cells (and their subsets), and by exploring the association of their ratio's with subsequent inflammatory disease activity in a cohort of interferon beta-treated RRMS patients. We defined disease activity according to commonly used markers, namely new or enlarging MRI lesions and clinical relapse. Additionally, we measured serum levels of neurofilament light chain (NfL), a new biomarker for axonal damage.[16]

MATERIALS AND METHODS

Patients

The complete set of in- and exclusion criteria for the SOLAR and its substudy SOLARIUM are described elsewhere.[17, 18]

In short, the SOLAR study (NCT01285401) recruited patients aged between 18 and 55 years, diagnosed with RRMS (according to the McDonald criteria 2005) confirmed by typical MS findings on MRI. The first clinical event must have been within 5 years prior to screening and signs of active disease must have been present in the last 18 months, but no relapse in 30 days before inclusion. Patients were excluded if they consumed more than 1,000 IU (25µg) of vitamin D3 supplements. All patients received IFNβ-1a 44µg s.c. three times weekly. They used IFN-β-1a at least 90 days but no longer than 18 months. After randomisation, the patients received either IFN-β-1a and a placebo or IFN-β-1a and vitamin D3 supplements (cholecalciferol, Vigantol®Oil, Merck KGaA, Darmstadt, Germany) of 7,000 IU daily for 4 weeks, followed by 14,000 IU daily up to week 48.

The SOLARIUM sub-study recruited patients from four of the five participating centers in the Netherlands without adding additional in- or exclusion criteria, being eligible when they agreed to participate in the sub-study. Peripheral blood samples were collected at baseline and after 48 weeks and analysed using flow cytometry. Written informed consent was acquired and the SOLARIUM study was approved by the Ethical Committee METC-Z (11-T-03; Heerlen, the Netherlands).

This is a further analysis of the data obtained in the SOLARIUM study, with the only requirement being a full measure of NK cell related markers at baseline and after 48 weeks.

For our measures of disease activity, the MRI and relapse findings as described in the SOLAR study were used,[18] as well as the NfL levels as measured from available material from the SOLAR study.[19] Since only 3/53 patients had more than one new MRI lesions within 48 weeks and 4/53 patients had more than one relapse within 48 weeks, data regarding MRI activity and clinical relapse were collected as a dichotomous yes/no outcome. Since this study included RRMS patients with a short disease duration and follow-up of 48 weeks, no clinically relevant disability progression was expected and therefore no correlations with EDSS progression were analysed. Plasma NfL levels were measured at baseline and 48 weeks using a single molecule array[19] in a subgroup of patients where samples were available. Since the baseline and week 48 levels were highly correlated (spearman rho=0.615, P<0.001), we calculated, where available, the mean NfL level to report the most representative level for the 48 week follow-up.

PBMC isolation

The acquirement and analysis of the peripheral blood mononuclear cells (PBMCs) is described elsewhere.[17] In summary, peripheral blood samples were collected from patients at baseline and week 48 of treatment. Blood was collected in a 10mL sodium heparin blood sampling tube (BD Biosciences, Breda, The Netherlands) and transported to Maastricht University Medical Center, the Netherlands, at room temperature. Within 24 hours PBMCs were isolated as described in previous publications.[17, 20]

Flow cytometry for NK cells, T cells and subtypes

Immediately after isolation, PBMCs were stained with a cocktail of monoclonal antibodies. The T cell staining is described elsewhere.[17] In short, PBMC were stained with a cocktail of monoclonal antibodies to define, among others, CD3+ T cells, CD3+CD4+ T cells and Treg cells. Cytokine expression of IFN- γ and IL-17A by CD3+CD4+ T cells was assessed after a 5h in vitro activation with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL, Sigma Aldrich, Zwijndrecht, The Netherlands) and ionomycin (1 μ g/mL, Sigma Aldrich) in the presence of monensin (1.25 μ g/mL, BD Biosciences). Expression of IL-10 was assessed after similar activation, but without the addition of monensin.[21]

The NK cell staining consisted of: NKp46-Horizon (Biolegend, Uithoorn, The Netherlands); CD56-PE (BD Biosciences, Breda, The Netherlands); CD3-PerCP (BD Biosciences); NKG2D-APC (Biolegend); CD16-PE-Cy7 (BD Biosciences). For FACS analysis (FACS Canto II flow cytometer (BD Biosciences)), NK cells were analysed for 100,000 events in the lymphocyte gate.

FACS DIVA software (BD Biosciences) was used to analyse the flow cytometry data. To identify both NK cell subsets, first CD3-NKp46+ cells (NK cells) were gated by accepting some contamination with CD3-NKp46- cells, because there is some overlap between both CD3- subsets. In order to adjust for this contamination, we defined the total NK cell population as the sum of CD56brightCD16- and CD56dimCD16- NK cells. Gating strategies are shown in **Figure 1A**.

Additional laboratory findings

During the SOLARIUM study, total white blood cell (WBC) counts were gathered to calculate absolute numbers of lymphocyte subsets (Covance, Princeton, NJ, USA).[17] These WBC counts were now used to calculate absolute cell numbers for subtypes. NfL levels were measured in plasma in duplicate with a single molecule array (Simoa).[19]

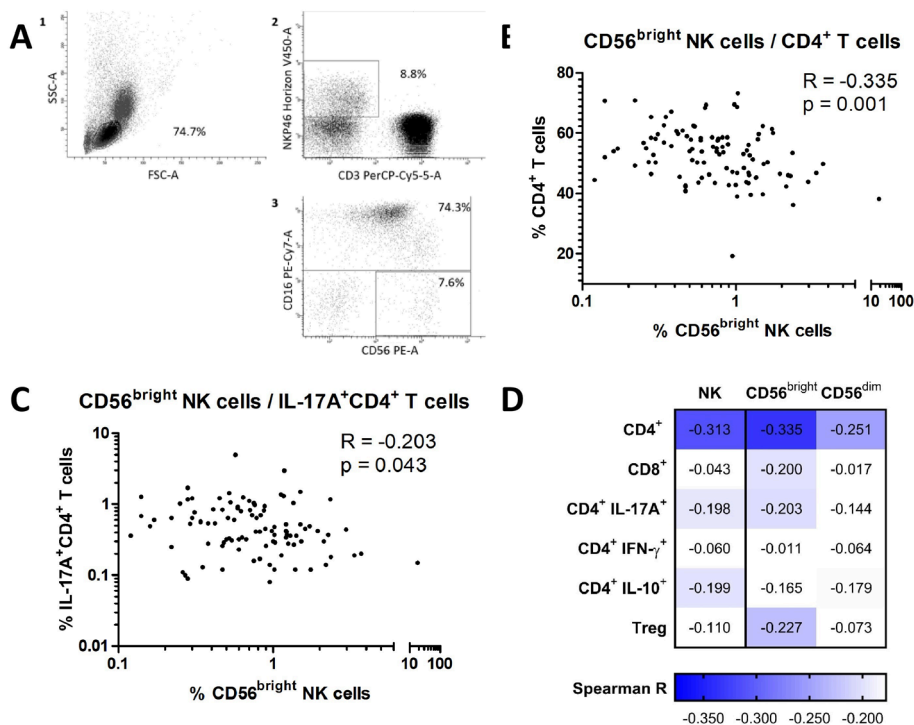


Figure 1. A: Gating strategy used to analyse NK cells and subsets. Step 1 shows the gating of lymphocytes from the PBMC population. Step 2 shows the gating of NK cells, defined as CD3- NKp46+. Step 3 shows the differentiation between the CD56dimCD16+ NK cells (above) and CD56brightCD16- NK cells (below). B: Correlation between the percentage of CD56bright NK cells and the percentage of CD4+ T cells. R shown is spearman rho. N=100 C: Correlation between the percentage of CD56bright NK cells and the percentage of IL17-A+CD4+ T cells. R shown is spearman rho. N=100 D: Heatmap of correlations between NK cells (subsets) and various T cell subsets. Correlations with a tinted background are statistically significant. The associations between NK cells and T cell subsets seem to be mainly caused by the CD56bright NK cells, despite them making up a small portion of the total NK cells. N=100

Statistical analysis

SPSS software (IBM SPSS, version 25.0. Chicago, IL) was used to assess the correlation between various NK cell subsets and T cell subsets, as well as the prognostic value of NK/T ratios and their subtypes for clinical outcomes and NfL levels. Normality of data was assessed by visual inspection of histograms with normal curves, skewness and kurtosis. Potential significant outliers were found using scatterplots with regression lines where a Cook’s Distance >1 was defined as a significant outlier. When a significant outlier was found, we performed an additional analysis without the outlier. Assessment of correlations between NK cell and CD4+ T cell subsets was done using Pearson r analyses or Spearman rho analyses, depending on normality of data distribution. To assess the association between ratios and MRI outcome/relapse outcome, independent t-tests or Mann-Whitney U tests were performed based on the distribution of data. Since in the

SOLAR trial, the presence of CUA was influenced by treatment arm,[18] a logistic regression analysis was conducted to correct the association between NK/T cell ratios and MRI endpoint for treatment arm allocation. If data was not normally distributed, ratios were logarithmically transformed in order to create normally distributed data. The presence of relapses[18] and plasma NfL levels[19] were not different between treatment arms, so no correction for these endpoints was applied. The correlation between ratios and NfL levels was assessed via the use of Pearson r or Spearman rho analyses, again based on data distribution. A p-value of ≤ 0.05 was considered statistically significant.

RESULTS

Patient characteristics

The SOLARIUM study included 53 patients, but due to incomplete staining at baseline or week 48 regarding NK cell related markers, 3 patients were excluded from the current study, leaving 50 patients for analysis. Baseline patient characteristics of both treatment arms are described in **Table 1**.

Additionally, 3 patients did not undergo a MRI examination at week 48, leaving 47 to be analysed for MRI activity. Clinical disease activity markers were equally distributed between patients with and without MRI activity at week 48 (**Table 1**). NfL levels were measured for 35 patients at baseline and 38 patients at week 48.

Table 1

	RRMS patients (N=50)	No MRI activity	MRI activity	p-value
Sex (N[%])				
Female	33 [66]	25 [69]	5 [45]	0.171
Male	17 [34]	11 [31]	6 [55]	
Age (years: median [interquartile range])	37.2 [31.7-44.3]	40.0 [32.8-45.3]	36.2 [29.6-43.9]	0.291
Body Mass Index (BMI) (N[%])				
≥ 25 kg/m ²	28 [56]	19 [53]	7 [64]	0.731
< 25 kg/m ²	22 [44]	17 [47]	4 [36]	
Disease duration (months: median [interquartile range])	7.4 [4.5-12.3]	7.3 [4.6-12.8]	8.4 [4.3-12.3]	0.851
Attacks during past 2 years at baseline (N[%])				
> 1	16 [32]	11 [31]	4 [36]	0.725
≤ 1	34 [68]	25 [69]	7 [64]	

Table 1 (continued)

	RRMS patients (N=50)	No MRI activity	MRI activity	p-value
Duration since last attack at baseline (months: median [interquartile range])	7.6 [5.0-10.5]	7.4 [4.4-10.4]	8.0 [7.2-11.3]	0.386
Treatment (N[%])				
Placebo	21 [42]	11 [31]	8 [73]	0.018
Vitamin D3	29 [58]	25 [69]	3 [27]	

Baseline characteristics of study participants, as well as a comparison of baseline characteristics between patients with and without MRI activity. Analysis of continuous data is done with a Mann-Whitney U test. Analysis of dichotomous data is done with a Fischer's Exact test.

NK cells correlate with CD4+ and IL-17A+CD4+ T cells

To explore the relationship between circulating NK and T cells, correlations were calculated between total NK cells, CD56bright NK cells and CD56dim NK cells on the one hand, and CD4+ T cells, CD8+ T cells, IL-17A+CD4+ T cells, IFN- γ +CD4+ T cells, IL-10+CD4+ T cells and Tregs on the other. Most notably, CD56bright NK cells correlated negatively with CD4+ T cells ($R=-0.335$; $p=0.001$) and CD4+IL-17A+ T cells ($R=-0.203$; $p=0.043$) (shown in **Figure 1B** and **Figure 1C**). Correlations between the CD56bright NK cell fraction and T cell-subsets were more prominent when compared to the CD56dim NK cell fraction (**Figure 1D**). These findings suggest that there is a biological association between NK and T cell proportions, supporting the assessment of ratios as proposed by Darlington et al.[15] Furthermore, these data suggests that the CD56bright NK cell subset is the most influential subset in these associations.

NK/ CD4+ T cell subset-ratios are lower in patients with new MRI lesions

To explore the relevance of NK/ T cell-subset ratios in the course of MS, we assessed the association of these ratios with the presence of new or enhancing MRI lesions (CUA) after 48 weeks follow-up as most sensitive marker for inflammatory disease activity.[22] When focussing on total CD4+ T cells, the total NK/ CD4+ T cell-ratio and the CD56dim/ CD4+ T cell-ratio were significantly lower in the group with MRI activity ($p=0.050$, corrected for treatment arm $p=0.071$ and $p=0.050$, corrected for treatment arm $p=0.061$; **Figure 2A**). Similarly, when analysing IL-17A+CD4+ T cells, patients with CUA had a lower total NK/ IL-17A+CD4+ T cell-ratio ($p=0.025$, corrected for treatment arm $p=0.029$) and CD56dim NK/ IL-17A+CD4+ T cell-ratio ($p=0.021$, corrected for treatment arm $p=0.026$) (**Figure 2B**).

NK/ IL-17A+ CD4+ T cell-ratios are lower in patients with subsequent relapses

To explore the consistency of this finding, we analysed other markers of disease activity. First, we compared patients who experienced a clinical exacerbation during the 48 week follow-up period with patients who did not experience a relapse. (**Figure 2C**). Focussing on the ratio's most clearly associated with radiological disease activity, we found a trend towards a lower CD56bright NK/ IL-17A+CD4+ T cell-ratio, whereas significantly lower total NK/ IL-17A+CD4+ T cell- ($p=0.006$) and CD56dim NK / IL-17A+CD4+ T cell-ratios ($p=0.005$) were observed in patients with exacerbations of disease.

NK/ CD4+ T cell subset-ratios are negatively correlated with NfL

Plasma NfL is a measure of clinical and MRI disease activity in MS, and strongly correlates with enhancing MRI lesions.[16, 23] Baseline total NK and CD56dim NK/ IL-17A+CD4+ ratios were negatively correlated with mean NfL levels ($R=-0.320$; $p=0.050$ and $R=-0.322$; $p=0.049$, **Figure 2D**). Notably, the same data point in all three correlation plots is a significant outlier (Cook's distance >1). Correlation analyses without this significant outlier were more clearly supportive of a negative correlation for IL-17A+CD4+ in a ratio with NK cells [$R=-0.422$; $p=0.009$], CD56bright cells [$R=-0.352$; $p=0.033$] and CD56dim cells [$R=-0.424$; $p=0.009$].

NK/ CD4+ subset ratios are not correlated with vitamin D

Low serum 25(OH)D levels are a biomarker for a higher risk of subsequent relapses.[24] First, baseline 25(OH)D did not significantly correlate with baseline NK/ CD4+ T cell subset-ratios (**Figure 3A**). Then, in order to assess an effect of vitamin D supplementation on NK/ CD4+ T cell subset-ratios, ratios at week 48 were compared between treatment arms, which did not show any influence of vitamin D3 supplementation (**Figure 3B**). Additionally, the relative in- or decrease in these ratio's during 48 weeks follow-up was analysed, stratified for treatment arm. There was no significant difference in distribution of any of the NK/ CD4+ T cell subset-ratios between vitamin D3 and the placebo arm (**Figure 3C**). Also, the relative and absolute NK cell (subset) proportions were not different between the treatment arms (**Supplementary figure 1**).

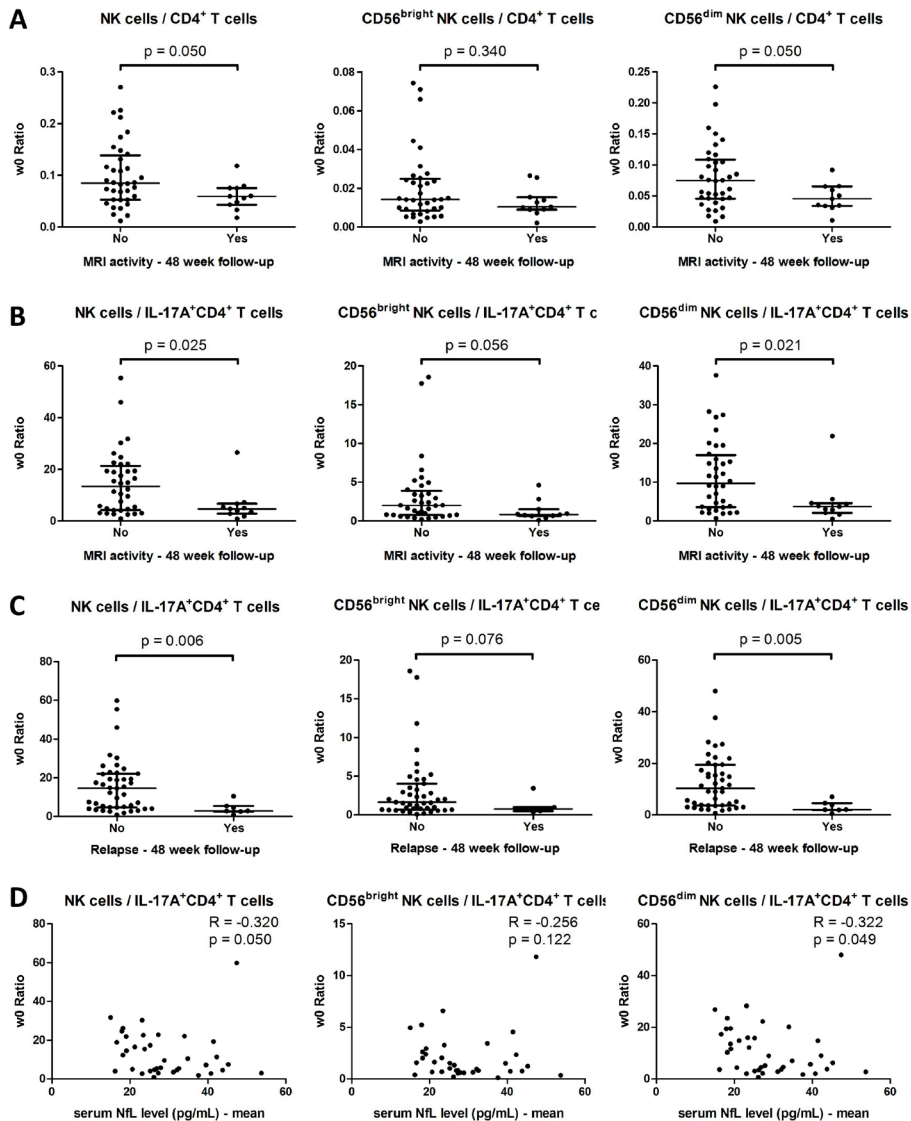


Figure 2. A: Differences in NK cells / CD4⁺ T cells ratio, CD56^{bright} NK cells / CD4⁺ T cells ratio and CD56^{dim} NK cells / CD4⁺ T cells ratio between patients with and without MRI activity after 48 weeks follow-up. P-value is calculated using a Mann-Whitney U test. Bars represent median with interquartile range. N=47 B: Differences in NK cells / IL-17A⁺CD4⁺ T cells ratio, CD56^{bright} NK cells / IL-17A⁺CD4⁺ T cells ratio and CD56^{dim} NK cells / IL-17A⁺CD4⁺ T cells ratio between patients with and without MRI activity after 48 weeks follow-up. P-value is calculated using a Mann-Whitney U test. Bars represent median with interquartile range. N=47 C: Differences in NK cells / IL-17A⁺CD4⁺ T cells ratio, CD56^{bright} NK cells / IL-17A⁺CD4⁺ T cells ratio and CD56^{dim} NK cells / IL-17A⁺CD4⁺ T cells ratio between patients suffering ≥ 1 relapses or no relapse during 48 weeks follow-up period. P-value is calculated using a Mann-Whitney U test. Bars represent median with interquartile range. N=50 D: Correlations between NK cells / IL-17A⁺CD4⁺ T cells ratio, CD56^{bright} NK cells / IL-17A⁺CD4⁺ T cells ratio and CD56^{dim} NK cells / IL-17A⁺CD4⁺ T cells ratio at baseline and mean NFL values measured in pg/mL. R shown is spearman rho. N = 3

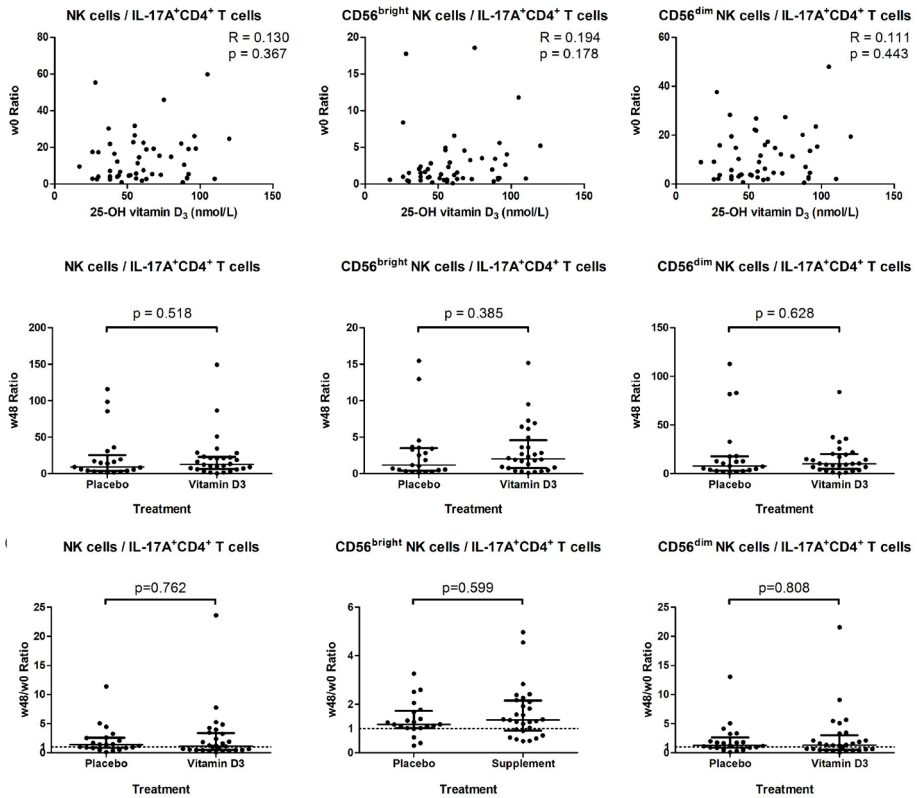


Figure 3. A: Correlation between 25-OH vitamin D levels at baseline and NK cells/ IL-17A+CD4+ T cells ratio, CD56^{bright} NK cells/ IL-17A+CD4+ T cells ratio and CD56^{dim} NK cells/ IL-17A+CD4+ T cells ratio. R shown is spearman rho. N=50 B: Comparison of NK cells/ IL-17A+CD4+ T cells ratio, CD56^{bright} NK cells/ IL-17A+CD4+ T cells ratio and CD56^{dim} NK cells/ IL-17A+CD4+ T cells ratio at week 48 between patients receiving a placebo or vitamin D3 supplements. P-value is calculated using a Mann-Whitney U test. Bars represent median with interquartile range. N=50 C: Comparisons of the evolutions of NK cells/ IL-17A+CD4+ T cells ratio, CD56^{bright} NK cells/ IL-17A+CD4+ T cells ratio and CD56^{dim} NK cells/ IL-17A+CD4+ T cells ratio. Evolution was calculated by dividing the ratio at week 48 by the ratio at baseline. An evolution >1 means the ratio has increased after 48 weeks, whereas a ratio <1 means the ratio has decreased. P-value is calculated using a Mann-Whitney U test. Bars represent median with interquartile range. N=50

DISCUSSION

We investigated the prognostic value of NK/ CD4+ T cell subset-ratios for disease activity in a cohort study of a homogenous group of interferon- β treated early RRMS patients.

First of all, we find an association between the relative presence of NK cells and the relative presence of CD4+ T cells and IL-17A+CD4+ T cells. This association is also found with NK cell subsets, where the CD56^{bright} subset of NK cells shows a stronger association than the CD56^{dim} subset, despite it making up a relatively small portion of the total

NK cell population. This stronger association with CD56bright NK cells may support the hypothesis of CD56bright NK cells fulfilling an immuno-regulatory role in MS by suppressing (autologous) activated T cells, as seen in daclizumab trials.[13]

Second, the relative presence of NK cells and subsets compared to CD4+ T cells and IL-17A+CD4+ T cells, expressed as a ratio, seems to be relevant for disease activity. Indeed, NK/CD4+ T cell subset-ratios are lower in patients with new and/or enlarging MRI lesions after 48 weeks follow-up. This effect is seen in ratios including CD4+ T cells and, perhaps more specifically, in ratios including IL-17A+CD4+ T cells. IL-17A+CD4+ T cells have been argued to constitute a subset involved in the pathogenesis of MS,[25-28] although other subsets based on other cytokines and surface markers have been shown to be involved as well. Nevertheless, our findings do reinforce the idea of a IL-17A+CD4+ T cell contribution to MS disease activity, and also supports a regulatory effect of NK cells on IL-17A+CD4+ T cells, as suggested by Darlington et al.[15] and daclizumab trials.[29] When specifically looking at the NK cell subsets, the prognostic value for disease activity seems predominantly driven by the CD56dim NK cell ratios. Given that the CD56bright NK cell subset is generally considered to be the regulatory subset and CD56bright NK cells correlate negatively with IL-17A+CD4+ T cell proportions, our clinical associations seem to be conflicting with this concept.[8] However, not only CD56bright NK cells, but also CD56dim NK cells show capability to kill activated T cells.[30] Over the last years, CD56bright NK cells have been the main focus for NK cell research in MS, but our findings highlight that the CD56dim population may be an important facet as well.

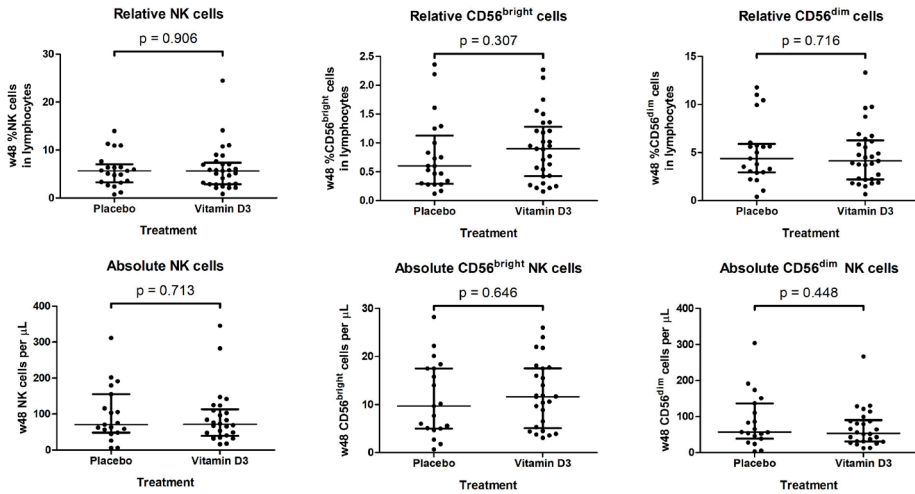
Our exploratory cohort study has some limitations. First, the SOLAR study was originally designed to investigate the effect of high dose vitamin D3 treatment,[18] while the SOLARIUM substudy was initiated to unravel the effect of high-dose vitamin D3 supplementation on the immune system.[17] This is also the reason for some missing data, in particular for NFL measurements.[19] Furthermore, MS patients included were selected based on having RRMS, short disease duration, and treatment with IFN- β . Therefore, extrapolation of the data requires extension of our findings in other MS patient cohorts. Finally, due to the initial research question of the SOLAR study, the number of patients is not based on a power calculation relevant to our question. Hence, we refer to our study as an exploratory study.

Interestingly, it has been shown in daclizumab studies that IL-2 is important in the interplay between NK and T cells[31] and other studies have shown that granulocyte-macrophage colony-stimulating factor (GM-CSF), a pro-inflammatory cytokine, is strongly induced by IL-2.[32] Unfortunately, we were unable to evaluate GM-CSF+CD4+

T cells due to too many missing data points at baseline.[17] Thus, the possible relation between GM-CSF, IL-2 and NK cells could not be investigated in this study.

In conclusion, we show a relation between NK cells (subsets) and CD4+ T cells (subsets) in RRMS. NK cells seem to exert a protective effect, hypothetically by controlling the T cell population. Not only the CD56bright NK cell population, known for its immunoregulatory properties, is involved, but also the CD56dim NK cell population seems to play a role in reducing disease activity. More research with independent data sets is needed to confirm the validity and use of NK/ CD4+ subset-ratios as a prognostic marker in RRMS.

SUPPLEMENTARY MATERIALS

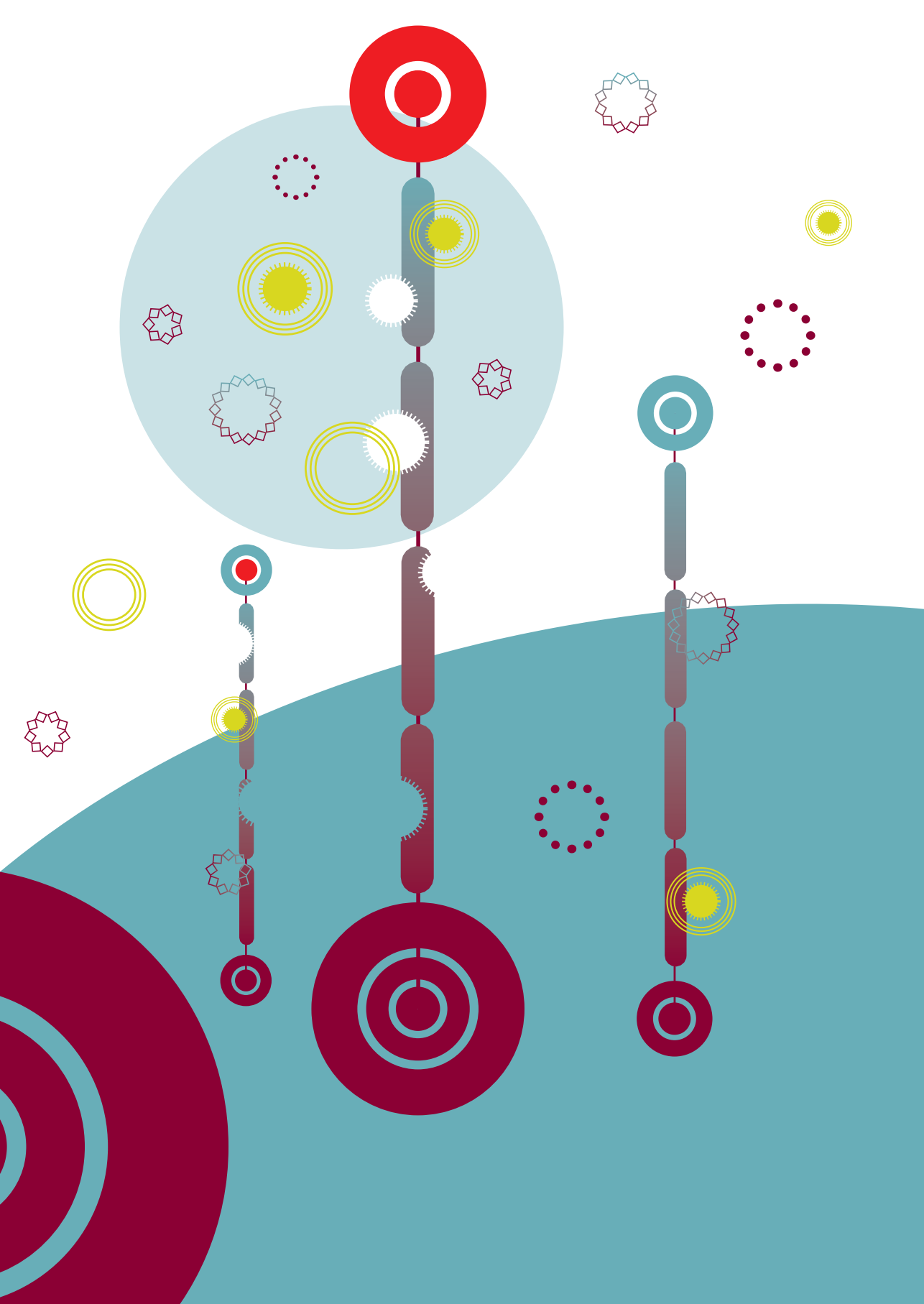


Supplementary figure 1. Comparison of relative NK cell (subset) numbers and absolute NK cell (subset) numbers per μ L at week 48, compared between treatment arms. P-value is calculated using a Mann-Whitney U test. N=45

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Chapter 6



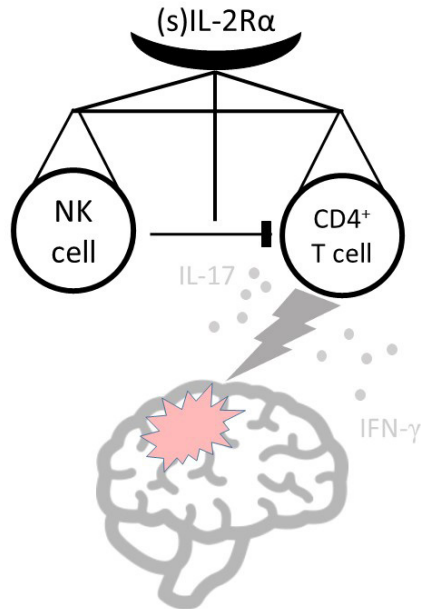
The IL-2 – IL-2R pathway influences NK – T cell interaction

Max Mimpfen, Linda Rolf, Anne-Hilde Muris, Oliver Gerlach, Geert Poelmans, Raymond Hupperts, Joost Smolders, Jan Damoiseaux.

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ABSTRACT

NK/T-cell ratios predict disease activity in relapsing remitting multiple sclerosis (RRMS). We investigated in 50 RRMS patients whether interleukin-2 receptor alpha-chain (IL-2R α) expression and shedding associates with NK/T-cell balance, as suggested by daclizumab-trials in RRMS. A subsample (N=31) was genotyped for *IL2RA*-associated MS risk SNPs. CD56^{bright} NK-cell/IL-17A⁺CD4⁺ T-cell ratios correlated negatively with plasma and PBMC-culture supernatant sIL-2R α -levels [R=-0.209; p=0.038 and R=-0.254; p=0.012, resp.], and with CD4⁺ T-cell CD25 MFI [R=-0.341; p=0.001]. Carriers of the rs3118470 risk-allele showed higher sIL-2R α -levels (P=0.031) and a lower CD56^{bright} NK-cell/IL-17A⁺CD4⁺ T-cell ratio (P=0.038). Therefore, IL-2R α may be involved in the interplay between NK-cells and T-cells.



INTRODUCTION

Multiple sclerosis (MS) is an immune-mediated inflammatory disease of the central nervous system.¹ Although not fully elucidated, many factors and pathways have been suggested to be relevant for MS disease activity. One of these pathways is the interleukin-2 (IL-2) / interleukin-2 receptor (IL-2R) pathway.^{2, 3} IL-2 is a cytokine with both pro- and anti-inflammatory effects on the immune system, well known as a survival cytokine for activated T cells, but also as important in the biology of regulatory T (Treg) cells and natural killer (NK) cells.⁴ The composition of the IL-2R is variable. Incorporation of the α -chain (IL-2R α , CD25) into a tri-molecular complex renders a receptor with high affinity for IL-2.⁵ Activated immune cells may shed IL-2R α and produce soluble IL-2R α (sIL-2R α), of which higher circulating levels are associated with MS disease activity.⁶ Whether sIL-2R α by itself has a more pro- or anti-inflammatory role, remains controversial.⁵

The IL-2/IL-2R pathway in MS gained renewed interest after the positive clinical results from an anti-CD25 therapy (daclizumab), aimed at reducing disease activity in relapsing remitting (RR)MS patients.^{7, 8} Daclizumab was shown to increase the NK cell population, specifically the immune-regulatory CD56^{bright} NK cell population, which is thought to kill activated T cells.⁹ This finding revealed the relevance of the interplay between NK and T cells in RRMS, with the IL-2R as possible mediator. Recently, we have shown in interferon beta-treated RRMS patients that disease activity is associated with lower NK/T-cell ratios,¹⁰ which not only points towards an interesting potential prognostic biomarker for disease activity, but also further implicates an influential role of NK/T-cell interplay in RRMS. The role of IL-2R α in these associations has not been explored previously.

We studied the association between sIL-2R α and NK/T cell ratios in MS. To further consolidate our finding, we extended this analysis by exploring several other IL-2R related markers, as well as the genetic status of our participants with regard to two known *IL2RA*-associated MS risk alleles.

METHODS AND MATERIALS

Patients

This study is a post-hoc extended analysis of the SOLARIUM study, which was a sub-study of the SOLAR study. The SOLAR study evaluated disease activity in interferon beta-treated RRMS patients using high dose vitamin D₃ supplements compared to placebo. The SOLARIUM study investigated the effect of high dose vitamin D₃ supplementations on the immune system composition. In- and exclusion criteria for the SOLAR and SOLAR-

IUM studies are described elsewhere.^{11,12} For inclusion in this analysis, all 50 participants of whom data regarding NK cells, T cells and IL-2R α were available were included. Retrospectively, 31 of these 50 participants provided consent for genetic analysis. Written informed consent was acquired and the SOLARIUM study was approved by the Ethical Committee METC-Z (11-T-03; Heerlen, the Netherlands).

PBMC isolation

The acquirement and analysis of peripheral blood mononuclear cells (PBMCs) is described elsewhere.¹² In summary, peripheral blood samples were collected from patients at baseline and at week 48 of treatment. Blood was collected in a 10mL sodium heparin blood sampling tube (BD Biosciences, Breda, The Netherlands) and transported to Maastricht University Medical Center, the Netherlands, at room temperature. Within 24 hours PBMCs were isolated as described previously.

IL-2 receptor alpha-chain gene expression, protein expression and shedding

Measurement of sIL-2R α is described elsewhere.¹³ In short, sIL-2R α levels in plasma were measured by a chemiluminescent assay, using a commercially available kit for quantitative measurement of sIL-2R α on the Immulite 2000 (Siemens; 95% reference range 158-623 U/mL; N=50). Additionally, sIL-2R α was measured in culture supernatant of PBMCs (100,000/well) which were cultured and stimulated by phytohaemagglutinin (PHA; 25 μ g/mL) for 72h (N=50). In addition, the process of obtaining gene expression data by PCR (N=39) and staining of PBMC for flow cytometry is described elsewhere.^{12,13} Flow cytometry was used to obtain MFI values of IL-2R α (CD25) on CD4⁺ T cells (N=50). The gating strategy is shown in **Figure 1A**. Genotyping the polymorphisms rs2104286 and rs3118470 was done using extracted DNA from buffy coats. This DNA was analysed on the Infinium PsychArray-24v1.3_A1 BeadChip (Infinium array technology) and the polymorphisms of interest were defined (N=31).

Statistical analysis

SPSS software (IBM SPSS, version 25.0. Chicago, IL) was used to assess the correlation between baseline and week 48 IL-2R α markers, as well as the correlation between IL-2R α markers and NK/T ratios. Normality of data was assessed by visual inspection of histograms with normal curves, skewness and kurtosis. Both correlations between baseline and week 48 IL-2R α markers, and IL-2R markers and NK/T ratios were performed using a Pearson r correlation or Spearman rho correlation test, depending on distribution of data. Differences in continuous variables between carriers of genetic risk-alleles were

analysed with a Mann Whitney U test. A p-value of <0.05 was considered statistically significant.

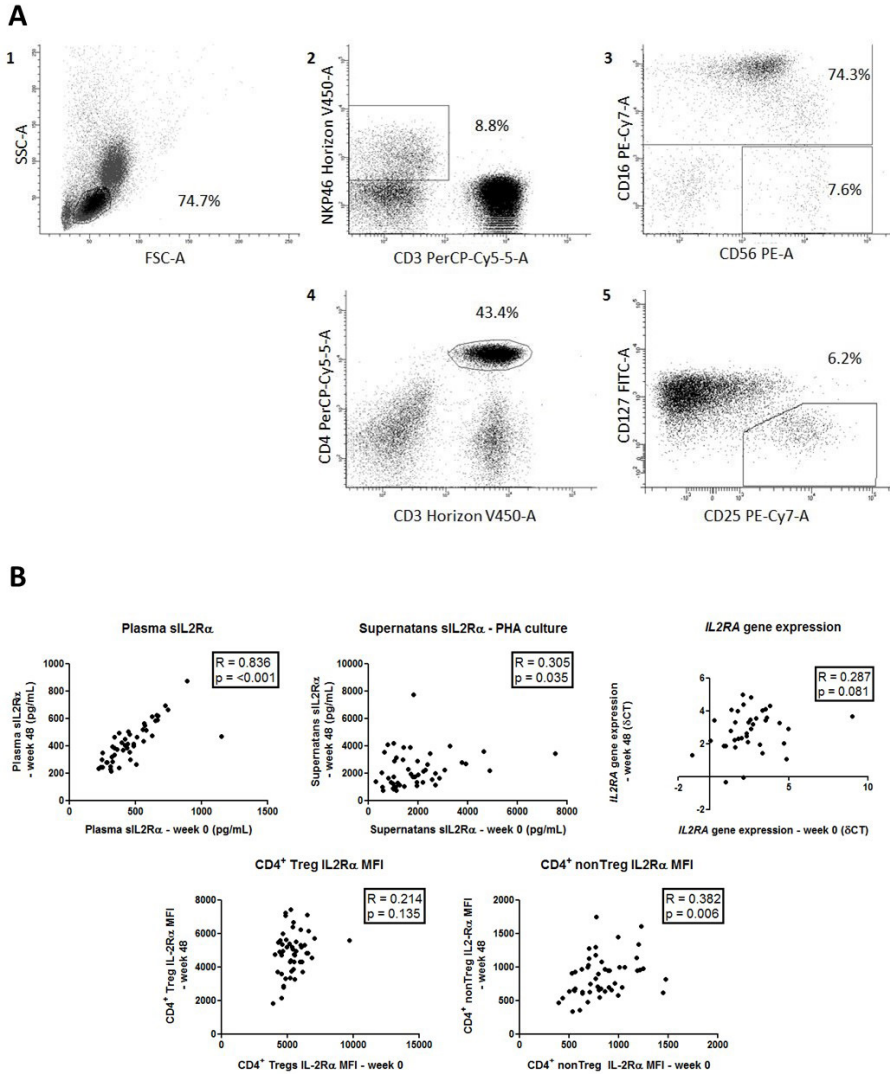


Figure 1. A: Gating strategy used to analyse NK cells, T cells and their respective subsets. Step 1 shows the gating of lymphocytes from the PBMC population. Step 2 shows the gating of NK cells, defined as $CD3^+NKp46^+$, from the lymphocyte population. Step 3 shows the differentiation between the $CD56^{dim}CD16^+$ NK cells (above) and $CD56^{bright}CD16^+$ NK cells (below). Step 4 shows the gating of $CD4^+T$ cells, defined as $CD3^+CD4^+$, from the lymphocyte population. Step 5 shows the differentiation between regulatory T cells (Treg), defined as $CD25^+CD127^-$ and other $CD4^+$ T cells (nonTreg). FSC: forward scatter; SSC: side scatter. **B:** Correlations between baseline and week 48 values of soluble IL-2 receptor alpha-chain (sIL2Rα) in serum, sIL2Rα in PBMC PHA culture, *IL2RA* gene expression, IL-2Rα MFI on Tregs and IL-2Rα MFI on nonTregs. R and p-value shown are based on Spearman rho analyses. MFI: mean fluorescence index; Treg: regulatory T cell; PHA: phytohaemagglutinin.

RESULTS

Strong correlation between baseline and week 48 plasma sIL-2R α levels

We analysed data on serum sIL-2R α levels, supernatant sIL-2R α levels, *IL2RA* gene expression and CD4⁺ T cell IL-2R α protein expression at baseline and at 48 weeks follow-up (**Figure 1**). While sIL-2R α levels in serum at baseline and week 48 correlated strongly ($R=0.836$; $p<0.001$), other IL-2R α related markers showed relatively low or statistically non-significant correlations (**Figure 1B**). Since these variables all show a biological variability, which also applies to the NK/T-cell ratios, we pooled our data of both time points.

Lower NK/T cell ratios associate with higher IL-2R α protein expression and shedding

As NK/IL-17A⁺CD4⁺ T cell ratios were most strongly associated with disease activity in our previous work,¹⁰ and IL-17⁺ T cells have been proposed as a pathogenic T cell subset in MS,¹⁴ this ratio was focus for our IL-2R α related analyses. We included ratios of the total, CD56^{bright} and CD56^{dim} NK cell subsets, to explore effects of these different subsets.

Most notably, all ratios involving IL-17A⁺CD4⁺ T cells showed a negative correlation with serum sIL-2R α levels (**Figure 2A**), as well as sIL-2R α shedding by PHA-stimulated PBMCs (**Figure 2B**). Since sIL-2R α has been hypothesized to be mostly shedded by activated CD4⁺ T cells,¹⁵ we also explored the correlation of NK/T-cell ratios with CD4⁺ T cell IL-2R α (CD25) MFI. Although IL-2R α MFI on Treg CD4⁺ cells did not correlate with NK/T-cell ratios (**Figure 2C**), IL-2R α MFI on non-Treg CD4⁺ T cells correlated negatively with all NK/T-cell ratios investigated (**Figure 2D**). *IL2RA* gene expression levels in total PBMCs were available but did not correlate with NK/T-cell ratios (**Figure 2E**). We conclude that in MS, IL-2R α protein expression and shedding by T cells correlate consistently with a, relative to NK cells, increased proportion of circulating (pathogenic) CD4⁺ T cells.

Presence of risk allele of rs3118470 is linked with higher sIL-2R α levels

To further consolidate the association of IL-2R α related endpoints with NK/T-cell ratios, we genotyped a subset of participants for *IL2RA*-associated MS risk SNPs rs2104286 and rs3118470.¹⁶ Notably, the rs2104286 risk-allele has been associated with higher serum sIL-2R α levels in MS.¹⁷ Of the N=31 participants of whom genetic data were available, N=30 were carriers of the rs2104286 risk allele. Comparing carriers and non-carriers of the rs3118470 MS risk allele (N=13 and N=18, respectively), carriers had a significantly higher level of sIL-2R α at baseline, as well as a lower CD56^{bright} NK/IL-17A⁺CD4⁺ T cell ratio with differences between other NK/T-cell ratios not being statistically significant (**Figure 3**).

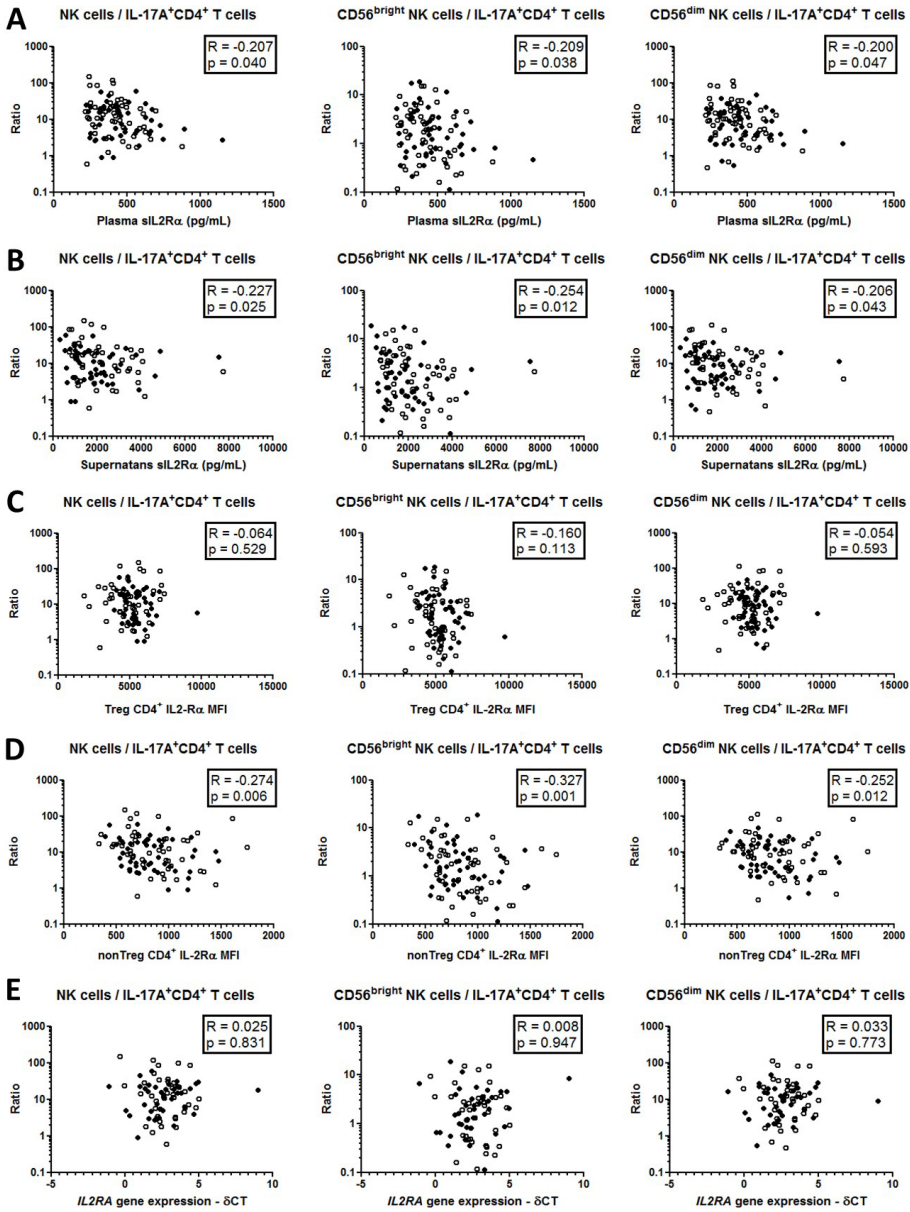


Figure 2. Correlations between NK/IL-17A⁺CD4⁺ T cells ratio, CD56^{bright} NK/IL-17A⁺CD4⁺ T cells ratio and CD56^{dim} NK/IL-17A⁺CD4⁺ T cells ratio, and **A:** soluble IL-2 receptor alpha-chain (sIL2Rα) in serum. **B:** soluble IL-2 receptor alpha-chain (sIL2Rα) in stimulated culture. **C:** IL2RA gene expression. **D:** IL-2 receptor alpha-chain expression on regulatory T cells. **E:** IL-2 receptor alpha-chain expression on non-regulatory CD4⁺ T cells. R and p-value shown are based on spearman rho analyses. Baseline and week 48 values are pooled. Baseline values are represented by black dots, week 48 values are represented by open dots. MFI; mean fluorescence index; sIL2Rα: soluble IL-2 receptor alpha-chain; Treg: regulatory T cell.

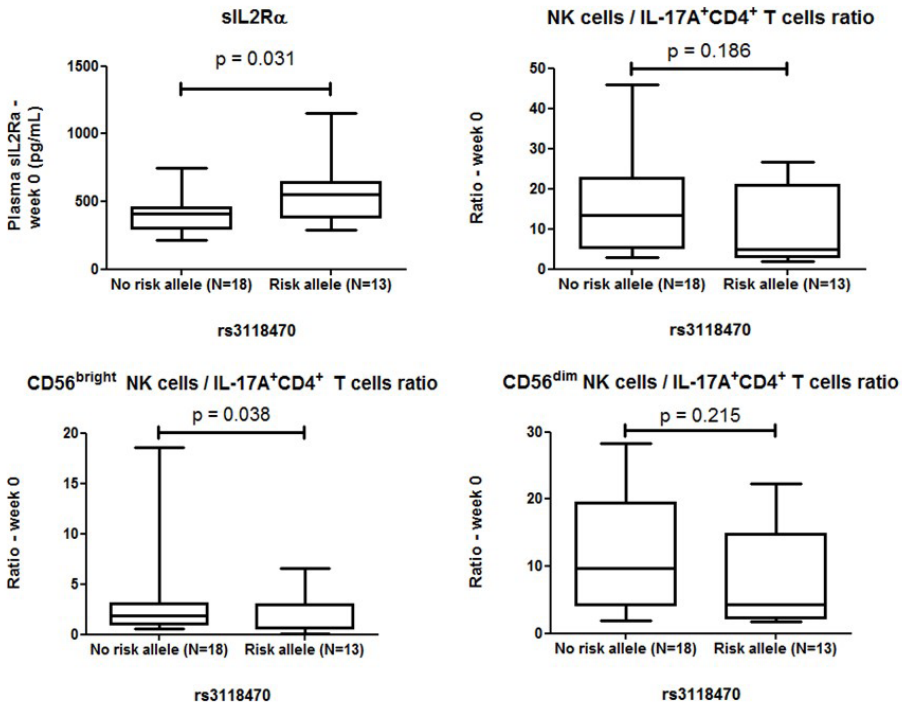


Figure 3. Influence of genetic profile on soluble IL-2 receptor alpha-chain (sIL2Ra) levels in serum, NK/IL-17A⁺CD4⁺ T cells ratio, CD56^{bright} NK/IL-17A⁺CD4⁺ T cells ratio and CD56^{dim} NK/IL-17A⁺CD4⁺ T cells ratio. All shown measurements are baseline values. Box and whiskers plots showing median and 5th-95th percentiles. P-value shown is calculated using Mann-Whitney U tests.

DISCUSSION

We investigated the role of IL-2Ra in the interplay between NK and T cells in a cohort study using a homogenous group of interferon beta-treated RRMS patients. We report associations between NK/IL-17A⁺CD4⁺ T cell ratios and sIL-2Ra protein shedding in vivo and in vitro, and IL-2Ra protein expression by non-regulatory CD4⁺ T cells. Furthermore, higher baseline serum sIL-2Ra levels and lower CD56^{bright}/IL-17A⁺CD4⁺ T cell ratios associate with the rs3118470 risk allele.

Our results may be interpreted in different ways. First, a higher sIL-2Ra level may simply be the result of a lower NK/T cell ratio. As a lower ratio would imply a reduced regulation of activated T cells by NK cells, this would mean that there are more activated non-regulatory T cells to shed IL-2Ra and thus increase sIL-2Ra levels. In this scenario, sIL-2Ra would be a biomarker of higher T cell activity, causing disease activity, which could then also be expressed as a lower NK/T cell ratio. Alternatively, sIL-2Ra could influence NK

cells and/or T cells in such a way that the NK/T ratio is altered, thus leading to a reduced regulation of activated T cells and paving the way for disease activity. Unfortunately, interpretation of our results is hindered by the poor understanding of the role of sIL-2R α in immune activation and regulation.⁵ Although our genetic data does not favour one interpretation over the other, it does further strengthen the implication that IL-2R α is involved in NK/T interplay.

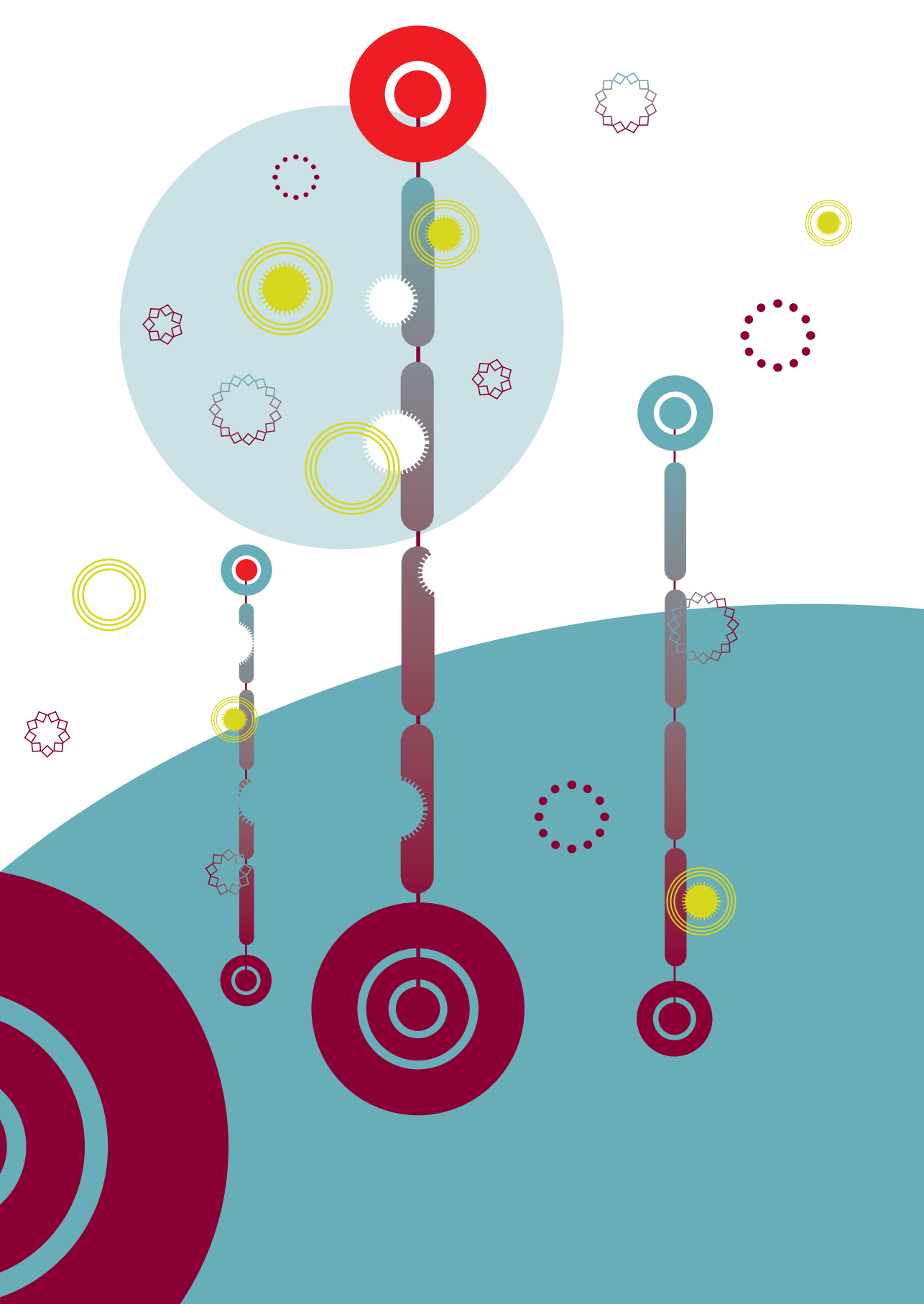
This study has some limitations. The initial research question of the SOLARIUM study was focused on vitamin D₃ supplementation, which makes the current analysis exploratory. Additionally, only a limited set of IL-2/IL-2R pathway-related variables was explored, with IL-2 levels lacking. Finally, our cohort consists of patients using interferon beta, that transiently affects IL-2R α expression on CD4⁺ T cells, which may have influenced our results.¹⁸

In conclusion, we report a significant association between IL-2R α protein expression and shedding and the interplay between NK cells and T cells. The exact context and influence remain to be elucidated and, as such, more research is needed to investigate the exact mechanism of action in MS.

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Chapter 7



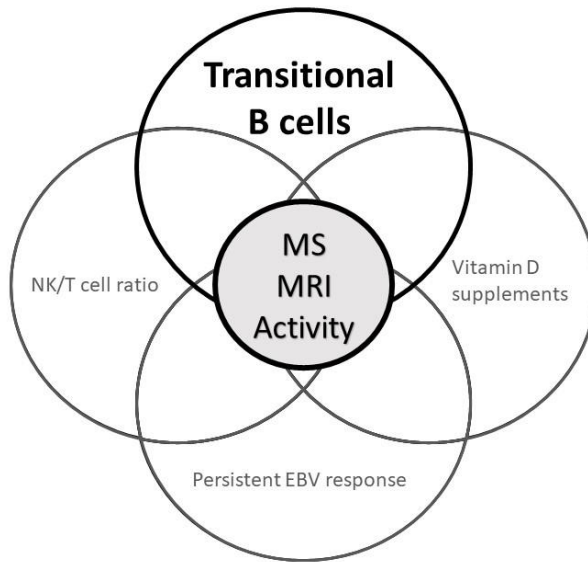
Prognostic value of transitional B cells: a step towards systems immunology

Max Mimpfen, Jan Damoiseaux, William van Doorn, Linda Rolf, Anne-Hilde Muris,
Raymond Hupperts, Marvin M. van Luijn, Oliver Gerlach, Joost Smolders.

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beta-treated multiple sclerosis patients. *Journal of Neuroimmunology*, 2021; 358: 577664.

ABSTRACT

B-cells contribute to MS pathogenesis. The association of circulating B-cell phenotypes with combined unique active lesions (CUA) on MRI at 48 weeks follow-up was investigated in 50 interferon beta-treated MS patients. Transitional B-cell proportions were lower in participants with CUA at week 0 and 48 [$p=0.004$, $p=0.002$]. A decrease in circulating anti-EBNA-1 IgG levels between week 0 and 48 associated with absence of CUA [$p=0.047$], but not with B-cell profiles. In a multi-factor model for CUA-risk, transitional B-cell proportions contributed independent from NK/T-cell ratio, change in anti-EBNA-1 IgG, and vitamin D supplements. Transitional B-cells may predict treatment response in MS.



INTRODUCTION

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS), of which the pathogenesis is driven by a complex interplay of environmental and genetic factors.[1] Although the exact underlying disease mechanisms of MS are not yet fully elucidated, several lymphocyte populations have been associated with its disease process. These include T helper 1 cells (CD4⁺IFN- γ ⁺ T cell, Th1 cell),[2] T helper 17 cells (IL-17A⁺CD4⁺ T cell, Th17 cell)[3, 4], natural killer (NK) cells[5-7], and B cells[8, 9]. Several environmental factors associated with MS onset are believed to modulate the functional or phenotypic characteristics of these lymphocytes, including vitamin D levels,[10-12] infection with Epstein-Barr virus (EBV),[13-15] smoking[16] and obesity.[13] Especially EBV, a B cell-tropic virus that remains latent in memory B populations, appears to be a prerequisite for developing MS.[17-19] Circulating antibodies against the EBV nuclear antigen type 1 (EBNA-1) are also associated with a higher risk of radiological MS activity.[15]

Of the lymphocyte populations, B cells have recently gained a lot of attention, largely due to the positive effects of B cell depleting CD20-targeted therapies on MS disease activity in both relapsing and progressive MS.[20, 21] Where B cells were traditionally viewed as a relatively passive population in the older MS disease models,[22] more recent models give them a more central role,[9, 23, 24] partially due to the growing appreciation for their antibody-independent functions in (auto)immunity.[25] This includes their role as antigen presenting cell (APC), their production of pro-inflammatory cytokines, as well as their involvement in establishing and maintaining meningeal tertiary follicles, as seen in secondary progressive MS patients.[26] These tertiary follicles in the secondary progressive (SP)MS phase are associated with an earlier onset of disease, as well as a more severe disease course.[27] Since EBV primarily infects (memory) B cells [14, 28] and EBV infected B cells have been found in the tertiary follicles by some,[29] but not all groups,[30, 31] it is suggestive that these factors are closely linked in the pathogenesis of MS.

Besides treatment of existing clinical manifestations, predicting subsequent disease activity is a challenge in MS. Accurate prediction would facilitate timely intervention, thereby reducing lesions and limiting permanent damage to the CNS. Recently, we showed that supplementation of vitamin D₃ for 48 weeks in interferon-beta-1a treated relapsing-remitting (RR)MS patients did not increase the proportion reaching no evidence of disease activity (NEDA), but was associated with a reduced proportion of combined unique active (CUA) lesions on week 48 MRI (*i.e.* new T2 or gadolinium-enhancing lesions).[32] In N=50 participants of a Dutch sub-study of this trial, we found that a low ratio between NK cells and IL-17A producing T helper cells (NK/IL-17A⁺CD4⁺ cell ratio) at

week 0 predicted the presence of CUA at 48 weeks.[6] Moreover, this ratio was associated with IL-2 receptor alpha chain expression and shedding.[33]

We now assess the prognostic value of circulating B cell phenotypes in the same cohort for MRI activity after 48 weeks. Furthermore, we explore how these phenotypes associate with anti-EBV serology, *i.e.* anti-EBNA-1 IgG and anti-VCA IgG, and other lymphocyte subsets.

METHODS

Patients

This study is a post-hoc extended analysis of the SOLARIUM study, which was a sub-study of the SOLAR study (NCT01285401). The aim of the SOLAR study was to evaluate disease activity in interferon beta-1a (IFN- β -1a) treated RRMS patients using high dose vitamin D₃ supplements versus placebo. Patients in the vitamin D₃ group received cholecalciferol drops (Vigantol Oil, Merck) 7000 IU/day in the first 4 weeks, followed by 14,000 IU/day up to week 48. The SOLARIUM sub-study investigated the effect of high dose vitamin D₃ supplementations on immune system composition. In- and exclusion criteria for the SOLAR and SOLARIUM studies are described elsewhere.[32, 34] In short, the SOLAR study recruited patients aged 18-55 years, diagnosed with RRMS (according to the McDonald criteria 2005) confirmed by typical MS findings on magnetic resonance imaging (MRI). The first clinical event had to be described within 5 years prior to study screening and signs of active disease must have been present in the last 18 months, but no relapse in 30 days before inclusion. Patients could not participate if they already consumed more than 1,000 IU (25 μ g) of vitamin D₃ supplements. All patients received IFN- β -1a 44 μ g s.c. three times weekly. Eligible participants had used IFN- β -1a at least 90 days, but no longer than 18 months. After randomisation, the patients received either IFN- β -1a and a placebo or IFN- β -1a and vitamin D₃ supplements.

Regarding the MRI outcome, procedures and findings as reported in the SOLAR trial were used.[32] In short, MRI assessments were performed at baseline and week 48. Scans included T2- and T1-weighted images (3mm slice thickness and 1mm in-plane resolution) before and after administration of IV gadolinium. MRI was used to find presence of combined unique active (CUA) lesions (new gadolinium-enhancing or new/enlarging T2 lesions) at week 48. Since only N=3 patients had more than 1 CUA after 48 weeks, MRI activity was measured as a dichotomous yes/no outcome.

The SOLARIUM sub-study recruited patients from four of the five participating centers in the Netherlands without adding additional in- or exclusion criteria, being eligible when they consented to participation in the sub-study. Written informed consent was acquired and the SOLARIUM study was approved by the Ethical Committee METC-Z (11-T-03; Heerlen, the Netherlands). Peripheral blood samples were collected at baseline (w0) and after 48 weeks (w48) and analysed using flow cytometry.

For the current study, N=50 participants of whom data regarding B cells and EBV antibody parameters were available could be included.

Peripheral blood mononuclear cells isolation

The acquirement and analysis of the peripheral blood mononuclear cells (PBMCs) is described elsewhere.[34] In summary, peripheral blood samples were collected from patients at baseline and week 48 of treatment. Blood was collected in a 10mL sodium heparin blood sampling tube (BD Biosciences, Breda, The Netherlands) and transported to Maastricht University Medical Center, the Netherlands, at room temperature. Within 24 hours PBMCs were isolated by gradient centrifugation as described in previous publications.[32, 34]

Flow cytometry

Immediately after isolation, PBMCs were stained with a cocktail of monoclonal antibodies in order to define B cells (CD19⁺ lymphocytes) and subsequently transitional B cells (IgD⁺CD27⁺CD38⁺⁺), naïve B cells (IgD⁺CD27⁺CD38⁺), non-isotype switched B cells (IgD⁺CD27⁺CD38^{+/}), isotype switched B cells (IgD⁻CD27⁺CD38^{+/}), plasmablasts (IgD⁻CD38⁺⁺) and senescent B cells (IgD⁻CD27⁻). The following fluorochrome-conjugated antibodies were used: IgD-FITC (BD Biosciences, Breda, The Netherlands); CD27-PE (BD Biosciences); CD19-PerCP-Cy5-5 (BD Biosciences); CD38-APC (BD Biosciences). Additionally, regulatory B cells (Bregs, CD19⁺IL-10⁺) were defined by IL-10 production upon stimulation with CpG, as described in an earlier publication.[34] For FACS analysis (FACS Canto II flow cytometer (BD Biosciences)), B cells were analysed for 100,000 events in the lymphocyte gate. FACS DIVA software (BD Biosciences) was used to analyse the flow cytometry data. Gating strategies, as well as phenotype definitions, are shown in **Figure 1**. The definition of transitional B cells and plasmablasts was validated in a subset of participants at week 48 using an alternative gating strategy (CD19⁺CD24⁺⁺CD38⁺⁺ and CD19⁺CD27⁺⁺CD38⁺⁺, respectively; **Supplementary Figure 1**).

Antibody measurements

From SOLARIUM participants, blood was drawn at baseline and after a 48-week study period for measurements of several markers. Levels of IgG against the EBV antigens

EBNA-1 and viral capsid antigen (VCA) were measured in plasma samples, which were stored at -20°C until analyses. Tests were performed using the quantitative LIAISON® EBNA or VCA IgG assays (DiaSorin, Saluggia, Italy), which use chemiluminescence immunoassay technology. Results $>22\text{ U/mL}$ were considered positive.

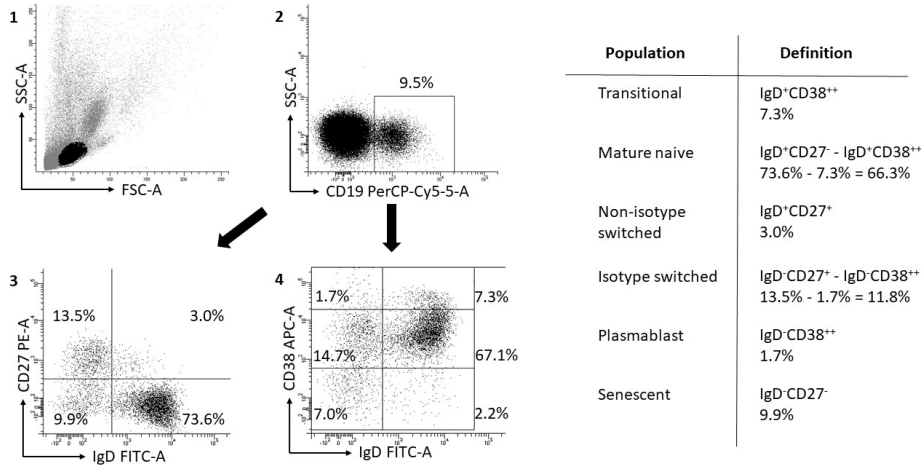


Figure 1. Gating strategy used to analyse and define B cells and subsets. Step 1 shows the gating of lymphocytes from the PBMC sample. Step 2 shows the gating of B cells, defined as CD19⁺ lymphocytes. Step 3 and 4 show distinctions made based on the IgD, CD27 and CD38 marker. Based on this strategy, six B cell phenotypes can be defined: transitional (IgD⁺CD38⁺⁺), naive (IgD⁺CD27⁻CD38⁺), non-isotype switched (IgD⁺CD27⁺CD38^{int}), isotype switched (IgD⁺CD27⁺CD38^{int}), plasmablasts (IgD⁻CD38⁺⁺) and senescent (IgD⁻CD27⁻). APC: allophycocyanin; FITC: fluorescein isothiocyanate; FSC: forward scatter; PE: phycoerythrin SSC: side scatter; PerCP: peridinin chlorophyll protein complex.

Statistics

SPSS software (IBM SPSS, version 25.0. Chicago, IL) was used to assess associations with disease activity. Normality of data was assessed by visual inspection of histograms with normal curves, skewness and kurtosis. To assess the association between B cell phenotypes and MRI activity after 48 weeks, independent t-tests or Mann-Whitney U tests were performed based on distribution of data. When assessing the role of multiple parameters for MRI activity after 48 weeks, a binary logistic model was used. If any parameters were not normally distributed according to our earlier mentioned criteria, they were log-transformed in order to normalise the data.

To determine cut-off points for an exploratory multi-factor model, ROC curves were plotted. The cut-off with the highest combined sensitivity and specificity was used. A p-value of <0.05 was considered statistically significant.

RESULTS

Baseline characteristics

The SOLARIUM cohort consisted of 53 RRMS patients, of which N=3 patients were ineligible due to incomplete immunostainings. Additionally, N=3 patients did not undergo an MRI examination after 48 weeks, leaving N=47 patients for analyses regarding MRI outcome. N=11 patients were positive for presence of CUA after 48 weeks follow-up, whereas N=36 were negative. Baseline characteristics did not differ between patients with and without MRI activity, except for a larger proportion placebo-randomized patients in the group with MRI activity (**Supplementary Table 1**). [6, 32] As reported earlier, all participants were EBV-seropositive: 92% of patients were anti-EBNA-1 positive while 96% were positive for anti-VCA, and none were negative for both markers. [35]

B cell compartment composition associates with MRI activity after 48 weeks

First, we assessed the association between circulating B cell subsets and the presence of CUA on week 48 MRI. At week 0, a lower proportion of transitional B cells [$p=0.004$] and, to a lesser extent, a higher proportion of isotype switched B cells [$p=0.030$] were found in patients with CUA on the week 48 MRI (**Figure 2A**). At week 48, similar associations were observed for transitional and isotype switched B cells [$p=0.002$ and $p=0.015$, respectively] (**Figure 2B**) with the addition of a higher percentage of non-isotype switched B-cells in participants with CUA on the week 48 MRI [$p=0.035$]. These data suggest higher proportions of transitional B cells, and to a lesser extent also lower proportions of isotype-switched B cells, to positively influence the risk for CUA in interferon beta-treated RRMS. Although our manuscript is focused on MRI activity of MS, as being the most sensitive marker for inflammatory disease activity for MS, [36] we also explored an association of B cells subsets with the absence of the clinical and MRI activity as expressed in the composite NEDA-3 endpoint. Patients with a NEDA-3 status at 48 week follow-up showed higher proportions of transitional B cells at baseline ($p=0.008$, Supplementary Figure 2). To exclude an effect of vitamin D supplementation on circulating B cell subsets, we explored effects of vitamin D supplementation on these phenotypes. None of the B cell subsets showed a significant change due to vitamin D₃ supplements (**Figure 2C**).

B cell subsets do not associate with anti-EBNA-1 IgG levels in serum

Since circulating antibodies against EBV antigens have been reported to be associated with MS MRI activity, [15] associations between B cell phenotypes and IgG responses EBNA-1 and VCA were explored. In our cohort, there was no difference in anti-EBNA-1 and anti-VCA antibodies between patients with or without CUA on week 48 MRI (**Figure**

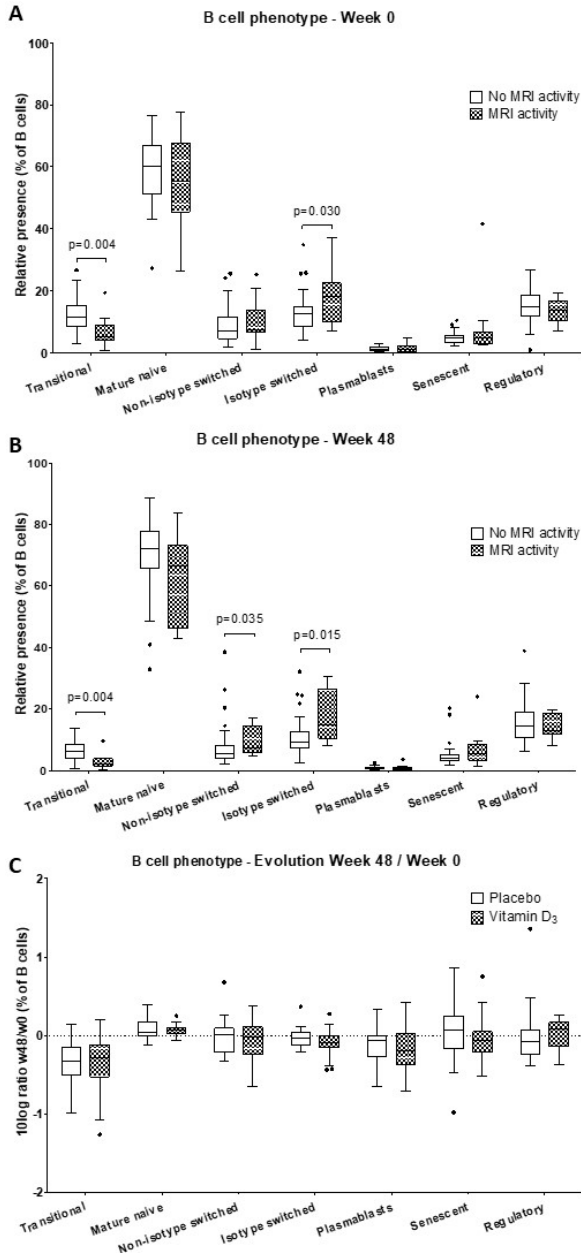


Figure 2. A: Differences in B cell phenotypes (as defined in Figure 1, with the addition of regulatory B cells) between patients with and without MRI activity, measured at baseline. Shown p-value is calculated using a Mann-Whitney U test. **B:** Differences in B cell phenotypes (as defined in Figure 1, with the addition of regulatory B cells) between patients with and without MRI activity, measured at week 48. Shown p-value is calculated using a Mann-Whitney U test. **C:** Differences in the evolution of B cells over 48 weeks between patients with and without vitamin D supplementation. Evolution is calculated as the log ratio of week 48/w0, where negative numbers represent a relative decrease of B cell phenotypes, while positive numbers indicate a relative increase in B cell phenotypes. Shown p-value is calculated using a Mann-Whitney U test. Dot-dotted lines represent the 0 value.

3A and 3B). Patients without MRI-activity at 48 weeks showed a more pronounced reduction in circulating anti-EBNA-1 but not anti-VCA IgG antibodies during 48 weeks of follow-up compared to patients with CUA (**Figure 3C**). Accordingly, vitamin D₃ supplementation was already shown to be associated with both a lower proportion of CUA at week 48,[32] and a reduction of circulating anti-EBNA-1 IgG antibodies.[35] In our cohort, circulating anti-EBNA-1 IgG antibodies did not correlate with B cell phenotype percentages at any time point (**Figure 3D**). This finding suggests that changes in B cell phenotypes and anti-EBNA-1 IgG in serum are independent contributors to CUA-risk in IFN- β -1a treated MS patients.

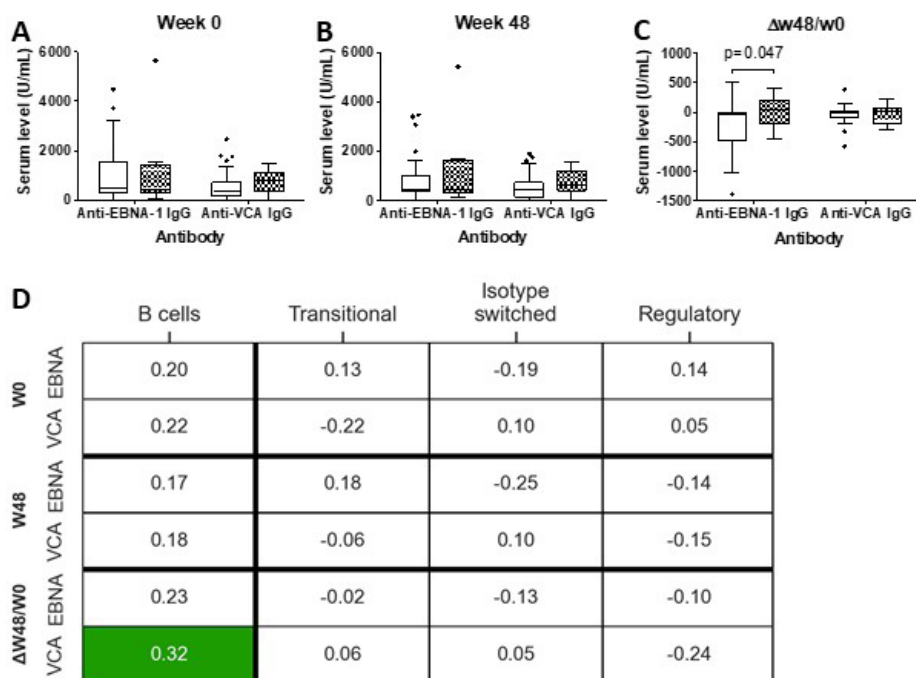
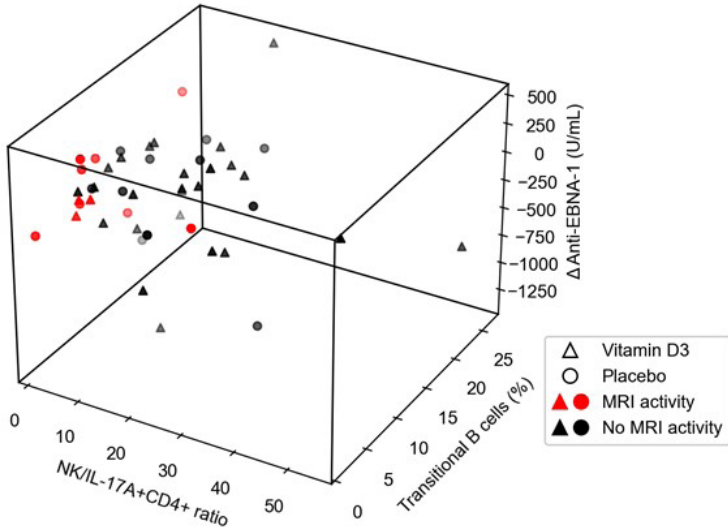


Figure 3. A: Differences in anti-EBNA-1 IgG and anti-VCA IgG between patients with and without MRI activity, measured at baseline. Shown p-value was calculated using a Mann-Whitney U test. **B:** Differences in anti-EBNA-1 IgG and anti-VCA IgG between patients with and without MRI activity, measured at week 48 **C:** Differences in anti-EBNA-1 IgG and anti-VCA IgG between patients with and without MRI activity, measured as the difference between week 48 and baseline. **D:** Heatmap showing correlations between anti-EBV serology and B cell subsets. Only statistically significant correlations are coloured. A green colour indicates a positive correlation. Correlation coefficient shown is a Spearman's rho.

Patients with MRI activity show distinct clustering for multiple prognostic parameters

Since B cell phenotypes appeared a correlate of neither vitamin D supplementation, nor anti-EBNA-1 IgG levels, we further explored how individual parameters contribute to CUA lesions at week 48. Our previous work showed that the week 0 ratio between NK

A



B

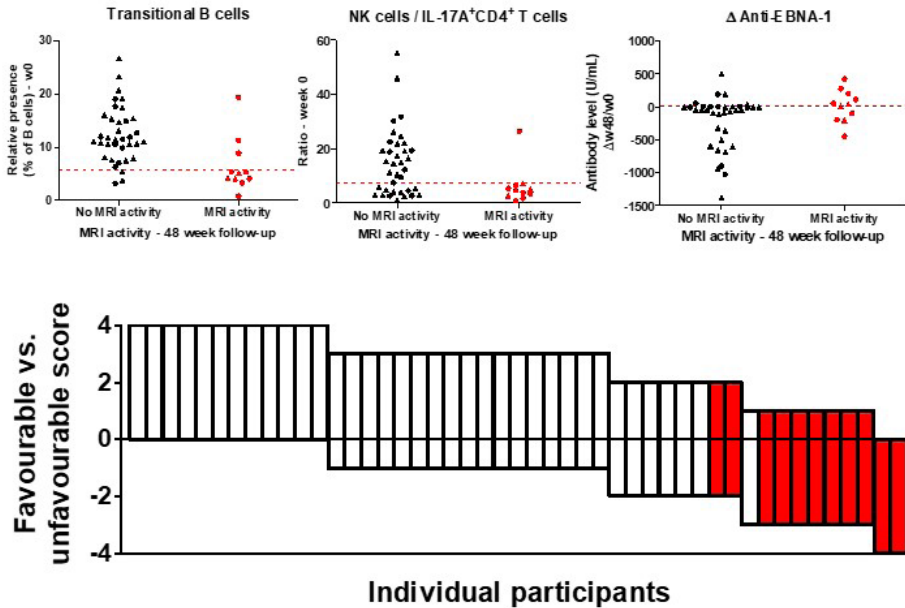


Figure 4. A: 3D plot using three prognostic markers: relative presence of transitional B cells in the B cell population, NK/IL-17A⁺CD4⁺ T cell ratio and the difference in anti-EBNA-1 IgG antibodies between week 48 and baseline. Red markers represent patients with MRI activity after 48 weeks, while black markers represent patients without MRI activity. Triangle markers represent patients who received high dose vitamin D₃ supplementation, while round markers represent patients who received placebo. **B:** Visualisation of multiple-hit model. When grouping patients based on the amount of favourable or unfavourable prognostic markers using cutoffs shown, patients with MRI activity (shown in red) tend to cluster around multiple negative markers.

cells and IL-17A⁺CD4⁺ T cells predicted the presence of CUA on week 48 MRI [6] and as such, this parameter was also included in our model. When introducing these 4 predictors of CUA on week 48 MRI in a 3D plot, patients with MRI activity tended to cluster together (**Figure 4A**). After log-transforming data that were not normally distributed, we introduced our parameters in an explorative binary logistic regression model where they explained 57.9% of the variance in CUA-activity in our cohort (Nagelkerke $R^2 = 0.579$), and all factors except Δ anti-EBNA-1 IgG ($\Delta w_{48}/w_0$) trended to contribute to this model (**Supplementary Table 2**). To visualize this interplay for all individual participants, we dichotomized all variables based on individual ROC-curves in a high- and low-risk profile for CUA (**Figure 4B**). Indeed, participants with CUA on week 48 MRI-scan tended to show a high-risk profile for multiple predictors.

DISCUSSION

We investigated the prognostic value of B cell subsets, and explored associations with other predictors of MS MRI activity in a homogenous cohort of interferon- β -1a treated RRMS patients. We show that low circulating proportions of transitional B cells associate with a lower risk of CUA on week 48 MRI, and that this is no correlate of proportions of IL-10⁺ Breg cells. Additionally, we show that this association of high transitional B cell proportions with a lower risk of CUA is not dependent on vitamin D supplementation, circulating anti-EBNA-1 IgG levels, or the ratio between NK and IL-17A⁺CD4⁺ T cells. Altogether, we conclude that the risk of MS MRI activity during interferon beta therapy is likely the result of many interacting phenotypes and effector characteristics of individual lymphocyte populations. Herewith, our data are a call for a systems biology approach to further understand the role of lymphocytes in the disease process of RRMS.

Our finding that transitional B cells may contribute to a protective effect in RRMS is in line with earlier research. Transitional B cells are shown to have regulatory properties. [37] As such, they have been implied in the pathogenesis of MS. Indeed, reduced transitional B cell numbers [38, 39] and regulatory function [40] and increased migratory capacity [38] have been reported in clinically isolated syndrome and MS. Additionally, some MS treatments associated with reduced disease activity in MS have been associated with increased transitional B cell proportions, including fingolimod [39, 41, 42] and interferon- β . [43] As such, one should exercise caution to extrapolate our findings to MS patients treated with other disease modifying therapies. Both fingolimod and IFN- β have been reported to increase transitional B cells through the increased circulating levels of B-cell activating factor of the TNF family (BAFF). [41, 44] However, since BAFF was not significantly affected by high-dose vitamin D₃ supplementation in a preceding

pilot-study [45], we did not include BAFF measurements in the current SOLARIUM study-design.

In several studies, the protective effect of transitional B cells has been attributed to their capability of producing IL-10.[39, 40, 42] In our study, an association with MRI-activity after 48 weeks follow-up was found only for transitional B cells, but not for IL-10⁺ B cells. This interesting contrast may be explained by the method to analyse IL-10⁺ B cells. In our study, B cells were stimulated with CpG for 24 hours, after which IL-10⁺ B cells were gated. One study shows that a combination of CpG and CD40L stimulation mainly expands regulatory B cells with a memory phenotype.[46] As such, it may be that transitional B cells exert their protective effect through a Breg phenotype, but that the transitional Breg population remains undetected by our induction method. Alternatively, transitional B cells may control CD4⁺ T cell proliferation and MS disease activity by other effector mechanisms than IL-10.[47]

Isotype-switched memory B cells are generally being viewed as an unfavourable cell subset in MS.[43] Our current results are in line with these notion, as higher percentages of isotype switched B cells are associated with a higher risk of CUA presence. The lack of correlation between anti-EBNA-1 IgG and isotype switched B cells is interesting, as EBV is known for its latent infection of memory B cells. One possible explanation may lie in recent evidence, suggesting that memory B cells only develop into antigen secreting cells after migrating to the CNS.[48] Isotype switched B cells as measured in peripheral blood may, therefore, give an incomplete view of anti-EBNA-1 production.

We showed that transitional B cell frequencies associate with MS CUA presence, as also observed for other immune-related predictors identified earlier in this cohort, including anti-EBNA-1 IgG,[35] vitamin D supplementation,[32] and NK/T cell ratios.[6] This observation fits the growing appreciation for the complex interplay between environmental factors and genetic background that influences cell subsets in (auto)immune diseases. [49-51] As such, the need for integrative models has increased, which led to an increase in popularity for a systems biology approach, where larger interacting systems are taken into account. For MS, a systems biology approach has been used to identify e.g. regulatory genetic pathways[52] as well as potential biomarkers for disease activity.[53] An approach on a cellular level remains poorly investigated. In our exploratory model, the combination of several prognostic factors, mainly the relatively increased or decreased presence of transitional B cells, NK cells and IL-17A producing T helper cells, leads to a model which currently explains over half of the variance in the prognosis of CUA in IFN- β -1a treated RRMS patients. To make more definitive claims on this approach, more biomarkers should be investigated and integrated into a larger model using a larger

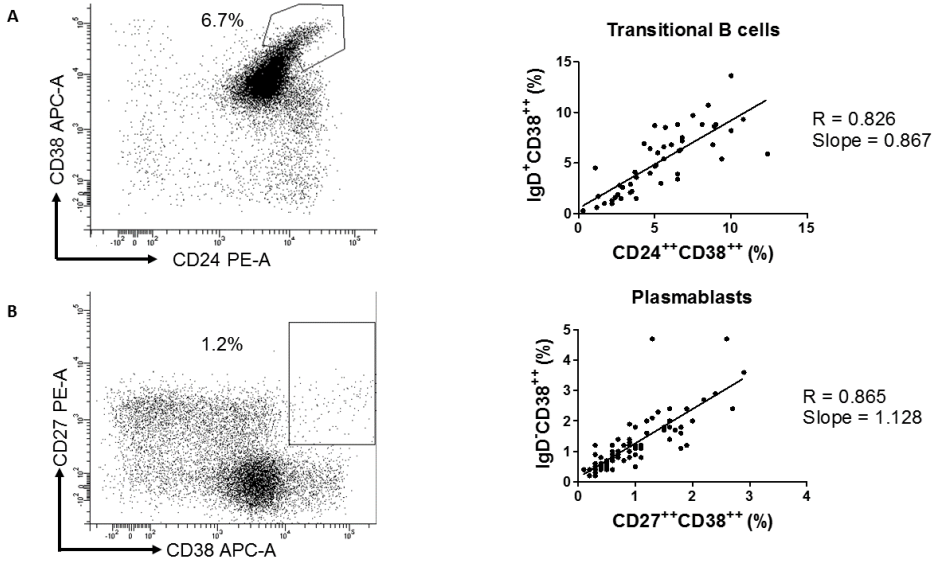
dataset. Nonetheless, our findings underline the importance of looking at the interaction rather than merely the presence of individual immune components to understand the complex pathogenesis of diseases like MS.

Our study has a some limitations. First, our data is derived from the SOLAR and SOLARIUM studies, which both were designed to answer a different research question. Thus, the exploratory nature of our research brings an increased risk for false negatives, in addition to the absence of a few potentially relevant parameters like the aforementioned BAFF levels. Additionally, our patients exclusively used interferon- β -1a. While this greatly increased homogenisation of the participants, it may influence the extrapolation of our data. Strengths of this study include its double-blinded nature, as well as its broad view of the B cell compartment.

In conclusion, our data underline the protective role for transitional B cells in IFN- β treated RRMS patients, as well as its potential as a prognostic biomarker for MRI activity. As noted earlier, our data suggests an interplay between several prognostic factors and calls for a systems biology approach to further grasp the interactions of lymphocyte subsets in RRMS. More research is necessary to confirm the prognostic value of transitional B cells in treatment-naïve RRMS patients and also to investigate other potential biomarkers in a systems biology approach.

SUPPLEMENTARY MATERIALS

Supplementary Figure 1



Supplementary Figure 1. Validation of gating strategy using alternative gating available for week 48 samples. Using a **A**: CD24⁺⁺CD38⁺⁺ phenotype and **B**: CD27⁺⁺CD38⁺⁺ phenotype, transitional B cells and plasmablasts were gated, respectively. Then, a Spearman rho test was performed to assess correlation with the transitional B cells and plasmablasts found using our main gating strategy based on IgD and CD38. A linear regression was performed to determine the slope.

Supplementary Table 1. Baseline patient characteristics

	RRMS patients (N=50*)	No MRI activity (N=36)	MRI activity (N=11)	p-value
Sex (N[%])				
Female	33 [66]	25 [69]	5 [45]	0.171
Male	17 [34]	11 [31]	6 [55]	
Age (years: median [interquartile range])	37.2 [31.7-44.3]	40.0 [32.8-45.3]	36.2 [29.6-43.9]	0.291
Body Mass Index (BMI) (N[%])				
≥ 25 kg/m ²	28 [56]	19 [53]	7 [64]	0.731
< 25 kg/m ²	22 [44]	17 [47]	4 [36]	
Disease duration (months: median [interquartile range])	7.4 [4.5-12.3]	7.3 [4.6-12.8]	8.4 [4.3-12.3]	0.851
Attacks during past 2 years at baseline (N[%])				
> 1	16 [32]	11 [31]	4 [36]	0.725
≤ 1	34 [68]	25 [69]	7 [64]	
Duration since start IFNβ-1a treatment (months: median [interquartile range])	4.7 [3.7-7.0]	4.9 [3.7-7.0]	4.6 [3.7-7.0]	0.930
Duration since last attack at baseline (months: median [interquartile range])	7.6 [5.0-10.5]	7.4 [4.4-10.4]	8.0 [7.2-11.3]	0.386
Treatment (N[%])				
Placebo	21 [42]	11 [31]	8 [73]	0.018
Vitamin D3	29 [58]	25 [69]	3 [27]	

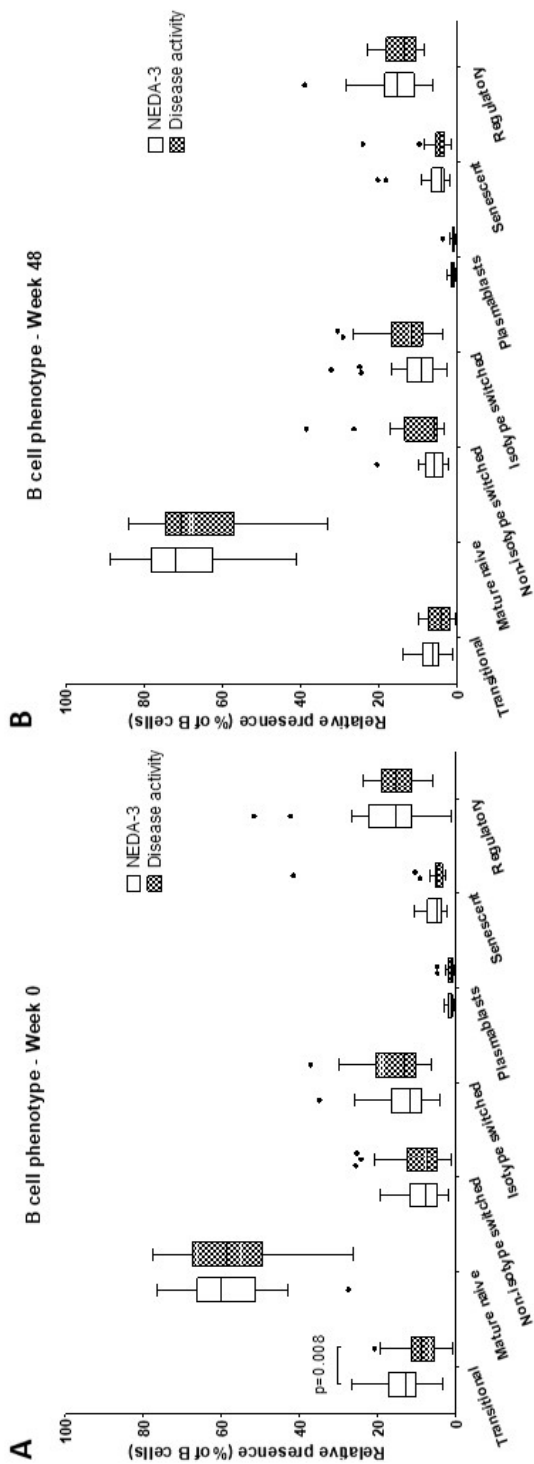
P-value is based on Mann-Whitney U test for continuous data and Fishers exact test for dichotomous data. * MRI data after 48 weeks follow-up were available for N=47 participants.

Supplementary Table 2. Odds ratios and p-values for individual parameters in an exploratory binary logistic regression model on MS CUA presence (yes/no)

Parameter	Odds ratio	95% confidence interval	p-value
Vitamin D₃ supplementation	0.202	0.027-1.532	0.122
Transitional B cells	0.764	0.606-0.963	0.023
NK/IL-17A⁺CD4⁺ T cell ratio*	0.081	0.006-1.049	0.054
Δ anti-EBNA-1 (w48/w0)*	2.680	0.580-12.389	0.207

* = log-transformed

Supplementary Figure 2



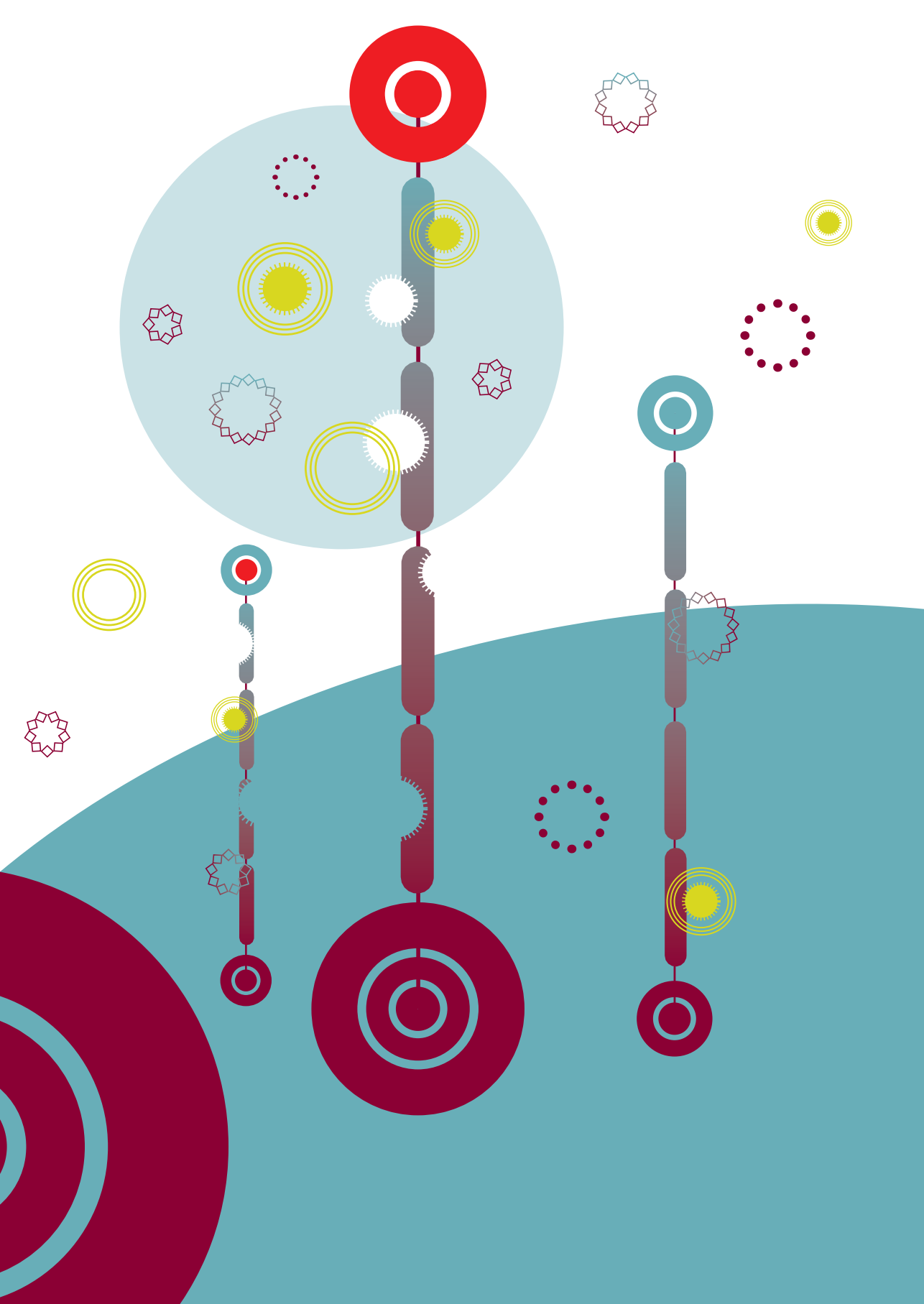
Supplementary Figure 2. Differences in B cell phenotypes at baseline (A) and week 48 (B) (as defined in Figure 1, with the addition of regulatory B cells) between patients with and without disease activity, measured using the NEDA-3 endpoint. Shown p-value is calculated using a Mann-Whitney U test.

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Chapter 8

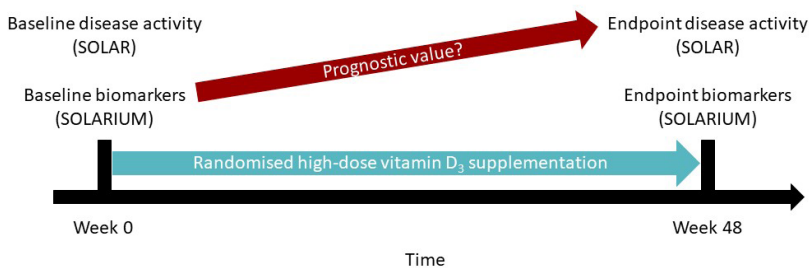
General discussion



The year 2011 marked the start of the SOLAR trial, which aimed to investigate the efficacy of high-dose vitamin D₃ supplementation in multiple sclerosis (MS) patients. [1] This study was born from an increasing scientific interest in vitamin D, especially in MS.[2-5] Later, the SOLARIUM sub-study evaluated the immunological effects of vitamin D₃ supplementation in MS patients.[6] Prior investigations from our group, and other groups as well, mostly focussed extensively on T cells.[6-8] However, with new research pointing towards roles for B cells and natural killer (NK) cells,[9, 10] combined with the publication of the clinical outcomes from the parent study (SOLAR), new questions arose.

First, the SOLAR trial did not reach its primary endpoint, yet showed improvement on secondary magnetic resonance imaging (MRI) endpoints.[1] To further explore these hints of benefit, the putative effects of vitamin D₃ supplementation on the plasma neurofilament light chain (NfL) biomarker of RRMS disease activity were investigated. Furthermore, although SOLAR missed its primary endpoint, subgroups of patients may benefit from supplements. Since vitamin D-associated genes were shown to contain MS risk alleles,[11] and patients with MS were shown to more frequently carry single nucleotide polymorphisms (SNPs) associated with low 25(OH)D levels,[12] we hypothesised that these SNPs could define subgroups with a distinct biochemical and possibly also clinical response in our studies.

Furthermore, since T cell phenotypes were not convincingly affected by vitamin D₃ supplementation in association with RRMS disease activity, we hypothesized that other lymphocyte subsets were most likely to contribute to the risk of encountering disease activity of RRMS as a patient, as well as the putative protective effects of vitamin D₃ supplements. The combination of immunological data at baseline from the SOLARIUM study, and clinical outcome measures after 48 weeks follow-up from the SOLAR study, gave us the opportunity to explore the prognostic value of lymphocytes for disease activity in RRMS, as visualised in **Figure 1**. Additionally, effects of vitamin D supplements on these lymphocyte subsets were investigated.



VITAMIN D

The original aims of the SOLAR and SOLARIUM studies were to investigate the effect of high-dose vitamin D₃ supplementation on absence of clinical and radiological disease activity during 48 weeks follow-up (no evidence of disease activity, NEDA) and lymphocyte composition after 48 weeks, respectively. The SOLAR study reported a reduction in MRI activity, but not in relapse rate, nor disability progression in RRMS patients using interferon-beta-1a and receiving high-dose vitamin D₃ supplementation.[1] Therefore, it did not reach its primary endpoint of showing an increased proportion of interferon beta-treated individuals reaching NEDA with high-dose vitamin D supplements. Accordingly, the SOLARIUM study reported no changes in lymphocyte composition, mainly focused on T cells, after high-dose vitamin D₃ supplementation.[6] Since MRI disease activity is a sensitive biomarker of inflammatory MS activity,[13] the improved secondary MRI endpoints can be interpreted as a signal of benefit. In addition to clinical symptoms and MRI characteristics, circulating NfL levels are increasingly recognized as sensitive biomarker of inflammatory disease activity in MS.[14] A positive effect of vitamin D₃ supplementation on this biomarker would further consolidate its anti-inflammatory effects. This biomarker was not measured in participants of the SOLAR trial. In **Chapter 2**, the effects of vitamin D₃ supplementation on serum levels of NfL in the SOLARIUM cohort were described. No significant effect of vitamin D supplementation on circulating NfL levels was reported. These findings appear congruent with other reports, such as the group of Holmøy et al.[15] and Hänninen et al.[16] which reported no significant associations between high-dose vitamin D₃ supplementation and NfL levels in serum. Therefore, in this small study, no additional arguments supporting a profound anti-inflammatory effect of vitamin D supplements were found. Nevertheless, small effects detectable in larger groups of patients, with more active disease or lower baseline 25(OH)D levels, or genetic subgroups of patients, cannot be excluded.

The effects of vitamin D may be most pronounced in a subgroup of participants of the SOLAR. There is an increasing interest in pharmacogenetics to enable prescription of drugs considering genetic background for dosing and risk mitigation. For neuro-immunologists, most clear examples are dosing of azathioprine with consideration of the patients TPMT-genotype, and the genotyping of CYP2C9 in RRMS patients opting for treatment with the sphingosine phosphate receptor inhibitor siponimod to predict metabolism and adjust dosing accordingly.[17] Several pharmacogenetics studies show genetics to have interesting potential for predicting treatment success, particularly for interferon-beta-1a and glatiramer acetate.[18] One example for interferon-beta-1a is the rs9828519 SNP status, which is likely tied to the *SLC9A9* gene. Presence of the rs9828519 risk allele shows an increased risk of relapses under interferon-beta-1a treatment (OR

1.48).[19] Similarly, for glatiramer acetate, a combination of risk alleles (*DRB1*15* + *TGFB1*T* + *CCR5**) was associated with a 14 to 15 times increased risk of therapy ineffectiveness, which was defined as any relapses and/or sustained progression of EDSS during at least two years of treatment.[20] In other words, genetics have already shown their importance in MS treatment and it would therefore be prudent to assess similar genetic effects on vitamin D₃ supplementation.

On a population level, several SNPs have been identified influencing serum 25(OH)D levels, which also have been shown to be enriched in people with MS. Additionally, MS risk alleles have been identified in regions associated with major vitamin D-metabolism genes.[11] Therefore, in the context of precision medicine, these genes could be relevant for biological response to vitamin D supplements. The influence of genetics on vitamin D levels is discussed in **Chapter 3**. Two SNPs, rs7041 and rs12368653, previously associated with an increased risk of developing MS, are thought to have influenced the serological effectiveness of high-dose vitamin D₃ supplementation. Thus, genetic subgroups in our RRMS cohort were identified which respond differently to high-dose vitamin D₃ supplementation. Whether this difference is also reflected by response to vitamin D supplementation in terms of MS disease activity remains uncertain. No skewed distribution of MRI-activity between genetic subgroups was observed, keeping in mind that our study is underpowered to unequivocally detect such an association. If these genetic variations did influence the outcome of the SOLAR(IUM) studies, it raises the question on how the results of vitamin D₃ supplementation studies in patients with different genetic backgrounds should be interpreted. In other words, can results from a study from an American or Egyptian population still be easily translated to a Dutch or Swedish population. To address this query, vitamin D-related genetic risk profiles should be included in future supplementation studies to correctly account for genetic influences.

Taken together, the influence of high-dose vitamin D supplementation on clinical outcome markers was investigated in our cohort. Since no significant effects were measured on NfL in the SOLARIUM, and on clinical endpoints in the SOLAR trial, it was deemed valid to explore the effects of lymphocyte subsets on the development of these endpoints in the SOLARIUM cohort. Associations with MRI-endpoints were adjusted for use of vitamin D supplements, considering the findings of the SOLAR study.

BIOMARKERS OF MULTIPLE SCLEROSIS DISEASE ACTIVITY

Currently, several body fluid biomarkers are used to support clinical decision making for treatment of MS patients. To put our findings into perspective, currently used immunological body fluid biomarkers are listed, and an overview of several promising body fluid markers is presented.

One of the earliest body fluid biomarkers in MS is the presence of IgG oligoclonal bands in cerebro-spinal fluid (CSF), which is associated with a higher risk of converting to MS in patients with clinical isolated syndrome (CIS), the precursor of MS.[21] The presence of two or more IgG oligoclonal bands unique to the CSF (compared to serum),[22] is indicative of an abnormal intrathecal B cell response, which supports an MS diagnosis. [23] CSF diagnosis has been an integral part of MS diagnostics for the last few decades and, although not always mandatory in the newest version of the diagnostic McDonald criteria, still play a major role in diagnosing MS.[24]

Another body fluid biomarker used in MS diagnostics is the presence of antibodies against aquaporin-4. Anti-aquaporin-4 antibodies are measured to distinguish MS from other MS mimicking diseases, namely neuromyelitis optica spectrum disorders (NMOSD), which selectively affect the spinal cord and optic nerves. Hence, MS and NMOSD have significant clinical overlap and are hard to distinguish based on clinical presentation alone. Anti-aquaporin-4 is present in 75-90% of patients with NMOSD and is almost always absent in MS patients, showing a 91% specificity for differentiating the two.[25] Differentiation between MS and NMOSD is important, as prognoses and treatments differ between the diseases.[26-29]

Several body fluid biomarkers that are used in clinical care of MS patients are related to disease modifying therapies. They are antibodies against the John Cunningham (JC) virus, antibodies against natalizumab and antibodies against interferon-beta-1a. The first biomarker, antibodies against JC virus, is used to assess the risk of developing progressive multifocal leukoencephalopathy (PML), a devastating complication of natalizumab treatment.[30] When antibodies against the JC virus, whose reactivation causes PML, rise above a certain threshold, clinicians will be urged to monitor their patients more closely, or switch to another DMT entirely to avoid this complication.[31]

The second biomarker is again related to natalizumab. In roughly 6% of all natalizumab users, antibodies against the biopharmaceutical are developed which increasingly hinder the effectiveness of the treatment.[32] As such, the presence of these antibodies

is monitored and, when detected, the clinician can switch therapies before the treatment becomes ineffective and the patient suffers clinical deterioration due to increased disease activity.

In a similar vein, antibodies against interferon-beta-1a have been described, although the reported prevalence varies. A prevalence from 3% up to 44% has been described, with no clear reason for this large variation, although intramuscular administration of the drug does appear to induce fewer cases of neutralising antibodies compared to intravenous administration.[32] Again, these antibodies hinder the effectiveness of the therapy and consequently cause an increase in relapses and MRI activity. Monitoring these antibodies gives an early sign of their presence and gives the clinician time to switch therapies in order to ensure effective treatment.

The neurofilament light chain biomarker for neuro-axonal loss has already been mentioned as a biomarker for MS disease activity.[33] Although not yet commonly used in clinical practise, there is sufficient evidence for its added value in clinical decision making.[34]

With NfL being an effective tool of measuring disease activity, most research has focussed on its practical use as an add-on to current diagnosis and monitoring methods. This means that most research evaluates the association between NfL levels and simultaneous disease activity, and not with future risk of disease activity. Early reports do indicate that NfL may be linked with later disease activity and severity, although this requires further investigation.[35-37]. Additionally, effective treatment with a DMT appears to lower NfL levels,[38, 39] which further supports the role of NfL as a body fluid biomarker for disease activity in MS.

Many other potential biomarkers in MS have been proposed, with varying degrees of evidence supporting them. A few of these potential biomarkers are listed in **Table 1**. Markers are characterised based on four types: predictive, diagnostic, disease activity and treatment-response, as put forward by Comabella and Montalban.[40] The term prognostic is not used here, as all these types carry an inherent form of prognosis.

Body fluid biomarker	Relation	Remarks
<i>Extracellular vesicles (EVs)</i> <i>Microvesicles (MVs)</i>	<p>Diagnostic: Increased quantity of EV in MS patients compared to healthy controls[41, 42]</p> <p>Disease activity: Increased quantity during relapse,[41, 42] increase in CSF associated with higher risk of disease activity[43]</p> <p>Treatment-response: Decrease associated with better outcome in patients using interferon-β-1a[44]</p>	<p>EVs are a collection of small vesicles of varying size, density, genesis and content.[45] The most commonly investigated EV is the microvesicle (MV). They play a significant role in physiological processes like remyelination,[46] but also appear to be involved in cellular activation by facilitating the crossing of the blood-brain-barrier.[47]</p> <p>Although elevated in RRMS patients, progressive MS patients show levels comparable to healthy controls, thus implying MVs to be a marker of short term inflammation.[42] EVs are the main carriers of miRNA, although the relation between the biomarkers remains insufficiently investigated.</p>
<i>Micro RNA (miRNA)</i>	<p>Diagnostic: hsa-miR-92a-1* differs between RRMS versus SPMS and RRMS versus HC. The let-7 family of miRNAs differentiated SPMS from HC and RRMS from SPMS. Hsa-miR-454 differentiated RRMS from SPMS. Hsa-miR-145 differentiated RRMS from HC and RRMS from SPMS.[48] Hsa-miR-96 is higher in HC versus RRMS.[49]</p> <p>Disease activity: hsa-miR-22.3p and hsa-miR-345.5p are upregulated in patients showing signs of inflammation on MRI, while hsa-miR-361.5p shows a protective effect against lesions and atrophy. [50] hsa-miR-96 is associated with remission.[49]</p>	<p>miRNAs are single-stranded forms of non-protein coding RNA, comprised of approximately 22 nucleotides. They play an important role in gene-regulation by cleaving and repressing messenger RNA.[51] Among others, it appears to be involved in myelination and inflammation.[52, 53] Although results have been promising, one major controversy surrounding miRNA studies is their contradicting results. a review by Piket et al.[54] shows this heterogeneity, as only 27.5% of all MS-associated miRNAs show dysregulation in the same direction in two or more studies. When this threshold increases to three studies, only 9% of miRNAs remained valid.</p> <p>Again, miRNA is usually carried by EVs, although their relationship is poorly investigated.</p>
<i>Anti-EBNA-1 IgG</i>	<p>Diagnostic: Increased titre is linearly associated with increased risk of MS.[55]</p> <p>Disease activity: Increase associated with increased risk of MRI activity[56] (although controversial[57, 58])</p> <p>Treatment-response: Decrease possibly associated with higher chance of success of natalizumab treatment[59]</p>	<p>Although the role of EBV in MS pathogenesis has become established, the value of anti-EBNA-1 IgG titres as a biomarker for MS remains controversial. Though well supported in its association with risk of developing MS, its other associations show conflicting results and, as such, it has not reached clinical use.</p>
<i>Uric acid</i>	<p>Diagnostic: Uric acid levels are lower in MS patients compared to HC.[60] They are also negatively correlated with disease duration.[61]</p> <p>Disease activity: Uric acid levels are negatively correlated with relapse and MRI activity,[62] as well as EDSS. [61, 63]</p>	<p>Uric acid is a strong natural scavenger of reactive oxygen and nitrogen species, which play an important part in inflammation, demyelination and axonal injury in MS.[64] Conflicting results regarding uric acid exist, which may be based on the use of 'other neurological disorders' as controls, as well as low statistical power due to small sample sizes.</p>

<i>Angiopoietin-like proteins (ANGPTLs)</i>	<p>Disease activity: ANGPTL6 associated with lower EDSS scores.[65]</p> <p>Treatment-response: ANGPTL4 is higher in patients responsive to natalizumab[65]</p>	ANGPTLs are structurally similar to angiopoietins and are involved in lipid metabolism, angiogenesis and inflammation.[65] Although investigated in other inflammatory diseases, they appear relatively unknown in multiple sclerosis studies.
<i>Soluble Fas</i>	<p>Diagnostic: Higher in PPMS patients compared to HC and RRMS patients. [66]</p> <p>Disease activity: Increased both in patients with MRI activity and EDSS progression.[66]</p>	Fas is an apoptosis mediator. Defects in this receptor have been reported in the T lymphocytes of RRMS patients, which may promote survival of autoreactive T cells.[67] The soluble form of Fas is shown to inhibit apoptotic events in T cells.[68]
<i>Soluble IL-2 receptor alpha chain (sIL-2Rα)</i>	<p>Diagnostic: Increased in MS patients compared to HC in serum and CSF. [69-71] Increased in RRMS patients with malignant disease course compared to benign disease course. [70]</p> <p>Disease activity: Increased during relapses.[69-71]</p>	sIL-2R α is the soluble variant of CD25, or the alpha unit of the IL-2 receptor (IL-2R).[72] Although its mechanism of release remains unclear, it appears to be associated with inflammation, either as a marker of increased inflammatory processes, or as a marker of increased regulation as a response to inflammation. Conflicting reports about the physiological stability of sIL-2R α have impeded its clinical application as a biomarker.[70, 73, 74]

Interestingly, the prognostic value of circulating lymphocyte characteristics has received limited attention in scientific research. This is remarkable, since all registered DMTs deplete or modulate functional behavior of these subsets. Indeed, one study investigating NMDAR, AMPAR, GABA β , IgLON5, LGI1 and CASPR2 auto-immune encephalitis reports that an increased neutrophil-to-lymphocyte ratio was associated with greater odds of first line treatment failure, thus showing a prognostic value.[75] In MS patients, Peeters et al. reported a significant association between a higher percentage of CD4 $^{+}$ cytotoxic T cells and higher disease severity at a later date, although this association was not very strong.[76] Quirant-Sánchez et al. reported an association between a higher percentage of CD4 $^{+}$ central memory T cells and subsequent relapses in fingolimod treated patients. [77] Finally, Demirci et al. and Yetkin et al. showed in RRMS patients that neutrophil-to-lymphocyte ratios predicted future disease activity in terms of relapses and treatment escalation, [78, 79] although these results could not be replicated.[80] We conclude that many potential biomarkers for disease activity are under investigation, although, with the possible exception of NfL, none appears robust or well-established enough for practical use. Additionally, despite lymphocytes being the main disease mediators in MS, research into their use for prediction of disease activity is, at current stage, quite preliminary.

NK cells

NK cells show great potential for regulatory activity in MS, as shown by the daclizumab studies.[81] In one of these studies, an expansion of CD56 bright NK cells was directly as-

sociated with treatment success, *i.e.* a reduction of the presence of MRI activity after start of therapy.[82] These findings point towards a possible prognostic value of NK cells in MS. Considering the relatively new appreciation for NK cells and the surge in NK cell-related studies in MS, we first reviewed the role of NK cells in MS in **Chapter 4**. A few points in this review should be discussed, as they highlight some potential gaps in our knowledge of NK cells in MS, as well as some limitations in our investigations.

The current view of NK cells in MS is that they are a regulatory cell subset, capable of killing (autologous) activated and putative autoreactive T cells. Many studies report on the anti-inflammatory capabilities of NK cells, such as their production of IL-10 and the aforementioned cytotoxic capabilities.[83] Additionally, while the daclizumab studies have highlighted the CD56^{bright} NK cell subset in particular, it seems that CD56^{dim} NK cells are similarly capable of killing activated T cells and thus may be of equal protective value.[84] However, the specifics of this cytotoxic mechanism, such as the exact location and the activating factors, remain partially unelucidated. As such, it is not clear if the two NK cell subsets differ in these characteristics and, by extension, fulfil differing roles in the suppression of autologous activated T cells. Likewise, it remains to be investigated if these two NK cell subsets react differently to different DMTs. In conclusion, while both the CD56^{bright} and the CD56^{dim} NK cell subsets appear to be capable of killing autologous activated T cells, their possible differences in activation and location of action make further research into the distinctions between the subsets an interesting topic.

Despite these regulatory capabilities, it may also be possible that NK cells have a damaging effect in MS as well. This possibility was first shown in EAE models where a study reports that removal of NK cells in later stages of EAE actually ameliorates the disease.[85] In humans, a post-mortem study suggests that myelin damage is based on an antibody-dependent mechanism, *i.e.* ADCC, which is mainly driven by NK cells, suggesting that they may be responsible for myelin damage in MS.[86] A recent study reports the presence of CD56^{bright} NK cells in periventricular lesions and the choroid plexus of MS patients with a NK cell signature specific to MS, whereas patients without MS did not show any NK cell presence.[87] Although these NK cells may be a regulatory reaction to the inflammatory process in the brain, they could just as likely be part of the inflammatory process itself. Thus, the role for NK cells in MS may be more ambiguous than suggested by daclizumab studies. Indeed, in several other auto-immune diseases, such as type 1 diabetes, rheumatoid arthritis, systemic lupus erythematoses and Sjögren's syndrome, NK cells appear to play both a protective and a destructive role.[88] Although most NK cell related research in MS points towards a regulatory role, a possibility of a destructive effect of NK cells, perhaps at a different point in the disease, is plausible and should be considered in later studies.

Finally, one of the key characteristics of the NK cell, and the mechanism that allows it to exert cytotoxic properties without prior sensitisation or antigen presentation, is its repertoire of activating and inhibiting receptors. In short, NK cells have a varying set of receptors that differ within individuals and between individuals. The balance between activating signals and inhibitory signals decides whether an NK cell releases its cytotoxic contents and kills the presenting cell.[89] Research into these receptors shows interesting alterations in these receptors, for example that NK cells are less capable of killing activated T cells in MS patients because of an increased expression of human leukocyte antigen E (HLA-E) on T cells, which acts as a ligand for the inhibitory NKG2A receptor.[90] Additionally, infection with EBV, one of the prerequisites of developing MS, elicits a phenotype change in NK cells, with an upregulation of NKG2A.[91, 92] Infection with cytomegalovirus (CMV), which shows a protective effect for developing MS,[93] causes an upregulation of the NKG2C receptor.[94, 95] This all implies the receptor repertoire of NK cells to be relevant when evaluating the role of NK cells in MS, and may even point towards the receptor repertoire as a possible prognostic marker in MS. However, due to the original design of the SOLARIUM study, the NK cell receptor composition was not analysed and as such these hypotheses could not be tested during our investigations.

NK/T cell ratios

With the potential of NK cells highlighted in the previous chapter, we moved to apply this knowledge in a clinical cohort, as described in **Chapter 5**. First, the absolute and relative presence of NK cells in the circulation showed no significant relation with disease activity. In an effort to better represent the alleged protective function of NK cells, the relation between NK cells and subsequent disease activity was explored with a ratio between NK cells and T cell (subsets). This ratio showed prognostic value for disease activity after 48 weeks.

In **Chapter 5**, an effort was made to better represent the complex interplay of the immune system in a parameter. While most groups reporting on NK cells use the relative or absolute presence of NK cells,[96, 97] this single measurement may not provide the full picture regarding the activity of the NK cell compartment. As currently understood, the main mechanism of regulation by NK cells in MS is the suppression of activated T cells. As such, presenting NK cell figures without T cell figures does not wholly represent the current balance of pro-inflammatory CD4⁺ T cells and suppression by NK cells, *i.e.* the current level of 'unregulated inflammation'. As proposed by Darlington et al.,[97] an attempt was made to provide a more comprehensive representation of the immunological imbalance in RRMS patients by using an NK/T cell ratio.

Darlington et al. reported only on the total NK cell population, as well as the Th1 and Th17 subsets. To further specify this NK/T ratio, we first correlated NK cells (subsets) with CD4⁺ T cells (subsets) in order to find the most directly linked populations and use these as our marker of immunological (im)balance. Eventually, we found the strongest correlation between NK cells on the one hand, and both the total CD4⁺ T cell population and the IL-17 producing CD4⁺ T cell subset on the other.

One may argue that correlating the relative presences of two lymphocyte subsets is redundant, as a proportional increase of one subset would implicate a proportional decrease in the other. Two arguments challenge this view. For one, NK cells and the IL-17 producing T helper subset are a numerically small fraction of the total lymphocyte population.[98] As such, an expansion of one subset should numerically hardly interfere with the presence of the other. The only exception to this would be if one subset showed a biological pathway to interfere with the presence of the other, which brings us to the second argument. As reviewed earlier, there exists a proven, biological pathway for NK cells to interact with T cells.[99] More specifically, CD56^{bright} NK cells have been proven to kill activated CD4⁺ T cells as part of their regulatory capabilities.[100] Because of this proven interaction, it is warranted to correlate NK cells (subsets) with CD4⁺ T cells (subsets) to find which NK cells oppose which (pro-inflammatory) CD4⁺ T cells the most. Significant negative correlations indicate a form of regulation, which justifies their use in an NK/T cell ratio.

NK/T cell ratios appear to be a relatively unknown biomarker. In MS, the prognostic value of a NK/T cell ratio appears in one recent observational study with patients after immune reconstitution following alemtuzumab treatment.[101] Here, they report that patients without disease activity showed a greater NK/T cell ratio. To the best of our knowledge, this appears to be, besides our own reports, the only study reporting on the prognostic value of NK/T cell ratios in MS patients.

Studies in other (auto-immune) diseases are more common and also show promising potential for NK/T cell ratios. For example, in severe aplastic anaemia, NK cells exhibit strong cytotoxicity towards autologous CD8⁺ T cells, which are detrimental in the auto-immune disease.[102] Similar to MS, this cytotoxicity is dependent on the activating NKG2D receptor. As another example, NK cells appear to show cytotoxic behaviour towards autoreactive CD4⁺ T cells in primary biliary cirrhosis. [103] Finally, NK cell cytotoxicity is negatively correlated with CD8⁺ T cells in fertile women, but not women with recurrent pregnancy loss, implying a lack of regulatory capacity of NK cells which causes risk of pregnancy loss.[104] To summarise, NK-T cell interaction does not appear to be an MS-specific phenomenon, but rather a physiological balance that may show regulatory

potential in a variety of (auto-immune) conditions. Findings of NK – T cell interactions in other diseases should be monitored closely, as they may further our understanding of the regulatory function of NK cells in MS. Conversely, we expect that research into the aforementioned diseases will benefit from our introduction of NK/T cell ratios as a biomarker in clinical studies.

The NK/CD4⁺ T cell ratio, and more specifically, the NK/IL-17⁺CD4⁺ T cell ratio, appears to be relevant for subsequent disease activity. Indeed, lower ratios were reported to be associated with a higher chance of disease activity, as measured in MRI activity, clinical relapses and NfL levels. It should be noted that, like many prognostic factors in MS, NK/CD4⁺T cell ratios explain only a part of the variation in disease activity.

A further specification of the NK/IL-17⁺CD4⁺ T cell ratio underlines our earlier statement about the regulatory capacity of CD56^{dim} NK cells, as the prognostic effect appears to be stronger with CD56^{dim} NK cells as opposed to CD56^{bright} NK cells. This again highlights the possible difference in mechanism of action and/or location of action between the two NK cell subsets and is more ground for further investigation. The reported lack of effect when analysing CD56^{bright} NK cells may also be due to their low presence in the circulation. This may highlight one of the restrictions of the SOLARIUM study, that being the limited sample size which increases the risk of false negative results and may underestimate the effect of CD56^{bright} NK cells. Additionally, the SOLARIUM study is based on lymphocyte analyses in the circulation, thus we are not able to comment on the function of CD56^{bright} NK cells in, for example, lymph nodes or CSF.

The IL-2/IL-2R pathway

After the positive clinical results of daclizumab,[105-107] an anti-CD25 (IL-2R α) antibody, and our reported prognostic value of the NK/CD4⁺ T cell ratio, the IL-2 – IL-2R pathway appeared increasingly interesting to investigate as an underlying mechanism.[108] As such, in **Chapter 6**, the influence of the IL-2 – IL-2R pathway on NK/CD4⁺ T cell ratios was investigated. Here, an association between levels of sIL-2R α and NK/IL-17⁺CD4⁺ T cell ratios was reported, where a decrease in ratio was associated with an increase in sIL-2R α .

In earlier publications, sIL-2R α levels in serum were reported to spontaneously fluctuate, showing peaks that were most often not related to MS disease activity, thus making it an unreliable biomarker.[73] In our cohort, however, baseline sIL-2R α levels correlated quite strongly with levels after 48 weeks follow-up, challenging this earlier report. There are some notes about this finding. For example, the 48 week follow-up period had no interval measurements. Additionally, the overall disease activity in the SOLARIUM cohort appears to be relatively low as compared to other studies using RRMS disease

activity as an outcome. As an example, 7 out of 50 patients (14%) in the SOLARIUM cohort reported a relapse in the one year of follow-up, whereas the annualised relapse rate for interferon- β -1a users in a trial comparing ocrelizumab and interferon was 0.29. [109] As such, the RRMS patients in our cohort appear to mostly show signs of a stable disease, possibly explaining the stable levels of sIL-2R α in our cohort. Nevertheless, the stability of sIL-2R α levels warrants further investigation, as it makes the use of sIL-2R α as a potential biomarker for inflammation and, possibly, disease activity far more attractive.

Additionally, an association between sIL-2R α levels and presence of the rs3118470 risk allele, located in the *IL2RA* gene, was reported in **Chapter 6**. Together with *IL7RA*, the *IL2RA* gene was the first genetic region associated with MS outside of the HLA region on chromosome 6. In later genome wide association studies (GWAS), different rs-numbers are reported within haplotype blocks, but the *IL2RA* region is consistently associated with an increased risk of development of MS.[11, 110] The fact that the MS-related *IL2RA* haplotype is associated with sIL-2R α levels in our cohort, and that these sIL-2R α levels are in turn correlated with NK/IL-17⁺CD4⁺ T cells ratios, underlines the involvement of the IL-2 – IL-2R pathway in our immunological analyses and the necessity to investigate this pathway further.

Since the daclizumab trials, it has become increasingly clear that the IL-2 – IL-2R pathway is involved in the MS disease process. Efforts of targeting this pathway through daclizumab were met with mixed results showing a positive effect on relapse rate and MRI activity, but carrying a risk of severe side effects.[111, 112] IL-2 is a cytokine involved in the activation and survival of a plethora of immune cells.[113] In order to regulate responses to IL-2, different cells express different receptors with varying affinities for IL-2. Inactive effector T cells and most NK cells express the medium-affinity IL-2R, whereas most regulatory T cells express the high-affinity IL-2R. This implies that, in a relative scarcity of IL-2, only cells with a high-affinity receptor have access to IL-2. Normally, this ensures that regulatory T cells can survive (as they are dependent on IL-2 for survival), while other immune cells dependent on IL-2 remain in a resting state. This mechanism is the theory behind low-dose IL-2 therapy in auto-immune diseases, which aims to stimulate regulatory T cell production while not activating effector T cells.[114] NK cells, like effector T cells, mostly express the medium-affinity IL-2R, although they are capable of expressing the high-affinity IL-2R. As the daclizumab trials demonstrated, the increased availability of IL-2 by blocking the high-affinity IL-2R does significantly expand the NK cell population, although this comes with many potential side effects. Developing ways to selectively direct IL-2 to NK cells, such as finding a mechanism to stimulate their expression of the high-affinity IL-2R, would be an interesting way to increase the NK cell

population, which appears to regulate the autoreactive T cell population in MS, without the hindrance of the regulatory T cell population.

Ideally, IL-2 levels would have been explored to further elaborate on the effects of the IL-2 – IL-2R pathway regarding NK/CD4⁺ T cell ratios. However, the SOLARIUM study did not initially aim to investigate this cytokine and as such, no IL-2 data were collected. This means that our findings cannot be supported with one of the key components of the IL-2 – IL-2R pathway, i.e., IL-2, which should be considered when interpreting our results. As sIL-2R α is theorised to be released as a result of shedding through an activated IL-2 – IL-2R pathway, it is used as our main measure of IL-2R upregulation.[72] Additionally, sIL-2R α shedding under stimulation, baseline CD25 expression on T cells and *IL2RA* gene expression by peripheral blood mononuclear cells (PBMC) were used to further enhance our understanding of the IL-2 – IL-2R pathway. Except for *IL2RA* gene expression, all our markers associated in some way with NK/IL-17⁺CD4⁺ T cell ratios. The reason that *IL2RA* gene expression did not associate with NK/IL-17⁺CD4⁺ T cell ratios may lie in the methodical approach. *IL2RA* gene expression was measured in PBMC and, as such, is confounded by the presence of other lymphocyte subsets that also may express IL-2R α . Nevertheless, a decreased level of the NK/T ratio appears to be associated with an increased measure in most of the IL-2 – IL-2R pathway markers. With our markers being markers of an activated IL-2 – IL-2R pathway, the increased IL-2 – IL-2R pathway measurements may either be the cause of a reduced NK/T cell ratio, or the result of it. Whichever may be true, the IL-2 – IL-2R pathway is concluded to be involved in the NK – T cell interplay and is considered relevant in understanding the prognostic value of the NK/T cell ratio in MS.

B cells

Besides the current attention for NK cells in MS, B cells have also been revealed as a key player in the MS disease model.[115] The discovery of their antigen presenting functionality, combined with the therapeutic success of anti-CD20 therapy,[116] has shifted the view of B cells from a secondary effector population towards a central contributor and regulator of the disease. Additionally, as the role of EBV becomes more pronounced, and with B cells being the main host for this Herpes virus, B cells are advocated to be involved in the MS disease process in several ways.[117] How EBV exerts its effect on MS risk remains uncertain, but it has been hypothesized that presence of EBV-infected cells in the CNS may offer antigens to infiltrating T cells, or that EBV infection potentiates antigen presentation and inflammatory characteristics of B cells.[115, 118] It appears similarly uncertain what effect EBV has on the composition of the B cell compartment. Reports of hematopoietic stem cell transplantation recipients show that unregulated EBV infection is associated with an increase in memory B cells,[119, 120] but reports

in immuno-competent patients are lacking. Since anti-EBNA-1 IgG levels may correlate with a higher risk of MS MRI activity,[121] the humoral response directed against EBV appears relevant for disease activity in MS. Despite this, the association of anti-EBNA-1 IgG in circulation and composition of circulating B cell compartment in MS was to our knowledge not explored.

The prognostic value of B cells and B cell subsets, as well as their relationship with high-dose vitamin D₃ supplementation and the relation between B cells and EBV related serology, was explored in **Chapter 7**. Here, the transitional B cell subset was reported to show protective value for RRMS disease activity, as higher proportions of transitional B cells were associated with a lower risk of developing MRI activity after 48 weeks. Additionally, a lack of decrease in anti-EBNA-1 serology was reported to be associated with a higher risk of MRI activity. Finally, the integration of the prognostic factors we identified within our dataset thus far was explored, which shall be discussed later.

Besides our findings pointing towards the transitional B cell compartment, which has been established to at least contain a regulatory subset, our results also show a role for isotype switched B cells, which are part of the memory B cell compartment. It is interesting to speculate as to why only this subset shows association with disease activity and not, for example, non-isotype switched B cells, which are also part of the memory component. One simple explanation may again be that our sample size and exploratory nature increase the chances of false negative results. Alternatively, the isotype switched subset may represent the B cells that are activated by the pathological Th population,[122] causing their isotype to be switched and thus being a surrogate marker of pathological Th activity. Finally, interferon-beta-1a is known to affect memory B cells in MS,[123] which may distort the relative numbers of memory B cells enough to obscure any associations, especially considering our sample size.

Interestingly, the regulatory B cell subset, defined as CD19⁺IL-10⁺ lymphocytes after CpG stimulation, showed no direct correlation with disease activity, even though other groups have implicated regulatory B cells (Bregs) in the disease process.[124, 125] Our group has reported a decrease in Bregs in MS patients compared to healthy controls, as well as a differing composition of Bregs between MS in remission and MS in relapse. [126] However, the earlier work reports on the difference between healthy controls and MS patients, which differs greatly from the internal comparison in our generally stable MS cohort and this may explain the conflicting results.

Lastly, one thing to keep in mind is that presence of lymphocytes was measured in the circulation in order to determine inflammatory activity in the CNS. It may be possible

that Bregs migrate past the blood-brain barrier or to the lymph nodes to exert their regulatory effects locally, thus removing them from the circulation, although reports on this are lacking.

An interesting association between the risk of MRI activity after 48 weeks, and the evolution of anti-EBNA-1 serology within those 48 weeks was found. Patients without MRI activity appeared to show a decrease in anti-EBNA-1 IgG, while patients with MRI activity tended to show stable elevated anti-EBNA-1 serology. This finding adds to the controversy surrounding anti-EBNA-1 as a prognostic marker in RRMS. Although circulating anti-EBNA-1 IgG levels have been extensively investigated in cross-sectional and longitudinal studies, the evolution of anti-EBNA-1 over a period of time is not commonly used for investigations.

As the key role of EBV in MS pathogenesis becomes more established, it becomes increasingly probable that measurements like anti-EBNA-1 have different meanings in MS patients compared to healthy controls. Where a decrease in anti-EBNA-1 may be part of physiological fluctuation in healthy controls, in MS patients a decreasing level of anti-EBNA-1 may imply a regulatory effort by the immune system. Likewise, where healthy controls may show stable levels of anti-EBNA-1 in a physiological setting, in MS patients a stable anti-EBNA-1 level may suggest a certain level of uncontrolled inflammation and an inadequate capacity to regulate the latent infection. It may also be a marker for the vitamin D status of the MS patient, as vitamin D₃ supplementation has been associated with both reduced MRI activity and reduced anti-EBNA-1 IgG levels after 48 weeks. [127] Further research into the relation between EBV and B cell composition should also incorporate EBV viral load, as this has been shown to influence B cell reconstitution in MS patients after bone marrow transplantation.[120]

Conclusions on lymphocytes as prognostic markers in multiple sclerosis

In the second part of this thesis, several cellular prognostic markers of disease activity in RRMS were investigated. Both a prognostic value for T cells and NK cells, in the form of the NK/IL-17A⁺CD4⁺ T cell ratio, and B cells in the form of relative presence of transitional B cells were reported. These factors, however, do cumulatively explain part of the variance in RRMS MRI activity and, as such, other prognostic factors should be investigated to add to this model. Nevertheless, the prognostic potential that lies in the lymphocyte composition in blood was outlined.

In our investigations, blood samples were used to assess lymphocyte composition. Given that MS is an inflammatory disease of the CNS, an interesting question arises regarding the location of the reported regulatory effect by NK cells and transitional B

cells. For NK cells, a clear distinction can be made between CD56^{dim} and CD56^{bright} NK cells. CD56^{dim} NK cells are most abundant in the circulation,[128] which may explain our results favouring the protective effect of CD56^{dim} NK cells over CD56^{bright} NK cells. However, CD56^{bright} NK cells are most abundant in lymph nodes[128] and are also best equipped to cross the blood-brain barrier.[129] Indeed, when looking at the lymphocyte composition of MS patients in the CSF, nearly all NK cells appear to have a CD56^{bright} phenotype.[130, 131] Thus, while a circulating NK/IL-17A⁺CD4⁺ T cell ratio shows real potential as a prognostic biomarker, it may undervalue the regulatory effects of CD-56^{bright} NK cells in other compartments than the circulation. Similarly, transitional B cells have just left the bone marrow and are on their way to the lymph nodes as part of their maturation path. While the regulatory effects of transitional B cells in the circulation have been noted, the prognostic value of transitional B cells in the lymph nodes is not well established. In conclusion, our blood markers show a prognostic value for disease activity. Further research is required to assess the possible regulatory effects of these cell subsets, including research into the lymphocyte composition at different locations, e.g. lymph nodes and CSF.

AN INTEGRATIVE MODEL

When assessing the figures regarding our prognostic data, a pattern appears to emerge. Some patients showing an unfavourable level of one prognostic factor, e.g. a low NK/IL-17⁺CD4⁺ T cell ratio, show no signs of disease activity, while other patients that do show signs of disease activity appear to have a favourable level of said prognostic factor. When noting the data for transitional B cells, a similar observation can be made. This prompted us to investigate this distribution further. Interestingly, patients without disease activity that showed unfavourable levels of one prognostic marker, usually had favourable levels of another to compensate. Again, conversely, patients that showed signs of disease activity but had favourable levels of one prognostic marker, usually had unfavourable levels of another. This finding, illustrated in **Figure 2**, led to the construction of the 3D figure as depicted in **Chapter 7**, attempting to illustrate the distribution of prognostic markers for patients with and without disease activity. As can be seen, patients with disease activity tend to cluster around multiple unfavourable factors, whereas patients without disease activity usually only had one unfavourable marker

This finding illustrates that there are several ways for a patient with RRMS to be at risk of, or protect from developing new disease activity. Some patients have insufficient NK cells compared to their T cells, whereas others may have too few transitional B cells to exert anti-inflammatory activity. It can also be noted from **Figure 1** that these two markers are

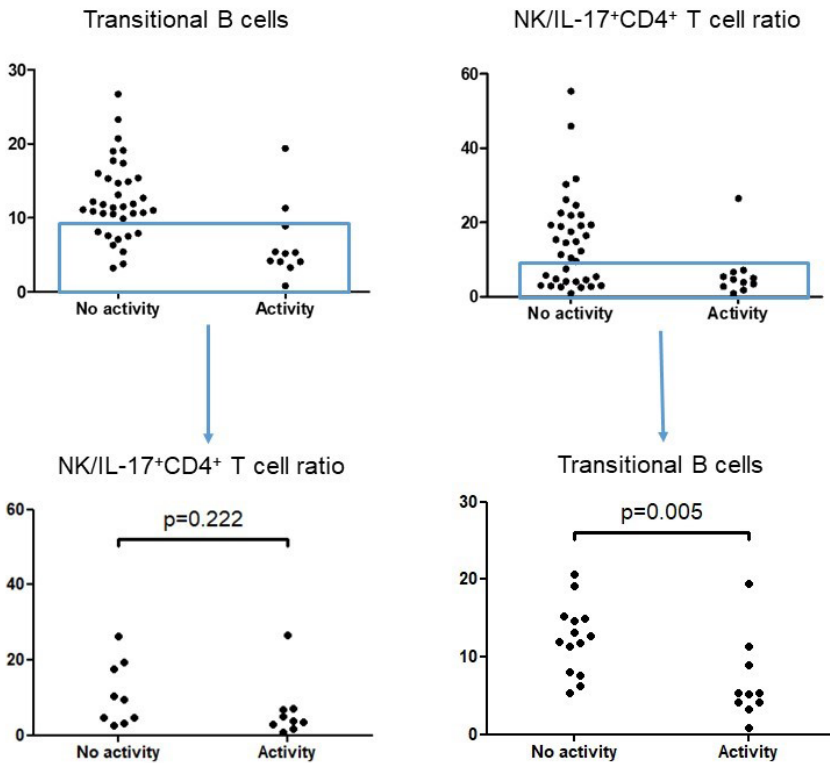


Figure 2. Illustration of prognostic marker distribution in study cohort. Although patients without disease activity tend to have a favourable level of the prognostic marker (NK/IL17⁺CD4⁺ T cell ratio or transitional B cell percentage), some patients still show unfavourable results. The inverse is also true, some patients with signs of MRI activity have favourable levels of the prognostic marker. When comparing the patients with unfavourable levels of one prognostic marker, the patients without MRI activity tend to have favourable levels of the other prognostic marker. Meanwhile, patients with MRI activity tend to show unfavourable levels of the other prognostic marker as well, suggesting that multiple unfavourable factors may be necessary before RRMS disease activity can occur. Cut-offs are the same as the cut-off values used in Chapter 7, determined by manual grouping by visual inspection.

not sufficient to explain all the variance in disease activity. As such, it appears that there are more factors that increase or decrease the risk of disease activity. In **Chapter 7**, two other factors are applied (vitamin D supplementation from the SOLARIUM study and evolution of anti-EBNA-1 levels from **Chapter 7**) to further strengthen this model. While it increases its accuracy, it does still not fully explain the variance in disease activity. This calls for a wider investigation of the immune system to find additional factors which may add to this prognostic model of RRMS disease activity. While our research has focussed on lymphocytes in RRMS, other cell subsets such as myeloid cells have been shown to be involved in the MS disease process.[132, 133] As such, further research into these prognostic factors should have a wide scope and include more than just lymphocytes.

Additionally, it should be noted that our cohort showed relatively little signs of disease activity, all used interferon- β -1a, and that the effects of vitamin D were corrected for. Thus, our findings provide a limited view of prognostic factors for future MS disease activity.

For a disease as complex as MS and with as many affected cell types as in MS, it seems logical that prognosis of its activity stems from a plethora of factors. In fact, an emerging view in MS states that immunological research should be focused on the immune system as a whole, rather than individual components.[134] Using this systems biology approach, one would evaluate the entire composition of the immune system to find patterns or profiles which are associated with an increased or decreased risk of disease activity. To do this, one would need access to a large cohort of patients and visualise as much of the immune system as possible to find the relevant markers or subsystems. In fact, to make proper use of the systems biology approach, more than just the immune system should be visualised. Characteristics like sex, age, genetics, metabolic state and hormone levels could all influence MS directly or indirectly and should thus be included in systems biology models.[135-139]

The concept of systems biology also introduces a discussion on the view of MS as a whole. As mentioned, early disease models used Th1 cells as the central player and suggested that aberrations in this particular cell subset were responsible for the disease. Now that more cell sets have been shown to be involved and taken together with our proposition that there are several ways for a patient to be at risk of disease activity, another more integrative model can be constructed.

The fact that not one, but several prognostic markers need to be affected to create disease activity, implies that the immune system has a certain redundancy. Akin to the anastomoses seen in the circulation, this redundancy in the form of multiple immune cell sets ensures that, no matter the biological variation between individuals, or in case of failure of one specific component, the function of the immune system remains intact. Despite this redundancy, MS patients appear to be vulnerable to uncontrolled inflammation, or a lack of regulatory capacity, which in the case of MS manifests as inflammatory lesions in the CNS. Restoring this redundancy through DMTs appears to increase the regulatory capacity enough for most patients to show reduced inflammation, and for a select few to halt the inflammation entirely. As such, MS may not be a disease based on a specific cell or pathway dysfunction, but rather a combined vulnerability of the regulatory capacity, or a limited redundancy.

Disease modifying treatments in MS show the delicate balance of the immune system. Where patients with MS tend to show a vulnerability to inflammation, treating them with an immune-modulatory component increases the risk of side effects like viral and bacterial infections. In cancer research, the opposite is true. New potent drugs aimed at enhancing the immune systems capability of eliminating tumour cells, e.g. checkpoint inhibitors, come with a substantial risk of auto-inflammatory complications. These examples serve to illustrate the point that balance is key in immunity. The immune system could be visualised as a system that moves within the confines of tolerance and immunity. Some factors may cause the immune system to lean towards one end of this spectrum, i.e. pathogens causing a pro-inflammatory reaction, while stress may cause a reduced vigilance of the immune system, and a vulnerability to disease. No matter the cause, the immune system is capable of resolving this upset balance and then return to a balanced medium, using its regulatory buffer. In the case of a reduced regulatory buffer, as may be the case in MS, this balance is far more difficult to maintain and regular stressors (perhaps the re-activation of EBV) may cause the immune system to go past the limits of tolerance, thus inducing autoimmunity. This point is illustrated in **Figure 3**. The fact that this decreased regulatory buffer appears limited to the intrathecal compartment in MS patients, and not in the entire body, may be due to a reduced access of regulatory immune cells beyond the blood-brain-barrier, or may be due to EBV presence in the brain, as found in post-mortem studies.[140-142] Nevertheless, although MS only presents itself in the CNS, there may be signs of reduced regulatory buffer in the rest of the body in MS patients. Indeed, patients with MS have an increased risk of other autoimmune diseases such as thyroid disease, inflammatory bowel disease, and psoriasis.[143] Similarly, families where MS is prevalent show a higher incidence of Hashimoto thyroiditis, inflammatory bowel disease and psoriasis in other members of the family.[144] The genetic risk profile of MS also shares several genes with other auto-immune diseases, such as *IL2RA*, *SH2B3*, *KIF5A*, and *CD226*, which are risk genes for MS, type 1 diabetes, and rheumatoid arthritis.[145] These findings point towards a general susceptibility in regulatory capacity, where MS may be a specific phenotype of this susceptibility based in the CNS.

One of the interesting factors in this model would be cataloguing the factors which shift the immunity balance towards the bounds of tolerance. When more of these factors become known, a patient may be better aware as to which behaviour or stressors cause risk of autoimmunity and/or disease activity. Furthermore, it is tempting to speculate that even pre-clinical measurements may already point towards a limited regulatory buffer and highlight an increased risk of autoimmunity or even specifically MS. Naturally, this remains speculative and warrants further investigation.

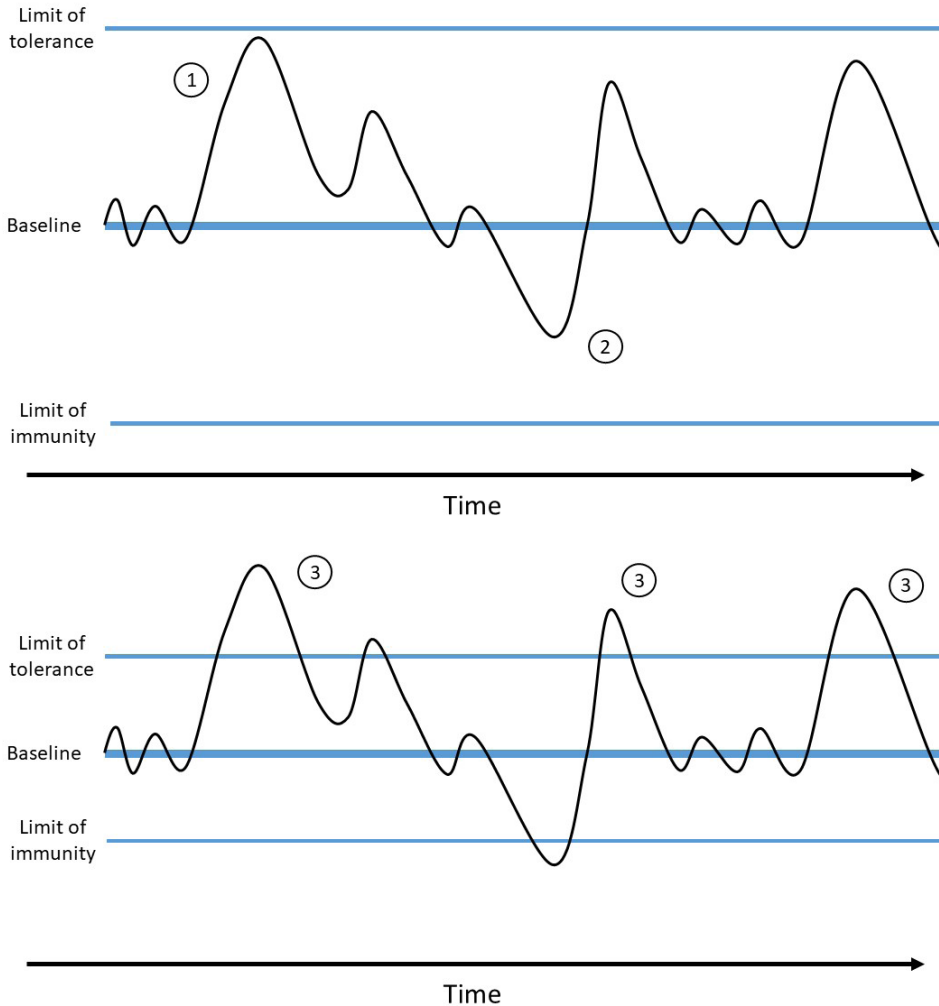


Figure 3. Illustration of homeostatic capacity in a healthy individual compared to an individual with MS. The immune system shows a capacity of self-regulation up to a point. Certain triggers, such as pathogens, may activate an inflammatory response to combat this trigger. As long as this reaction remains within the limit of tolerance (1), the immune system will be able to control the inflammatory process and bring the immunological activity back to a baseline level on its own. Similarly, other triggers, such as stress, may cause the immune system to underperform, giving opportunity for pathogens to manifest in the host. Again, as long as this depression in activity does not exceed the limit of immunity, the host will be able to control the pathogen and return immunological activity back to baseline. Following this model, MS can be seen as a disease which strongly limits the regulatory and homeostatic capacity of the host. As such, a trigger which would normally be under the level of tolerance in a healthy individual is now far more difficult to control and exceeds the lowered threshold of tolerance (3). Beyond the limit of tolerance, the inflammatory process is far less controlled and may lead to things like immune responses to the host. Because of the limited regulatory capacity, this period of uncontrolled inflammation is hard to correct for and may take more time than usual. These periods of uncontrolled inflammation may present themselves in the patient as a clinical relapse or MRI activity.

FUTURE DIRECTIONS

This thesis provides an exploration into the use of lymphocyte subsets, particularly NK cells and B cells, as a prognostic tool for disease activity in RRMS. Ultimately, the results described in this thesis raise many new questions, although these questions show exciting paths for MS research to take.

First and foremost, the results from this thesis come from a patient cohort using interferon- β -1a (and possibly high-dose vitamin D₃ supplementation). Since the inception of the SOLAR study, from which the SOLARIUM cohort is derived, there has been a large influx of new and highly effective therapies. Pharmaceuticals like ocrelizumab (anti-CD20), natalizumab (anti- α 4 integrin) or fingolimod (S1P receptor-1 inhibitor) are now more commonly prescribed due to their superior efficacy. As such, our results should be reproduced with other DMTs to extrapolate our findings to the general RRMS population. In addition, similar investigations should be performed using a cohort of progressive patients, especially now that treatments for progressive MS are beginning to emerge. Of course, certain therapies will be unsuitable for the investigation of certain cell types. For example, ocrelizumab, which is used to deplete most B cells including transitional B cells, is inherently less suitable to measure normal levels of transitional B cells in MS patients, although it is an excellent intervention to evaluate the prognostic value of the re-emergence of (transitional) B cells.

Another challenge will be to expand the list of involved lymphocyte subtypes that show prognostic value for disease activity. In this thesis, pharmaceutical studies were used to find cell types that seem to exhibit a protective effect, such as CD56^{bright} NK cells. In a similar vein, many cell types seem to be affected by modern disease modifying therapies and, as such, may hold prognostic value in RRMS patients. For example, dimethyl fumarate modifies the activation profile of monocytes, which may imply a prognostic value of monocyte subsets. Likewise, macrophages are heavily implied in the inflammatory process of MS and may hold similar prognostic value.[146] Future prospective studies investigating prognostic factors, should include these factors to investigate their prognostic potential.

If more prognostic factors can be found and catalogued, an effort could be made to design a prognostic model for future disease activity. This could be a simple point-based system where every unfavourable marker adds a point, similar to our last figure in **Chapter 7** and similar to systems currently used in the risk stratification of primary myelofibrosis, for example.[147] Using a cut-off point, a score above a certain threshold

would indicate a substantial risk of subsequent disease activity and may prompt the treating clinician to intensify treatment or to switch DMTs.

If the systems biology approach is followed, two other prognostic approaches could be imagined. First, the system biology approach may identify a number of immunological 'profiles' based on the relative abundance or shortage of certain cell types. These profiles could then be categorised as high-risk, medium-risk or low-risk profiles. Based on the profile found by, for example, yearly blood analysis, clinical decision making could be assisted. Going one step further, an algorithm could be designed to calculate a percentage risk score of disease activity, which could also support clinical decision making. This would more closely resemble the system used by urologists to determine the percentage chance of prostate cancer based on several parameters.[148, 149] Again, a cut-off may be determined upon which a clinician is urged to reconsider the current treatment, or perhaps an increase in percentile risk could prompt further investigation. Naturally, many factors would have to be introduced in such an algorithm, such as the type of DMT used, comorbidities, patient characteristics such as age and sex, and perhaps vitamin D levels or even genetic variations. The vast amounts of data required to establish these profiles require a new and innovating approach, which can be found in the field of artificial intelligence. Specifically, deep learning strategies, like currently used in radiology to enhance detection of abnormalities like tumours,[150] could be used to assess what combination of factors increases or decreases the risk of MS disease activity.

Another interesting future prospect, albeit more speculative and distant, can be derived from the findings in **Chapter 7**. As illustrated in **Figure 3**, the disease MS may best be described as a lack of homeostatic capacity, with varying cell types seemingly affected. Our findings as shown in **Figure 2** suggest that different MS patients may have different combinations of aberrations in their immune system, with accumulating aberrations leading to a higher risk of MS disease activity. As an example, one patient may show a lowered transitional B cell level with a normal NK/T cell ratio, while another patient may show the inverse. If this were the case, then the first case may also be classified as a 'B cell oriented/deficient MS' and the second as a 'NK cell oriented/deficient MS'. This leads to an interesting new application for circulating lymphocytes in RRMS: using them as guidance in choice of therapy. Patients with NK cell oriented MS may benefit more from treatments like dimethyl fumarate, which suppress $CD4^+$ T cells but not NK cells.[151] By contrast, a patient with B cell oriented MS, may show additional benefit from therapies that alter the B cell compartment, like glatiramer acetate does.[152] If the earlier mentioned research into prognostic factors in the immune system does indeed show certain patients to exhibit certain profiles, then efforts should be undertaken to match profiles to their best-suited therapies and evaluate whether this increases the

odds of therapy success. Matching these therapies to the initial immune aberrations might even prevent some patients from having to escalate to second line therapies such as ocrelizumab or natalizumab, which carry more risk of side effects.[153] One promising lead may be found in a study attempting to find predicting factors for therapeutic response to fingolimod treatment. This study reports that lymphocyte subpopulation compositions were already different between responders and non-responders before the start of fingolimod,[77] alluding to the fact that certain treatments may indeed have a higher chance of success for certain lymphocyte profiles.

Taken together, this thesis lays a foundation for exciting new ventures in MS disease management. If future trials show the mentioned prospects to be true, a future could be foreseen where lymphocyte assessments play a major role in therapy choice and follow-up. Using MRI scans to illustrate past disease activity, and NfL values to display current disease activity, blood analysis could be used to determine future disease activity. The added benefit of this prospective measurement is easy to envision: if a patient would show an increased risk of disease activity, the clinician would have a window of opportunity to intensify treatment, thus possibly preventing new inflammatory activity and reducing overall clinical disability in patients. Again, this remains speculation, and more research is needed to confirm the clinical usefulness of lymphocyte phenotyping in MS.

CONCLUSIONS

Multiple sclerosis is a debilitating auto-immune disease with many involved cell types and inflammatory mechanisms. Currently, much is known about the varying changes and shortcomings of the immune system and especially lymphocytes in MS patients, although the use of lymphocytes in clinical practice remains under-investigated. Based on our exploratory research, a prognostic role for lymphocytes may be plausible and may help in prescribing the best DMT for the patient, or help predicting disease activity before it happens, thus allowing a window for intervention. This thesis should be considered as a starting point for subsequent research to solidify the use of lymphocytes to predict treatment response and optimize patient care in RRMS.

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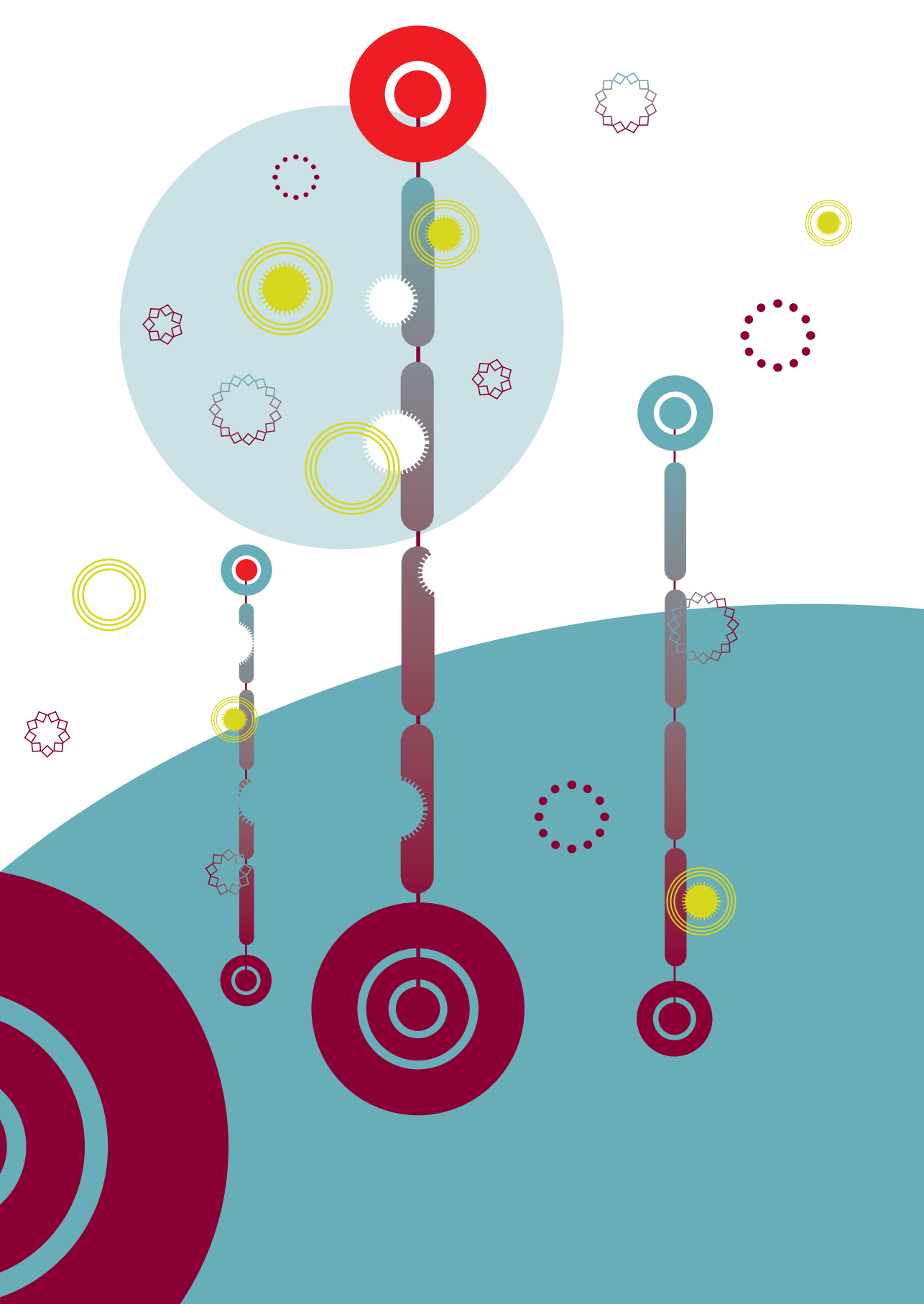
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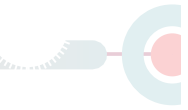
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IMPACT PARAGRAPH

1. What is the main goal of the research reported in the thesis and what are the most important results and conclusion?

Over the last few decades, the clinical care of multiple sclerosis (MS) patients has evolved dramatically. An influx of new disease modifying treatments, as well as the introduction of brain imaging through magnetic resonance imaging (MRI), has provided the clinician with many more options to monitor and treat MS patients. Despite these advancements, much remains unknown or suboptimal in our knowledge of MS. One of these things is the lack of a prospective biomarker for disease activity in MS. Currently, no curative treatment for MS exists, and as such patients are committed to lifelong use of immune modulating therapies to suppress further disease activity. Currently, the choice to intensify treatment is based on clinical relapse or MRI activity, which are both retrospective measurements, *i.e.*, the choice to intensify treatment can only come after new activity is recorded. Thus, damage to the CNS has already occurred and may have caused permanent disability in the MS patient. Identification of predictive markers may help make choices regarding treatment before disease activity occurs, which could prevent or delay disability progression.

To find such a marker, it is tempting to look at the cells which effectuate the damage in MS. Classically, MS was considered to be caused mainly by aberrant T-cells. However, more recent insights have shown roles for other lymphocytes, such as B cells and NK cells, in the MS disease process. Despite these new insights, very little is known about the significance of the presence of these lymphocytes in the serum of MS patients. In other words, while we better understand which cells contribute in which manner to the MS disease process, we still do not know what the relative presence of these cells in the blood means for MS patients. If the inflammatory process of MS is mediated by these lymphocytes, then early alterations in the composition of lymphocytes may show prognostic value for disease activity.

The main goal of this research was to explore prognostic value of the lymphocyte composition in the blood of MS patients, with a focus on the lymphocyte subsets which gained recent attention as key players in the disease process. As we performed this research in a cohort receiving high-dose vitamin D₃ supplementation, we also aimed to further elucidate the effects of vitamin D₃ in MS.

Starting with vitamin D, we report no association between vitamin D levels and serum levels of the neurofilament light chain biomarker for axonal loss, implying this biomarker

can be used in our cohort without the need for correction for vitamin D supplements. Second, we report that certain genetic subgroups in our cohort had different serological responses to high-dose vitamin D₃ supplementation, with some showing a lower increase in serum vitamin D than others. This has implications for present and future vitamin D₃ supplementation studies, as it implies that genetic subgroups of patients may benefit differently from supplements. It may be true that only a specific subgroup benefits from vitamin D₃ supplementation, which may explain the varying results from earlier vitamin D₃ supplementation studies and would aid MS therapy by supplementing vitamin D₃ to specific MS patients.

Moving on our lymphocyte related results, we report a prognostic value of a natural killer (NK) / T cell ratio for MS disease activity. Not only does this finding underline the interaction between NK cells and T cells in MS, it also proposes a new and accessible biomarker for MS disease activity. Then, we go on to show that this NK/T cell interaction is partially mediated through the IL-2 – IL-2 receptor pathway. This finding adds to the understanding of the NK/T cell interaction in MS.

To continue, we report a prognostic value of transitional B cells for MS disease activity. Again, this finding both shows the potential protective role of transitional B cells in MS, as well as providing another potential new and accessible prognostic marker for MS disease activity. Additionally, we find that a persistence of the elevated anti-EBNA-1 titre in serum is associated with an increased risk of MS disease activity.

Finally, taking these mentioned prognostic markers from our results, we construct a rudimentary prognostic model, showing fact that MS patients without disease activity that have unfavourable levels of one prognostic marker, appear to compensate this by having favourable levels of another marker. By contrast, patients with unfavourable levels of multiple prognostic markers show a higher risk of MS disease activity. Here, we propose that by finding more involved prognostic markers, one may construct a predictive model involving markers in the circulation that indicates a risk of MS disease activity in the next year. We conclude that certain lymphocytes show prognostic value for disease activity in MS patients, and that by searching for additional markers we may not only further our understanding of the MS disease process, but also add to the prognostic model in order to more accurately determine risk of disease activity.

2. What is the potential contribution of the results from this research to the scientific community, and if applicable to public sectors and public challenges?

Our vitamin D related research contributes to later high-dose vitamin D trails, especially our results concerning the difference in serological response between genetic subgroups. Considering high-dose vitamin D₃ supplementation reduces MRI lesions, as shown in the SOLAR study, this finding could imply that certain patients have a better response to supplementation than others based on their genetics. Later vitamin D supplementation studies should include these genetic variations in order to further establish the exact effects of these genetic variations, which may ultimately point towards a genetic subgroup of patients that benefits most from high-dose vitamin D₃ supplementation.

Generally, our results regarding the prognostic value of lymphocytes may be used in two ways. First, we describe prognostic values for several cell subsets for disease activity in MS. These cell subsets may either show an association with an increased or a decreased risk of MS disease activity. Although this implicitly implies their respective detrimental or protective roles, it remains unclear how these roles are carried out *in vivo*. As such, our results may prompt researchers to further investigate our reported cell subsets to find their mechanism of action in MS disease activity, ultimately aiming to uncover new therapeutic targets for MS treatment. The second way our work contributes to the scientific field is by laying the foundation for a predictive model of MS disease activity using serological markers. While our model at the end of this thesis is quite preliminary, it does illustrate the potential of serological markers as predictors of disease activity. As such, other groups may use this knowledge to find more circulating markers, or to extend and/or refine our currently proposed model.

3. For whom may the results of this research be interesting and/or relevant? And why?

Our results may be of interest to a multitude of groups thanks to the broad implications of our research.

First, clinical researchers should find our results interesting for several reasons. We report a genetic impact in our cohort receiving high-dose vitamin D₃ supplementation, which implicates that these genetic factors should be considered/corrected for in further vitamin D studies. Additionally, our findings regarding the prognostic value of several lymphocyte subsets should prompt researchers to include these immunological parameters in later prognostic studies. Furthermore, the results of **Chapter 7** should interest clinical researchers investigating systems biology, as it lays a foundation for immune profiling in MS patients.

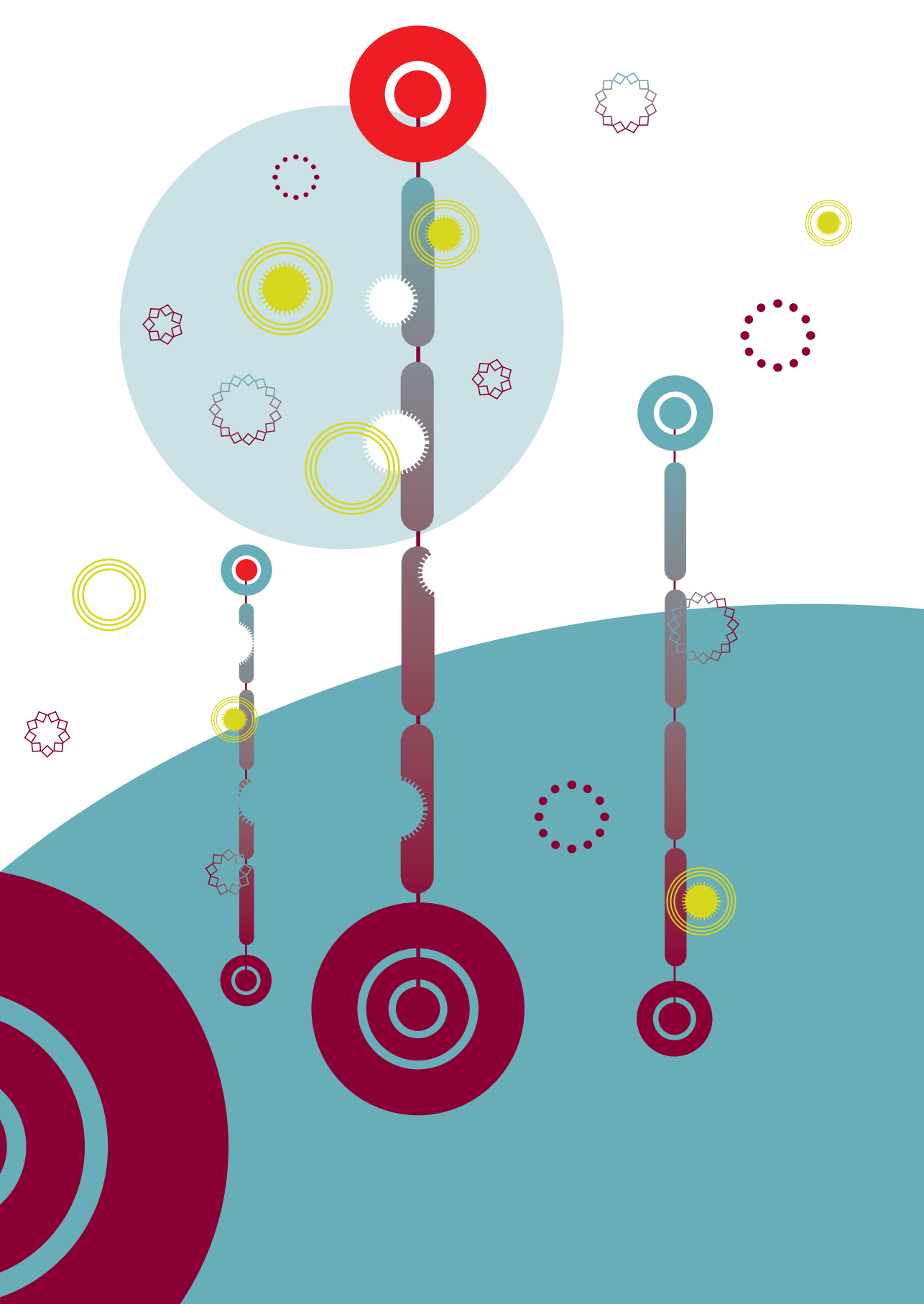
Laboratory researchers should be interested in our efforts to further elucidate the NK – T cell interaction, especially through the IL-2 – IL-2 receptor pathway. Additionally, as we show that patients with a unfavourable NK/T cell ratio may compensate with a favourable level of transitional B cells, the interaction of B cells with NK cells and T cells (The NK – T- B cell interaction) is shown to be an interesting and relevant topic and may prompt researchers to elucidate it further.

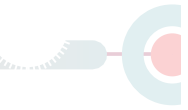
Next, our results are also of interest to clinicians, as they provide further insights into the workings of a commonly prescribed dietary supplement in MS patients (*i.e.* vitamin D). Additionally, clinicians should be excited for the prospect of a possible prognostic/predictive tool for MS disease activity. Although this tool is at the very early stages, if it indeed reaches clinical practise, then it is easy to imagine the significant impact it would have on clinical decision making.

Finally, MS patients may be interested in the developments surrounding vitamin D supplementation, especially the implication that some MS patients may show genetic predisposition to a stronger response to vitamin D supplementation, which may in turn show clinical benefit, although this remains to be investigated. Additionally, we provide insights in what the composition of the immune system in blood samples means for the patient and how we may use it to treat their chronic disease more effectively.

4. In what way could these target audiences be involved in and informed of the results of this research, so that the gained insights can be used in the future?

First and foremost, all our research described in **Chapter 2** through **Chapter 7** has been published in established peer-reviewed scientific journals and is thus readily accessible to the scientific community at a trustworthy source. Additionally, most of our chapters have been presented at national or international conventions, even though due to the COVID-19 pandemic, the number of presentations has been limited. Regardless, other renowned MS research teams have submitted proposals to further investigate NK cells in multiple sclerosis, which may be partially inspired by our published and/or presented results. Finally, a summary of some chapters has been published on the website of the Dutch national MS foundation (Nationaal MS fonds), which makes our results accessible at patient level for people with MS.





Nederlandse Samenvatting

NEDERLANDSE SAMENVATTING

Multiple sclerose (MS) is een auto-immuun aandoening van het centrale zenuwstelsel, oftewel de hersenen en het ruggenmerg. Door een fout in het immuunsysteem worden zenuwcellen (neuronen) en hun isolerende laag (myeline) aangevallen. Als gevolg van deze aanvallen kunnen de zenuwen signalen niet meer goed doorgeven, waardoor patiënten klachten krijgen zoals zwakte, gevoelsstoornissen, pijn, problemen met het zien en balansproblemen. Het grootste deel van de MS patiënten heeft relapsing-remitting MS (RRMS). Dat wil zeggen dat klachten tijdens een aanval ontstaan (een relapse), waarna ze (gedeeltelijk) wegtrekken (remissie). Bij een kleiner deel van de MS patiënten is er sprake van geleidelijke achteruitgang zonder tussentijds herstel, dit wordt progressieve MS genoemd. Als de progressieve vorm direct aanwezig is zonder dat er relapsen aan vooraf zijn gegaan, dan wordt dit primair progressieve MS (PPMS) genoemd. Indien de progressieve fase vooraf wordt gegaan door een relapsing-remitting beloop, wordt dit secundair progressieve MS (SPMS) genoemd. Naast het bijhouden van klinische klachten kan MS ook opgevolgd worden met MRI scans van de hersenen. Hierop kunnen lokale ontstekingen in de hersenen te zien zijn, welke gezien kan worden als een teken van actieve ziekte, of ziekteactiviteit. Op dit moment is er geen genezing voor MS, maar wordt door middel van medicijnen die het immuunsysteem beïnvloeden gepoogd om de ziekteactiviteit zo veel mogelijk te remmen, om achteruitgang zo veel mogelijk te voorkomen.

Het is nog niet duidelijk hoe de fout in het immuunsysteem die MS veroorzaakt kan ontstaan. Waarschijnlijk zorgt een ongelukkige combinatie van genetische factoren en omgevingsfactoren voor de fout. Uit onderzoek blijken enkele omgevingsfactoren als belangrijke spelers in het ontstaan van MS naar voren te komen. Dit betreft een tekort aan vitamine D, een infectie met het Epstein-Barr virus (EBV, bekend als de veroorzaker van de ziekte van Pfeiffer), roken en obesitas.

Zoals gezegd is MS een auto-immuun ziekte, wat betekent dat er afweercellen betrokken zijn in de oorsprong van de ziekte. Lang werd gedacht dat één soort afweercel de hoofdrolspeler was in MS, namelijk de T cel (vernoemd naar het orgaan waar hij gemaakt wordt, namelijk de thymus) en in het bijzonder de T helper cel. Recenter onderzoek toont dat naast de T cel, ook de B cel (vernoemd naar het beenmerg waar hij gemaakt wordt) en de NK cel (natural killer, of natuurlijke doder, omdat hij niet door een andere cel geactiveerd hoeft te worden voordat hij zijn functie uit kan voeren) actief een rol spelen in het ziekteproces bij MS. Door dit recente onderzoek zijn we veel te weten gekomen over hoe deze cellen werken in MS patiënten, maar desondanks is er weinig bekend over wat de aanwezigheid van deze cellen betekent voor de patiënt. Oftewel:

Is er een waarde voor de patiënt om naar de samenstelling van het immuunsysteem te kijken? Zegt dit bijvoorbeeld iets over de mate van ziekteactiviteit die ze te wachten staat? Deze kwesties worden verkend in dit proefschrift.

Hoofdstuk 1 geeft een algemene introductie omtrent de aandoening multiple sclerose en een overzicht van de bekende factoren die bijdragen aan het ontstaan en beloop van multiple sclerose. Dit hoofdstuk beschrijft de doelstellingen en de opzet van dit proefschrift.

Het hoofdstuk benoemt onder andere vitamine D. Dit is een belangrijke stof die we voornamelijk binnen krijgen door UV straling van zonlicht. Het staat vooral bekend om zijn rol in botregulatie en calcium huishouding, maar lijkt ook een rol te spelen in het immuunsysteem. Omdat lage vitamine D waarden zijn geassocieerd met het ontstaan, en met een slechter beloop van MS, hebben verscheidene studies het effect van vitamine D supplementies bij MS patiënten onderzocht. Een van deze studies was de internationale SOLAR studie, welke uitzocht of MS patiënten die één jaar lang hoge dosis vitamine D suppletie kregen, minder tekenen van ziekteactiviteit hadden dan MS patiënten die één jaar lang een placebo namen. Dit werd gemeten in onder andere hoeveelheid aanvallen en in nieuwe ontstekingshaarden op MRI scans na één jaar. Een kleiner deel van de patiënten in de SOLAR studie werden ook benaderd voor een aanvullende analyse (de Nederlandse SOLARIUM substudie). Bij deze patiënten werd ook bloed geprikt vóór ze begonnen aan de suppletie danwel placebo, en na één jaar. In dit bloed werd gekeken naar de samenstelling van het immuunsysteem bij deze patiënten, met als doel om te kijken of patiënten die vitamine D suppletie kregen een meer gebalanceerd immuunsysteem lieten zien, ten opzichte van patiënten die placebo kregen. Uit de SOLAR studie bleek dat patiënten die vitamine D suppletie kregen, minder MRI afwijkingen lieten zien dan patiënten met een placebo. De SOLARIUM studie toonde geen significant verschil in de samenstelling van het immuunsysteem tussen patiënten die wel of geen vitamine D suppletie hadden gekregen. Om ons te verdiepen in de onderzoeksvragen, hebben we de gegevens van de SOLAR studie en de SOLARIUM studie gecombineerd. Zodoende kijken we of de samenstelling van het immuunsysteem aan het begin van het jaar, iets zegt over de ziekteactiviteit aan het einde van het jaar. Omdat we ons baseren op vitamine D studies, wordt eerst de rol van vitamine D in MS verder uitgediept.

Hoofdstuk 2 beschrijft de relatie tussen vitamine D en een nieuwe marker van ziekteactiviteit: neurofilament lichte keten (neurofilament light chain, NfL). Deze relatief nieuwe biomarker geeft een indruk van de mate van zenuwschade en verhoogde NfL waarden zijn geassocieerd met een ernstiger MS ziektebeloop. Gezien vitamine D suppletie geassocieerd is met een afname van MRI afwijkingen vergeleken met een placebo, werd

onderzocht of eenzelfde gunstig effect op NfL waarden bestaat. Bij onze 40 patiënten werd geen significant verschil in NfL waarden gevonden tussen beide behandelingen. Na correctie voor leeftijd werd bij hogere NfL waarden een trend gezien richting een verhoogd risico op MRI activiteit. Deze uitkomst ondersteunt het gunstige effect van vitamine D suppletie niet. Wel geeft het aan dat we NfL in latere analyses kunnen gebruiken zonder te corrigeren voor vitamine D suppletie.

Hoofdstuk 3 verdiept zich verder in vitamine D en beschrijft de invloed van genetica op het effect van hoge-dosis vitamine D suppletie, zoals in de SOLARIUM studie. In het SOLARIUM cohort werd, waar mogelijk, het dragerschap van vier risico-allelen bepaald welke allen geassocieerd zijn met vitamine D metabolisme. Vervolgens werd onderzocht of dragerschap van deze risicoallelen geassocieerd is met een hogere of lagere hoeveelheid vitamine D in het bloed, vergeleken met niet-dragers. Dit werd gemeten bij de start van het onderzoek en na één jaar hoge-dosis vitamine D suppletie. Bij de start van het onderzoek werd geen verschil gevonden in vitamine D waarden tussen dragers en niet-dragers. Na één jaar suppletie blijken dragers van het rs7041 risico-allel een verhoogde concentratie vitamine D in het bloed te hebben, terwijl dragers van het rs12368653 risico-allel een verlaagde vitamine D concentratie te hebben. Verder onderzoek moet uitwijzen of deze veranderde vitamine D waarden ook klinisch een verschil maken tussen dragers en niet-dragers van de risico-allelen. Ook moet blijken of dit gemeten verschil ook aanwezig is bij suppletie in lagere, meer fysiologische doseringen. In ieder geval zouden deze risico-allelen moeten worden meegenomen in toekomstige (hoge-dosis) vitamine D suppletie studies om te kunnen corrigeren voor genetische opmaak.

Na de verdieping in vitamine D, wordt de focus verlegd richting afweercellen en hun klinische waarde.

Hoofdstuk 4 is een overzicht van onze huidige kennis over NK cellen. NK cellen zijn relatief recent beschreven als sleutelspelers in het MS ziekteproces. Er wordt ingegaan op de functie, subtypering en werking van NK cellen, waarin we met name stilstaan in het unieke activatiemechanisme van NK cellen. NK cellen dragen een verzameling activerende en inhiberende receptoren bij zich, welke gebruikt worden om gezonde cellen van ongezonde cellen (bijvoorbeeld geïnfecteerd met een virus, of een kankercel) te onderscheiden. Door dit mechanisme heeft de NK cel geen andere cel nodig die hem activeert, zoals wel het geval is bij B en T cellen. Na deze algemene beschrijving wordt ingegaan op de rol van NK cellen in MS. Eerst wordt ingegaan op het bestaande bewijs voor de rol van NK cellen in MS, waarna de (veranderde) functie van NK cellen in MS besproken wordt. De bekendste rol voor NK cellen in MS lijkt een regulerende rol te zijn, waarbij NK cellen de capaciteit hebben om geactiveerde T cellen (en vooral diegene die

het eigen lichaam aanvallen) te doden en zo auto-immuniteit tegen te gaan. Echter lijken NK cellen ook betrokken bij de schade die veroorzaakt wordt door de ontstekingsreactie in het centrale zenuwstelsel. Ondanks hun voornamelijk beschermende rol, is het dus te kort door de bocht om te zeggen dat NK cellen alleen maar beschermend zijn in MS. Vervolgens wordt gekeken naar de effecten van de meest bekende MS omgevingsfactoren en NK cellen. Hier wordt gezien dat de meeste omgevingsfactoren een directe of indirecte relatie met NK cellen hebben. NK cellen hebben bijvoorbeeld een vitamine D receptor en reageren dus op een verlaagd vitamine D niveau. Daarnaast zijn NK cellen, als een van de eerste beschermingen tegen virusinfecties, nauw betrokken bij infecties met EBV. Als laatste worden de effecten van verschillende MS medicamenten op de NK cel populatie benoemd. Hoewel ze bijna allemaal niet ontwikkeld zijn met het doel om NK cellen te beïnvloeden, hebben de meeste therapieën direct of indirect invloed op de NK cel populatie, vaak in gunstige zin. Het is dus mogelijk dat deze therapieën een deel van hun succes te danken hebben aan een, nog onderkend, effect op NK cellen. Samenvattend zijn NK cellen nauw betrokken in het MS ziekteproces, voornamelijk als regulerende entiteiten, welke veelbelovend is om het MS ziekteproces beter te begrijpen en uiteindelijk beter te kunnen behandelen.

Hoofdstuk 5 beschrijft de prognostische waarde van NK cellen en T cellen in het bloed, uitgedrukt als de NK/T cel ratio. Zoals beschreven in hoofdstuk 4 is de veronderstelde beschermende functie van NK cellen in MS toe te schrijven aan hun mogelijkheid om geactiveerde T cellen te remmen. Derhalve lijkt met name hun relatieve aanwezigheid ten opzichte van T cellen relevant, in plaats van hun absolute aantal. In dit retrospectieve onderzoek met 50 patiënten wordt een relatieve toename van NK cellen geassocieerd met een relatieve afname van bepaalde T cellen, namelijk T helper cellen en in het bijzonder T helper 17 (Th17) cellen. Zoals eerder genoemd zijn T helper cellen kernspelers in het MS ziekteproces en Th17 cellen in het bijzonder zijn gemerkt als schadelijke cellen in MS. Dit ondersteunt de notie dat NK cellen deze schadelijke cellen kunnen onderdrukken. Vervolgens werden de patiënten verdeeld in patiënten die na één jaar tekenen van ziekteactiviteit lieten zien en patiënten die dat niet hadden. Dit gebeurde op basis van MRI scans, klinische relapsen en NfL waarden. Bij deze patiënten werd onderzocht hoe hun NK/Th17 cel verhouding was aan het begin van de studie. Na correctie voor vitamine D suppletie hebben patiënten met nieuwe MRI afwijkingen na één jaar gemiddeld een lagere NK/Th17 cel ratio aan het begin van de studie dan patiënten zonder ziekteactiviteit. Dit fenomeen wordt ook gevonden als wordt gekeken naar klinische relapse en NfL waarden. NK/Th17 cel ratio's werden niet beïnvloed door vitamine D suppletie. We concluderen dat patiënten die binnen één jaar ziekteactiviteit ontwikkelen over het algemeen een ongunstiger NK/Th17 cel ratio hebben in hun bloed dan patiënten die geen ziekteactiviteit ontwikkelen. Deze ratio is dus een mogelijke

biomarker voor ziekteactiviteit en zou met verder onderzoek gebruikt kunnen worden om het risico op ziekteactiviteit binnen een jaar beter in te schatten.

Hoofdstuk 6 verdiept zich in de interactie tussen NK en T cellen. Eén van de grote doorbraken op het gebied van NK cellen in MS is gekomen door een MS geneesmiddel genaamd daclizumab. Dit middel blokkeert een onderdeel van de interleukine-2 (IL-2) receptor (IL-2R), namelijk de alfa keten van de receptor, ook wel bekend als CD25. Door deze receptor te blokkeren hoopte onderzoekers geactiveerde T cellen te remmen, aangezien deze IL-2 nodig hebben om te kunnen overleven. Hoewel T cellen niet geremd leken te worden door blokkade van CD25, zorgde het wel voor een toename van NK cellen, welke uiteindelijk T cellen remde en dus zorgde voor een afname van ziekteactiviteit in behandelde MS patiënten. Het IL-2 – IL-2R pad lijkt dus belangrijk in de balans tussen NK en T cellen, welke bijdraagt aan de kans op ziekteactiviteit zoals beschreven in hoofdstuk 5. Om dit te onderzoeken zijn onderdelen van het IL-2 – IL-2R pad gemeten in 50 MS patiënten en vergeleken met hun NK/Th17 cel ratio's, om een mogelijk verband aan te tonen. De onderdelen van het IL-2 – IL-2R pad zijn de oplosbare vorm van CD25, zowel gemeten in bloed als in supernatant na T cel stimulatie, CD25 expressie op T cellen, en uitdrukking van het *IL2RA* gen, welke codeert voor CD25. Er is bloed afgenomen op week 0 en week 48 van het onderzoek. Allereerst werden IL-2 – IL-2R markers op week 0 en week 48 vergeleken, welke een hoge mate van correlatie toonde. Door deze hoge correlatie konden metingen van week 0 en week 48 samengenomen worden, om zo een grotere onderzoeksgroep te krijgen. Vervolgens werd gekeken naar de correlatie tussen IL-2 – IL-2R markers en NK/Th17 cel ratio's. Oplosbaar CD25, zowel in bloed als in supernatant, alsook CD25 expressie op niet-regulatorische T cellen, zijn negatief gecorreleerd met NK/Th17 cel ratio's. Dit ondersteunt een relatie tussen het IL-2 – IL-2R pad en NK cel – T cel interactie. Het verband tussen verhoogde IL-2 – IL-2R markers en een verlaagde NK/Th17 ratio kan op twee manieren geïnterpreteerd worden. Enerzijds kan een verlaagde ratio een gevolg van de verhoogde markers zijn, wat zou betekenen dat de markers ervoor zorgen dat T cellen geactiveerd worden of NK cellen juist geremd worden, bijvoorbeeld door IL-2 weg te vangen van NK cellen. Anderzijds kan de verlaagde ratio een oorzaak zijn van de verhoogde markers, wat zou betekenen dat er door een slechtere balans tussen geactiveerde T cellen en regulerende NK cellen, er een situatie ontstaat waarin het IL-2 – IL-2R pad meer geactiveerd wordt en meer markers meetbaar zijn. Welke interpretatie de juiste is zal moeten blijken uit toekomstig onderzoek.

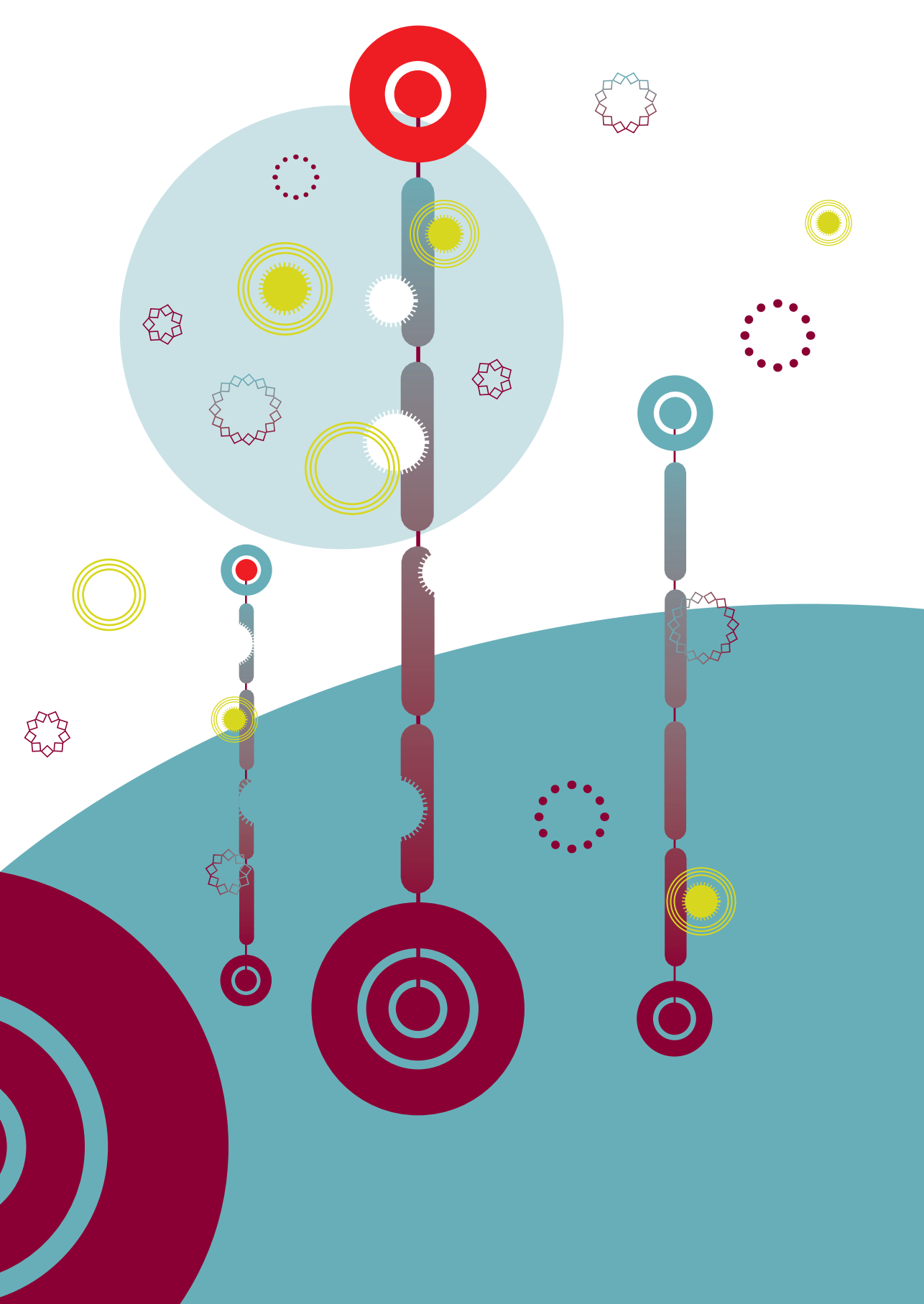
Hoofdstuk 7 betreft een nieuw celtype, namelijk de B cel, in het proefschrift. Net zoals de NK cel zijn B cellen relatief recent ontdekt als hoofdrolspelers in het MS ziekteproces. Dit komt voor een groot deel door het goede klinische effect van ocrelizumab, een MS medicament welke CD20 blokkeert, een marker die zich op de meeste B cellen bevindt

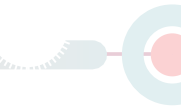
en deze cellen remt. B cellen zijn het meest bekend als de cellen die antistoffen produceren, echter is er recent meer aandacht gekomen voor hun antistof-onafhankelijke functies, welke juist van grote invloed lijken in het MS ziekteproces. Een eerder genoemde omgevingsfactor bij MS is besmetting met het Epstein-Barr virus (EBV). Dit virus blijft na initiële infectie sluimerend aanwezig in B cellen. Op deze manier zijn B cellen dus ook betrokken in het MS ziekte proces. In dit hoofdstuk wordt de voorspellende waarde van B cellen en B cel subtypes onderzocht, net zoals dit bij NK/Th17 cel ratio's is gedaan. Daarnaast worden afweerstoffen tegen EBV gemeten en vergeleken met de aanwezigheid van B cellen en B cel subtypes, alsook het risico op nieuwe MS ziekteactiviteit. Allereerst hebben patiënten met nieuwe MRI afwijkingen binnen één jaar over het algemeen lagere proporties transitionele B cellen, en hogere proporties isotype geswitchte B cellen, dan patiënten zonder nieuwe MRI afwijkingen. De transitionele B cel is een jonge vorm van de B cel welke immuun regulerende capaciteiten heeft. De isotype geswitchte B cel is een geheugen B cel die in eerder onderzoek benoemd is als een schadelijke cel in het MS ziekteproces. De proporties transitionele B cellen en isotype geswitchte B cellen kunnen gezien worden als een marker voor MS ziekteactiviteit, welke kan helpen een inschatting te maken van het risico op MRI activiteit binnen één jaar. De proporties van B cellen worden niet beïnvloed door vitamine D suppletie. Antistoffen tegen EBV zijn niet geassocieerd met B cel proporties. Patiënten zonder nieuwe MRI afwijkingen lieten een daling van antistoffen tegen EBV zien over één jaar, terwijl patiënten met nieuwe MRI afwijkingen geen afname lieten zien. Dit betekent dat een daling van EBV antistoffen of het gebrek aan daling ook een mogelijk marker voor toekomstige ziekteactiviteit is.

Tijdens de analyse van deze markers viel een interessant verdelingspatroon op. Zo waren er patiënten met nieuwe MRI activiteit die juist gunstige waardes van transitionele B cellen in het bloed hadden, terwijl er ook patiënten waren zonder nieuwe MRI activiteit die ongunstige waarden hadden. Deze verdeling werd ook teruggezien bij de analyse van NK/Th17 cel ratio's. Verdere verdieping toonde dat patiënten zonder nieuwe MRI afwijkingen en een ongunstige waarde van transitionele B cellen, vaak wel gunstige waarden van andere markers voor ziekteactiviteit, zoals NK/Th17 cel ratio's hadden. Patiënten met nieuwe MRI activiteit binnen één jaar hadden vaak ongunstige waarden van meerdere markers van ziekteactiviteit. Dit impliceert dat patiënten met één ongunstige waarde in hun immuunsysteem geen MRI afwijkingen hoeven te ontwikkelen, zolang ze dit kunnen compenseren met andere regulatoire mechanismen. Het echte risico op MRI afwijkingen ontstaat dus bij meerdere afwijkingen in het immuunsysteem, waardoor dit compensatiemechanisme wegvalt. Deze theorie wordt ondersteunt door een laatste analyse, waarbij we patiënten scoren op vier markers voor ziekteactiviteit, namelijk NK/Th17 cel ratio, proportie proportionele B cellen, wel of geen afname van EBV antistoffen, en het wel of niet ontvangen van vitamine D suppletie. Alle patiënten met nieuwe MRI

afwijkingen hadden een ongunstige waarde op minstens twee van deze onderdelen. Alle patiënten die vier ongunstige waarden hadden, hadden nieuwe MRI afwijkingen, en dit gold ook voor nagenoeg alle patiënten met drie ongunstige waarden. Van alle patiënten die drie of vier gunstige waarden hadden, had geen enkele nieuwe MRI afwijkingen. Concluderend hebben transitionele B cellen, isotype geswitchte B cellen en de afname van EBV antistoffen prognostische waarde voor MS ziekteactiviteit. Er lijkt een meervoud aan ongunstige afwijkingen in het immuunsysteem nodig te zijn om nieuwe MRI activiteit uit te lokken.

Hoofdstuk 8 geeft een overzicht van de eerdere hoofdstukken en stelt de bevindingen ter discussie. Er wordt bij MS onderzoek uitgebreid gezocht naar markers voor toekomstige ziekteactiviteit, hoewel dit opvallend weinig wordt onderzocht in de context van circulerende afweercellen. Onze bevindingen tonen dat de samenstelling van het immuunsysteem een mogelijke marker is voor het risico op nieuwe ziekteactiviteit. Daarnaast geven onze bevindingen aanleiding om te speculeren dat MS ziekteactiviteit wordt veroorzaakt door een gebrek aan compensatiemechanismen in het immuunsysteem. Hier worden de bevindingen in de huidige wetenschappelijke context geplaatst, alsook suggesties voor vervolgonderzoek aangedragen.





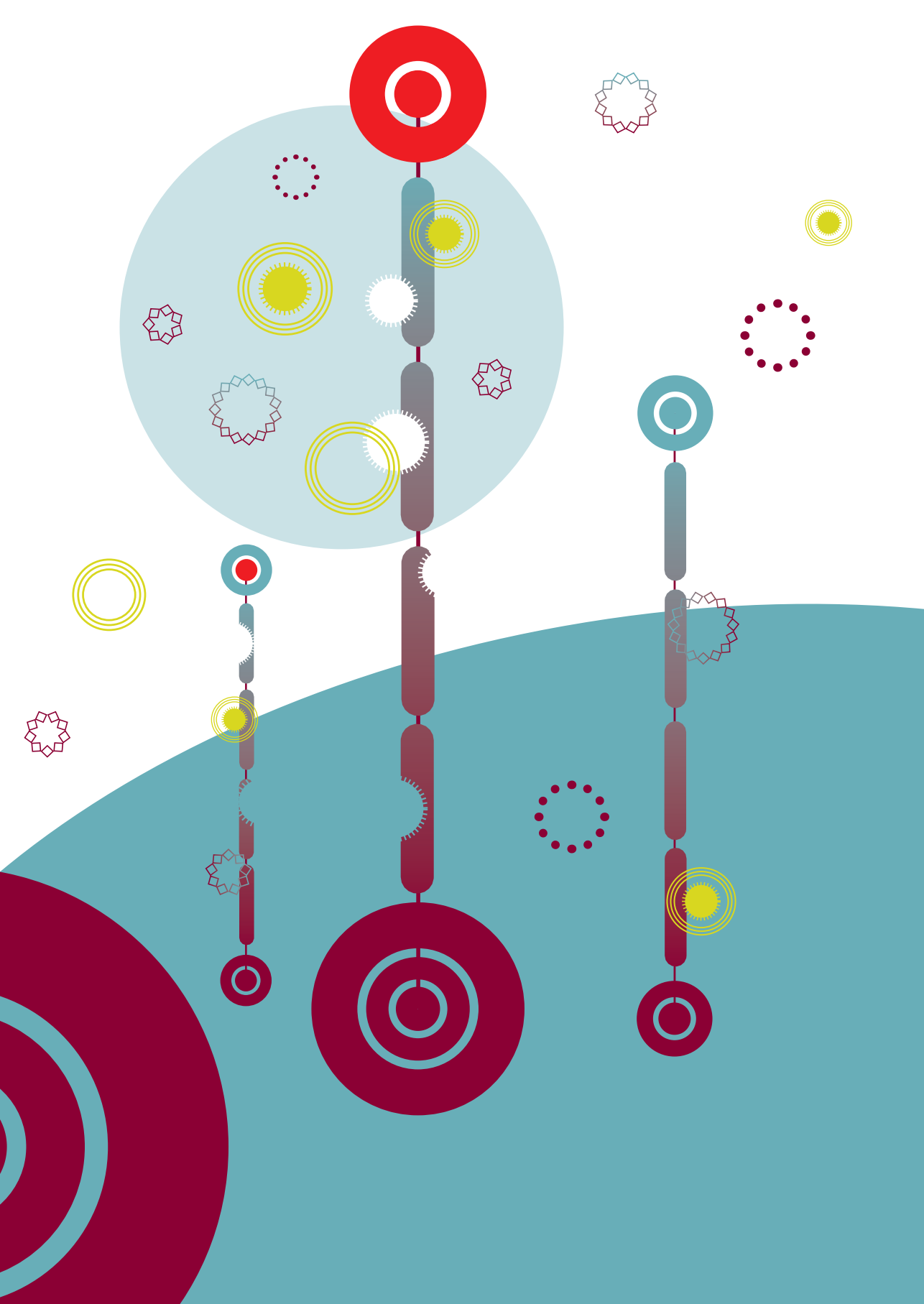
Curriculum Vitae

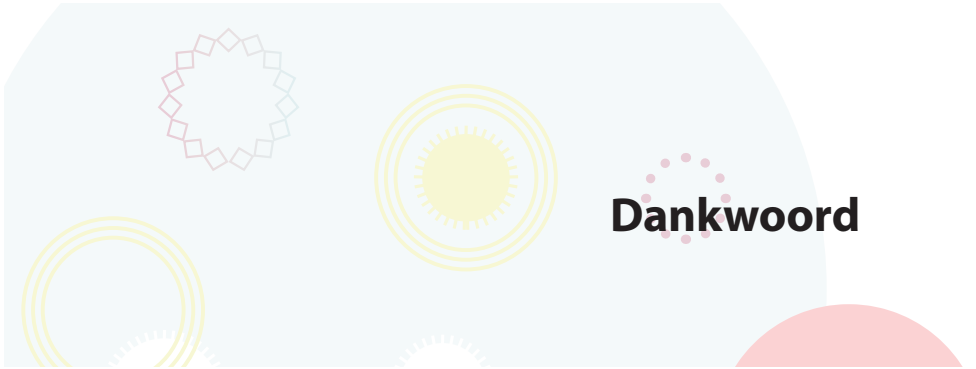
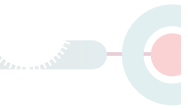
CURRICULUM VITAE



Max Mimpen was born on February 21st 1995 in 's-Hertogenbosch (The Netherlands), and grew up in Drunen (province of Brabant). He graduated from secondary school in 2013 (d'Oultremontcollege, Drunen) and started attending medical school (Maastricht University, The Netherlands) in the same year. During his elective rotations as well as his senior internships, Max developed a great interest and fascination for neurology. Following an initial taste of academic research through an internship on the prognostic value of NK cells in multiple sclerosis, he started his PhD studies at Maastricht University in

2019, working closely with the Maastricht University Medical Center and the Academic MS Center Zuyderland. He investigated the relationship between environmental factors and lymphocytes in multiple sclerosis, as well as the prognostic value of lymphocytes for disease activity in multiple sclerosis. The results of this PhD trajectory, supervised by prof. dr. Raymond Hupperts, dr. Jan Damoiseaux, dr. Joost Smolders, and dr. Oliver Gerlach, are presented in this thesis. He aims to continue working in the field of Neurology, both as a medical doctor and a researcher. Max has started working as a clinical physician in October 2021, currently working at the department of Neurology at Zuyderland Medical Center.





 **Dankwoord**

DANKWOORD

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