Vitamin A, D and E and inflammation in multiple sclerosis

Doctoral Thesis

Egil Rørvik Røsjø

Faculty of Medicine, University of Oslo, Oslo, Norway

Department of Neurology, Akershus University Hospital, Lørenskog, Norway

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Content

1. General summary

Multiple sclerosis (MS) is a chronic immune-mediated disease of the central nervous system (CNS) characterized by detrimental demyelination and inflammation that vary both in space and time. Recent disease prevalence estimates are about 2.1 million worldwide and just above 10000 in Norway, but seem to be rising due to better case ascertainment, longer survival and perhaps increasing disease incidence. MS usually present during early adulthood with a relapsing and remitting (RRMS) disease course followed by a secondary progressive (SPMS) phase, while a minority of cases, often at a somewhat older age, presents with a primary progressive (PPMS) disease course. Currently, the disease etiology remains largely unknown, and the primary aims of the rather recently developed medical therapies are to modify and delay the natural disease course.

Even though the exact disease etiology is unclear, it is evident that a genetic susceptibility exists whereupon environmental factors may act and ultimately lead to a harmful immune response against tissues in the brain and the spinal cord. An essential part of this response is the development of both a general and a CNS restricted inflammation. The inflammatory process involves a multitude of different factors that promote either tissue injury or healing of already afflicted tissue. In addition, some factors have varying properties during different phases of the inflammatory response.

The fat-soluble vitamins A, D and E serve important functions in the human body that are clearly demonstrated by medical illnesses such as night blindness, rickets and peripheral neuropathy, which arise when the levels of these vitamins are critically low. However, their functions are not limited to specific organs, and low levels of vitamin A, D and E have all been linked to an increased vulnerability to infections. This suggests that they are essential for a well-functioning immune system. In concordance with this, experimental research has shown that vitamin A, D and E can modulate immune cell development, composition and function, and the way non-immune cells interact with the immune system during both normal and inflammatory conditions. Furthermore, epidemiological studies in MS have supported a possible anti-inflammatory effect of vitamin A, D and E, as high levels of these vitamins have been associated with reduced disease risk (excluding vitamin E), activity and worsening. However, initial small-scale intervention trials with vitamin D in MS have generally struggled to translate prior observed associations into clinically consistent results, and the clinical effect of vitamin A and E supplementation is largely unknown.

The primary aim of this Thesis was to examine the possible influence of vitamin A, D and E on biological markers of systemic inflammation in patients with RRMS. Furthermore, we wanted to explore if the potential effects were modified by or interacted with interferon (IFN)-β treatment, and if the selected inflammation markers could reflect clinical disease parameters. To address this, we obtained demographic information, serum samples and clinical data from a total of 156 RRMS patients from two independent clinical trials that each lasted two years. In addition, brain magnetic resonance images (MRIs) were included from 88 patients from one of the trials. The serum samples were subjected to measurements of vitamin A, D and E and a panel of 11 biological markers of systemic inflammation.

The results indicate that the serum levels of vitamin A, D and E are associated with distinct markers of systemic inflammation in RRMS patients, and that these relationships are modulated by IFN-β therapy. However, the inflammation markers do not seem to differentiate between patients with or without relapses or disease worsening. Moreover, the previously reported anti-inflammatory serological and radiological effects of IFN-β treatment in RRMS are confirmed

and extended, as it seems that they are independent of the patients' vitamin D status. Furthermore, the anti-inflammatory effect associated with high serum concentrations of vitamin D in an observational setting appears to be small when compared to the anti-inflammatory effect of IFN-β treatment. Lastly, two years of high-dose oral vitamin D_3 supplementation does not seem to affect serum markers of inflammation in RRMS.

In summary, the findings reported in this Thesis suggest that serum levels of the fat-soluble vitamins A, D and E may affect inflammation in RRMS. However, the anti-inflammatory effect associated with increasing vitamin D levels is most likely modest, and may depend on whether the elevation is a consequence of natural variation or nutritional intake. This implies that the advocacy of vitamin D_3 supplementation in RRMS should primarily be based upon the known beneficial effects of vitamin D on bone health. Furthermore, the conflicting findings regarding the role of vitamin D in inflammation in an observational and an interventional setting suggest that caution should be taken when studies with fundamentally different designs are compared, and that future studies should address confounding factors related to the serum level of vitamin D. Finally, the results indicate that the role of vitamin A and E should be further examined in RRMS, however, it must be underlined that there is currently no evidence supporting the need for vitamin A and E supplementation beyond the established official recommendations.

2. Acknowledgments

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3. Abbreviations

$1.25(OH)_2D_3$	1.25-dihydroxyvitamin D_3
25(OH)D	25-hydroxyvitamin D
ALCAM	Activated leukocyte adhesion molecule
APC	Antigen presenting cell
ARR	Annual relapse rate
BBB	Blood-brain barrier
Biomarker	Biological marker
BMI	Body mass index
CCL21	Chemokine (C-C motif) ligand 21
CCR7	Chemokine (C-C motif) receptor 7
CD	Cluster of differentiation
CIS	Clinically isolated syndrome
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTL	Cytotoxic T cell
CXCL16	Chemokine (C-X-C motif) ligand 16
CXCR6	Chemokine (C-X-C motif) receptor 6
DBP	Vitamin D binding protein
DC	Dendritic cell
DMT	Disease modifying therapy
DSS	Disability status scale
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EDSS	Extended disability status scale
FS	Functional system
Gd	Gadolinium
Gd^+	Gadolinium-enhancing
HDL	High density lipoprotein
HLA	Human leukocyte antigen
HPLC	High-performance liquid chromatography
IFN	Interferon
IL	Interleukin
IL-1R	Interleukin 1 receptor
IL-1Ra	Interleukin 1 receptor antagonist
ILC	Innate lymphoid cell
LAP	Latency-associated peptide
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MSFC	Multiple Sclerosis Functional Composite
MSSS	Multiple sclerosis severity scale
Nabs	Naturalizing antibodies
NAWM	Normal appearing white matter
OFAMS	Omega-3 Fatty Acid Treatment in Multiple Sclerosis
OPG	Osteoprotegerin
OPN	Osteopontin
OR	Odds ratio
PML	Progressive multifocal leukoencephalopathy
PPMS	Primary progressive multiple sclerosis
PRR	Pattern recognition receptor
PTX3	Pentraxin 3
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RANKL	Receptor Activator of Nuclear Factor κ B ligand
RBP	Retinol binding protein
RCT	Randomized controlled trial
RRMS	Relapsing-remitting multiple sclerosis

RXR	Retinoid X receptor
sFRP	Secreted Frizzled-related protein
SPMS	Secondary progressive multiple sclerosis
sTNF-R1	Soluble tumor necrosis factor receptor 1
TGF	Transforming growth factor
Th	T helper cell
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
Treg	Regulatory T cell
TTP	Tocopherol transport protein
UNN	University Hospital of North Norway
UVR	Ultraviolet radiation
VDR	Vitamin D receptor
VLA	Very-late antigen
VLDL	Very-low density lipoprotein
VRE	Vitamin D response element
Wnt	Wingless/Int

4. Publications

- Article I

  Increasing serum levels of vitamin A, D and E are associated with alterations of different inflammation markers in patients with multiple sclerosis

  Egil Røsjø, Kjell-Morten Myhr, Kristin I. Løken-Amsrud, Søren J. Bakke, Antonie G. Beiske, Kristian S. Bjerve, Harald Hovdal, Finn Lilleås, Rune Midgard, Tom Pedersen, Jūratė Šaltytė Benth, Øivind Torkildsen, Stig Wergeland, Annika E. Michelsen, Pål Aukrust, Thor Ueland, Trygve Holmøy. *Journal of Neuroimmunology 2014 Jun 15;271 (1-2): 60-5.*

- Article II

  Vitamin D status and effect of interferon-β1a treatment on MRI activity and serum inflammation markers in relapsing-remitting multiple sclerosis

  Egil Røsjø, Kjell-Morten Myhr, Kristin I. Løken-Amsrud, Søren J. Bakke, Antonie G. Beiske, Kristian S. Bjerve, Harald Hovdal, Finn Lilleås, Rune Midgard, Tom Pedersen, Jūratė Šaltytė Benth, Øivind Torkildsen, Stig Wergeland, Annika E. Michelsen, Pål Aukrust, Thor Ueland, Trygve Holmøy. *Journal of Neuroimmunology 2015 Mar 15; 280:21-8.*

- Article III

  Vitamin D supplementation and systemic inflammation in relapsing-remitting multiple sclerosis

  Egil Røsjø, Linn H. Steffensen, Lone Jørgensen, Jonas C. Lindstrøm, Jūratė Šaltytė Benth, Annika E. Michelsen, Pål Aukrust, Thor Ueland, Margitta T. Kampman, Øivind Torkildsen, Trygve Holmøy. *Journal of Neurology. 2015 Dec; 262 (12): 2713-21.*

5. Introduction

5.1. Immunology and inflammation

5.1.1. General aspects of the immune system

The main purpose of the immune system is to protect the body from foreign and internal threats (1). Cells of the immune system are therefore embedded within all tissues of the body and circulate between the tissues, the lymphatics and the blood on constant alert for signs of alien presence and potential harm.

The innate immune system is present at birth and consists of tissue-protective barriers together with the plasma cascade systems, protective enzymes and cellular components that come in to play upon breach of the structural hindrances (1;2). Innate immune cells only discriminate between self and non-self through a limited set of conserved pattern recognition receptors (PRRs) (3), but are highly effective once activated, crucial for the initiation of inflammation, and central in activation of and cooperation with the adaptive immune system (4).

The adaptive immune system develops after birth and is shaped through interaction with the environment (1). Consequently, the adaptive immune cells are able to recognize minute differences between pathogens and changes in the bodies' tissues, while simultaneously adapting their behaviour to the setting present in the tissues.

5.1.2. Innate immune cells

The innate immune cells are the macrophages, dendritic cells (DCs), granulocytes, mast cells and innate lymphoid cells (ILC). The innate immune cells are derived from hematopoietic stem cells in the bone marrow, and constitute together the frontline defence of the immune apparatus (1;2). They can be broadly divided into cells that are mainly stationary or endlessly on patrol. Resident macrophages and DCs survey the integrity of the tissues and may acquire specific characteristics reflecting their location like the microglia in the CNS and the Langerhans cells in the skin (5-7). Upon activation by their PRRs, these cells can mount an immunological and inflammatory reaction through recruitment of circulating immune cells or migration to lymphatic tissues and interaction with adaptive immune cells (2;4). Mast cells are also generally situated within tissues close to small blood vessels and may amplify tissue inflammation by releasing substances that attract circulating immune cells and promote their ability to enter the inflamed tissues (8). The granulocytes, which are normally largely dominated by the neutrophil granulocytes, are in constant circulation and can rapidly migrate into distressed tissues in high numbers and act as the foot soldiers of the immune system (9). The ILCs represent a recent addition to the innate immune cells and differ in both function and main residence. It is believed that they may represent an evolutionary link between innate and adaptive immunity (10), exemplified by natural killer cells that express a limited and similar set of surface receptors in addition to receptors that may interact with tissue specific antigen complexes known as major histocompatibility complexes (MHCs) or human leukocyte antigens (HLAs).

5.1.3. Adaptive immune cells

The B cells and the T cells constitute the adaptive arm of the immune system (1). Like the innate immune cells, B cells and T cells arise from hematopoietic stem cells in the bone marrow, but the T cell progenitor cells leave the bone marrow at an early stage and settle in the thymus (11). The maturation of the B cells and T cells is focused on the development of a functional surface antigen receptor through a process of positive and negative selection (11;12). To survive this selection, they have to express a surface antigen receptor cable of recognizing normal tissue-proteins displayed within MHCs (positive selection), but this recognition can only be of moderate strength to avoid deletion (negative selection). During the development of the surface receptors, B cells and T cells undergo a process of somatic recombination that equips each cell with a receptor with a unique variable region, which generates the diverse specificity of the B cell and T cell compartments. Furthermore, the negative selection ensures the central tolerance development needed to limit generation of detrimental autoreactive B cells and T cells.

Mature, naïve B cells circulate between the vascular system and the secondary lymphoid organs that are comprised of the lymph nodes, spleen, mucosal associated lymphoid tissues and Payer's patches of the intestine (1). The secondary lymphoid organs collect information from the surrounding tissues by drainage of extra vascular fluid trough lymphatic vessels. This fluid may contain foreign and native entities recognized by the naïve B cell, and this recognition will in an inflammatory setting lead to B cell maturation and proliferation after collaboration with T cells present in the secondary lymphoid tissues (13). Through this process, a vast amount of identical daughter cells arises together with further differentiated B cells (plasma cells) that secret soluble B cell antigen receptors (antibodies).

During thymic maturation, the pre-T cells develop a unique T cell surface antigen receptor and co-stimulatory surface molecules called cluster of differentiation (CD) 4 and CD8 (11). However, they lose expression of one of these molecules and become single positive naïve $CD4^+$ or $CD8^+$ T cells before leaving the thymus. The naïve T cells travel between the blood and the secondary lymphoid organs where they encounter antigen-presenting cells (APCs) in the form of migrated tissue resident innate immune cells and macrophages, DCs and B cells that inhabit these organs (1). During this encounter, the APCs present degraded foreign and familiar proteins within their MHCs that may be recognized by the T cell's antigen receptor and, depending on the state of the APC, this recognition leads to T cell activation, anergy (unresponsiveness) or death (13). If activated, $CD4^+$ T cells mature to T "helper" (Th) cells (1), while $CD8^+$ T cells become cytotoxic T cells (CTLs) (14), and both subsets attain the ability to leave the circulation and accesses the surrounding tissues (15). A further description of the different subtypes of Th cells will be presented in section 5.1.7.

5.1.4. The immune system of the central nervous system

The CNS constitutes the brain and the spinal cord and is protected behind the outer confinements of the skull and the spinal column, and the inner confinements of the meninges and the cerebrospinal fluid (CSF). In addition, a further layer of internal protection is provided by the blood-brain barrier (BBB) in the capillaries of the CNS (16). This provides a stable milieu for the nerve cells in the CNS, as only a restricted set of fluid factors and cells are normally cable of crossing the BBB (16;17). Within the CNS, large numbers of innate microglia cells survey the tissues and rapidly react to changes in the CNS homeostasis in order to confront internal or external threats (18). In addition, perivascular and meningeal APC that are situated at the interface between the circulation and the CNS serve as an important connection between the immune system in the periphery and the CNS (19).

The limited access of the peripheral immune cells to the CNS, the lack of secondary lymphoid structures within the CNS and its low expression of MHCs restrict the immunological surveillance of the brain and the spinal cord under normal conditions (19). Furthermore, an intact BBB also prevents the direct release of CNS specific molecules to the systemic circulation (16). Together, these circumstances paved the way for the idea of the immune privileged CNS, which suggested that CNS injury could initiate a chronic immune response against the CNS due to the release of CNS proteins to the peripheral circulation and the curtailed central tolerance induction against CNS antigens (20;21). However, in contrary to this belief, it has been shown that that insults to the CNS can elicit an acute immune response toward CNS antigens without leading to a sustained reaction against the brain or the spinal cord (22;23). A reason for this may be that the peripheral immune cells are not so unfamiliar with CNS antigens as earlier perceived, as the prior assumption that the CNS was devoid of lymphatic vessels has been proven wrong with the recent discoveries of drainage of extracellular CNS fluids to the CSF by the glial lymphatic system and the further drainage of the CSF through lymphatic vessels to the deep cervical lymph nodes (21;24-26).

5.1.5. General aspects of inflammation

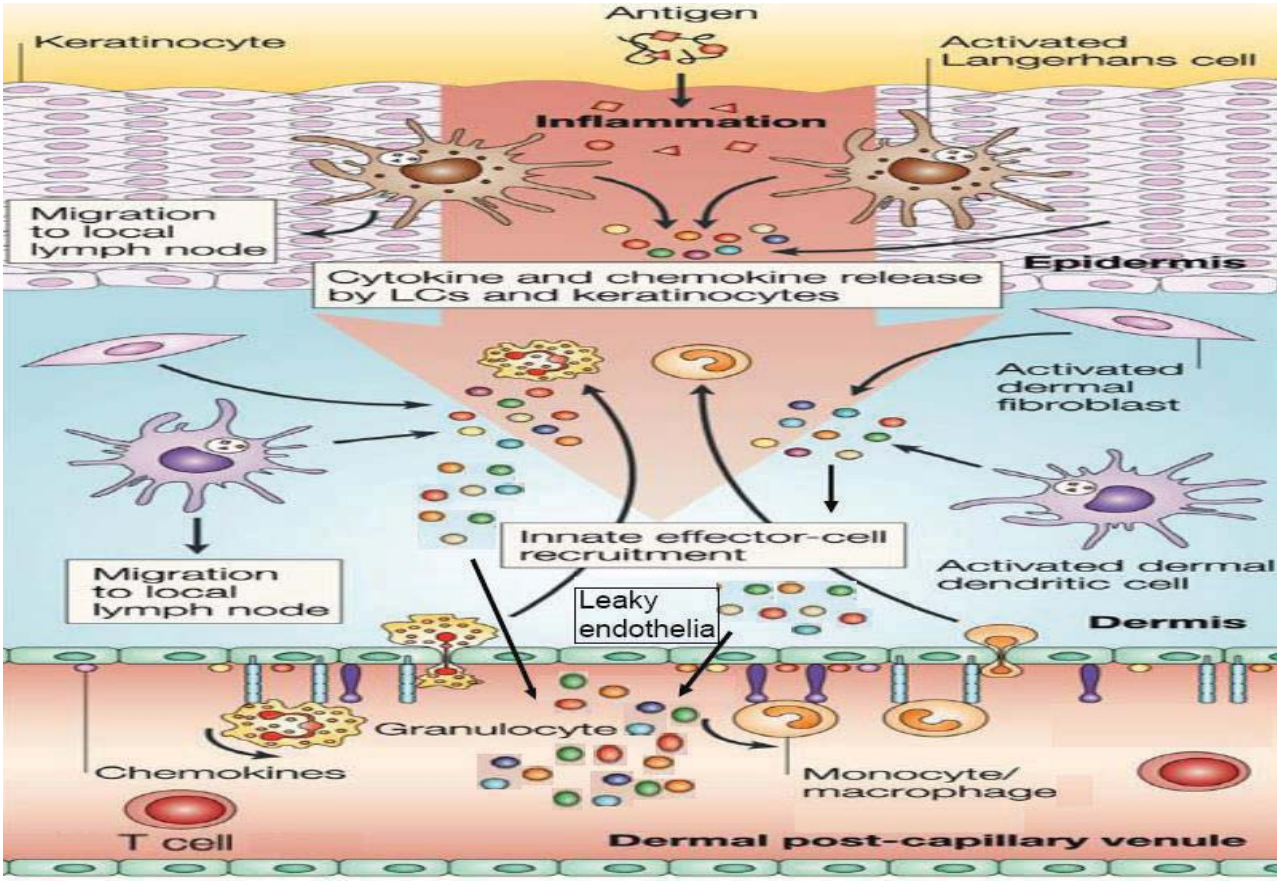
The ability to mount an inflammatory response against harmful stimuli is a prerequisite for survival and essential for restoring homeostasis in damaged tissues (7), and the concept of inflammation was already appreciated in ancient times (27). The word inflammation is derived from the Latin *inflammare* that means to burn and reflects the heat and redness accompanying superficial inflammations like bacterial skin infections. In addition, inflammation leads to swelling, pain and loss (alteration) of function, that together with heat and redness are collectively referred to as the five cardinal signs of inflammation (28).

5.1.6. The inflammatory process

Initially, inflammation may seem like a quite simple concept to grasp. However, when examining inflammation in more detail it becomes clear that this process has a complexity that is hard to comprehend. A comprehensive account of this complexity is therefore beyond the scope of this Thesis, but some details of paramount importance may serve as main principals for the understanding of the overall process.

Disruption of tissue integrity lies at the heart of all acute inflammatory processes (2;7), and initiates a series of actions involving both local and distant defence mechanisms that confine and remove the harmful stimuli before restoring tissue homeostasis (Figure 1). At first, the damaged tissue cells release soluble factors that activate tissue resident immune cells, increase the permeability of the tissues and dilate the surrounding vasculature. This releases attractant factors to the systemic circulation and promotes migration of circulating immune cells toward the afflicted tissues. The process is further amplified by soluble factors secreted by the activated tissue resident immune cells, leading to an accumulation of extravascular fluid and an influx of primarily granulocytes and macrophages from the blood. This series of events was first observed and described by Cohnheim in 1867 (29), and explains the basis for the increased heat, swelling, redness and the altered function of the inflamed tissue. The arrival of recruited cells initiates the phase focused upon removal of

Figure 1. The acute inflammatory process in the skin



Modified from Kupper and Fuhlbrigge 2016, reprinted with permission from Nature Publishing Group

the entity that triggered the inflammation. During this phase, noxious mediators are released from the immune cells, dying cells and, if present, foreign pathogens (2;7). These mediators act in concert with tissue swelling to irritate peripheral nerve endings, which again leads to pain and constriction of smooth muscles in the neighbouring vasculature. Circulating adaptive immune cells may be present in the tissues shortly after the innateimmune cells if the pathogen has been faced before, but they are further reinforced during this phase by activated cells arriving from the secondary lymphoid organs. With the presence of both innate and adaptive cells, the pathogenic stimuli will normally be removed rather quickly and the acute inflammatory process subsides.

With the resolution of the acute inflammation, the immune cells and the plasma cascade systems (the complement, kinin, coagulation and fibrinolysis systems) set the stage for the tissue repair phase (1;7). During this phase, a shift in the balance between pro- and anti-inflammatory factors occurs that favours reestablishment of tissue integrity. The hallmarks of this reparatory phase are the suspension of further immune cell recruitment, egress or death of the innate immune cells present in the tissues (with the exception of the tissue-resident macrophages and DCs), clearance of cellular debris and production of growth factors that promote tissue regeneration, organization and vascularization.

However, in some instances, the perceived threat cannot be overcome, and the acute inflammatory response evolves into a chronic process (7). Chronic inflammation is defined by a prolonged immune cell extravasation and local immune activation that leads to concurrent tissue destruction and repair. Over time, this detrimental development may result in an irreversible disruption of tissue homeostasis and the formation of fibrosis that limits the inflammation to the

already afflicted tissues. Still, a compartmentalization is not always possible or sufficient to avoid an ensuing secondary immunologically independent degeneration of the surrounding tissues.

5.1.7. Soluble inflammatory mediators

The inflammatory response is influenced throughout its course by soluble mediators released from the tissues and the immune cells. These mediators can eliminate harmful entities, modulate tissue structure, attract immune cells, manage recruited cells and induce tissue healing. Together, they constitute an extensive network where each factor can initiate the release of a new set of factors that simultaneously promotes further inflammation and limits release of the preceding factor. In addition, eventually a multitude of substances is released that affects several levels of this cascade and inhibits further inflammation and promotes tissue healing.

Large amounts of soluble inflammatory mediators are released when immune cells are activated. In brief, the innate immune cells release factors that directly affect pathogens, attract adaptive immune cells and promote their activation (2), while CTLs release factors that can kill pathogens and tissue cells and promote further inflammation (14). Once activated, the Th cells mature into different cellular subsets depending on influences from the APCs and the soluble factors in their vicinity. The Th subsets are central in coordinating the ongoing immune response through their production of different soluble signalling molecules called cytokines. The Th cells are primarily subdivided into Th1, Th2, Th17 and regulatory T (Treg) cells that are distinguished by their cytokine secretion with Th1 and Th17 cells being mainly pro-inflammatory and Th2 and Treg cells being mainly anti-inflammatory (1). More specifically, Th1 cells secret interleukin (IL)-2, IFN-γ and tumour necrosis factor (TNF) α and β that activate CTLs and innate immune cells, Th2 cells secret IL-4, IL-5, IL-9, IL-13 and granulocyte macrophage colony-stimulating factor that promote B cell maturation and granulocyte and mast cell activation, Th17 cells secret IL-17, IL-21, IL-22 and IL-26 that promote inflammatory responses against pathogens and osteoclast differentiation (30), and Treg cells secret IL-35 and transforming growth factor (TGF)-β that inhibit pro-inflammatory immune cells and promote survival of existing and production of new Treg cells (31). In addition, Treg cells release IL-10 that inhibits maturation of DCs and subsequent induction of Th1 and Th17 cells, and is crucial for maintenance of peripheral tolerance. However, even though the subdivision of the Th cells may help in the understanding of different immunological process, it is also crucial to acknowledge that the Th cells may adapt to their environment by changing between subdivisions (32)

5.2. Multiple sclerosis

5.2.1. History, epidemiology and disease impact

MS is a chronic inflammatory disease afflicting the CNS of individuals with a perceived genetic disposition (33). Historical descriptions of possible disease cases date back to the end of the 12th century (34), however, the first recognized medical description dates from 1840 (35). The disease was first depicted pathologically by Carswell in 1838 and clinicopathologically by Cruveilhier in 1842 (36;37), but the first comprehensive account of the disease and naming of the illness is attributed to Charcot in 1868 (38).

The estimated worldwide prevalence of MS was 2.3 million cases in 2013 (39). However, the prevalence differs considerably between regions from >100 cases/100000 inhabitants in North America and parts of Europe to 0-5 cases/100000 inhabitants in parts of Asia. Norway has one of the highest disease prevalence estimates in the world with ~205 cases/100000 inhabitants (i.e. more than 10000 cases in total) (40;41). Furthermore, the prevalence estiamtes are increasing in some regions possibly due to improved case ascertainment (42), prolonged survival (43), and changes in environmental risk factors (see section 5.3.7.). Interestingly, the prevalence also differs with age and between genders, as the disease rarely debuts before early adulthood and is more frequent among females (42).

As MS is a lifelong disease with a peak incidence and prevalence around 30 and 50 years of age, respectively, it is clear that the illness has ample implications for both the patients and the society (42). Studies focusing on disease implications for the patients have reported reduced overall health-related quality of life compared with the general population from an early time point (44), considerable negative social and financial consequences (45), frequent psychiatric commodity (46), and an especially increased relative risk of death before the age of 60 that contributes to a reduced life expectancy compared to the general population (47). Additionally, the disease may lead to a significant burden on the patients' caregivers (45), and substantial healthcare expenditures (48).

5.2.2. Clinical description and natural course

The clinical description of MS varies between patients with respect to initiating symptoms, disease course, rate of development and response to therapy. However, some onset symptoms are more frequent than others (i.e. optic neuritis, sensory disturbances, motor impairments and brain-stem dysfunction) (49), and a set of disease phenotypes may be used to classify cases with respect to clinical course and treatment availability (50). These disease phenotypes are the clinically isolated syndrome (CIS), RRMS, PPMS and SPMS. The CIS precedes MS development, as it is defined as the onset of clinical symptoms related to inflammatory CNS demyelization without fulfilling the diagnostic criteria for MS (presented in section 5.3.4). About 85% of MS patients are initially diagnosed with RRMS (49), which is characterized by recurrent episodes of disease activity followed by partial or complete recovery and a step-wise increase in disability (50). In contrast, progressive MS exhibits a gradual accumulation of disability either from onset (PPMS), or following an initial RRMS phase (SPMS).

Natural history studies have shown that the majority of RRMS patients develops SPMS within 20 years from their initial symptoms (51). This is also reflected in declining annual relapse rates (ARRs) with increasing disease duration (52). Moreover, as the time to conversion is shorter and the relapse frequency decreases faster among patients with onset after compared to before 40 years of age, it has been suggested that the conversion to progressive disease is age dependent (51;52). This is further supported by the findings that about ¾ of the patients develop SPMS within 50 years of age, and that PPMS cases are usually about 10 years older than RRMS cases and have a higher degree of disability at diagnosis (51).

5.2.3. Pathological description

The definite initiating cause of MS remains unknown, but several lines of evidence point toward an early involvement of the immune system (53). However, two main opposing hypotheses exist regarding the timing of the immune response

during disease development. The first hypothesis states that the immune activation is initiated in the periphery and manifests subsequently in the CNS (primary peripheral debut), while the other states that the immune response represents a secondary response to a detrimental process arising within the CNS (primary central debut). The notion of a primary peripheral debut is supported by findings in an experimental animal model of MS called experimental autoimmune encephalomyelitis (EAE). EAE is driven by an adaptive immune response against the CNS that is usually triggered by peripheral immunization and re-exposure to structural components of the CNS, or when T cells from challenged animals are transferred to the peripheral circulation of naïve recipients (54;55). This disease model has been central for the development of several of the disease modifying therapies (DMTs) in MS, however, with time it is has also become evident that EAE is not an animal equivalent of MS (56;57). The primary central debut theory on the other hand is supported by histological findings of oligodendrocyte death without involvement of resident or peripheral immune cells in newly forming CNS lesions (58), and the presences of an influx of immune cells during primary neurodegenerative diseases like adrenoleukodystrophies (59). Nevertheless, this view is weakened by the lack of common genetic variations among MS patients in genes known to be involved in neurodegenerative diseases (60), no unequivocal histopathological findings of infectious agents in the CNS of MS cases (61), and no findings of other definite environmental causes that can elicit the CNS pathology found in MS (62).

The gross histopathological aspects of MS were depicted over 150 years ago and consist of widespread (multiple) demyelinating lesions and axonal loss, infiltration of peripheral and local immune cells, and glial scaring (sclerosis) (36;63). The disease affects both the grey and the white matter of the CNS, but the illness was for a long time perceived to be dominated by white matter pathology due to the clear visibility of white matter lesions on histopathological examination and MRI scans of the CNS (63;64). The white matter lesions have a predilection towards the periventricular, juxtacortical or infratentorial areas of the brain in addition to the optic nerves and the spinal cord (65), and the active lesions are characterized mainly by an oval shape with adjacent perivascular inflammation, local BBB breakdown, accumulation of activated T cells, B cells, macrophages and microglia, distressed astrocytes, dying oligodendrocytes, loss of neural axons and gliosis (63). However, this general description may not portray the heterogeneity of active white matter lesions between patients (66), and it has been proposed that four distinct patterns of demyelination with varying degree of immune cell and antibody involvement, oligodendrocyte loss and remyelination may persist within patients over time (67). With the development of new staining methods and imaging techniques, it has become clear that grey matter demyelination is less restricted to specific areas and is at least as extensive as the white matter demyelination (64). Furthermore, it seems partly independent of white matter disease, is more evident during progressive phases and has a greater association with long-term disability (68). The general description of the grey matter lesion also differs from the white matter lesion, as there is seldom immune cell infiltration and antibody involvement, the BBB seems intact and the remyelination is extensive in spite of a substantial oligodendrocyte loss (64). However, the demyelization of the grey matter is also heterogeneous, and seems to vary with localization. Still, it is unknown if the process changes with time, and the cause remains obscure despite speculations regarding excessive local production of noxious substances by microglia or diffusion of detrimental factors from adjacent inflamed meningeal tissues (69;70).

5.2.4. Diagnostic criteria

The diagnostic criteria for MS have undergone radical changes since the conception of the Charcot criteria of nystagmus, intention tremor and scanning speech in 1868 (38). In brief, several major revisions have been undertaken during the last

60 years that have incorporated increasing knowledge regarding disease symptoms and course, and paraclinical findings related to the disease (71-76). However, to maintain brevity, only the MacDonald criteria will be further commented upon.

The 2001 McDonald criteria were the first criteria to include MRI evaluation in the diagnostic work-up (72). Although other paraclinical evaluations had been included earlier (75), the preceding criteria had all depended heavily on clinical factors that can be summarized as the presence of ≥2 objective clinical findings involving separate parts of the CNS (i.e. dissemination in space), ≥2 episodes or step-wise disease worsening (i.e. dissemination in time), and the exclusion of other similar disease processes. The clinical criteria were carried forward in the MacDonald criteria, but due to the inclusion of MRI evidence a MS diagnosis could also be given to a CIS patient if a follow-up MRI after 3 months demonstrated a new gadolinium (Gd) contrast enhancing lesion in a new location. The introduction of MRI findings did, however, overshadow the importance of the earlier established clinical criteria, and in the 2005 revision emphasis was again made upon that the diagnosis could only be made if clinical evidence was present (73). Furthermore, as a consequence of the increasing knowledge regarding pathological MRI findings, a third revision was undertaken already in 2010 that made it possible to diagnose MS with only a total of two separate MRI lesions if these showed simultaneous presence of Gd enhancement (Gd^+) and non-enhancement, or when new lesions arose at any time in follow-up scans of CIS patients (74).

5.2.5. Disease evaluation

A central issue in medicine besides accurate diagnosis and description of the disease, is the ability to objectively and reproducibly describe the consequences of the illness. With this in mind, Kurtzke developed in 1955 a "scale for evaluating disability in MS" with 11 steps ranging from normal neurologic examination (0) to death due to MS (10) (77). The scale became known as the Disability Status Scale (DSS), and was later included in the Kurtzke's Expanded Disability Status Scale (EDSS) (78). The EDDS consists of the DSS and an examination of a specific set of neurological functions (Functional Systems, FS) together with an evaluation of ambulation. The FS are made up of 8 functional groups (pyramidal, cerebellar, brain stem, sensory, bowl and bladder, visual, cerebral or mental, and other) that are graded from 0 (normal) to 5 or 6 (maximal impairment) except the category of "other" that is scored as 0 (none) or 1 (present). The highest obtained FS grade will usually determine the DSS score between steps 0 and 3, as the affliction of ambulation takes precedence from DSS step 4 and beyond. The EDSS has been a principal outcome measure in most clinical trials in MS, but concerns have been raised regarding the scales' inter- and intra-rater scoring agreement (79), heavy influence of ambulatory ability on the higher steps (80), rather crude evaluation of cerebral and mental function together with the limited importance of other symptoms, and the non-linear change of the DSS with increasing FS grades (79). Furthermore, the ability of the EDSS to determine disease worsening is dependent on longitudinal examinations. To overcome these shortcomings, several additional clinical evaluation tools like the Multiple Sclerosis Severity Score (MSSS), Multiple Sclerosis Functional Composite measure (MSFC) and the Fatigue Severity Scale are commonly utilized in clinical MS trials (81-83). However, further details regarding these tools are excluded from this Thesis, as results from these evaluations were either of minor importance or not included in the conducted analyses.

In addition to the clinical evaluation, paraclinical investigations are a central part of both the diagnosis and follow-up of MS patients. As indicated in the previous section, MRI is the corner stone in this paraclinical evaluation.

MRI utilizes radio-frequency radiation emitted after the exposure of nuclei of hydrogen atoms (i.e. protons) to a magnetic field (84). The protons have both an inherent spin and magnetic field that are altered upon placement in a uniform magnetic field, before they revert back to their original state when this field is removed. MRI measures the resonant absorption and re-emission of radio waves by the protons during their return to their normal state. This process generates two sets of images reflecting the time it takes the protons to become out of line with each other (the T2 relaxation time), and the time it takes them to return to their original position (the T1 relaxation time). Due to differences in tissue composition (i.e. densities of mobile protons), the T1 and T2 relaxation times will also differ between tissues. Furthermore, a detailed spatial resolution may be obtained by adding an extra magnetic field with a varying gradient that together with the background magnetic field will generate signals from each proton that reflects their individual spatial localization. Dependent on the three principal MRI parameters proton density and T1 and T2 relaxation times, it is possible to construct images that are proton density-, T1- or T2-weighted. As MRI scans of the CNS clearly delineate grey mater from white mater due their differing water- and fat-content (i.e. myelinisation), it was not surprising that MS was one of the earliest CNS diseases to be studied by MRI (85). In MS, T1-weigheted images will show lesions as iso- or hypointense due to intracellular oedema, gliosis, extracelluar widening and axonal loss, while the lesions in T2-weighted images will appear as hyperintese due to demyelination and increased extracelluar water (86). A further increased sensitivity for detection of lesions on T1-weighetd images can be achieved by the employment of contrasts agents like Gd (87), which will accumulate in areas of perivascular immune cell infiltration and BBB breakdown. With the introduction of MRI, it became possible to demonstrate pathological abnormalities on a scale similar to what was found on necropsy (85), and it was quickly apparent that new MRI lesions were 5-10 times more frequently detected than new clinical attacks (87). The further value of MRI in MS has been demonstrated by the clear correlations between MRI lesions and specific established functional and cognitive deficits, the appearance of coinciding MRI lesions and clinical relapses, and the reduction in both clinical and MRI activity after use of DMTs (88;89). However, as conventional MRI sequences primarily detect white matter abnormalities, the grey matter affliction has been underappreciated (64). In addition, although white matter pathology is related to clinical relapses, it is only weakly associated with long-term disability (68;90). A phenomenon that was initially labelled as the “clinico-radiological paradox” (86). Still, recent advances in grey matter MRI have suggested that imaging may also have the ability to predict long-term disability (68).

5.2.6. Disease modifying therapies

The treatment of MS consisted of symptomatic therapies with doubtful long-term effects like the corticosteroids in the era preceding the introduction of the MS specific DMTs in the 1990s (91). This changed with the pivotal phase III randomized placebo-controlled trials (RCTs) of IFN β-1a, IFN β-1b and glatiramer acetate for RRMS patients between 1993-1998 (92-95). The initial DMTs had all similar effects on reduction of short-term ARR and CNS MRI activity, however, it was evident that the effects were quite limited (96). Moreover, their ability to alter the disease course beyond the duration of the original RCTs (i.e. after 2 years) still seems uncertain (43;97;98). A further step in the evolution of the DMTs came with the phase III RCT of the monoclonal antibody natalizumab in 2004 (99), as a substantial reduction in both MRI and clinical disease activity was noted in the intervention arm during the two-year trial period. Although seen as major break-through in MS patient care, it soon became clear that natalizumab severely affected the immune surveillance of the CNS, and the marketing approval of the treatment was voluntary suspended for one year following the death of one patient from progressive mutifocal leukoencephalopathy (PML) (100). This illustrated that the increasing

efficacy was closely related to the treatment's ability to interfere with normal immune function, which again has been used to categorize DMTs into first-line and second-line treatments with regards to their risk of serious side effects. During the last decade several new first-line (teriflunomide and dimethyl fumarate) and second-line therapies (fingolimod and alemtuzumab) have gained approval (101-104), extending the DMT armamentarium to include both injectable and *per oral* formulations.

IFN-β (IFN β-1a or β-1b) was the predominant DMT utilized by a large majority of the patients included in this Thesis. The IFNs are naturally occurring cytokines produced especially during exposure to external pathogens (105). Their primarily perceived functions were to block uptake of virus to neighbouring cells and to promote production of intracellular molecules that interfered with viral replication in infected cells. However, it was later found that this was only one of their many wide-ranging immunological and non-immunological effects. IFN-β is a class I IFN mainly produced by tissue cells and immune cells with an overall anti-inflammatory effect (105;106). This complex effect has been partly demonstrated in MS patients, where subcutaneous injection of recombinant IFN-β has been associated with a simultaneous increase of anti-inflammatory and decrease of pro-inflammatory soluble serum factors that are most likely related to a reduction of co-stimulatory molecule expression on T cells and T cell differentiation (107;108). In addition, it has been found that IFN-β therapy may confine lymphocytes to the periphery by increasing their expression of specific chemokine receptors and reducing their expression of endothelial adhesion molecules like very-late antigen (VLA) 4 (109;110). Moreover, IFN-β may also promote BBB integrity and CNS repair, as T cells from MS patients on IFN-β treatment have been found to increase production of neuronal growth factors from human brain microvascular endothelial cells *in vitro* (111).

Due to the limited amount of patients on glatiramer acetate or natalizumab therapy in only one of the studies included in this Thesis, only a brief summary will be provided regarding their background and methods of action. Glatiramer acetate is a random synthetic polymer consisting of four amino acids commonly found in one of the main myelin proteins (112). It triggers a beneficial immune response characterized by increasing serum levels of anti-inflammatory mediators due to a presumed alteration of APC function and promotion of among other Treg cells (113;114). Furthermore, it may also promote CNS tissue repair, as T cells from treated patients have been reported to produce beneficial neurotropic factors (115). In contrast to this purely chemical compound, natalizumab is a humanized recombinant monoclonal antibody produced in murine cell culture directed towards VLA-4 (116). Its major function is to block the binding of VLA-4 to its ligands that are among other expressed on inflamed endothelia cells. The intravenous injection of natalizumab has been shown to be highly effective in decreasing lymphocyte recruitment across the BBB in addition to reducing the amount of Th cells in the blood over time (117;118).

5.2.7. Risk factors

MS development is believed to be due to an intricate interplay between nature (i.e. genes), nurture (i.e. environment) and stochastic factors. The genetic and environmental contributions to the overall disease risk have been debated for several decades (119;120). However, based upon the concordance rate of MS in monozygotic twins, environmental factors and genetic factors seem to have more or less similar importance (121).

The field of MS genetics has been rapidly expanding since the introduction of the genome wide-association studies in 2007 (122). Nevertheless, the most important genetic risk factor for MS was discovered three decades earlier

when a consistent HLA-association was found among MS patients with a North European background (123). This HLA-association has later been mapped to the HLA allele DRB1*15:01 and seems convey a near 3-fold increased likelihood of disease development compared to not having this haplotype (124). However, the HLA-association cannot solely explain the increased familial risk of MS, which was noted already 120 years ago (125). When compared to a general population prevalence of 100 cases/100000 inhabitants, the lifetime risk of MS is increased in a monozygotic twin of an affected individual by 300-fold and a sibling or offspring by 20-40-fold (119). Although the HLA-association in itself points toward an involvement of the immune system in MS, further support for this notion has been provided by the predominance of immunological genes among the over 100 additional MS-associated gene variants identified by the recent genome wide-association studies (60;122;124). However, each individual gene variant only seems to increase the risk of MS by about 10-30% (60).

An enormous amount of data has been accumulated on the potential importance of different environmental entities on MS risk (62). However, within this myriad of widely different potential risk factors, some main areas of research have arisen over time. One of these is the investigation into the importance of Epstein-Barr virus (EBV) infection in MS. Infection with EBV usually leads to subclinical primary illness during childhood with a subsequent establishment of viral latency within the B cell compartment (126). Although the prevalence of EBV infection is high in the general population (127), the EBV seropositivity (i.e. presence of serum antibodies against the virus) rate has been found to be even higher among MS patients (128). This has led to the hypothesis that prior exposure to EBV may be a prerequisite for MS development (129). Further support for this has been provided by the findings that both the presence and the level of antibodies against EBV in serum are exceedingly associated with subsequent emergence of MS (130-132). In addition, the individual's genetic make-up and age at primary infection seem to modulate this association, as the presences of HLA-DRB1*15:01 and infection during adolescence or early adulthood (i.e. mononucleosis) may increase the disease risk to a large extent (133). However, a lower seroposotivity rate has been found among paediatric than adult MS cases (134), and a heightened level of serum antibodies against EBV was not found to be predictive of later development of MS among over 400 CIS patients aged 23-37 years (135).

Studies concerning the impact of the patient's life-style on disease risk has led to several important findings in a variety of medical research fields, highlighted by the finding of the causal association between smoking and lung cancer in the 1950s (136). However, the effect of smoking on MS risk was largely uncharted until the last decades (137). Nevertheless, it is now evident that smokers have an increased risk of MS of about 50-60% compared to never smokers (138). Moreover, the confidence in this relationship has been strengthen by the finding of an association between the cotinine (a nicotine metabolite) level in prospective serum samples and the hazard of developing MS (139). This study reported that the increased disease risk was again in the scale of 50%, but even more strikingly, a close to 2-fold elevation in risk was found among young adults (below 27 years). Interestingly, another recent study also suggests that early nicotine exposure might explain this association, as increased serum cotinine levels in CIS patients older than 22 years were not associated with later conversion to MS (135).

Another environmental risk factor for MS found to be associated with adolescence and early adulthood is obesity. During the last years, several studies have examined the relationship between overweight in younger age and subsequent emergence of MS (140-143), partly based on the notion of an inflammatory link between the two conditions (144). These studies have reported that a body mass index (BMI) value over compared to below 30 before 21 years of age is associated with up to a doubling in disease risk (140;142;143). Furthermore, this association seems to be substantially strengthened

among those expressing HLA-DRB1*15:01 or having prior mononucleosis (141;145). However, as obesity affects a range of biological parameters, it remains to be shown if it has a direct effect on MS risk.

An additional environmental entity that has received much attention in epidemiological studies of MS risk is latitude. As mentioned in section 5.2.1., a clear disparity exists between the disease prevalence in different parts of the world (39). Although it is evident that this may be related to ethnicity, it was noted more than 50 years ago that the abundance of MS seemed to rise together with the latitude (i.e. increasing distance from equator) (146). When considering the genetic background, one of the most interesting findings in this research field is the presence of a latitudinal gradient in MS risk in Australia (147;148). As Australians are predominantly of Northern European origin, it is clear that environmental risk factors related to latitude must be considered when explaining why a ~10° increase in latitude is associated with a close to 2.5 times increased disease prevalence (147). Additional support for an effect of latitude on MS risk has come from studies focusing on the effect of ultraviolet radiation (UVR) on the hazard of MS. Notwithstanding the fact that several diverse factors are related to latitude, the importance of UVR seems paramount due to its close relation to the distance from equator (149). As UVR may have a systemic immuno-suppressive effect (150-152), it is interesting to note that the MS prevalence in different regions of the world has been found to be highly related to the amount of UVR the regions receive (153). Moreover, several retrospective epidemiological studies have found that self-reported low UVR exposure during childhood and adolescents is associated with later development of MS (154-156). In addition, actinic skin-damage, which is closely related to long-term UVR-exposure, has also been found to be reduced among MS patients (156). Nevertheless, the relationship between latitude and MS risk seems to be either declining or non-existing in several regions (42). Furthermore, UVR exposure also leads to production of vitamin D (157), which has immunological properties of its own that will be elaborated upon in the next section.

5.3. Vitamin A, D and E

5.3.1. Vitamin D

The vitamins are divided into fat-soluble (A, D, E and K) and water-soluble (B and C) vitamins, reflecting their chemical structure. Together they constitute a collection of organic chemical compounds dependent on external supply due to their lack of or insufficient synthetization in the human body. Vitamin D is unique among the vitamins, as it can obtained by either oral intake of food-sources like fatty fish or produced from 7-dehydrocholesterol in the skin after UVR exposure and thermal isomerization (157;158). Two main vitamin D precursors exist and are known as vitamin D_2 (ergocalciferol) and D_3 (cholecalciferol) (158). Following intake (vitamin D_2 and D_3) or production (vitamin D_3), both forms are bound to a specific vitamin D-binding protein (DBP) and transported with the blood to the liver where they undergo conversation by the 25-hydoxylase enzyme to 25-hydroxyvitamin D_2 ($25(OH)D_2$) and 25-hydroxyvitamin D_3 ($25(OH)D_3$), respectively. $25(OH)D_2$ and $25(OH)D_3$, collectively denoted 25(OH)D, represent the storage forms of vitamin D, with $25(OH)D_3$ being the dominant of the two due to the usually higher amount of vitamin D_3 available and its superior biological potency (159). $25(OH)D_3$ is again bound to DBP and transported with the circulation to tissues throughout the body where it may undergo further conversions. However, the first step of this process takes mainly place in the kidneys with the generation of active vitamin D, 1.25-dihydoxyvitamin D_3 (calcitriol, $1.25(OH)_2D_3$) through the action of the 1-α-hydroxylase enzyme (158). Active vitamin D acts either directly upon the cells responsible for its production in an autocrine manner, neighbouring cells in a paracrine fashion, or distance tissues in a hormonal mode after transport by

DBP. The amount of circulating active vitamin D is tightly regulated by the kidneys and further production is down-regulated in response to elevated serum concentrations of active vitamin D, parathyroid hormone, calcium and phosphorus. Active vitamin D exerts its effect through intracellular binding to the vitamin D receptor (VDR), which preferentially dimerizes with the retinoid X receptor (RXR) and they bind together to vitamin D response elements (VREs) present in large collection of genes. Trough further metabolism, $1.25(OH)_2D_3$ is converted by the 24-hydroxylase enzyme into an inactive vitamin D metabolite, which again undergoes several additional hydroxyaltions before being ultimately secreted in urine in the form of calcitroic acid.

Current recommendations regarding vitamin D sufficiency advocate a serum level of 25(OH)D ≥50 nmol/L (160). However, several studies have indicated that levels above 75 nmol/L may be more beneficial (161). Still, no clear evidence exists regarding what is the optimal level for good health, and the concentrations may further be affected by among other age, BMI and genetic make-up (162-164).

The main task for vitamin D is the maintenance of the calcium homeostasis through its action on intestinal cells and osteoblasts in the skeleton (158). A normal serum level of calcium is crucial for the proper function of cells in bone, muscles, blood and the nervous system, and the importance of vitamin D in humans became first apparent following the recognition of rickets in Europe from the mid-17th century (165). Even though vitamin D was not discovered before 1922 (166), both oral intake of cod-liver oil (which is rich in vitamin A, D and E) and exposure to sunlight had been suggested as treatments for this condition since the early 19th century (167;168). However, the finding of VDRs in immune cells in the early 1980s strongly indicated that vitamin D also had an effect on the immune system (169). This effect became evident with the later *in vitro* findings of inhibition of APC maturation and lymphocyte proliferation (170;171), promotion of Th2 and Treg cell differentiation (172;173), and reduction of antibody and pro-inflammatory cytokine production after exposure to active vitamin D (170;171). Furthermore, the anti-inflammatory effect of active vitamin D has been shown *in vivo* in the setting of the EAE model, where supplementation prior to disease induction reduced its development and application after disease onset ameliorated its progress (174).

It was quite early hypothesized that MS could be related to low exposure to sunlight and that this could be further attributed to insufficient vitamin D levels (146;175). The latter has later been supported by a multitude of publications (176;177). However, in order to maintain brevity, only some landmark studies will be referred to. One of these was the finding of significantly lower levels of vitamin D in biobank serum samples from patients that later developed MS than among healthy controls (178). More specifically, an increase in 25(OH)D levels by 50 nmol/L was associated with a close to 40% reduction in MS risk, and only 1 of 39 cases with an age below 20 years at sampling had a 25(OH)D level ≥100 nmol/L. Similar results were later reported in a Swedish patient cohort (179), where it was found that 25(OH)D levels over compared to below 75 nmol/L were associated with close to 60% reduction in risk of disease. A further indication for an important role for vitamin D in reducing MS risk came from a retrospective case-control study of close to 200000 American nurses, where the intake of vitamin D supplementation was associated with a reduced hazard for MS by close to 40% compared to no supplementation (180). However, the vitamin D supplementation in this study was only extrapolated from the use of multi-vitamin supplements and unspecified vitamin D supplements, and no relationship was found between prior total dietary intake of vitamin D and MS risk.

Moving on from the possible effect of vitamin D on disease risk, vitamin D has also been reported to affect established MS, as the vitamin D status has been associated with relapse rates, disease worsening and MRI activity (181-186). In more detail, observational studies of seasonally adjusted serum 25(OH)D levels among RRMS patients have

noted a near 2 fold reduction in clinical activity between patients with concentrations <50 nmol/L and >100 nmol/L 25(OH)D, and a 10-15% reduction in the relapse risk with each 10 nmol/L increase (181;184;185). However, results from a study utilizing estimated monthly 25(OH)D levels found that only patients on IFN-β treatment had an apparent effect of having ≥50 nmol/L compared to <50 nmol/L on the hazard of clinical attack (187). Still, each 10 nmol/L increase in the 25(OH)D level has been found to be associated with a close to 10% reduction in the amount of new MRI lesions among patients both with and without IFN-β treatment (181-183). Lastly, patients with a 25(OH)D value of ≥50 nmol/L have been reported to accumulate lower disability after 4 years compared with those with lower levels (181). However, neither clinical activity nor disease worsening were reported to be associated with vitamin D status in a very recent observational study of nearly 1500 RRMS patients on IFN-β therapy (182). Nevertheless, results from earlier open-label studies of per oral vitamin D_3 in RRMS patients on immunomodulatory treatment had indicated that increased vitamin D levels reduced both relapses rates and the amount of Gd^+ lesions (188-190). Furthermore, vitamin D supplementation of MS patients had prior to the initiation of this doctoral work been found to be associated with anti-inflammatory effects such as dampened T cell proliferation (188;191;192), reduced Th1:Th2 ratio (193), and increased serum levels of transforming growth factor β (TGF-β) and IL-10 (192-194). However, the majority of the examined parameters in these studies were unaltered. In addition, some methodological concerns could be raised regarding these studies, as significance levels were kept at 5% in all but one of the studies despite the simultaneous assessment of 4-15 parameters (195), and the use of potentially confounding immunomodulatory treatments was either not controlled for or not reported in three of these studies (188;191;194). Moreover, despite the encouraging results from the earlier open-label studies, several small-scale double-blinded RCTs of vitamin D intervention among RRMS patients on DMTs (192;196-198), including one of the studies elaborated upon in this Thesis (199), failed to show any clinical effects compared to placebo (200;201). In addition, only one of three trials that included evaluation of MRI disease activity obtained results that collaborated the prior finding of fewer Gd^+ lesions during vitamin D supplementation (192;197;198). Still, the effect of vitamin D_3 supplementation on clinical, radiological and immunological endpoints in demyelinating conditions remains uncertain until results from the ongoing larger trials are presented (202-205) (Table 1).

Table 1. Ongoing double-blinded RCTs of vitamin D_3 supplementation in MS or CIS patients, ordered by initiation date

Trial registration	Trial name	Start/end (month, year)	Trial intervention	Patient type	Main outcome
NCT01198132	CHOLINE	01.2010/ 03.2015*	100000 IU or placebo every other week for 96 weeks	129 RRMS[1]	Reduction of relapse rate during the trial period
NCT01024777	NA	03.2010/ 01.2013*	10000 IU or 400 IU daily for 6 months	40 MS[2]	Effect on serum immune markers during the trial period
NCT01285401	SOLAR	03.2011/ 04.2015‡	14000 IU or placebo daily for 48 weeks	232 RRMS[1]	Proportion without disease activity at trial conclusion
NCT01440062	EVIDIMS	12.2011/ 03.2015†	20400 IU or 400 IU every other day for 18 months	80 RRMS or CIS[1]	Cumulative number of new T2 lesions at trial conclusion
NCT01490502	NA	03.2012/ 12.2014†	5000 IU or 600 IU daily for 24 months	172 RRMS[3]	Proportion with relapse during the trial period
NCT01728922	CISAVID	11.2012/ 05.2015*	10000 IU, 5000 IU or placebo daily for 24 weeks	45 CIS[2]	Effect on serum immune markers during the trial period
NCT01753375	NA	01.2013/ 07.2014§	50000 IU or placebo weekly for 12 months	200 RRMS[4]	Reduction in relapse rate during the trial period
2012-004602-97	VITADEM	01.2013/ ?	4000 IU or placebo daily for 12 months	100 RRMS[5]	Proportion with relapse during the trial period
NCT01768039	NA	03.2013/ 08.2014§	50000 IU or placebo weekly for 12 months	240 RRMS[1]	Change in EDSS after 6 months
NCT01817166	D-Lay-MS	07.2013/ 07.2019	100000 IU or placebo every other week for 24 months	316 CIS[2]	Conversion to MS during the trial period
ACTRN126120 01160820	NA	07.2013/ ?	10000 IU, 5000 IU, 1000 IU or placebo daily for 48 weeks	240 CIS[2]	Conversion to MS during the trial period

[1] On IFN-β; [2] On no DMT; [3] On glatiramer acetate; [4] Not specified DMT; [5] On IFN-β or glatiramer acetate

* Delayed, but completed without reported results; ‡ Delayed, but completed with alterations and without reported results; † Delayed, but ongoing; § Unknown current status

NA; Not available

5.3.2. Vitamin A

Vitamin A is, in contrast to vitamin D, only obtained through intake of produce or from animal sources (206). Vitamin A has a range of precursors that are subsequently converted into an array of different metabolites, however, only the retinyl esters and β-carotenes will be commented upon. β-carotenes (provitamin A carotenoids from plants) are absorbed directly by the intestinal epithelial cells, while retinyl esters (from animals that have already taken in and converted plant provitamin A carotenoids) are modified before to uptake by intestinal enzymes. The first step in the vitamin A metabolism for β-carotenes is oxygenation within the enterocytes, while the first step for the retinyl esters is intraluminal hydrolysis. Through these conversions the β-carotenes and the retinyl esters become all-trans-retinal and all-trans-retinol (referred to hereafter as retinal and retinol), respectively. Retinal is further converted in a similar manner as the retinyl esters to retinol, and retinol is esterified to retinyl esters in the enterocytes before being incorporated into chylomicrons. The chylomicrons are released via the lymphatics to the circulation, where they are converted into chylomicron remnants that transfer the retinyl esters either to peripheral tissues or to the liver. Normally, the liver stores up to 80% of the body's total amount of vitamin A and tightly regulates the release of vitamin A to the circulation. The retinyl esters are again converted to retinol in the liver cells and it is bound to a specific retinol binding protein (RBP) before being released to the circulation. The retinol-RBP complex transports retinol to a wide assortment of peripheral tissues including immune cells. Within the cells, retinol is converted to retinal and further irreversibly to active vitamin A (retinoic acid) by a series of alcohol dehydrogenases and retinal dehydrogenases, respectively. Active vitamin A binds to specific retinoic acid receptors (RARs) that mostly dimerizes with the RXR (similarly as the VDR), and the RAR-RXR heterodimer finally binds to retinoic acid response elements (RAREs) in a variety of genes. However, the heterodimeric complex can also bind to other transcription factors and thus increase the total amount of vitamin A responsive genes. Retinoic acid is further catabolized to inactive polar metabolites by different cytochrome P450 enzymes and discharged through urine, bile and faeces.

The serum level of retinol is kept in a narrow range by strict regulation and do under normal circumstances not reflect the body's vitamin A status (i.e. the amount of stored vitamin A in the liver) (207). Furthermore, additional vitamin A supplementation will primarily change the serum retinol level only among individuals with reduced pre-supplementation values. With this in mind, the current recommendations for an adequate serum retinol level is >1.05 µmol/L, however, it is clear that the concentration is influenced by age, gender, BMI and overall health (207;208).

Vitamin A has an important function during the organogenesis including the development and maturation of the CNS (206;209). After birth, it plays a crucial role in the regeneration of a range of tissues like the lungs, bone marrow, skin, and eyes in addition to being involved in the synaptic plasticity of the CNS. Although vitamin A deficiency can lead to a range of diverse clinical signs, the vital importance of the vitamin became first apparent through its effect on the visual system in the form of night blindness and subsequent xerophtalmia (inability to produce tears). Anecdotal evidence states that this condition may have been treated with intake of animal liver in the ancient Egypt, however, the first description of treatment with cod liver oil did not appear until the end of the 19th century (210). Vitamin A was first identified in 1915 through animal experiments that showed that rats on a vitamin A-deficient diet had reduced growth, and that this could be corrected with vitamin A supplementation (211). However, in contrast to vitamin D, it was quickly realized that vitamin A was important for the immune system, as the vitamin A-deficient animals were very susceptible to widespread infections (212). Still, the direct immunological effect of vitamin A was not clearly delineated until the mid-1980s, when it was found that active vitamin A is central for the differentiation of myeloid cells and affects the later

maturation and survival of DCs and macrophages (209). Furthermore, active vitamin A has been found to affect T cell trafficking and the differentiation of B cells and T cells by promoting anti-inflammatory B cells, Th2 and Treg cells, and inhibiting pro-inflammatory Th1 and Th17 cells (213-215). Similarly as for vitamin D, the *in vivo* effect of vitamin A has been demonstrated in the EAE model where it may both suppress disease manifestation and progress (216).

Despite the findings in the EAE model and the recent interest for Treg and Th17 cells in MS (216-218), the attention toward a possible effect of vitamin A in MS has been rather limited. However, it was hypothesized in the 1980's that MS may develop due to a deficient intake of vitamin A and E during infancy (219), and more recently that reduced sunlight-exposure may limit local production of active vitamin A in the retina and thereby promote CNS inflammation via reduced control of leukotriene synthesis and pathogenic Th17 cells (220;221). Nevertheless, only three studies have assessed if vitamin A may be related to MS risk (222-224). The results from these case-control studies were inconsistent, as intermediate levels of RBP (as a vitamin A proxy) in prospective serum samples and vitamin A intake in the form of cod liver oil during teenage years was found to be associated with a reduced disease development compared to low levels of RBP and no intake (222;223), respectively, while intake of carotenoids through multi-vitamin supplements was not associated with an altered disease hazard (224).

Epidemiological studies of serum retinol levels in patients with established MS have been far apart (225-228), and the results have been inconclusive with regards to showing differences when compared either to patients with other inflammatory neurological diseases or healthy controls. Longitudinal studies of serum retinol levels have found no difference between patients with or without clinical disease activity (227;229), but a close to 50% reduction in MRI activity was associated with each 1 μmol/L increase of retinol in one of our previous studies (229). In contrast, a more consistent anti-inflammatory effect has been found when active vitamin A was used *in vitro* with cells from MS patients or when cells were obtained from MS patients on vitamin A supplementation (230-234), as these studies reported reduced proliferation of T cells after exposure to myelin proteins (230;231), and skewing of T cell differentiation toward Th2 and Treg cells and away from Th1 and Th17 cells (231-234). Interestingly, four of the studies noted that vitamin A could have effects in conjunction with IFN-β use (230;232-234). Finally, to my knowledge, only one vitamin A interventional study has been conducted among RRMS patients (235). In this one-year double-blinded RCT, an improvement was noted in MSFC scores among patients on supplementation with retinyl palmitate (25000 IU/d for 6 months and 10000 IU/d for 6 months) as an add-on to IFN-β treatment compared to patients on IFN-β and placebo, but no differences in brain MRI activity, relapse rates or EDSS scores were found.

5.3.3. Vitamin E

Vitamin E is also a dietary essential mainly found in plants (236;237). The precursors of vitamin E are plentiful, but the tocopherol family is the most important in humans. α-tocopherol is the dominant member of this family, as it is efficiently taken up by the intestine, retained and transported in the body, and has the highest biological potency (237;238). In contrast to vitamin A and D, vitamin E has no need for further modification before exhibiting its biological effect. Upon dietary uptake, α-tocopherol is assembled together with triglycerides, cholesterol, phospholipids and apolipoproteins before being released as chylomicrons into the blood through the intestinal lymphatic vessels (237). α-tocopherol is rapidly removed from the chylomicron remnants when arriving the liver, where it becomes bound to a specific α-tocopherol transport protein (TTP) and is incorporated into high density or very large density lipoproteins (HDL and

VLDL, respectively). HDL and VLDL are transported with the circulation throughout the body and enter the tissues together with triglycerides and cholesterol metabolites. α-tocopherol is transferred into cells via scavenger receptors either directly from HDL or after release from VLDL during lipolysis. Once intracellularly, vitamin E can be quickly employed through shuttling to the Golgi apparatus, lysosomes and mitochondria, or it may directly affect gene transcription through effects on protein kinase C and several other transcription factors. If not utilized, α-tocopherol can be exported and again become incorporated into lipoproteins. The elimination of vitamin E may be in an unaltered form through the bile, or it can be metabolized to tocopheronic acid and tocopheronolactone that are further converted to glucuronides or sulphates and excreted in the urine.

Due to its high dietary-content, vitamin E deficiency is very rare except in conjunction with malabsorption syndromes or genetic abnormalities of molecules involved in the production or transport of α-tocopherol or lipoproteins (236). However, a serum level of α-tocopherol below 30 μmol/L may indicate an inadequate dietary intake. Still, the validity of using serum measurement as an indicator of vitamin E status may be questioned, as the serum level of α-tocopherol is usually kept stable by a range of regulatory mechanisms, and the concentration is also affected by gender, BMI and smoking status (239).

The main function of vitamin E is related to its anti-oxidative properties and it is regarded as the body's first line defence against lipid peroxidation (237;238). This function is mediated by its ability to neutralize peroxyl and reduce the production of toxic free radicals during processes such as cellular respiration. Vitamin E is vital during embryonic development and central in the maturation of the hematopoietic and nervous systems during infancy (238;240). In adult life, vitamin E is essential for the function of most tissues and it is especially needed in tissues with elevated production of oxidative substances due to high cellular turnover or metabolism. Although discovered in 1922 after recognition of its importance on the reproductive system in rats (241), the importance of vitamin E in humans did not become evident before 60 years later with the finding of neurological deficits among patients with hereditary abetalipopoteinemia (inability to synthesize chylomicrons or export VLDL) (240). Later, several other genetic abnormalities have been found to affect vitamin E transport and lead to ataxia, myopathies and peripheral neuropathy (236). In addition to neurological impairments, these patients may also display impaired cellular immunity (242), which indicates an important role for vitamin E in the immune system. Vitamin E has both oxidant and non-oxidant effects that are important during proliferation and maturation of all immune cells (237;238), and it has been shown to be crucial for macrophage function. Furthermore, vitamin E may affect lymphocyte function either through direct effects on naïve T cells or indirect effects on mature T cells and B cells via modulation of macrophage cytokine production (238). However, research on vitamin E in animal models of demyelination has been focused on its protective effect against oxygen radicals and its ability to promote reparation of damaged CNS tissues with findings of reduced demyelization and enhanced remyelization together with abated gliosis, CNS inflammation and myelin loss after injection of vitamin E or synthetic tocopherol (243;244).

Limited knowledge exists on the possible role of vitamin E in MS. However, as mentioned in the previous section, it has been hypothesized that a lack of vitamin A and E in infancy could contribute to disease development (219). Still, varying results have been found in studies examining the potential effect of vitamin E intake on MS risk (222;224). Furthermore, research into the possible role of vitamin E in MS has also been mainly focused on its anti-oxidative properties in established disease, as immune cell production of reactive oxygen species and plasma or serum indicators of lipid peroxidation have been reported to be elevated in MS patients compared to healthy controls (225;245;246). Still, the presence of ongoing systemic oxidative stress provides only circumstantial evidence for the importance of vitamin E

in MS. Unfortunately, only a few epidemiological studies have been published where the serum or plasma levels of α-tocopherol have been investigated among MS patients (225;228;247;248). These studies found significantly lower serum vitamin E levels in MS cases than healthy controls (225;228;247), lower levels during relapses than remissions (248), and increased levels after use of IFN-β therapy (248). Moreover, only two small-scale studies have examined the possible effects of α-tocopherol in conjunction with fatty acid treatment on RRMS (249;250). The results from these studies were largely in conflict, as the study by Pantzaris *et al.* reported that a combination containing α- and γ-tocopherol (22 and 760 mg, respectively) reduced the relative rate of relapses by 64% compared to placebo and the cumulative probability of disease worsening was 48% lower in the treatment than the placebo group (250), while our previous study found no difference in clinical activity between patients on combinations containing 13 or 22 mg α-tocopherol and the vitamin E serum levels did not differ between patients with or without relapses or disease worsening (249). However, we did find that each 10 μmol/L increase of α-tocopherol was associated with a ~35% reduction in new T2-lesions during IFN-β treatment.

5.4. Biological markers

5.4.1. Biological markers in multiple sclerosis

The term biological marker (biomarker) is assigned an entity that can be objectively measured and evaluated as an indicator of a normal physiological or pathological process, or a response to therapeutic intervention (251). A biomarker for a therapeutic response may be further characterized as a mediator that explains how or why the intervention mediates a clinical outcome, or as a moderator that affects the relationship between the intervention and the clinical outcome (252). Moreover, the utility of a biomarker can be evaluated by its precision and accuracy, meaning its ability to provide measurements that are reproducible and correlated with a specific clinical endpoint (251). This indicates that biomarkers with high utility may be used as proxies for clinical endpoints in the form of surrogate endpoints.

Biomarkers in MS are routinely used to evaluate different pathological aspects of the disease and to what degree DMTs can affect these processes (253;254). In addition, biomarkers are also used in relation to diagnosis (and differential diagnosis), monitoring and determination of risk for potential side-effects of treatment, and predicting primarily short-term prognosis. With this in mind, the already mentioned use of MRI can be characterized as biomarker with a high short-term utility for a both relapses and increasing disability in RRMS patients (89;255), while the measurements of oligoclonal bands in CSF and naturalizing antibodies (Nabs) against IFN-β and John Cunningham virus antibodies in serum are clinically useful biomarkers that may provide specific information about prognosis, efficacy of treatment and risk of developing PML, respectively, among CIS patients and subsets of MS patients (256-258). In addition, a range of additional biomarkers in serum has recently been suggested to be related to the pathological process or treatment of MS (253;259).

5.4.2. Biological markers of systemic inflammation

Inflammation is a central part of the disease process in MS (260). Although the inflammatory response in MS is directed against the CNS, it is clear that CNS inflammation is partly dependent on the influx of immune cells from the systemic circulation, and breaches in the BBB can together with the lymphatic drainage of the CNS lead to the release of locally

produced inflammatory mediators to the systemic circulation (259). Biomarkers of inflammation in the systemic circulation (hence fort referred to as inflammation markers) may therefore be seen upon as distal biomarkers of the disease process in MS. However, it must be underlined that the correlation between systemic and CNS levels of inflammation markers differ substantially between different markers (253;261). Furthermore, the inflammation marker field is enormous and encompasses a range of molecules involved in a multitude of different inflammatory pathways. Nevertheless, a simplified inflammation marker organization may be applied based on their perceived main effect and which inflammatory phase they mainly belong to. This organization is presented together with an allocation of the inflammatory markers examined in this Thesis in Table 2.

Table 2. Examined systemic inflammation markers, ordered firstly by main effect and secondly by alphabet

Inflammation marker	Alternative name	Main effect	Inflammation phase	Class	Primary expression or production	Primary function
ALCAM	CD166	Pro-inflammatory	Acute	Immunoglobulin (superfamily)	Epithelia, endothelia and unspecified CNS cells	Adhesion of immune cells expressing CD6
CCL21	SLC[1]	Pro-inflammatory	Acute and reparatory	CC chemokine (family)	Lymphoid tissue endothelia	Attraction of CCR7-expressing cells
CXCL16	SR-PSOX[2]	Pro-inflammatory	Acute and reparatory	CXC chemokine (family)	Macrophages, DCs, endothelia, microglia and astrocytes	Attraction of CXCR6-expressing cells
OPN	SPP1[3]	Pro-inflammatory	Acute	Small integrin-binding ligand N-linked glycoprotein (family)	Osteoblasts, innate immune cells and T cells	Ligation of VLA-4-expressing cells and attraction of several cell types
PTX3	TSG-14[4]	Pro-inflammatory (and anti-inflammatory)	Acute and reparatory	Long pentraxin (family)	Epithelia, macrophages, DCs and unspecified CNS cells	Neutralization, complement and cell activation and opsonization

[1] Secondary lymphoid-tissue chemokine; [2] Scavenger receptor for phosphatidylserine and oxidized lipoprotein; [3] Secreted phosphoprotein 1; [4] TNF-inducible gene 14 protein

Table 2. Examined systemic inflammation markers (continued)

Inflammation marker	Alternative name	Main effect	Inflammation phase	Class	Primary expression or production	Primary function
IL-1Ra		Anti-inflammatory	Acute	IL-1 cytokine (family)	Macrophages and DCs	Antagonist for type I IL-1 receptors
OPG	TNFRSF11B[5]	Anti-inflammatory	Acute	TNF-receptor (superfamily)	Osteoblasts and innate immune cells	Decoy receptor for RANKL and TRAIL
sFRP3	FRZB[6]	Anti-inflammatory	Acute and reparatory	SFRP (family)	Several cell types including unspecified CNS cells	Decoy receptor for Wnt
sTNF-R1	TNFRSF1A[7]	Anti-inflammatory	Acute	TNF-receptor (superfamily)	Monocytes and T cells	Decoy receptor for TNF
MMP-9	Gelatinase B	Context dependent	Acute and reparatory	Metallo-proteinase (family)	Immune cells and fibroblasts	Extracellular matrix degradation and protein cleavage
TGF-β		Context dependent	Acute and reparatory	TGF-β (superfamily)	Innate immune cells	T cell developmental factor

[5] TNF receptor superfamily member 11B; [6] Frizzled-related protein; [7] TNF receptor superfamily member 1A

5.4.3. Pro-inflammatory markers

The panel of inflammation markers examined in this Thesis was primarily selected to reflect different aspects of MS pathology by highlighting different inflammatory pathways, interactions between immune cells and different tissues, and factors involved in tissue repair. In light of the data available at the initiation of my doctoral studies, the following inflammation markers were judged to be primarily pro-inflammatory; Activated leukocyte cell adhesion molecule (ALCAM), chemokine (C-C motif) ligand 21 (CCL21), chemokine (C-X-C motif) ligand 16 (CXCL16), osteopontin (OPN), and pentraxin 3 (PTX3).

ALCAM is an adhesion glycoprotein belonging to the immunoglobulin superfamily (262). The molecule ligands with CD6, which is primarily expressed on activated T cells, B cells and APCs (263). ALCAM is mainly found on immune cells, epithelia, BBB endothelia and within the CNS and has been implicated in among other haematopoiesis, neurogenesis, neurite outgrowth and lymphocyte activation, differentiation and migration (262-264). ALCAM can be produced in a soluble isoform and the membrane bound form can be shed to the circulation by among other matrix metalloproteinases (MMPs) (265), but the potential role of soluble ALCAM seems uncertain. The role of endothelial bound ALCAM has been examined in EAE, where it was found that blockade of the molecule restricted Th cell migration to the CNS, delayed disease onset and reduced its severity (264). Furthermore, ALCAM expression has been shown to be higher in active MS lesions than in normal appearing white matter (NAWM) or CNS tissue from controls without neurological disease. Genetic variants associated with genes for ALCAM and its receptor CD6 have been identified as susceptibility loci for MS (122;266). However, as far as I know, prior to 2013 only our preceding study from one of the patient cohorts included in this Thesis had examined the serum levels of ALCAM among RRMS patients (267). This study found that the serum levels of ALCAM seemed unrelated to MRI activity and unaffected by initiation of IFN-β therapy.

CCL21 is a small cytokine belonging to the CC chemokine family (268;269). CCL21 binds primarily to the CC chemokine receptor 7 (CCR7) on among other T cells and APCs. It is constitutively present in secondary lymphoid organs and the CNS and is further upregulated during inflammation, but factors regulating the release of CCL21 to the circulation seem obscure. The main function of CCL21 is related to the trafficking of CCR7-expressing cells to and within secondary and tertiary lymphoid tissues (269). In addition, an increased expression had been found in cells responsible for tissue repair and fibrogenesis at the periphery of tertiary lymphoid follicles during chronic inflammation (270). Similarly, increased transcript levels were localized to inflamed endothelium in the periphery of affected CNS parenchyma in mice with EAE (271). Only a handful of studies had investigated the potential role of CCL21 in MS and the results were inconsistent as one study found barely detectable or no transcripts of CCL21 in control or MS CNS tissues, CSF or blood (272), another reported similar protein serum levels in MS patients and neurological controls without detecting CCL21 in CSF (273), and a third study of both serum and CSF protein levels found only elevated CCL21 levels in CSF of patients with inflammatory neurological illnesses (including MS) compared to patients with non-inflammatory neurological illnesses (274). Lastly, only our aforementioned study had examined serum levels of CCL21 in relation to radiological disease activity and IFN-β use (267). However, we found no association between the serum levels and MRI activity despite initiation of treatment being associated with a significant increased concentration.

CXCL16 is similarly to CCL21 a chemokine, but belongs to the CXC chemokine family (275). It is mainly found on the surface of macrophages, DCs, microglia and astrocytes (275;276), and binds to the CXC receptor 6 (CXCR6) expressed on T cells and innate immune cells (275). CXCL16 is primarily expressed in a membrane-bound form that is

upregulated in response to inflammation and released into circulation after being cleaved off by MMPs (277). It functions as a scavenger receptor for oxidized lipoproteins when membrane-bound and as a chemoattractant for CXCR6-expressing cells when in soluble form (275;278). The chemokine properties of CXCL16 had been examined in EAE, where it had been found to both promote and inhibit CNS lymphocyte influx depending on its place of expression (278;279). Furthermore, CXCL16 had been reported to be increased in demyelinating lesions, CSF and serum of MS patients (276;280), but elevated CSF and serum levels had also been found during other inflammatory neurological conditions (280). Our previous study found that the serum levels of CXCL16 were inversely associated with both simultaneous and subsequent MRI activity, and its concentration was significantly increased upon IFN-β initiation (267).

OPN is an extracellular matrix phosphoprotein belonging to the small integrin-binding ligand N-linked glycoprotein family and was initially discovered as non-collagenous bone matrix protein (281). However, its presence in bone has later been largely overshadowed by its expression on innate immune cells, T cells and B cells. OPN can ligand among other with VLA-4 after being modified by the activated coagulation factors thrombin or plasmin. This binding triggers a potent pro-inflammatory response by APCs characterized by increased IL-6 and IL-12 and decreased IL-10 an IL-27 production that promotes Th1 and Th17 cell maturation. Furthermore, OPN may be cleaved off by MMPs, generating a soluble form that acts as a chemoattractant. The pro-inflammatory effect of OPN had been clearly shown in EAE, where injections of OPN induced relapses (282), and increased expression of OPN and OPN receptors was found during disease progression on DCs and Th17 cells, respectively, in both the periphery and the CNS (283). The potential importance of OPN in MS had been addressed on the genetic level where certain OPN gene polymorphism were associated with disease susceptibility (284), the patohistological level where OPN expression was increased along with plaque activity (285), and in clinical studies where higher plasma levels of OPN were noted in active and progressing MS patients than in stable patients and healthy controls (286;287). Two longitudinal studies had also been carried out in addition to our previous study (267;288;289), reporting significantly higher CSF levels and a trend for higher serum levels of OPN levels during relapse than in remission (288;289), and an association between increasing serum levels and the subsequent amount of Gd^{+} lesions (289). However, no association was found between increasing serum concentrations of OPN and simultaneous radiological activity in the latter study and in our initial exploration (267). Furthermore, in contrast to earlier studies (286;287), our study found that IFN-β therapy was associated with an increased serum level of OPN.

PTX3 belongs to the long pentraxin family of the acute phase proteins (290;291). PTX3 is a fluid PRR that can bind directly to foreign or internal entities and its production in endothelia, epithelia, macrophages, DCs and the CNS is elevated by inflammatory mediators. Upon binding, it can act as a direct neutralizing agent, an activator of the complement and coagulation cascade systems, and an opsonizing agent that promotes phagocytosis (290). However, PTX3 may also have a beneficial role in late phases of inflammation, as IL-10 promotes its production (292), and animal experiments have shown that PTX3 may limit phagocytosis of damaged neurons and reduce immune cell migration into inflamed tissues (293;294). Prior to 2013, only one study had investigated the role of PTX3 during EAE, finding peak transcription in conjunction with the active phase of the condition (295). A similar finding had been made in a longitudinal study of MS patients, where serum levels of PTX3 were significantly elevated during disease activity compared to in remission and in neurological or healthy controls (296). Our earlier results were in concordance with this by finding an association between increasing PTX3 levels and MRI activity (267). However, a similar relationship was not noted during IFN-β treatment, as this was associated with a significantly increased PTX3 level.

5.4.4. Anti-inflammatory markers

The following inflammation markers were deemed to be chiefly anti-inflammatory; Interleukin-1 receptor antagonist (IL-1Ra), osteoprotegerin (OPG), soluble frizzled-related protein 3 (sFRP3), and sTNF receptor 1 (sTNF-R1).

IL-1Ra is a member of the IL-1 cytokine family and binds to the IL-1 receptors (IL-1Rs) (297). IL-1 was one of the first discovered cytokines in the early 1970s (298;299), and IL-1Ra was recognized about 10 years later due to its IL-1 inhibitory bioactivities (297;300). IL-1 and IL-1Ra are usually transcribed sequentially and *in vitro* studies had found the appearance of IL-1Ra to be slightly delayed compared to IL-1, but more prolonged (297). The production and release of the IL-1 cytokines are especially pronounced after macrophage and DC PRR stimulation, but it is also secreted by among other microglia, neurons, fibroblast and keratinocytes (297;301). IL-1 production can be regarded as a perquisite for the initiation of an inflammatory response, only being preceded by the activation of the inflammasome and the caspases (301). IL-1Ra competes with IL-1 for binding of the IL-1Rs, and its ligation do not induce intracellular signalling under normal conditions (297). With respect to the effect of IL-1 and IL-1Ra on demyelination, findings from EAE models had indicated that increased levels of IL-1 escalated disease severity (302), and that blockade of IL-1 signalling by among other IL-1Ra delayed disease onset and severity (302;303). In MS, IL-1 had been found to be expressed in MS lesions (304), but findings regarding its concentration in CSF or serum were variable with results ranging from elevated compared to healthy controls to rarely detectable (305;306). On the other hand, the results for IL-1Ra had been somewhat clearer with findings of IL-1Ra in perivascular macrophages and within active lesions (307), and reports of serum concentrations being significantly increased during clinical or MRI activity (308;309). However, CSF concentrations were reported to be decreased during clinical and radiological active disease (305;310), but remained higher among MS patients than in non-inflammatory neurological controls (305). In contrast to aforementioned studies (309;310), we previously found a strong trend for an association between fewer new Gd^+ lesions and increasing IL-1Ra levels in serum (267). Still, our earlier results were in line with prior studies that reported increased IL-1Ra concentrations after initiation of IFN-β treatment (308;311)

OPG is a cytokine in the TNF-receptor superfamily, but in contrast to most members in this family it does not possess a transmembrane region (312). It is normally found in high quantities in bone tissues, but can also be produced in substantial amounts by activated DCs and B cells (313). Besides its effects on osteoclasts, soluble OPG functions as a neutralizing decoy receptor for the pro-inflammatory molecules Receptor Activator of Nuclear Factor κ B ligand (RANKL) and TNF-related apoptosis-inducing ligand (TRAIL) (312). The impact of OPG and RANKL had seemingly not been examined in the context of EAE before 2013, but blockade of TRAIL in the CNS of mice with EAE led to a substantially reduced apoptosis of neural cells and clinical severity when matched to mice with EAE without blockade (314). Furthermore, limited investigations had been carried out on the potential effect of OPG in MS, but OPG transcripts had been found in human spinal cord and similar CSF levels were reported among patients with MS and other non-inflammatory disorders (315). Additionally, two cross-sectional studies had found serum OPG levels to be increased in MS patients compared to healthy controls (316;317), and in RRMS patient compared to SPMS patients (317). Lastly, serum levels of OPG had been found to be increased upon starting IFN-β (318). The latter was confirmed in our prior study together with the finding of an association between increasing OPG levels and reduced MRI activity during IFN-β treatment (267).

sFRP3 is a glycoprotein member of the secreted frizzled-related protein (sFRP) family (319). The sFRPs are closely related to the Frizzled receptors, which they share ligand properties with for the Wingless/Int (Wnt) proteins.

sFRP3 functions as a decoy receptor, as it binds Wnt in the extracellular compartment. The modulation of Wnt-signalling by sFRPs plays an important role during early embryonic development and affects Wnt-regulation of proliferation, differentiation and survival of T cells and CNS cells in adult life (320;321). However, the sources responsible for Wnt and sFRP3 production and secretion in adulthood seems uncertain (322), and the mapping of the effect of Wnt-signalling and sFRP3 modulation in the human CNS is in its infancy. Nevertheless, animal experiments had found that Wnt signalling could delay remyelination, and Wnt proteins were found to be upregulated in the spinal cord in mice with EAE (323;324). To my knowledge, the effect of sFRP3 had not been examined in the context of demyelination, but interference with Wnt-signalling trough other neutralizing molecules than sFRP3 had surprisingly shown inhibition of development of neurons, astrocytes and oligodendrocytes from neural stem cells (325). The possible role of sFRP3 in MS had not been addressed directly before the publication of Article I, but increased levels of Wnt had been found within MS lesions compared to control tissues and NAWM (323;326). Moreover, Wnt transcription levels in whole blood had been found to be elevated in MS patients compared to controls, and IFN-β treatment was associated with a down-regulation of Wnt gene expression (327).

sTNF-R1 is also a cytokine belonging to the TNF-receptor superfamily, but unlike OPG, its has both transmembrane and intracellular signalling domains (328). It is primarily expressed as TNF-R1 on monocytes and T cells (329), and it is believed that sTNF-R1 is produced by cleavage of the extracellular portion of the transmembrane TNF-R1 by trypsin-like proteases (328). Like OPG, sTNF-R1 is released during inflammation and functions as a neutralizing decoy receptor for the pro-inflammatory molecule TNF (330). The effects of TNF and various neutralizing substances including sTNF-R1 had been extensively investigated in murine EAE models. These studies found TNF transcription to be increased before the onset of clinical signs (331), and at its peak at the height of the illness (331;332). Furthermore, inhibition of TNF-activity by injections of sTNF-R1 had been found to prevent disease development after transfer of encephalitogenic T cells (333). The role of TNF and sTNF-R1 had also been quite vigorously examined in MS with findings of increased TNF-expression in endothelia, astrocytes and macrophages in CNS lesions compared to controls with other neurologic diseases (334), higher levels of TNF and sTNF-R1 in CSF among relapsing and progressing than stable patients or patients with non-inflammatory neurologic disease (335;336), and increased serum concentrations of sTNFR-1 in patients with active disease compared to stable patients or controls that were healthy or had other neurological conditions (337). However, result showing no change or a delayed increase in serum sTNF-R1 levels in patients compared to controls had also been reported (338;339). Lastly, a MS susceptibly allele had been found in the gene for sTNF-R1 that coded for a truncated form of sTNF-R1 that promoted monocyte responses to TNF (266;340). The latter could explain the detrimental effect of TNF-blockage among RRMS patients reported in a phase II double-blind RCT of a recombinant sTNF-R1 (341). Nevertheless, our prior investigation found a strong trend for reduced MRI activity with increasing serum levels of sTNF-R1 (267), and, in line with an earlier report (311), we also found raised concentrations in the period after compared to before initiation of IFN-β therapy.

5.4.5. Context dependent markers

Even though several of the aforementioned markers may exhibit both pro- and anti-inflammatory properties, their overall effects were determined to be dominated by one of these properties. However, two of the markers examined in this Thesis were judged to have pro- and anti-inflammatory properties of equal importance in different settings, and were therefore labelled as context dependent markers. These markers are MMP-9 and TGF-β.

MMP-9 is a zinc-dependent metalloproteinase enzyme that has its place in the zinc-metalloproteinase family (342). The main function of MMP-9 is related to its ability to degrade extracellular matrix, which is central in embryonic development and during angiogenesis, tissue remodelling and resolution of inflammation in adulthood. Additionally, MMP-9 modulates a range of chemokines, receptors, adhesion molecules and cytokines like IL-1 and TNF (342;343). The production of MMP-9 is specifically elevated by fibroblasts, lymphocytes and innate immune cells like microglia and astrocytes during inflammatory processes (342;344). It is primarily secreted as a pro-proteinase and is dependent on further modification by other MMPs, various oxidants, tissue proteinases or plasminogen (an activated factor from the fibrinolysis cascade system) before exerting its action. When activated, MMP-9 can be suppressed by the tissue inhibitors of metalloproteinases. The role of MMP-9 in EAE had been found to be mainly detrimental, as it was upregulated immediately preceding the onset of clinical disease and increased in parallel with the clinical severity (345). However, MMP-9 may also have a beneficial effect during the reparatory phase of the inflammatory process, exemplified by the earlier findings of reduced maturation of oligodendrocytes and remyelination in MMP-9 gene knockout mice after lysolecithin injection and prolonged injury and impaired functional recovery after MMP-9 blockade in mice models of stroke (346;347). In MS, MMP-9 had been found to be increased in macrophages, microglia and reactive astrocytes within acute lesions (344), and higher in the CSF in MS patients compared to neurologic controls (348). Furthermore, the level of MMP-9 had been reported to be increased in MS patients compared to neurological or healthy controls in serum (348;349), and higher in CSF and serum of patients with radiological or clinical activity compared to stable patients. Similarly, our earlier study found a strong trend for a positive association between increasing serum levels of MMP-9 and disease activity on MRI (267). However, in contrast to an earlier study (350), the MMP-9 concentrations seemed to increase during IFN-β treatment.

TGF-β is a cytokine belonging to the TGF-β superfamily with three different isoforms (351). After synthesis, TGF-β is bound in a complex with the latent TGF-β binding protein and the latency-associated peptide (LAP) before secretion, and its ability to bind to TGF-β receptors is first possible after its release from this complex by serum proteinases like plasmin (another activated factor from the fibrinolysis cascade system) and MMPs. TGF-β is secreted by many different cells types, but the chief amount is produced by innate immune cells like macrophages, DCs and granulocytes in the form of TGF-β1. The effects of TGF-β are pleiotropic and have been found to affect proliferation, differentiation, migration and survival of both innate and adaptive immune cells. The two-faced role of TGF-β in inflammation is shown by its necessity for the development of both the pro-inflammatory Th17 and the anti-inflammatory Treg cells. A wealth of data exists concerning the role of TGF-β in inflammation in a variety of animal models including EAE, where it had been found to prevent or delay disease development (352;353), and its transcription had been found to be increased at the peak of clinical symptoms and during remission phases (354). Despite the considerable interest for TGF-β in EAE, only a few studies had examined the possible role of TGF-β in MS prior to 2013. These studies reported elevated TGF-β expression in macrophages and astrocytes in both active and chronic MS lesions compared to control tissues (355), and higher CSF and serum levels in MS patients than non-inflammatory neurologic or healthy controls (356;357). In addition, IFN-β therapy had been found to augment TGF-β levels in serum (357), and inverse relationships had been found between transcript levels in blood monocytes and MRI activity (358). The latter finding was, however, not confirmed by our previous results (267), but a trend for higher levels during than before IFN-β treatment was found.

6. Background for studies

Our previous observational studies had reported associations between serum levels of vitamin A, D and E and MRI activity (183;229;249), and between MRI activity and the selected panel of serum inflammation markers in the RRMS patients included in the OFAMS study (267;359). However, the existence of direct associations between serum levels of vitamin A, D and E and the inflammation markers was uncertain. Furthermore, initiation of IFN-β treatment had been found to be associated with reduced MRI activity and changes in inflammation marker concentrations, but the possible common effects between vitamin A, D and E and IFN- β on inflammation in this patient cohort were unknown. Finally, findings from several other researchers had indicated a potential benefit of high-dose vitamin D supplementation on markers of systemic inflammation in MS (Table 3), but the results were inconsistent and hampered by possible methodological issues (188;191-194).

Table 3. Vitamin D intervention studies on systemic inflammation in MS prior to 2013, ordered by publication date

Study	Patient type	Total patient number	DMT	Intervention per day	Duration	Positive findings	Negative findings
Mahon *et al.* 2003	MS	39	Unknown	1000 IU[1]	6 months	↑ TGF-β	IFN-γ, IL-2, IL-13, TNF-α
Burton *et al.* 2010	MS	49	Mixed	10000 IU[2]	12 months	↓ T-cell proliferation	IFN-γ ,IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, MMP-9, TIMP[3], TNF-α
Smolders *et al.* 2010	RRMS	13	IFN-β	20000 IU[4]	3 months	↑ IL-10, ↓ Th1/Th2 ratio	IFN-γ, IL-4, IL-17
Kimball *et al.* 2011	MS	49	Mixed	10000 IU[2]	12 months	↓ T-cell proliferation	CRP[5], IFN-γ, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, Kallikrein-6, MMP-9, OPN, TIMP[3], TNF-α
Mosayebi *et al.* 2011	RRMS	59	IFN-β	10000 IU[6]	6 months	↑ TGF-β, ↑ IL-10, ↓ T-cell proliferation	IFN-γ

[1] Unspecified vitamin D; [2] Mean dose vitamin D_3 (initial escalation and subsequent down-titration 0-40000-0 IU daily); [3] Tissue inhibitor of metalloproteinase 1; [4] Vitamin D_3; [5] C-reactive protein; [6] Administered as 300000 IU vitamin D_3 monthly

7. Aims of studies

7.1. Main objectives

The primary objective of this Thesis was to explore the possible association between serum levels of the fat-soluble vitamins A, D and E and inflammation in RRMS. The secondary objective was to investigate the potential interaction between vitamin A, D and E and IFN-β therapy on inflammation in RRMS.

7.2. Specific aims

The work provided in this Thesis was carried in attempt to answer the following specific questions;

- Are serum levels of vitamin A, D and E associated with serum markers of systemic inflammation in RRMS?
- Does IFN-β therapy modulate the associations between vitamin A, D and E and markers of systemic inflammation in RRMS?
- Are markers of systemic inflammation associated with clinical activity in RRMS?
- Do serum levels of vitamin D modulate the effect of IFN-β therapy on markers of systemic inflammation and inflammatory MRI activity in RRMS?
- Does IFN-β therapy affect the serum level of vitamin D in RRMS?
- Does high-dose oral vitamin D_3 supplementation affect markers of systemic inflammation in RRMS?

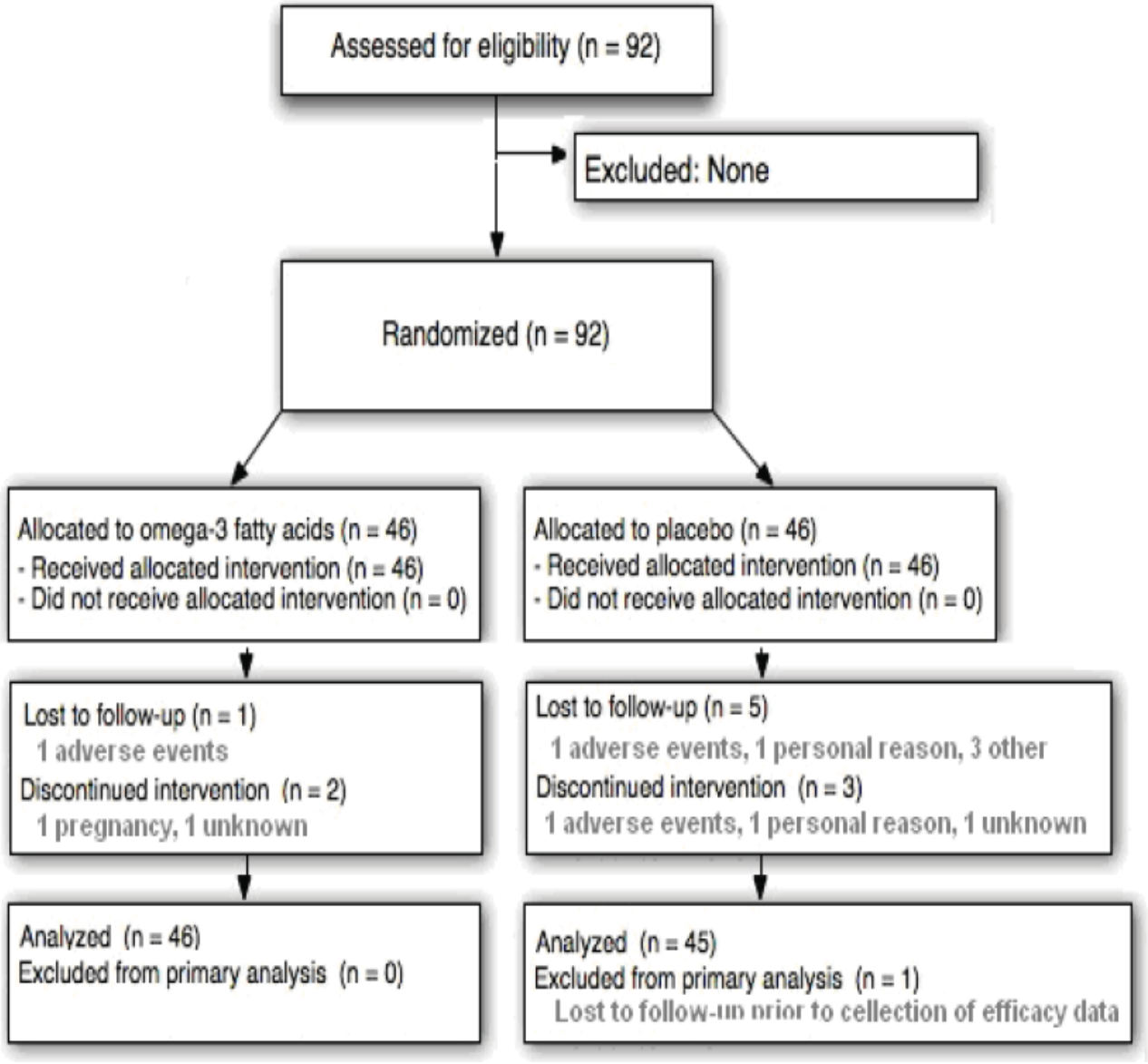
8. Methodological considerations

8.1. Trial designs

8.1.1. Patient cohorts and trial criteria

The 156 RRMS patients included in the investigations in Thesis stem from two RCTs conducted in Norway between December 2004 and February 2010. The first trial conducted was the OFAMS study that was originally designed to evaluate effects of omega-3 fatty acid supplementation alone (the first 6 months of the study) and in addition to IFN β-1a treatment (the last 18 months of the study) on clinical and MRI endpoints (359). The trial enrolled a total of 92 patients from 13 neurological departments across Norway (Figure 2). The patients were diagnosed with RRMS according to the 2001 McDonald criteria (72), were between 18 and 55 years of age, had an EDSS score ≤5.0, and had active disease

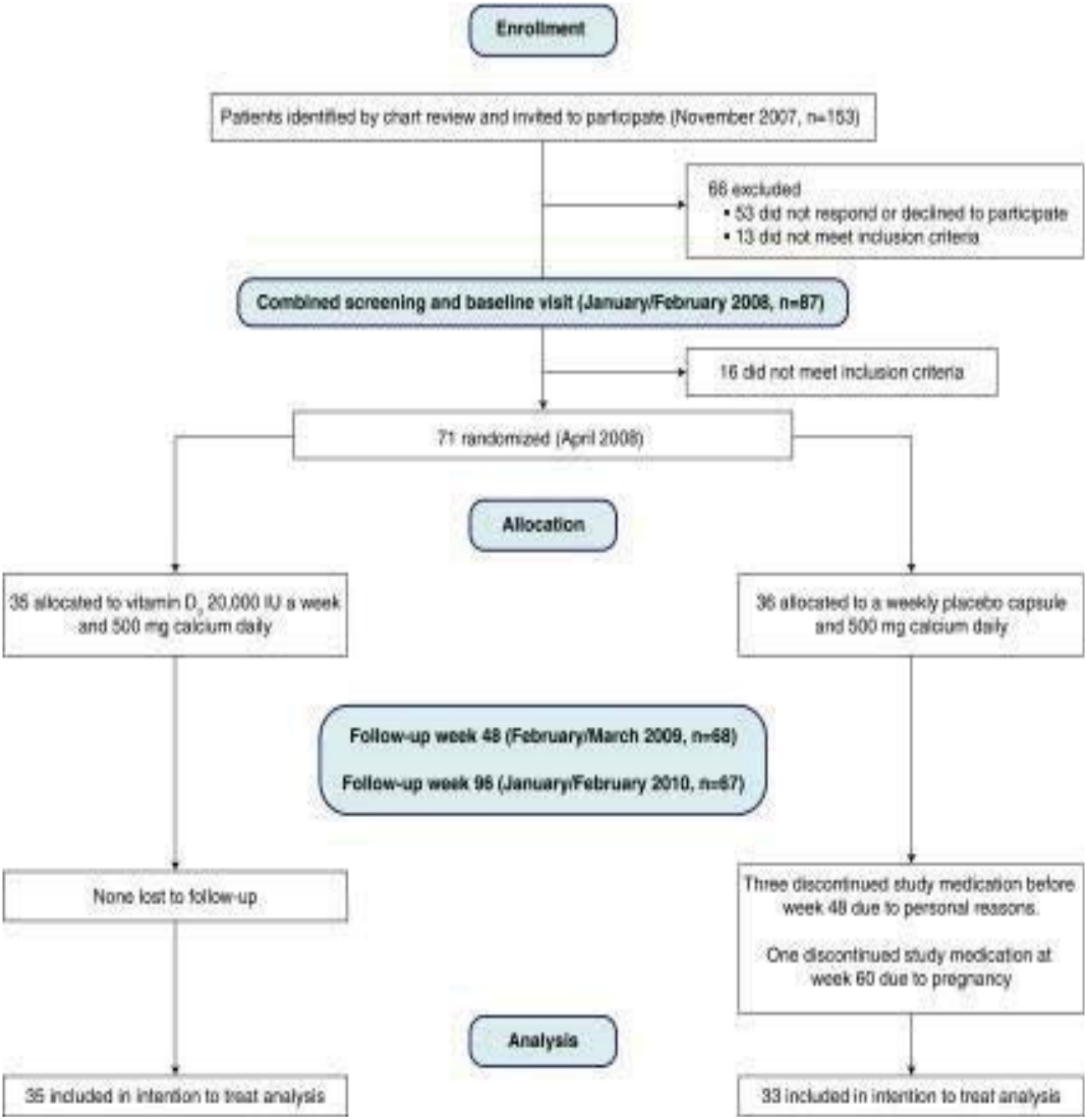
Figure 2. The Omega-3 Fatty Acid Treatment in Multiple Sclerosis study



within the year preceding enrolment (defined as ≥1 clinical relapse or MRI activity in the form of ≥1 new T1-weighted Gd^+ or T2-weighted lesion). The exclusion criteria were focused on safety, prior use of immunomodulatory or immunosuppressive treatment and clinical disease activity the last month before screening.

The second trial conducted was a single-center trial at the UNN that was originally devised to assess effects of high-dose vitamin D_3 supplementation on bone health and clinical disease activity (199;360). A total of 68 patients were included in the original trial and followed for 24 months (Figure 3). The enrolled patients fulfilled the 2001 McDonald criteria for the diagnosis of RRMS (72), were between 18 and 50 years old and had an EDSS score ≤4.5. No emphasis

Figure 3. The University Hospital of North Norway trial



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was made on clinical activity or use of immunomodulatory or immunosuppressive treatment before enrollment. The exclusion criteria were focused on safety and factors that could affect bone health. However, an original participant was left out due to missing measurements and replaced by a patient previously excluded do to lactation, as she received trial supplementation and was otherwise followed throughout the trial period in an equal manner as the originally included patients.

In brief, the included patients were 68% women (65% in OFAMS study and 71% in the UNN trial), had an average age of 39.5 years (38.6 and 40.4, respectively) and had a mean BMI of 25.9 (25.6 and 26.2, respectively). Furthermore, the EDSS scores were quite similar with a mean score of 1.9 in the OFAMS study and a median score of 2.3 in the UNN trial. However, the studies differed substantially with respect to mean disease duration (5.6 years in OFAMS study and 10.5 years in UNN trial) and *pre study* ARR (1.65 and 0.13, respectively).

8.1.2. Interventions, disease modifying therapies and follow-up

The patients in the OFAMS study were randomized and allocated 1:1 to daily omega-3 (Triomar, Pronova Biocare AS, Sandefjord, Norway) or placebo capsules for 24 months that contained 13 or 22 mg of α-tocopherol, respectively (359). Treatment with three times weekly 44 µg subcutaneous IFN β-1a (Rebif, Merck Serono, Geneva, Switzerland) was initiated at study-month 6 for all patients and continued throughout the remaining 18 months of the trial. No other DMTs were allowed during the study. The allocation to omega-3 or placebo was double-blinded and the trial was conducted in a parallel fashion. The participants underwent clinical evaluations with a full physical and neurological examination at baseline and months 1, 3, 6, 7, 9, 12, 18 and 24, EDSS evaluation every 6 months and height and weight assessments at baseline and study conclusion. Additional clinical follow-ups were carried out in case of relapses.

The patients in the trial at the UNN were randomized and allocated 1:1 to once weekly 20000 IU vitamin D_3 (Dekristol, Mibe Gmbh Arzneimittel, Brehna, Germany) or placebo capsules for 24 months (360). All patients also received daily 500 mg tablets of calcium (Weifa Kalsium, Weifa ASA, Oslo, Norway or Calcium Sandoz, Sandoz A/S, Odense, Denmark). Additional vitamin D supplementation used at enrollment was permitted further throughout the study. No limitations were set on the use of DMTs, and patients were allowed to start, switch or end treatment at any time. The assignment to the vitamin D_3 or placebo group was double-blinded and permanent. A full physical examination with height and weight assessment and a complete neurological evaluation with EDSS scoring were conducted at baseline and every 12 months during the study. Further follow-ups were conducted by telephone every 3 months and in the case of relapses needing hospital admittance. Additional information was gathered every 3 months through questionnaires. The questionnaires contained detailed enquires regarding several lifestyle factors, but only information regarding dietary habits, use of vitamin D supplementation and UVR exposure has been included in the analyses in this Thesis.

8.1.3. Ethics and approvals

All patients received written and oral information about the respective studies before giving signed consent to enrollment, and they were free to withdraw this consent without consequence at any time during the studies. The patients were covered by the Liability Insurance in Connection with Clinical Trials of Drugs, and specific attention was paid toward possible side-effects of the interventions and the immunomodulatory treatment through patient education in addition to blood test and enquiry every 3-6 months. The trials were granted approval from Regional Committees for Medical and Health

Research Ethics and the Norwegian Medicines Agency, conducted in accordance with the World Medical Association's Declaration of Helsinki and the European Medicines Agency Note for Guidance on Good Clinical Practice, and registered at ClinicalTrials.gov (NCT00360906 and NCT00785473).

In light of current knowledge regarding the potential ability of DMTs to change the disease course in demyelinating disease and the focus on early intervention (361), it may seem unethical that treatment with IFN-β was delayed for 6 months in the OFAMS study (359). However, it is pertinent to comment that regulations regarding the use of IFN-β in RRMS in Norway at the time the study was conducted demanded formal approval from the regulatory authorities. As the duration of this approval process was usually about 6 months, it is evident that earlier treatment initiation was at this time rarely possible.

8.2. Measurements

8.2.1. The Omega-3 Fatty Acid Treatment in Multiple Sclerosis study

Laboratory tests were carried out in conjunction with the planned clinical visits and whole blood and serum samples were shipped in a frozen state and stored at -80° until *post study* determination of HLA-DRB1 status and measurements of retinol, 25(OH)D (i.e. $25(OH)D_2$ and $25(OH)D_3$), α-tocopherol and 11 markers of systemic inflammation. HLA-DRB1 typing was carried out at the Department of Immunology and Transfusion Medicine, Oslo University Hospital Rikshospitalet, Oslo, Norway, with the SeCore DRB1 Locus sequencing kit (Invitrogen, Carlsbad, CA, USA) (183). 25(OH)D, retinol and α-tocopherol concentrations were measured at the Department of Medical Biochemistry, St. Olavs' Hospital, Trondheim University Hospital, Trondheim, Norway, with Reagent kits for high-performance liquid chromatography (HPLC) determination of s-retinol and alpha-tocopherol (CHROMSYSTEMS Instruments & Chemicals GmbH, München, Germany) on an Agilent 100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) (229;249), and the radioimmunoassay 25(OH)D RIA kit (Immunodiagnostic Systems, Boldon, UK) (183). The inflammation markers were measured with immunoassay kits from R & D Systems (Stillwater, MN, USA) at the Research Institute of Internal Medicine, Oslo University Hospital Rikshospitalet, Oslo, Norway (267). Lastly, Nabs against IFN-β were detected with a myoxvirius-resistant protein A messenger ribonucleic acid induction assay at the Department of Neurology, Haukeland University Hospital, Bergen, Norway (256). The personnel responsible for the laboratory analyses had no prior involvement in the study or knowledge of its results.

MRIs of the brain were acquired monthly with 1,5 Tesla scanners the first nine months of the study, and at months 12 and 24 following guidelines for use of MRI in trials in established MS (88). Blinded evaluation of images for occurrences of new T1-weighted Gd^+ lesions, new or enlarging T2-weighted lesions and the composite of these outcomes (labeled as combined unique activity) was carried out during the original study by two independent radiologists.

The vitamin and inflammation marker measurements had all an intra-assay coefficient below 10%, which indicated that technical issues were likely to account for only a small amount of the overall variation in the analyses. However, we cannot rule out that storage and handling may have had different effects on the stability of the different parameters, as the measurements were not repeated in different serum batches. With respect to the limited number of patients included in the study and a follow-up period of only two years, it is clear that the obtainment of MRI examinations was important due to its ability to detect subclinical disease activity (87). Furthermore, the frequent scans during both the

period before and after IFN-β initiation made it possible to evaluate the anti-inflammatory effect of serum vitamin D levels both alone and together with IFN-β. In addition, the inclusion of both T1-weigheted images after contrast administration and measurements of the alteration in size of the T2-weighted lesions made it possible to detect a larger amount of new or ongoing radiologic disease activity. However, as only conventional MRI techniques were applied, it is likely that the detection of grey matter disease activity was limited (68).

8.2.2. The University of North Norway trial

Routine blood tests were obtained every 3 months during the trial, but only serum samples from baseline and month 24 were stored for later analysis. The serum samples were kept at -70° during storage until transport on dry ice in conjunction with *per study* measurements of $25(OH)D_3$ levels and *post study* determination of serum inflammation marker concentrations. The $25(OH)D_3$ levels were assessed with a tandem mass spectrometry method on API 3000 mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA, USA) at the Hormone Laboratory, Haukeland University Hospital, Bergen, Norway (360). Serum inflammation marker measurements were obtained as described in the previous section. Again, all laboratory analyses were carried out in a blinded fashion.

As in the OFAMS study, the serum inflammation marker measurements had an intra-assay variation coefficient under 10% in the trial at the UNN. However, it is clear that the mean baseline values differed between the studies. Still, the differences seem to be related to varying inter-individual factors among the patients in the studies, as no clear overall pattern was evident between the studies (i.e. the levels were not consistently lower in one study than the other). Furthermore, the mean baseline vitamin D level was somewhat higher in the OFAMS study than the trial at the UNN. However, it seems unlikely that this is related to the measurement of only $25(OH)D_3$ levels in the latter study, as the $25(OH)D_2$ levels usually make a negligible contribution to the overall 25(OH)D serum level (159).

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8.3. Statistics

8.3.1. Article I

We included 85 of the original 92 OFAMS patients in this work, as seven patients were left out due to missing vitamin and/or inflammation marker measurements. Serum values of 11 inflammation markers and vitamin A, D and E were determined at 9 different time points (total number of measurements are provided in Table 2 of Article I in the Appendix). Both the omega-3 fatty acid and the placebo supplementation contained vitamin E (α-tocopherol), while differences in vitamin A (retinol) and vitamin D (25(OH)D) concentrations were only driven by individual and seasonal factors. This means that the results obtained with regards to vitamin A and D can be characterized as observational. Furthermore, the patients were analyzed collectively, as no difference had been found in the serum levels of the markers between patients on omega-3 supplementation or placebo in the initial investigation of the inflammation markers in this cohort (267).

The associations between the level of each inflammation marker and each vitamin were examined with linear regression models for hierarchical data after adjusting estimates for clustering. Three sets of models were developed, corresponding to the whole trial period, the period before and the period after initiation of IFN-β treatment. Log-transformed inflammation marker values were employed in the models, as most of the values were not normally

distributed. All models were estimated with the SAS MIXED procedure that incorporated both fixed (non-random) effects for vitamin values and random effects for patients. The models were used to obtain crude estimates for the change in inflammation marker levels associated with increasing levels of the different vitamins (Table 2 and 3 in Article I in the Appendix). Results were presented as the associated percent changes in the inflammation marker concentrations with every whole-study mean standard deviation increase for each vitamin (the latter is found in Supplementary Table 1 of Article I in the Appendix). Similar supplementary models were developed with adjustment for vitamin levels at baseline (vitamin A and E) or whole-study mean levels (vitamin D). Finally, multivariate models were estimated that controlled for gender, age, BMI, HLA-DRB1*15 positivity and study arm.

The models that estimated the crude associations between the whole-study levels of the vitamins and the inflammation markers were selected as the primary outcome measures of the study, as these models included the largest datasets and therefore had the highest statistical power. The significance level was lowered with a Bonferroni correction due to the large amount of simultaneously assessed outcomes, and only p-values ≤ 0.0015 were considered significant (3 vitamins and 11 inflammation markers that together constitute 33 tests) (362).

8.3.2. Article II

This paper was again based upon measurements from the OFAMS study, however, the number of patients and measurements included varied between the analyses as 3 patients with missing inflammation marker values had MRI and vitamin D measurements. Thus, data from 88 patients were utilized in analyses of serum vitamin D levels and MRI activity (total numbers of measurements can be found in Figure 1 and 2 and Table 2 and 3 in Article II in the Appendix), while 85 patients were included in analyses of serum levels of vitamin D and 10 inflammation markers (the total amounts of measurements are provided in Table 4 in Article II in the Appendix).

Four separate statistical strategies were devised to analyze the relationship between serum levels of vitamin D and the anti-inflammatory effect of IFN-β treatment. Primarily, a hierarchical logistic regression model was estimated to assess the association between MRI activity (present or absent) and crude serum level of vitamin D during the whole study by using the SAS GLIMMIX procedure. The model incorporated fixed effects for continuous vitamin D values and dummy variables for each study month and their interaction with the vitamin D level. Only coinciding measurements of serum vitamin D and MRI activity were utilized and pooled together from all the patients at each of the 8 time points. The model estimated the MRI activity associated with 50, 75, 100 and 125 nmol/L 25(OH)D at 4 time points before and after initiation of IFN-β therapy (Figure 1 in Article II in the Appendix). A second hierarchical regression model was estimated to assess the association between serum vitamin D level and MRI activity in the period before and after introduction of IFN-β, and the reduction in the MRI activity between the two periods. This model was based on all available MRI scans and serum vitamin D values (measurements from 12 and 9 different time points, respectively). The vitamin D measurements were first adjusted for seasonal fluctuations with a previously developed model for this variation in the OFAMS cohort (363), and individual mean values for the whole study period were subsequently calculated and ordered in ascending fashion before being divided into quartiles (i.e. the 1st quartile contained the 22 patients with the lowest and the 4th quartile the 22 patients with the highest mean levels). Furthermore, the model also incorporated fixed effects for quartiles, a dummy variable for IFN-β treatment and random effects for patients. The model estimated the MRI activity for the different quartiles before and after introduction of IFN-β and the reduction in MRI activity between the

periods (Table 3 in Article II in the Appendix). In addition, *p*-values for trends in the reduction in MRI activity were calculated in order to examine if the reduction differed between the quartiles (i.e. was affected by the serum vitamin D level). Thirdly, a similar hierarchical regression model was fitted to assess if the seasonally adjusted serum vitamin D levels in the period before IFN-β initiation affected the MRI activity in the subsequent period during treatment. However, only vitamin D measurements from baseline until study month 3 and MRI measurements from study month 7 to 24 were included to avoid coinciding measurements and to take in to account that the effect of IFN-β therapy on MRI activity is usually first evident after about 1 month (364). The MRI activity was estimated with fixed effects for quartiles and random effects for patients, and *p*-values were calculated for difference between activity in the 4th quartile and the 1st, 2nd and 3rd quartiles (Figure 2 in Article II in the Appendix). Lastly, linear mixed models were estimated to compare the serum levels of vitamin D and the 10 inflammation markers between the two study periods (measurements from 4 time points before and 5 time points after initiation of IFN-β therapy). The patients were again categorized into quartiles by their deseasonalized whole-study vitamin D averages and the models contained random effects for patients and fixed effects for a dummy variable that indicated the presence or absence of IFN-β treatment. The mean values of vitamin D and the different inflammation markers were estimated before and after introduction of IFN-β for each quartile (Table 2 and 4 in Article II in the Appendix). Additionally, *p*-values for trends between mean ratios of the inflammation markers and patient quartiles were calculated to asses if vitamin D status affected the alterations of the inflammation markers (Table 4 in Article II in the Appendix). All models employing patient quartiles were adjusted for patient age and omega-3 or placebo allocation.

The primary outcomes were defined as the differences in reduction of MRI activity between the patient quartiles, as the models used to estimate these results incorporated the largest amount of available data. The significance level was again adjusted for multiple testing, but a *p*-value ≤0.01 was deemed significant as the MRI outcomes were not considered to be independent values (195).

8.3.3. Article III

The results reported in this article stem from 68 patients from the UNN trial and included measurements of serum levels of vitamin D and 11 inflammation markers at study onset and conclusion. As the amount measurements in this study was small compared to the amounts in the studies in Article I and II, and we no longer examined different associations, a more simplistic statistical approach was devised.

The effect of vitamin D_3 supplementation on systemic inflammation was investigated by comparing the alteration of the inflammation markers during the study between the patients receiving high-dose oral vitamin D_3 and the ones on placebo. In addition, a similar comparison was made between patients in the vitamin D group that were on or off DMTs at the initiation of the study. Furthermore, as the UNN cohort was made up of an equal amount of patients with and without immunomodulatory treatment at baseline, we also assessed the possible effect of immunomodulatory treatment on the initial inflammation marker concentrations. In order to provide comparable results between this and the earlier studies, we continued to report mean values for vitamin D and the inflammation markers (Table 2 and 3 in Article III in the Appendix). However, the comparison between the values were calculated with non-parametric Mann-Whitney tests, as the distribution of the measurements were mostly skewed (Table 2, 3, 4 and 5 in Article III in the Appendix). Moreover, linear regression models for each of the inflammation markers were estimated to compare the mean change in the markers

during the study between the vitamin D and the placebo group (Table 4 in Article III in the Appendix). These models were adjusted for baseline inflammation marker values, change in use of DMTs during the study, high UVR exposure (use of tanning bed or vacation to lower latitudes) during the last 6 months before study initiation, and high dietary vitamin D intake (>7.5 μg/day) the last 12 months before study initiation.

The main results in this article were obtained with the linear regression models. As 11 different inflammation markers were examined, we once more adjusted the significance level and only considered *p*-values ≤0.005 to be significant (195).

9. Results

9. 1. Article I

The main finding from the examination of the relationship between the serum levels of vitamin A, D and E and the panel of systemic inflammation markers in the OFAMS cohort were that the vitamins were associated with distinct inflammatory pathways. More specifically, the analysis of the whole study period showed an inverse association between PTX3 and vitamin A (-9.4% decrease with each 0.5 μmol/L increase of retinol), positive associations between both sFRP3 and IL-1Ra and vitamin D (5.5% and 11.3% increase, respectively, with each 26.8 nmol/L increase of 25(OH)D), and a positive association between CXCL16 and vitamin E (5.8% increase with each 8.0 μmol/L increase of α-tocopherol) (Table 2 in Article I in the Appendix). Furthermore, these associations were sustained after controlling for patient characteristics (age, gender, BMI and HLA-DRB1*15-positivity), study arm, and individual baseline vitamin levels (vitamin A and E) or mean vitamin levels throughout the study (vitamin D).

Secondary analyses showed that the relationships seemed to be modified by IFN-β therapy, as the associations differed between the period before and after initiation of IFN-β (Table 3 in Article I in the Appendix). Moreover, only the association between increasing vitamin A and decreasing PTX3 concentrations was evident when the periods were analyzed separately, while the associations between vitamin D and IL-1Ra and sFRP3 and vitamin E and CXCL16 were isolated to the period before and after initiation of treatment, respectively. Lastly, the levels of PTX3, IL-1Ra, sFRP3 and CXCL16 were neither different between patients with or without relapses nor between patients that were clinical stable or worsening (EDSS score increasing by ≥0.5 during the study).

9.2. Article II

In order to explore the potential interaction between vitamin D status and IFN-β therapy on inflammation among the patients in the OFAMS study, we analyzed associations between serum vitamin D levels and MRI activity in addition to systemic inflammation marker levels. The primary result was that IFN-β treatment had a profound anti-inflammatory effect on both the MRI activity and the inflammation markers, but no further beneficial effect was found among patients with high compared to low serum levels of 25(OH)D. In more detail, the initiation of IFN-β therapy was accompanied by a drop in odds ratio (OR) for all MRI outcomes to about 0.4 (Figure 1 in Article II in the Appendix) and an OR for reduction of all MRI outcomes between 2.7-4.8 across patient quartiles based upon ascending individual deseasonalized mean 25(OH)D levels (Table 3 in Article II in the Appendix). A similar result was found when the patients were categorized in accordance to their 25(OH)D levels in the period preceding the initiation of IFN-β (Figure 2 in Article II in the Appendix). Furthermore, the patients' mean serum levels of sTNF-R1, CXCL16, IL-1Ra, OPN and OPG were all higher and MMP-9 lower in the period during IFN-β treatment than in the period without treatment, and the change in the concentrations was not dependent on their vitamin D status (Table 4 in Article II in the Appendix). Adjustment of the categorical analyses for age at enrolment and omega-3 or placebo allocation led to marginally altered results with no effect on the overall conclusions.

In contrast to our earlier report (183), this investigation was not specifically designed to examine the relationship between the serum level of vitamin D and CNS inflammation. Nevertheless, we observed that the ORs for MRI activity were consistently lower among patients with the highest compared to the lowest crude or seasonally adjusted 25(OH)D levels (Figure 1 and Table 3 in Article II in the Appendix). However, the magnitude of difference between the ORs in 1st and the 4th quartiles in both study periods was 1.5-2.5 folds lower than the difference within these quartiles between the period before and after IFN-β initiation. Similar observations were made with regards to the serum inflammation markers, as patients in the 1st compared to the 4th quartile had higher concentrations of IL-1Ra, OPN, CCL21 and sFRP3, and lower concentrations PTX3, but again these differences were small compared to the effect associated with initiation of IFN- β therapy (Table 4 in Article II in the Appendix). Finally, we assessed the effect of IFN-β initiation on the seasonally adjusted vitamin D levels. As shown in Table 2 in Article II in the Appendix, the mean 25(OH)D levels rose after initiation of therapy, but the increase was very modest.

9.3. Article III

As vitamin D was associated with several inflammatory pathways in the RRMS patients in the OFAMS study, we investigated if vitamin D_3 supplementation had a similar effect on the systemic inflammation markers among the RRMS patients in the independent RCT at the UNN. The chief outcome was that two-years of high-dose oral vitamin D_3 supplementation led to a marked elevation of the serum 25(OH)D_3 levels in the vitamin D group, but had no effect on the panel of serum markers inflammation markers when compared to placebo. A more meticulous presentation of the results is that the serum level of 25(OH)D_3 in the vitamin D group increased by about 120% (~70 nmol/L increase) during the 24-month trial period (Table 2 in Article III in the Appendix), while only the serum level of sTNF-R1 increased by about 10% (Table 4 in Article III in the Appendix). However, the rise in the sTNF-R1 concentration was not due to the vitamin D_3 supplementation, as a coinciding increase of roughly 7% was found in the placebo group. These findings were unaltered by adjustments made in order to control for baseline inflammation marker levels, factors associated with a high baseline vitamin D level, and change in use of immunomodulatory therapy during the study.

In light of these and earlier results, an auxiliary investigation was carried out to determine if IFN-β treatment still had a major impact on the examined serum inflammation markers in this cohort of RRMS patients. This analysis confirmed several of the earlier findings, as patients on DMTs at baseline (91% on IFN-β) had higher levels of CXCL16 and IL-1Ra and a strong trend for lower levels of MMP-9 when compared to patients without treatment (Table 3 in Article III in the Appendix). Lastly, the lack of an apparent interacting anti-inflammatory effect between high serum vitamin D levels and DMT use was also seemingly confirmed, as the change in serum inflammation markers values was similar among the patients in the vitamin D group that were on or off immunomodulatory therapy at the beginning of the trial (Table 5 in Article III in the Appendix).

10. Discussion

10.1. The relationship between vitamin D and systemic inflammation

The results from the OFAMS cohort in Article I indicated that increasing serum levels of vitamin A, D and E could possibly affect a range of inflammatory pathways in RRMS. However, the observational findings of positive associations between vitamin D and the anti-inflammatory markers IL-1Ra and sFRP3 were perhaps the most interesting result. This was further corroborated by the results in Article II, that showed that the mean concentrations of IL-1Ra and sFRP3 were consistently higher in the 4th than in the 1st quartile (i.e. among the patients with the highest compared to the lowest vitamin D levels).

The association with IL-1Ra suggests that vitamin D can limit inflammation already during its initiating phases by constraining the pro-inflammatory effects of IL-1 (365). The systemic effect of IL-1 is far reaching, but its ability to promote the maturation of pro-inflammatory Th17 cells may be especially important in RRMS where the expression of IL-17 has been found to be increased in the blood during relapses and in active MS lesions (366-368). Moreover, as *in vitro* studies have found that active vitamin D can inhibit expression of IL-17 in Th cells (369), it is possible that high vitamin D levels may reduce disease activity by inhibiting the induction of Th17 cells both directly and indirectly by limiting the effect of IL-1 through an elevation of the IL-1Ra level. Thus, the finding of a positive association between IL-1Ra and vitamin D levels may contribute to the explanation of why relapse rates seem to be reduced when the naturally occurring vitamin D levels are high (370). Furthermore, the promoting effect of vitamin D on IL-1Ra production may have played a part in the finding of a difference in change in serum IL-17 levels between the high-dose oral vitamin D_3 groups and the low-dose vitamin D_3 or placebo groups in two recent RCTs among RRMS patients on IFN-β treatment (371;372). Still, the effect of vitamin D intake on IL-17 concentrations in RRMS seems uncertain, as the results were heterogeneous with respect to alterations in the IL-17 level in the high-dose group in the study by Golan *et al.* (371), and the difference between the study arms was driven by an increase in the placebo group in the study by Toghianifar *et al.*(372). In addition, a trend for an increasing IL-17 concentrations was noted in a third contemporary RCT with a similar design (373). In light of these discrepancies, it is noteworthy that the association between vitamin D and IL-1Ra differed substantially between the period before and after IFN-β initiation in Article I. This will be further commented upon in section 10.3.

The finding of an association between increasing levels of vitamin D and sFRP3 may also implicate vitamin D in the regulation of Wnt-signaling. Although the relationship between vitamin D and the Wnt-pathway has not been examined in MS, it has been found that active vitamin D can reduce Wnt-signaling in human cancer cells *in vitro,* and that a similar effect was found in intestinal cells obtained from patients with sporadic colorectal adenomas after vitamin D_3 supplementation (374). It is therefore plausible that vitamin D can modulate the Wnt-pathway in human T cells and cells in the CNS. With regards to the potential effect of sFRP3 on T cells, it has been shown that Wnt-signaling is especially important for the proliferation and survival of memory CTLs (321). As the CTLs are the dominant T cell population in active MS lesions (63), and CTL clones isolated from brain biopsies of MS patients have been found to persist in their CSF and blood (375), it is possible that vitamin D may reduce disease activity by inhibiting the proliferation and survival of these detrimental CTLs though increasing the sFRP3 concentration both within the CNS and in the systemic circulation. Furthermore, in animal models of demyelination, increased Wnt-signaling has been found to inhibit

maturation of oligodendrocyte progenitors (376), while addition of active vitamin D may promote this maturation (377). This indicates that vitamin D may have beneficial effect during remyelination, as it may interfere with the Wnt-pathway either directly or indirectly by increasing the sFRP3 level. Even though the 25(OH)D level in the human CNS is usually low, it has been shown that the level in the CSF correlates with the serum concentration and increases with loss of BBB integrity (378). This suggests that patients with active RRMS and high serum levels of 25(OH)D could also have elevated levels of active vitamin D and sFRP3 within the CNS, as VDR expression and vitamin D metabolizing enzymes are found throughout the human CNS. If this is the case, sFRP3 and active vitamin D may both contribute to the mending of the apparent failure of myelin repair in MS (379). With this in mind, it is interesting to note that a 50 nmol/L increment of serum 25(OH)D was associated with a reduced brain volume loss over 5 years in a recent epidemiological study of about 500 CIS patients (181). However, it could be that vitamin D only has an effect on brain atrophy during early phases of the disease, as a similar result was not found in a subsequent epidemiological study of nearly 1500 RRMS patients (182).

10.2. The effect of vitamin D_3 supplementation on systemic inflammation

To address the possibility that the observed associations between vitamin D and IL-1Ra and sFRP3 in the OFAMS cohort (Article I and II) could be due to a direct effect of vitamin D on the inflammation markers, the serum inflammation markers were measured before and after two years of high-dose oral vitamin D_3 intervention in UNN cohort in Article III. As expected, the 25(OH)D_3 concentration changed drastically in the vitamin D group after two years with the mean level reaching above 120 nmol/L. Moreover, a minor, but significant, mean increase of 6 nmol/L was also noted in the placebo group that may be attributed to an increased awareness of the enrolled patients toward vitamin D obtainment, as high dietary intake of vitamin D and UVR exposure were common among them already at baseline (380). However, despite similar inflammation maker levels in the vitamin D group and the placebo group at baseline and a substantial difference in 25(OH)D_3 level between the study arms at study conclusion, no differences were found when the mean change in inflammation marker levels was compared between the groups. This finding contrasted with the expected increase of at least 10-20% for IL-1Ra and sFRP3 based on the effect size of the associations in Article I. Nevertheless, a similar result was reported by Burton *et al.* and Kimball *et al*. in an earlier one-year open-label trial of escalating doses of oral vitamin D_3 supplementation in a cohort of 49 MS patients with a comparable distribution of patients on and off DMTs, as no alterations were found with respect to a total of 15 systemic markers of inflammation (including MMP-9 and OPN) in spite of an increase of 25(OH)D to over 150 nmol/L (188;191). Furthermore, a recent study on serum inflammation markers among 59 RRMS patients that received 20000 IU/week oral vitamin D_3 or placebo as add-on to IFN-β treatment for one year found no differences between the vitamin D group and the placebo group for 13 inflammation markers (including LAP (as a proxy for TGF-β) if adjusting the significance level for multiple testing (195;373).

It is tempting to primarily attribute the differing observational and interventional results to an insufficient amount of patients included in the intervention trial, too few and widely spaced serum samplings in the intervention trial, the much lower preceding disease activity in the UNN cohort than the OFAMS cohort, and differences in the design of the studies. However, the results could also suggest that more fundamental issues are to blame. More specifically, can the effect associated with an increased vitamin D level in an observational setting really be compared to the effect of an increased vitamin D level attained trough supplementation, does the vitamin D metabolism in immune cells differ from that in renal tissues, and can low levels of vitamin D rather be a result than a cause of inflammation? These topics will be further discussed in section 10.6.

10.3. Vitamin D status and interferon-β therapy

The results from our initial investigations of the systemic inflammation markers in the OFAMS cohort indicated that initiation of IFN-β treatment had a profound effect on the serum concentration of the majority of the markers (267). However, it was not clear if this effect was shared by all the patients, as an earlier study claimed that a good vitamin D status was a prerequisite for a clinical effect of IFN-β treatment (187). Furthermore, our earlier investigation into the relationship between serum vitamin D levels and MRI activity in the OFAMS study found an inverse association between these parameters only prior to the initiation of IFN-β therapy (183).

In light of these reports, it was clear that a further exploration was needed in order to assess the possible existence of interacting effects between serum vitamin D levels and IFN-β therapy among RRMS patients. The results in Article I provided some support for the earlier finding of an anti-inflammatory effect of increasing vitamin D levels only before treatment onset (183), as the positive associations between vitamin D and IL-1Ra and sFRP3 in the period before treatment initiation were lost during treatment. However, the associations were still evident when samples from the whole study period were analyzed together. To expand upon this, we devised in Article II several new approaches to evaluate the effects of both the serum vitamin D status and the IFN-β treatment on MRI activity and systemic inflammation. Although investigated in a new manner, the initial finding of a reduced MRI activity with increasing seasonally unadjusted vitamin D values before initiation of IFN-β treatment was expected due to the utilization of the same measurements as in our previous analysis (183). Nevertheless, the substantial drop in MRI activity across a range of vitamin D levels after initiation of IFN-β therapy was a new finding even though it also reflected earlier findings of a pronounced effect of IFN-β on MRI activity in the original OFAMS study (359). Still, the initial analysis in Article II was not adjusted for the potential seasonal variation in MRI activity among MS patients (381). A second analysis was therefore conducted with the patients divided into quartiles according to their mean seasonally adjusted vitamin D level. The result form this analysis clearly indicated that the initiation of IFN-β treatment was associated with a significant reduction for all MRI outcomes irrespective of the patients' vitamin D level. In addition, a similar finding was made when the patients were divided into quartiles by their mean seasonally adjusted vitamin D level in the period before treatment. These results opposed the results reported by Stewart *et al*. (187). However, it must be emphasized that the findings are not directly comparable, as the focus in the latter study was directed against the effect of vitamin D status and IFN-β treatment on clinical disease activity Nevertheless, the findings in Article II were in line with results from two earlier small-scale RCTs of high-dose vitamin D supplementation in RRMS patients on DMTs that reported no difference in the number of new Gd^+ lesions between patients on intervention and either placebo or maintenance doses of vitamin D (192;198).

Despite the earlier findings of associations between several of the inflammation markers and the MRI activity (267), it was still imaginable that the effect of IFN-β on the serum inflammation markers could be dependent on the serum vitamin D level. The effect of IFN-β initiation on the inflammation markers was therefore also analyzed with patient quartiles based on mean seasonally adjusted vitamin D levels for the whole study period. As reported previously, commencement of IFN-β treatment was associated with a significant increase in the serum levels of CXCL16, IL-1Ra, OPG and OPN and a decrease in the MMP-9 level. Moreover, the alterations were similar across patient quartiles, again implying that the effect of IFN-β on the inflammation markers was also independent of the patients' vitamin D status. In addition, the finding of a close to 60% higher IL-1Ra concentration after compared to before treatment strongly suggested that the effect of IFN-β on this marker largely overshadowed that of vitamin D. Furthermore, the patients on DMTs

(chiefly IFN-β) in the trial at the UNN (Article III) were also found to have significantly higher levels of CXCL16, IL-1Ra and OPN and lower levels of MMP-9 compared to the patients without treatment. In addition, even though the analysis only included a limited number of cases, the increasing vitamin D levels did not seem to enhance the effect of IFN-β on the systemic inflammation markers among these patients neither.

Finally, it had been suggested that the anti-inflammatory effect of IFN-β was partly due to a modulation of vitamin D metabolism (187). However, no difference had been noted between the vitamin D level before or after initiation of IFN-β therapy in our earlier study of the OFAMS cohort (183). Still, this earlier analysis did not take into account that most of the patients were enrolled during late winter and early spring. This meant that a majority of the vitamin D measurements obtained after IFN-β initiation would primarily reflect the naturally decreasing vitamin D levels during autumn and wintertime. By seasonally adjusting the measured vitamin D levels, the skewed inclusion was better accounted for and we found that IFN-β initiation was associated with a 3 nmol/L mean increase in the 25(OH)D level for the whole cohort. Interestingly, an about 2 nmol/L higher baseline $25(OH)D_3$ level was also noted among patients on DMTs compared to the patients on no medication in Article III. Nevertheless, according to the noted relationship between vitamin D and IL-1Ra in Article I, this increase in the vitamin D level would have only increased the IL-1Ra level by about 1%.

In summary, the findings clearly indicated that the substantial anti-inflammatory effects of IFN-β treatment on both MRI activity and systemic inflammation markers were independent of the patients' vitamin D status. Still, it may be argued that the results could be due to the limited number of patients included in the studies. However, according to the estimated effect of increasing vitamin D levels on MRI activity from the recent observational studies of CIS and RRMS patients on IFN-β, a close to 50% difference in MRI activity between the 1st and the 4th patient quartile would have been expected in Article II if the effect of IFN-β was dependent on the patients' vitamin D status (181;182). Furthermore, the finding of no interacting effect between vitamin D status and IFN-β therapy on the serum inflammation markers is concordant with the large majority of negative results reported for examinations of markers of systemic inflammation in studies of vitamin D_3 supplementation among RRMS patients on IFN-β therapy (193;373). A reason for this may be that vitamin D and IFN-β act on similar inflammatory pathways, and that the effect of IFN-β on these pathways largely outweighs that of vitamin D. The latter is illustrated by the findings regarding IL-1Ra in the OFAMS cohort, and a similar situation may also be suspected to be the case for a wide range of other inflammation markers as a 98% overlap between vitamin D- and IFN-β-regulated genes has been reported (382). Nevertheless, the assumption that an increasing vitamin D levels do not interact with the anti-inflammatory effects of IFN-β treatment is in conflict with findings from the aforementioned studies among CIS and RRMS patients (181;182), and one RCT of high-dose vitamin D_3 intervention among RRMS patients (197). Possible explanations behind this discrepancy will be provided in section 10.6.

10.4. The relationship between vitamin A and E and systemic inflammation

Earlier studies conducted in the OFAMS cohort showed that the serum level of vitamin A and E were quite stable throughout the study period, even though the latter was somewhat increased by both the omega-3 and the placebo tablets (229;249). It may therefore be suggested that this modest variation reduced our ability to find associations between these vitamins and the systemic inflammation markers, and that this also reduced the effect size of the uncovered associations. Nevertheless, the findings of an inverse association between PTX3 and vitamin A levels and a positive association

between CXCL16 and vitamin E levels in Article I point toward a role for these vitamins during the inflammatory process in RRMS.

The finding of declining PTX3 levels with increasing vitamin A levels is in line with earlier *in vitro* results showing that vitamin A can affect monocyte differentiation to mature DCs (209), which constitutes one of the main cellular subsets driving PTX3 production during inflammation (292). Moreover, the inhibitory effect of vitamin A on production of mature DCs may subsequently affect T cell activation and could be a reason behind the finding of a larger reduction in the T cell response against a CNS antigen in the vitamin A group than the placebo in a recent small-scale RCT of vitamin A supplementation in RRMS patients on IFN-β therapy (230). Still, the potential beneficial effect of vitamin A supplementation may only be temporary, as follow-up studies from the same research group found that 6 months of intervention led to a reduction in RAR expression in blood monocytes from these patients (383), and after 12 months no difference was found between patients on supplementation or placebo with regards to clinical or radiological disease activity or change in EDSS (235). However, it could also be speculated that the lack of difference after 12 months of intervention may be related to a reduction in the PTX3 mediated inhibition of immune cell trafficking to the CNS, neural protection, and CNS remodeling and repair that counteracted the beneficial effect of vitamin A on T cell activation (293;294;384).

Despite the low effect size of the association between increasing vitamin E and CXCL16 levels, it is still evident that this association may be of clinical importance as the level of CXCL16 was found to be associated with reduced MRI activity in our initial investigation (267). The potential role of CXCL16 in demyelination has been somewhat difficult to comprehend, as one of its main functions is to attract activated T cells (275). However, elevated serum levels of vitamin E may promote higher CXCL16 levels in the peripheral circulation and thereby hamper T cell migration to the CNS. Support for this has been found in EAE, where increased peripheral expression of CXCL16 after pre-treatment with a strong immunological stimulus led to a diversion of encephalitic T cells away from the CNS (279). Furthermore, as IFN-β therapy may increase the serum concentration of vitamin E (248), it could be suggested that the increased serum levels of CXCL16 during IFN-β use was related to an increased vitamin E level. However, no clear increase in the vitamin E level was seen upon introduction of IFN-β in this cohort (249), indicating that the treatment did not promote CXCL16 production solely via vitamin E. Nevertheless, the finding of an association between vitamin E and CXCL16 only during IFN-β treatment implies interacting effects between vitamin E and IFN-β on CXCL16. Interestingly, in our earlier study of the relationship between serum vitamin E levels and MRI activity among the OFAMS patients (249), we also found an inverse association between these measurements only during IFN-β therapy. This again suggests that the joint action of vitamin E and IFN-β on CXCL16 may be clinically relevant. In addition to its potential peripheral effects, it is also possible that CXCL16 plays a beneficial role within the CNS. Support for this notion comes from experiments in mice that have found increased CXCL16 production in both astrocytes and microglia during inflammatory conditions, and that this ultimately reduced neuronal death (385). As vitamin E can be transported across the BBB (237;386), it may be envisioned that an increasing serum vitamin E level could lead to a subsequent increase of vitamin E and CXCL16 within the CNS. If this is the case, vitamin E may have neuronal protective effect either thorough its anti-oxidative properties or its effect on CXCL16 production. Furthermore, the lack of these protective effects may provide a partial explanation to the findings of atrophy of the spinal cord and reduction of Purkinje cells in the cerebellum among patients with genetic abnormalities that affect vitamin E transport (386). However, it remains uncertain if this has any clinical relevance in MS, as, to my knowledge, the possible relationship between CNS atrophy and vitamin E or CXCL16 levels within the CNS or systemically has yet to be examined in patients with MS.

Although not significantly associated with increasing serum levels of vitamin A, it was also intriguing to find trends for positive relationships with CXCL16, OPG and TGF-β and an inverse relationship with IL-1Ra. This underlines the potential complex role vitamin A may play in inflammation in RRMS and indicates that vitamin A and D may have opposing effects on IL-1Ra production, while vitamin A and E may both promote higher CXCL16 levels. The possible conflicting effects of vitamin A and D on IL-1Ra production may be related to a competition between VDR and RAR for their common nuclear receptor partner RXR and between the VDR-RXR and RAR-RXR hetrodimers for common gene response elements. An earlier *in vitro* examination of the latter showed that gene transcription was governed by complex interactions with interference between the hetrodimes for some genes (387), but it is unknown if this also applies for genes in the IL-1 cytokine family. Finally, the finding of an association between vitamin A and CCL21 only after initiation of IFN-β treatment complicates the picture even more, and indicates that vitamin A may act in concert with IFN-β when it comes to regulating T cell trafficking in RRMS (110;268).

10.5. Association between systemic inflammation and clinical activity

The findings in Article I and II indicated that vitamin A, D and E were primarily associated with anti-inflammatory markers. This result was in line with our prior finding of an associated beneficial effect of increasing vitamin A, D and E levels on MRI activity (183;229;249). Based on this, it was somewhat surprising that the levels of the inflammation markers were not associated with clinical disease activity. However, this concurred with the earlier reports of no difference in the vitamin levels between the patients with or without relapses or disease worsening in the OFAMS study. Moreover, it is evident that the OFAMS study was most likely underpowered with concern to addressing if the selected inflammation markers could be employed as clinically useful biomarkers. In addition, these results could also be due to the prescheduled timing of the serum sampling (i.e. not during relapses) and the low relapse rates and limited disease worsening during the studies (199;359).

10.6. General methodology

10.6.1. Confounding

Medical research is often focused upon examining the effect of a specific exposure on a specific outcome. However, an outcome is rarely the result of a single exposure. A key aspect is therefore the identification of other exposures that are associated with both the specific exposure and the specific outcome, but do not represent a link between them (388). Furthermore, these exposures can also be associated with the specific outcome in the absence of the specific exposure. The non-specific exposures are usually denoted as confounding factors or simply confounders. The identification of confounders makes it possible to adjust the conducted analyzes for these factors and thus find the true effect of the specific exposure on the specific outcome. However, the uncovering of a confounder may be a challenging task, as it presupposes that the relationships between the exposure, the outcome and the potential confounder are already known.

In the studies included in this Thesis, possible confounding factors were identified primarily through their known association with the vitamins or the inflammation markers, or their differing distribution between the patient groups. More specifically, the analyses in Article I were adjusted for gender, age and BMI at baseline, as these factors had been shown to affect the vitamins and other serum markers of inflammation (162;164;208;239;389), HLA-DRB1*15, as it had been

found to possibly interact with vitamin D status (163), and allocation to omega-3 or placebo supplementation, as these supplements differed in vitamin E content (249). In Article II, no adjustments were made for gender, BMI and HLA-DRB1*15, as the results in Article I were not altered in a meaningful fashion by adjustment for these factors and thus indicated that these factors did not represent influential confounders in his patient material. However, adjustments were made for the patients' age at study inclusion and allocation to omega-3 or placebo supplements, as the distribution of these factors differed between the patient quartiles. With concern to Article III, adjustments were applied for prior high vitamin D intake and high UVR exposure, as these factors had been found to be closely associated with the baseline vitamin D status in this patient population (390). In addition, we controlled for change in use of DMTs, as this had been found to affect the inflammation markers in Article I and in prior studies (267;308;311;318;350;357). Even though several factors were controlled for, it is evident that the list of identified potential confounders was not exhaustive. Furthermore, it is possible that the findings in Article I and II could have been altered by adjustments for high UVR exposure. Similarly, adjustments for the expression of HLA-DRB1*15 and vitamin A and E levels could have affected the results in Article III.

In addition to the adjustments mentioned, the models in Article I were also corrected for the patients' individual baseline vitamin A and E values and their individual mean vitamin D values, as these values differed substantially between the patients in the OFAMS cohort and could have influenced the results. The adjustment for the mean vitamin D levels rather the baseline levels was founded upon the fact that the latter was highly affected by the patients' season of enrollment. Similarly, the models in Article III were adjusted for the patients' baseline inflammation maker values, as there was a marked intra-individual variation for most of the inflammation markers in both the vitamin D group and the placebo group. Lastly, as the serum samples in the OFAMS study were obtained throughout the year, we adjusted the vitamin D measurements in Article II for season by utilizing a model established for the seasonal variation of the vitamin D level in this cohort (363). A similar approach has been employed in earlier and recent observational studies (181;182;184;185), however, some disagreement exists regarding how this adjustment should be made (391). Moreover, to further reduce the potential confounding effect of season on the results, we used the individual adjusted vitamin D averages of each patient when dividing the patients into quartiles. This ensured that patients enrolled during the same season were less likely to be allocated to the same quartile.

In Article III, all serum samples were obtained during late winter, which limited the potential effect of seasonal factors. However, in order to further limit the influence of other factors than the vitamin D_3 supplementation on the vitamin D levels, we also included among other an adjustment for high UVR exposure. In contrast, it seems that adjustment for varying UVR exposure has neither been applied in the earlier and current epidemiological studies nor in the earlier RCTs of vitamin D in RRMS (182;183;185;186;192;196-199). Although seasonality was addressed in different ways in these observational studies, it is evident that UVR exposure cannot be completely adjusted for by using deseasonalized vitamin D levels. It is therefore possible that the largely differing results between these observational and interventional studies may be partially related to differing UVR exposure between these cohorts, as it has been clearly indicated that UVR may have immunomodulatory effects that are independent of vitamin D (150-152;392). Additional support for this independent effect has been provided by the findings of associations between UVR exposure and disease activity in RRMS even when vitamin D levels were controlled for (393;394), and in experimental models of demyelization where the beneficial effect of UVR exposure may be largely or totally attributed to other factors than vitamin D (395;396). Furthermore, it is also noteworthy that the large majority of the vitamin D in serum is usually derived from vitamin D production in the skin after UVR exposure (397). In light of this, it seems safe to assume that UVR exposure was the

primary determinant of the vitamin D levels in the OFAMS cohort. This again indicates that the findings related to the increasing vitamin D level in Article were most likely confounded by the patients' UVR exposure. Moreover, it may also be suspected that this has been the case in the previous and more recent epidemiological studies of the effect of increasing vitamin D in RRMS (182;183;185-187). If this is true, it seems unreasonable to compare the effects of increasing vitamin D levels between studies with fundamentally different designs without adjusting for factors related to how the increase is obtained.

10.6.2. Causality

A main outcome in epidemiological research is the finding of an association. The finding of an association is central when developing new hypothesis regarding the effect a specific exposure may have on a specific outcome, but an association does no more than suggest that the specific exposure may have a relation to the specific outcome. It is therefore crucial to further investigate the basis for this association, as an association can arise through confounding, causality, reverse causality, bias and random variation (398). The confounding factors addressed in this Thesis have been covered in the previous section, and it is clear that our analyses were not free of residual confounding. However, the anti-inflammatory associations noted for vitamin D in the OFAMS cohort and in a vast amount of other MS cohorts cannot be ruled to be a result of confounding solely based on the results in Article III. It is therefore valid to evaluate if the association between vitamin D status and inflammation in RRMS can be explained in another way.

Causality can be defined as the relationship between something that happens or exists and the thing that causes it. To aid in the evaluation of whether or not an association could be due to causality, a set of guidelines was put forward by Sir Brardford Hill in 1965 that has later been referred to as the Hill's causality criteria (399). According to these criteria, the claim of causality can be made if the association has a sufficient strength, consistency, analogy, experimental backing, coherence, specificity, dose-response gradient, biological plausibility, and a correct temporality. However, excluding correct temporality, none of these criteria are absolute for establishing causality (398). The strength of an association can be measured by the relative risk between the exposure and the outcome (i.e. the likelihood of the outcome for an exposed individual compared an unexposed individual). As elaborated upon in section 5.2.7, 5.3.2, 5.3.3. and 5.3.4., several factors have been found to be associated with the risk of disease activity in MS. Currently, a low serum level of vitamin D seems to be among the associations with the largest risk for high disease activity in RRMS (182). This is also supported by the findings of positive associations between increasing levels of vitamin D and the anti-inflammatory markers IL-1Ra and sFRP3 in Article I and II. The inverse association between vitamin D levels and disease activity has also been a consistent finding in a variety of patient populations (177). However, the findings in Article III and the results from the original trial at the UNN and several other vitamin D intervention trials suggest that this may not be case for all RRMS patients (192;196;198;199). Furthermore, a claim of analogy cannot be made for vitamin D with regards to disease activity, as no other factor has been shown to have a causal relationship with this in MS. Moreover, vitamin D has not been found to have any definite beneficial effects on a wide range of other inflammatory diseases (400). Nevertheless, there are ample results from animal experiments that support a beneficial effect of elevated vitamin D levels on CNS inflammation, as active vitamin D can reduce the progression of EAE and promote remyelination after toxically induced demyelization (174;244;401). In addition, as mentioned in section 5.3.1., some studies have reported reduced MRI activity among MS patients after vitamin D_3 supplementation (189;197). Coherence can also be made between a declining serum vitamin D level and an increasing latitude (157), which has also been associated with clinical disease activity in RRMS

(370). Although specificity was initially included in the criteria, it has largely been abandoned with the knowledge that a disease can arise from more than one cause or by several factors acting in concert. On the other hand, the criteria of a dose-response gradient may have become more important with time, and several findings, including the results from the initial analysis in Article II, suggest a decreasing disease activity with rising serum levels of 25(OH)D (181-184;186). However, it remains uncertain if an increase of the inactive 25(OH)D can be directly translated to an increase in active vitamin D, as different regulatory principals and mechanisms govern the regulation of these vitamin D metabolites (158). More specifically, the 25(OH)D level has been found to reach a plateau first after several months in conjunction with repeated high UVR exposure or high dietary intake of vitamin D (402), while active vitamin D seems to stabilize within a much shorter time period (193;403). With this in mind, it is also noteworthy that measurements of active vitamin D in serum have not been found to be associated with MS activity (404), and that the beneficial effects of vitamin D found in EAE have mostly been achieved with supraphysiological doses of active vitamin D (405). When continuing to the criteria for biological plausibility, it is clear that an increased intake or production of vitamin D will lead to a higher serum level of 25(OH)D and a large supply of vitamin D that can be activated within cells containing the necessary metabolic apparatus (406). In this respect, it has been found that immune cells like macrophages possess the capability to convert 25(OH)D into active vitamin D, and the finding of considerably elevated levels of active vitamin D in medical conditions with sustained macrophage activation indicates that this process is not limited by the normal regulatory mechanisms found in renal tissues (158;407). However, in contrast to what is the case for the macrophages, *in vitro* studies of human B cells, T cells and DCs have found that exposure to a high level of 25(OH)D do not only increase 1-α-hydroxylase transcription and promotion of active vitamin D production, but it also increases transcription of the active vitamin D degrading enzyme 24-hydroxylase (406;408;409). Furthermore, supplementation of $25(OH)D_3$ to patients with end-stage kidney disease has shown that the catabolism of active vitamin D may also be rapid and highly inducible in the extra-renal tissues that possess this degrading capability (410). In summary, this indicates that B cells, T cells and DCs are able to increase their catabolism of active vitamin D, and consequently, it seems unlikely that a high extracellular level of 25(OH)D would lead to a sustained elevation of active vitamin D within these cells. The latter assumption could further suggest that the immunological effect of an increasing vitamin D level is transient. This may explain why earlier studies with a duration of 6 months or less have found an increase in TGF-β and IL-10 in MS patients on vitamin D (192-194), while similar studies with a time frame of one year or more have not been able to verify these results with certainty (188;191;373). Further support for this proposal is provided by the finding of a passing alteration in the production of several inflammation markers among healthy individuals on repeated high-dose vitamin D supplementation (411), and our recent observation of a transitory reduction in the serum antibody level against EBV nuclear antigen 1 after one year in the UNN trial (380). However, these results may rather be viewed upon as an expansion than an exclusion of the biological plausibility of a causal association between vitamin D and inflammation in MS.

10.6.3. Reverse causality

The most central criteria for establishing causality is the finding of a correct temporality of the exposure and the outcome. This means that the exposure has to precede the outcome and not be a result of the outcome. If this is not the case, despite the fulfillment of all other criteria, the association is attributed to reverse causality. The order of the exposure and the outcome may sometimes be difficult to assess in medical research. With respect to the association between vitamin D and disability in MS, several studies have shown that a higher EDSS score is associated with a lower serum value of vitamin

D (404;412). However, does this necessary mean that a poor vitamin D status leads to increasing disease worsening? The answer is no, as this may be a result of reverse causality where the low vitamin D values are a result of disease worsening due to the increased likelihood that severely impair patients spend less time outside. This shows that the relationship between a low vitamin D value and a presumed outcome may not always be straightforward. Therefore, it may also be appropriate to discuss if a low vitamin D level causes inflammation or if the inflammatory process leads to reduced vitamin D levels. With concern to the latter, it has been found that the inflammatory response arising after elective surgery is associated with a significant drop in serum vitamin D concentration (413;414). In addition, it is well known that vitamin A levels drop during inflammation due to the fact that RBP is a negative acute phase protein (i.e. reduced liver production in response to inflammation) (208). As the DBP is also primarily produced in the liver and over 90% of vitamin D in the circulation is bound to DBP (415), it is also possible that a similar reduction will occur for vitamin D during inflammatory conditions like MS. This potential alteration in DBP production may partly explain the finding of a lower DBP level in CSF among RRMS patients (416;417). Furthermore, both vitamin D and DBP may be consumed during the inflammatory process due to the increased metabolism of vitamin D in immune cells along with the binding to complement factors and the clearance of actin from damaged CNS cells by DBP (406;409;418-420). Still, with respect to DBP, it seems uncertain if the inflammatory process in the CNS also leads to a lower systemic level of DBP, as similar serum levels of DBP have been found between RRMS patients and healthy controls (421).

10.6.4. Bias and random variation

To test if there may be an association between a specific exposure and a specific outcome it is common to initially examine if this is the case for a limited population. Ideally, this limited population sample should reflect the overall population. However, systematic or random errors may sometimes disturb this sampling. These errors may also be called bias and random variation, respectively. The different forms of bias and random variation will be commented upon in the following together with the steps that were taken to avoid these errors in the included studies.

Several different types of bias exist. However, in clinical studies like the ones conducted in this Thesis, the most important forms of bias are information bias, selection bias, performance bias, detection bias and attrition bias. The information bias deals with misclassifications like the inclusion of a healthy individual when studying a specific disease. With regards to this, the included patients in the trials in this Thesis were all diagnosed with specific criteria for MS aimed at removing misclassification. Selection bias refers to the inclusion of individuals that are not representative of the overall population (i.e. biased sampling) or differing characteristics between the patients in the different study arms. Concerning biased sampling, it cannot be ruled out that the included patients in the current studies were more prone to attend neurological outpatient clinics and were therefore also more likely to be included than other patients. However, almost 30% of the managed MS patients at the UNN were included in the trial in Article III (422), and the participants in Article I and II were recruited from a large number of neurological departments from across Norway. Furthermore, the baseline characteristics were evenly distributed among all patient groups in the included studies by means of computerized randomization. A bias in performance or detection arises if the included patients do not receive the same exposure or if the outcome in the study arms is assessed in a different manner, respectively. However, these factors can more or less be ruled out if the study is carried out in a double-blinded fashion like the OFAMS study and the UNN trial. Lastly, attrition bias is related to the loss of included patients during the study. This can lead to an overestimation of the effect of the exposure, as included patients with little effect will be more likely to dropout than the ones with a good effect. However,

it seems very unlikely that this had a substantial effect on the results in this Thesis, as only about 7% of the included patients dropped out of the OFAMS study and the trial at the UNN.

An association can also be caused by random variation, which is an inherent factor in all biological measurements. The random variation arises due to irregular or erratic fluctuations or by chance and cannot be controlled for. However, the random variation can in general be decreased by increasing the size of the sample population. The sample populations included in this Thesis were quite small, and it is therefore expected that the random variation may have had an important impact on the findings. Nevertheless, it seems unlikely that this variation could explain the consistent findings of an effect of immunomodulatory treatment on CXCL16, IL-1Ra, MMP-9 and OPN concentrations in Article II and III.

10.6.5. Reliability and validity

The reliability of a result is fundamental in the search for new scientific proof regarding the relationship between a specific exposure and a specific outcome. This can only be obtained through the demonstration that the same result can be generated in independent studies with the same conditions. In this Thesis, the earlier result regarding the associated effect of IFN-β treatment on IL-1Ra in RRMS patients has been replicated in Article II and to a large extent in Article III (as more than 90% of the patients on immunomodulatory treatment used IFN-β in the UNN trial) (308;311). It may therefore be concluded that it is likely that IFN-β therapy has an effect on the IL-1 pathway in RRMS, and that IL-1Ra may be consider as a biomarker for a therapeutic response to IFN-β treatment. Furthermore, the finding of an increase in the serum level of 25(OH)D during IFN-β therapy in Article II and III is also in line with results from another independent research group even though the magnitude of the elevation was small in comparison with the earlier results (187). In addition, the results in Article II again confirm the earlier findings of an anti-inflammatory effect of IFN-β on MRI activity (96). Lastly, the finding of no synergistic effect between high vitamin D levels and IFN-β in Article II was also found in Article III, indicating that this was not evident in two independent patient cohorts. However, it remains to be seen if this finding and the findings of associations between vitamin A and PTX3, vitamin D and IL-1Ra and sFRP3, and vitamin E and CXCL16 may be replicated by other research groups.

Validity determines if the applied measurements truly measures the intended outcome. In this respect, it may be argued that the included panel of inflammation markers does not measure all aspects of the inflammatory process. However, with exclusion of CCL21 and CXCL16 and OPG and sTNF-R1, the selected markers represent an extensive variety of different inflammatory pathways. Furthermore, the measurement of additional inflammation markers would have further limited our ability to detect associations due to the restricted number of patients enrolled in the studies. In addition, the validity can be further dived into internal and external validity, where internal validity is related the quality of the research and the external validity refers to how well the findings may apply to another setting. With concern to the internal validity, the inclusion of data from both the OFAMS study and the UNN trial strengthens this Thesis overall ability to assess the relationship between vitamin D and systemic inflammation in RRMS, as these studies complement each other with the findings in the OFAMS study being primarily attributed to the natural variation of serum vitamin D and the findings in the UNN trial being chiefly related to the oral intake of vitamin D_3. Moreover, the associations between vitamin A, D and E and the inflammation markers are all strengthened by the predefined and stringent study conduction and the well-defined patient cohorts that together restrained random effects and patient heterogeneity. Additionally, the

application of statistical methods specifically devised to deal with challenges arising in longitudinal studies reduces the likelihood that the reported findings are spurious, and the seemingly similar effect of IFN-β therapy on the systemic inflammation markers in both studies implies that it is unlikely that the results can be attributed to technical issues. Lastly, the studies have individual strengths with the OFAMS study including frequent serum sampling and MRI imaging both before and after initiation of IFN-β therapy, and the UNN trial confining serum sampling to one season and including a detailed survey of potential confounding factors. On the other hand, it is clear that confounding, especially by UVR exposure in Article I and II, may reduce the internal validity of the findings in this Thesis. Furthermore, the results are weakened by the small numbers of patients included in the different analyses and the rather low disease activity during the trials It is also likely that the obtainment of measurements at predefined time points severely restricted the chance of detecting alterations related clinical activity, and the varying inclusion criteria and study designs limit the ability to compare the results across the studies. Finally, the studies have individual weaknesses with the OFAMS study being vulnerable to a natural reduction in disease activity as it included patients with recent disease activity (i.e. an regression to the mean effect), and the trial at the UNN having a limited number of available serum measurements. Regarding the external validity of the current results, it is apparent that the patient populations included in this Thesis do not reflect the multifaceted general MS population. Still, the findings from the studies in this Thesis may highlight some important aspects that need to be accounted for when future RCTs of vitamin D_3 supplementation in RRMS are analyzed.

11. Implications for current clinical practice

The results in this Thesis may have several clinical implications. However, the manner of how these findings were obtained and the limited scope of the conducted investigations only permit the making of cautious statements.

With respect to the relationship between vitamin D and inflammation in RRMS, it is evident that the results reported in this Thesis and the current available evidence do not allow a claim of causality to be made between a poor vitamin D status and an increased level of inflammation. However, as stated by the astrophysicists Carl Sagan; "The absence of evidence is not evidence of absence", and further judgment on this association cannot be made before the ongoing RCTs of vitamin D_3 supplementation in RRMS are presented (202;203;205). Still, in light of current knowledge, it seems appropriate to focus the advocacy of vitamin D_3 supplementation or obtainment of vitamin D through exposure to UVR among RRMS patients toward the beneficial effects this may have on bone health (161), which has been found to be reduced among RRMS patients from an early time point (423). With this in mind, it should be noted that the current recommendation regarding a daily intake of 400 IU vitamin D_3 in the Nordic countries seems to be insufficient with respect to obtaining the vitamin D level needed to ensure efficient absorption of calcium from the diet (160;161). In light of this, it may be advisable that the daily intake is somewhat higher than 400 IU vitamin D_3. Additional support for this was also found in an earlier investigation of the potential of vitamin D_3 supplementation in the OFAMS cohort, as an additional intake of 400 IU/day or 800 IU/day would be enough to ensure at least one 25(OH)D measurement above 75 nmol/L during the year for about 10% and 70% of the patients, respectively (424).

Another key result in this Thesis was the finding that the anti-inflammatory effects of IFN-β treatment was seemingly not increased by high vitamin D levels obtained either naturally or after supplementation. Moreover, the results in Article II suggest that the anti-inflammatory effect of an increasing vitamin D levels is small when compared to IFN-β treatment. This implies that the anti-inflammatory effects of vitamin D may not be evident if the patients are already using IFN-β. Furthermore, the results also indicate that vitamin D_3 supplementation should not be considered as an alternative to first-line treatments like IFN-β.

Although the studies included in this Thesis were most likely too small to address if the examined markers of systemic inflammation could be implemented as clinically useful biomarkers in RRMS, it is clear that the consistent finding of an associations between IFN-β treatment and IL-1Ra could indicate that this marker may be utilized to evaluate the efficacy of IFN-β treatment. However, as the serum level of IL-1Ra level may also be related to other factors like the concentrations of vitamin A and D, it is uncertain if IL-1Ra measurements may have the ability to provide as reliable results as the current assessment of Nabs against IFN-β (256).

The results in Article I indicate that both vitamin A and E may play a beneficial role in MS. This suggests that oral supplementation with vitamin A and E may be considered as an additive to existing DMTs in RRMS. In light of its popularity in Norway and high content of vitamin A, D and E, it may be suggested that cod liver oil is the ideal supplement for patients with RRMS. However, current knowledge suggests that the official recommendation for daily intake of especially vitamin A should not be exceeded, as exaggerated supplementation with this vitamin may among other cause serious neurologic side-effects (425).

12. Future perspectives

Although the findings in this Thesis suggest little effect of increasing vitamin D levels on the examined inflammatory pathways in RRMS, it cannot be ruled out that vitamin D may affect other important pathways or that it may have a more pronounced effect among CIS patient or in RRMS subgroups like patients with higher disease activity or a poor vitamin D status. However, it should be noted that the conflicting findings in the observational OFAMS study and the intervention trial at the UNN, together with reports of discrepancies between the effect of high vitamin D levels in observational studies and RCTs of vitamin D supplementation in a range of medical conditions with an perceived inflammatory component (400), may indicate that the data from these fundamentally different study methods are not directly comparable. To put it more bluntly, it could be that we are "comparing apples and oranges" when direct comparisons are made between observational and interventional data regarding the role of vitamin D in inflammation. To avoid this problem, it seems appropriate that especially future observational explorations of the anti-inflammatory effects of vitamin D in RRMS should be designed to take account for confounding factors like the effect of UVR exposure on inflammation. One way to partly accomplish this is to recommend that studies should restrict themselves to a limited geographical region and be conducted within one specific season. Furthermore, it could be suggested that the use of questionnaires regarding environmental factors related to the vitamin D level like UVR exposure are incorporated into the study design. Lastly, it may be advisable that measurements of vitamin D are accompanied by measurements of cis-urocanic acid, which can be directly related recent UVR exposure (152). Even though environmental factors related to the vitamin D level are less likely to affect the results in vitamin D supplementation trials, it may also be suggested that interventional studies should try to limit and, if possible, incorporate adjustments for these factors when the results are analyzed.

Another fundamental issue that should be addressed in future studies of the role of vitamin D in inflammation in RRMS is the possibility that vitamin D may rather be an indicator than a generator of the inflammatory process. This may perhaps be best accomplished by conducting longitudinal observational studies with measurements of vitamin D and established biomarkers of inflammation like C-reactive protein both at frequent prescheduled times and in conjunction with disease activity. Moreover, the obtained measurements should primarily be compared within each patient over time, as the individual variation in vitamin D and inflammation marker levels seem to differ substantially between individuals. Finally, it may again be advisable that the study design takes account for other environmental factors related to the vitamin D concentration, as suggested in the previous paragraph.

As IFN-β may modulate and largely overshadow the effects of vitamin D on inflammation in RRMS, it seems apparent that future RCTs of vitamin D_3 intervention among patients on IFN-β therapy should be designed in a way that permits separate evaluations of the effects of vitamin D and IFN-β. However, the design utilized in the OFAMS study may now be considered unethical, as it would delay treatment and potentially promote disease evolution. An alternative approach could therefore be to delay supplementation in order to first establish the effect of IFN-β on the trial outcomes. Moreover, use of several study arms with different vitamin D_3 doses may contribute to differentiate between the effect of the vitamin and the DMT. Additionally, as the effect of vitamin D may be transient, it also seems advisable that frequent assessments are carried out throughout a study period of at least one year.

Even though the effects of vitamin A and E on inflammation in RRMS still seem uncertain and complex, it is clear that the results in this Thesis and the earlier small-scale interventional studies indicate that further investigation is warranted. An initial focus in these studies should be to determine the overall effects of these vitamins on inflammation,

as the findings in this Thesis suggest that especially vitamin A may modulate both anti-inflammatory and pro-inflammatory pathways. Furthermore, as the knowledge regarding the potential side-effects and the pharmacokinetics of supplementation with vitamin A and E seems limited, it is recommendable that additional small-scale studies are carried out in order to establish the safety and biological properties of the interventions. Additionally, it may be suggested that further observational studies are conducted in order to determine the extent of the interactions between vitamin A, D and E and IFN-β on the herein examined inflammation markers, and the possible effects of vitamin A and E on additional inflammatory pathways.

Lastly, despite the apparent lack of clinical relations for the included panel of systemic inflammation markers, current and earlier results indicate that some of these markers are associated both disease treatment and pathology. It may therefore be suggested that especially measurements of CXCL16, IL-1Ra, MMP-9 and OPN are included in future studies of serological inflammatory biomarkers in RRMS.

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