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ABSTRACT

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Vitamin D in the Prevention and Treatment of Multiple Sclerosis

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Background: Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system (CNS); it is the most common cause of neurological disability in young adults. The cause of MS is unknown but it is believed to be precipitated by both genetic and environmental risk factors. An association between vitamin D deficiency and increased risk of MS has been detected in several epidemiological and case-control studies and is further supported by immunological and genetic studies. It is still unclear whether there is any special age at which exposure to vitamin D deficiency increases the MS risk e.g. is in utero vitamin D deficiency particularly detrimental. Serum levels of vitamin D are associated with both clinical and magnetic resonance imaging (MRI)-assessed disease activity but there have been very few randomized placebo-controlled trials (RCTs) investigating the benefits of vitamin D supplementation in established MS patients.

Aims of the study: This study initially investigated the association between vitamin D status during early pregnancy and the risk of MS in the offspring in the Finnish Maternity Cohort. Secondly, we performed the first RCT with vitamin D in MS, evaluating the safety and efficacy of 500 ug weekly dose of vitamin D supplementation or placebo in 68 MS patients receiving interferon beta-therapy. We also studied the mechanism of action of vitamin D supplementation in our study participants. Finally, we updated the MS prevalence and studied the fracture risk and the role of vitamin D in the risk of fractures in MS patients in Southwest Finland.

Results: Maternal vitamin D deficiency during early pregnancy was associated with a nearly 2-fold increased risk of MS in their children in comparison with children born to mothers who were non-deficient for vitamin D. In the vitamin D supplementation study there was a statistically significant decrease in the total number of T1 Gadolinium (Gd) enhancing lesions in patients treated with vitamin D in comparison with placebo treated patients. Serum levels of transforming growth factor beta (TGF-β), an immunoregulating cytokine, indirectly measured by a latency-associated peptide (LAP) immunoassay, increased significantly in the vitamin D treated group but not in the placebo group. The prevalence of MS in southwest Finland was $212/10^5$. The relative risk was 1.3 for all types of fractures and 1.5 for osteoporotic fractures in patients with MS compared with matched controls.

Conclusions: Correcting vitamin D deficiency during pregnancy may exert a beneficial effect on the risk of the offspring developing MS. Vitamin D supplementation at 500 ug/week influenced the MRI-assessed activity in patients with MS. The immunoregulatory effects of TGF-β may play a role in the improved MRI outcomes observed in our vitamin D treated MS patients. At a mean serum level of 110 nmol/l of 25-hydroxyvitamin D (25(OH)D), its immunological effects can already be detected. We observed a statistically significantly elevated risk of osteoporotic fractures in patients with MS. Several conclusions emerge from our results. First, we recommend correcting vitamin D deficiency during pregnancy at the population level but especially in MS patients planning to become pregnant. Second, we recommend that vitamin D levels should be analyzed in all MS patients after the diagnosis of MS and vitamin D supplementation should be initiated at a dose of 50 to 100 ug/day, targeting serum levels above 100 nmol/l.

Keywords: Multiple Sclerosis, vitamin D, pregnancy, vitamin D supplementation, TGF-β, fractures

TIIVISTELMÄ

Julia Åivo

D-vitamiini MS-taudin ehkäisyssä ja hoidossa

Turun yliopisto, Lääketieteellinen tiedekunta, Neurologian oppiaine, Turun yliopiston kliininen tohtoriohjelma

Tausta: Multippeliskleroosi eli MS-tauti on keskushermoston krooninen autoimmuunisairaus ja yleisin liikunta- ja toimintakykyyn vaikuttava neurologinen sairaus nuorilla aikuisilla. MS-taudin aiheuttajaa ei tiedetä, mutta sen uskotaan syntyvän perintötekijöiden ja ympäristöriskitekijöiden yhteisvaikutuksesta. D-vitamiinin puute on noussut tärkeäksi MS-taudin ympäristöriskitekijäksi useissa epidemiologisissa ja tapaus-verrokkitutkimuksissa sekä immunologisissa ja geneettisissä tutkimuksissa. Vielä on epäselvää, missä iässä koettu D-vitamiinin puute lisää MS-taudin riskiä ja vaikuttaako esimerkiksi äidin raskaudenaikainen D-vitamiinin puute lapsen riskiin sairastua MS-tautiin. Seerumin D-vitamiinitasojen on todettu olevan yhteydessä MS-taudin kliiniseen ja magneettikuvauksella (MRI) todettavaan aktiivisuuteen, mutta D-vitamiinilisän tehoa jo puhjenneessa MS-taudissa on tutkittu vasta vähän satunnaistetussa lumekontrolloidussa asetelmassa.

Tavoitteet: Tämän tutkimuksen tavoitteena oli tutkia äidin raskaudenaikaisen D-vitamiinitason yhteyttä lapsen MS-tautiriskiin Äitiysneuvolakohorttiin kuuluvilla naisilla. Tavoitteenamme oli myös tutkia 500 ug viikossa annosteltavan D-vitamiinilisän tai lumevalmisteen vaikutusta aivojen magneettikuvauksella todettavaan ja kliiniseen tautiaktiviteettiin MS-potilailla, jotka käyttävät interferoni beetaa, sekä selvittää D-vitamiinin immunologisen vaikutuksen mekanismeja näillä potilailla. Tavoitteenamme oli myös päivittää MS-taudin esiintyvyysluvut ja murtumariski MS-potilailla Varsinais-Suomen sairaanhoitopiirin alueella ja tutkia D-vitamiinin yhteyttä MS-potilaiden murtumariskiin.

Tulokset: Äidin raskaudenaikainen D-vitamiinin puute oli yhteydessä lapsen lähes kaksinkertaiseen riskiin sairastua MS-tautiin verrattuna niihin lapsiin, joiden äidillä ei ollut D-vitamiinin puutetta. D-vitamiinilisän vaikutuksia selvittäneessä tutkimuksessa D-vitamiinilisää saaneilla potilailla aivojen MRI:ssä nähtävät T1-painotteiset gadoliniumilla (Gd) tehostuvat muutokset vähenivät tilastollisesti merkitsevästi verrattuna lumevalmistetta saaneisiin potilaisiin. Immunologisessa osatutkimuksessa todettiin immuunivastetta vaimentavan sytokiinin, transformoiva kasvutekijä beetan (TGF-β), pitoisuutta mittaavan latency-associated peptide LAP:n pitoisuuden nousevan tilastollisesti merkitsevästi enemmän D-vitamiini- kuin lumeryhmässä. MS-taudin esiintyvyys Varsinais-Suomessa oli $212/10^5$ vuonna 2012. Murtumariski MS-potilailla oli 1.3-kertainen ja osteoporoottisten murtumien riski 1.5-kertainen verrokkeihin nähden.

Johtopäätökset: Äidin raskaudenaikaisen D-vitamiinin puutoksen korjaamisesta saattaa olla hyötyä lasten MS-taudin ehkäisyssä. D-vitamiinilisä 500 ug viikossa vähensi aivojen magneettikuvauksella todettavaa MS-taudin aktiivisuutta. TGF-β:n immuunivastetta säätelevillä vaikutuksilla saattaa olla osuus MRI-tuloksiin, jotka havaittiin D-vitamiiniryhmässä. Immunologisia vaikutuksia voitiin todeta keskimäärin D-vitamiinitasolla 110 nmol/l. MS-potilaiden riski saada osteoporoottinen murtuma on merkitsevästi kohonnut. Tulostemme pohjalta suosittelemme D-vitamiinin puutoksen korjaamista raskausaikana sekä väestötasolla että erityisesti MS-potilailla, jotka suunnittelevat raskautta. Suosittelemme D-vitamiinitason määrittämistä kaikilta MS-potilailta ja D-vitamiinilisän aloittamista annoksella 50-100 ug päivässä tavoitteena seerumin D-vitamiinitaso yli 100 nmol/l.

Avainsanat: Multippeliskleroosi, D-vitamiini, raskaus, D-vitamiinilisä, TGF-β, murtuma

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ABBREVIATIONS

1,25(OH)2D	1,25-dihydroxyvitamin D
1α(OH)ase	1-α hydroxylase
24(OH)ase	25-hydroxyvitamin D 24-hydroxylase
25(OH)D	25-hydroxyvitamin D
9HPT	9 hole peg test
APC	Antigen presenting cell
ARR	Annual relapse rate
BBB	Blood-brain barrier
BMD	Bone mineral density
BMI	Body mass index
BOD	Burden of disease
CIS	Clinically isolated syndrome
CNS	Central nervous system
CSF	Cerebrospinal fluid
DBSS	Dried blood spot sample
DC	Dendritic cell
DIS	Dissemination in space
DIT	Dissemination in time
DMT	Disease modifying therapy
DXA	Dual-energy X-ray absorptiometry
DZ	Dizygotic
EAE	Experimental autoimmune encephalitis
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
EDSS	Expanded disability status scale
FDE	First demyelinating event
FGF	Fibroblast growth factor
Foxp3	Forkhead box P3
GA	Glatiramere acetate
Gd	Gadolinium
HLA	Human leukocyte antigen
ICD	International Classification of Diseases
IFN	Interferon
IL	Interleukin
IM	Infectious mononucleosis
LAP	Latency-associated peptide
LTBP	Latent transforming growth factor beta binding protein

LTGF-β	Latent transforming growth factor beta
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MSFC	Multiple sclerosis functional composite
MxA	Myxovirus A
MZ	Monozygotic
NAB	Neutralizing antibodies
NEDA	No evidence of disease activity
OR	Odds ratio
PASAT	Paced auditory serial addition test
PPMS	Primary progressive multiple sclerosis
PTH	Parathyroid hormone
RANK	Receptor activator nuclear factor kappa
RANKL	Receptor activator nuclear factor kappa ligand
RCT	Randomized controlled trial
RRMS	Relapsing remitting multiple sclerosis
RXR	Retinoid X receptor
SPMS	Secondary progressive multiple sclerosis
T25FW	Timed 25 foot walk
TGF	Transforming growth factor
Th	T helper cell
TNF	Tumor necrosis factor
Treg	T regulatory cell
TTW10	Timed 10 foot tandem walk
UVB	Ultraviolet B
UVI	Ultraviolet intensity
UVR	Ultraviolet radiation
VDR	Vitamin D receptor
VDRE	Vitamin D response element

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1 INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) but its cause is still unknown. A genetic predisposition explains only a fraction of the MS risk and the most widely accepted hypothesis is that MS is an autoimmune disease that is precipitated in genetically susceptible individuals as a consequence of a complex interplay between the genetic risk factors, environmental exposures and infectious agents (Sospedra, Martin 2016).

Vitamin D deficiency is a major environmental factor associated with the risk of developing MS. The MS frequency increases with increasing latitude and latitude is strongly inversely correlated with ultraviolet radiation (UVR) intensity and duration (Ascherio, Munger 2007b). Ultraviolet B (UVB) radiation exposure in turn is the most important source of vitamin D for most people (Holick 2010). Furthermore, increased childhood and occupational sun exposure is associated with a decreased risk of MS (Ascherio, Munger et al. 2010). In addition to UVR exposure, increased vitamin D intake (Munger, Zhang et al. 2004) and increased serum levels of vitamin D (Munger, Levin et al. 2006) are associated with a decreased risk of MS. The role of vitamin D in MS is further supported by immunological studies showing immunoregulatory effects of vitamin D (Peelen, Knippenberg et al. 2011). Moreover, genetic studies have revealed an association between the MS risk and polymorphisms in genes involved in vitamin D metabolism (Sundqvist, Bäärnhielm et al. 2010, Rhead, Bäärnhielm et al. 2016).

There is substantial data supporting the association between MS risk and sun exposure during childhood and adolescence (Handel, Giovannoni et al. 2010) but also in utero exposure to vitamin D deficiency may be relevant for the MS risk. In the northern hemisphere, MS patients are more likely to be born in the spring, especially April or May, after winter months of low sunlight and consequently low gestational serum levels of vitamin D (Torkildsen, Grytten et al. 2012). In the southern hemisphere, the pattern is reversed, with the excess of MS births occurring in November and December (Staples, Ponsonby et al. 2010). A higher vitamin D intake during pregnancy has also been associated with a lower risk of MS in the offspring (Mirzaei, Michels et al. 2011).

Vitamin D status has been linked with disease activity in several studies. For example, MS patients have lower vitamin D levels during relapse than remission (Soilu-Hänninen, Airas et al. 2005, Soilu-Hänninen, Laaksonen et al. 2008, Correale, Ysrraelit et al. 2009) and magnetic resonance imaging (MRI) activity has been shown to inversely correlate

with vitamin D levels (Løken-Amsrud, Holmøy et al. 2012, Mowry, Waubant et al. 2012). Higher vitamin D levels early in the course of the disease have been observed to predict a lower level of clinical progression (Ascherio, Munger et al. 2014).

Vitamin D deficiency also has negative impact on bone health (Holick 2007a) and MS patients are at increased risk of osteoporosis and consequent fractures (Hearn, Silber 2010).

Safety data suggests that high dose vitamin D supplementation is well tolerated and no serious adverse events have been reported (Kimball, Ursell et al. 2007, Burton, Kimball et al. 2010). Nonetheless, so far, clinical trials performed with vitamin D in MS, irrespective of the doses used, have failed to demonstrate efficacy on clinical endpoints, but have suggested an effect on disease activity as assessed by MRI.

2 REVIEW OF LITERATURE

2.1 Multiple sclerosis

2.1.1 Epidemiology

2.1.1.1 Prevalence

MS is the most common chronic inflammatory demyelinating disease of the CNS. After trauma, it is the leading cause of neurological disability in young adults. It is estimated that about 2.5 million people have MS worldwide; in Europe the estimate is 400 000 (MS international federation 2013). In Finland, the prevalence of MS has been estimated to be 8000, but there is no published up-to-date country-wide prevalence data. The most striking epidemiological feature of MS is its uneven geological distribution (Kurtzke 1977). Northern Europe and North America are considered areas with high (>30 per 100000), Southern Europe and Southern USA with medium (5-30 per 100 000) and Asia and Africa with low (<5 per 100 000) prevalences (Kurtzke 2000). The total estimated prevalence rate of MS in Europe has been 83 per 100 000 for the past three decades (Pugliatti, Rosati et al. 2006). Finland belongs to a high risk region with prevalence of 100-200 per 100 000 in different areas (Sumelahti, Tienari et al. 2001, Sarasoja, Wikström et al. 2004, Krökki, Bloigu et al. 2011).

2.1.1.2 Incidence

In addition to the true frequency, prevalence estimates can be affected by survival time, diagnostic accuracy and ascertainment probability. Consequently, incidence is a better measure of MS risk (Koch-Henriksen, Sørensen 2010). In a recent meta-analysis, annual incidence rates in Europe varied widely from <1 per 100 000 to >10 per 100 000 (Kingwell, Marriott et al. 2013). The estimated mean annual MS incidence in Europe is 4.3 per 100 000 (Pugliatti, Rosati et al. 2006). In Finland, the age-adjusted incidence varies from 6.7 per 100 000 in Pirkanmaa to 12.5 per 100 000 in Seinäjoki (Holmberg, Murtonen et al. 2013).

2.1.1.3 Change in incidence and sex ratio

Results of meta-analyses suggest an overall increase in MS incidence over time. This seems to result primarily from an increase in the incidence among women (Koch-Henriksen, Sørensen 2010, Kingwell, Marriott et al. 2013, Alcalde-Cabero, Almazán-Isla et al. 2013, Alonso, Hernán 2008). This increase in the female/male sex ratio has been demonstrated in many countries. In a global database study, there was a general increase in the sex ratio from 2.35 to 2.73 and from 1.96 to 4.55 in northern European countries over six decades (Trojano, Lucchese et al. 2012). In a large Canadian study, the sex ratio increased from 1.9 in patients born in 1936-40 to 3.2 in patients born in 1976-80 (Orton, Herrera et al. 2006). In Oslo, Norway there was an increase in the sex ratio from 1.48 in 1910 to 2.3 in 1980 (Celius, Smestad 2009). This increase in female incidence has also been reported from Germany, France and Australia (Koch-Henriksen, Sørensen 2010) and Finland (Krökki, Bloigu et al. 2011, Sumelahti, Holmberg et al. 2014). The reason for the increased incidence in women but not in men is unclear but it indicates the existence of an environmental influence on the risk of MS (Koch-Henriksen, Sørensen 2010).

2.1.2 MS subtypes and clinical course

The course of MS is characterized by two different clinical phenomena, acute relapses and disease progression. In 85% of MS patients, the disease starts with a relapsing-remitting course (RRMS) with succeeding relapses (Confavreux, Vukusic 2014). A relapse is characterized with occurrence, recurrence or worsening of neurological symptoms lasting at least 24 hours (McDonald, Compston et al. 2001) and is considered to be a clinical expression of acute, focal inflammation and demyelination within the CNS (Youl, Turano et al. 1991). The first acute episode or relapse is referred to as a clinically isolated syndrome (CIS) if the criteria for MS are not readily met. CIS is usually monosymptomatic, most commonly affecting the optic nerve, brainstem or spinal cord. All patients with CIS do not develop MS, but if there is at least one clinically silent white matter MRI lesion, the risk of definite MS rises to 65% in 7 years and 80% in 20 years' follow-up (Tintoré, Rovira et al. 2006, Fisniku, Brex et al. 2008). Cerebrospinal fluid (CSF) oligoclonality increases the risk of second relapse 1.7-fold independently of baseline MRI (Tintoré, Rovira et al. 2008).

Majority of the patients with RRMS will ultimately convert to secondary progressive MS (SPMS) with steady disability progression. In several large series, the median time to conversion has been about 19 years. In 15% of patients, the disease course is primary progressive (PPMS) with disease progression from the onset without the preceding relapsing-remitting phase (Confavreux, Vukusic 2014). MS is the cause of death in about 50% of MS patients and they have excess mortality rates also from other diseases, except cancer, and from accidents and suicide (Brønnum-Hansen, Koch-Henriksen et al. 2004). The mortality is almost three fold higher in patients with MS compared with the general population (Sumelahti, Hakama et al. 2010) and MS is responsible for a reduction in the life expectancy of between 5 to 10 years (Brønnum-Hansen, Koch-Henriksen et al. 2004, Lunde, Assmus et al. 2017).

2.1.3 Diagnosis and follow-up

2.1.3.1 Diagnosis

The first diagnostic criteria for MS were published by Schumacher et al. in 1965 in order to select subjects for clinical trials. Only clinical, no laboratory data, was incorporated into the diagnostic criteria (Schumacher, Beebe et al. 1965). The 1982 review of diagnostic criteria by Poser et al. included also paraclinical evidence and laboratory support (Poser, Paty et al. 1983).

In 2001, new diagnostic criteria, known as the "McDonald criteria", were issued by the International Panel on the Diagnosis of Multiple Sclerosis. The principle of diagnosis is to demonstrate dissemination of lesions in space (DIS) and time (DIT) and to exclude alternative diagnoses. Diagnosis can be based on clinical evidence alone or on integration of clinical evidence and MRI. No single clinical feature or diagnostic test alone is sufficient for the diagnosis of MS and diagnostic criteria include a combination of clinical and paraclinical assessments (McDonald, Compston et al. 2001, Polman, Reingold et al. 2005, Polman, Reingold et al. 2011).

The McDonald criteria have been revised twice, in 2005 and 2010. In previous versions, Barkhof-Tintore MRI criteria were used to demonstrate DIS. In the 2010 revision, Barkhof criteria were replaced by the simplified criteria devised by Swanton et al. (Swanton,

Fernando et al. 2006). In addition, the criteria for DIT were simplified in the 2010 revision. In the 2010 revision, a new T2 lesion can establish DIT irrespective of the timing of the baseline scan and DIT can also be demonstrated by simultaneous presence of asymptomatic gadolinium-enhancing and non-enhancing lesions on the same scan. Thus, in some CIS patients diagnosis can be made based on a single MRI (Polman, Reingold et al. 2011). The updates of McDonald MRI criteria for DIS and DIT are shown in table 1.

Table 1. MRI criteria for DIS and DIT in McDonald updates from 2001 to 2010, modified from Milo et al. 2014.

	2001	2005	2010
DIS	Three of the following 1) ≥1 Gd enhancing lesion or ≥9 T2 lesions 2) ≥3 periventricular lesions 3) ≥1 juxtacortical lesion 4) ≥1 infratentorial lesion	Three of the following 1) ≥1 Gd enhancing lesion or ≥9 T2 lesions 2) ≥3 periventricular lesions 3) ≥1 juxtacortical lesion 4) ≥1 infratentorial lesion	≥1 T2 lesion in ≥2 of 4 areas 1) periventricular 2) juxtacortical 3) infratentorial 4) spinal cord
DIT	≥1 enhancing asymptomatic lesion ≥3 months after CIS onset ≥1 new T2 lesion on a scan obtained ≥3 months after CIS onset	≥1 enhancing asymptomatic lesion ≥3 months after CIS onset ≥1 new T2 lesion on a scan obtained ≥ 30 days after CIS onset	1) new T2 and/or Gd enhancing lesion on follow up MRI 2) simultaneous presence of asymptomatic Gd enhancing and non-enhancing lesions at any time

CIS-clinically isolated syndrome; DIS-dissemination in space; DIT-dissemination in time; Gd-Gadolinium; MRI-magnetic resonance imaging

In the 2001 and 2005 revisions of the McDonald criteria, a positive CSF finding could be used as part of DIS criteria. In the 2010 McDonald criteria, the CSF status is not included in the assessment of DIS but can support the inflammatory nature of the underlying disease and be of importance in the differential diagnostics (McDonald, Compston et al. 2001, Polman, Reingold et al. 2005, Polman, Reingold et al. 2011).

The 2010 McDonald criteria for PPMS require disease progression of one year. In addition to this, 2 of the 3 following criteria must be met: A) Evidence of DIS in the brain, based on at least one T2 lesion in at least one area typical for MS B) Evidence of DIS in the spinal cord with two or more T2 lesions in the cord and C) a positive CSF finding (Polman, Reingold et al. 2011).

2.1.3.2 Follow-up

2.1.3.2.1 Clinical outcome measures

Relapses are the most characteristic clinical feature of RRMS and the annual relapse rate (ARR) has been used as the primary outcome measure in most clinical phase III trials in RRMS patients (Lim, Constantinescu 2010). Surprisingly, the association between relapse rate and longtime prognosis is not clear; it seems that most of the long time disability is not attributable to the relapse rate (Confavreux, Vukusic 2006, Scalfari, Neuhaus et al. 2013).

The expanded disability status scale (EDSS) is a method of qualifying the disability in MS and the most commonly used outcome measurement scale in MS clinical trials (Uitdehaag 2014). It is a 20 point scale ranging from 0 (normal neurological examinations) to 10 (death due to MS) with 0.5 unit increments. Scoring is based on assessment in seven functional systems: visual, brainstem, pyramidal, cerebellar, sensory, bowel/bladder and cerebral. In addition, the ability to walk up to 500 m and the use of walking aids are considered in scoring (Kurtzke 1983). In an attempt to devise a more responsive outcome measure, the Multiple Sclerosis Functional Composite (MSFC) was developed in the 1990s. MSFC is a composite measure consisting of three performance tests. The 9 hole peg test (9HPT) for arm function, the timed 25-foot walk test (T25FW) for leg function and the paced auditory serial addition test (PASAT) for cognition. Measures are converted to standard scores (z-scores) and then averaged to form a single MSFC score. There do seem to be correlations between MSFC and disease stage, EDSS, MRI and quality of life. (Cutter, Baier et al. 1999, Fischer, Rudick et al. 1999).

2.1.3.2.2 Radiological outcome measures

MRI is more sensitive in detecting disease activity than clinical monitoring and can identify new inflammatory lesions more frequently than simply counting the numbers of relapses (Stone, Albert et al. 1995, Barkhof, Scheltens et al. 1992). Acute inflammation can

be measured by Gadolinium (Gd) enhancing T1 lesions or active T2 lesions. Gd enhancement in T1 weighted sequences is a marker of blood-brain barrier (BBB) breakdown and is associated with inflammation and demyelination in patients with MS (Brück, Bitsch et al. 1997, Kermode, Thompson et al. 1990). New lesions show enhancement for only around 3 weeks (Cotton, Weiner et al. 2003); for this reason, detecting disease activity cannot be based solely on Gd enhancing lesions. T2 weighted hyperintense lesions are non-specific, e.g. they can be caused by edema, demyelination or axonal loss (Brück, Bitsch et al. 1997). Even though they are non-specific, T2 lesions provide information of past and new disease activity on serial imaging and active T2 lesions (new or enlarging lesions between two scans) can be used to measure disease activity (Simon 2014).
The total volume of T2 lesions in brain or spinal MRI is called the burden of disease (BOD). Recent evidence shows that MRI lesion load in early disease course is a high-impact prognostic factor for disability accumulation (Tintore, Rovira et al. 2015). A higher number of T2 lesion in CIS patients (Fisniku, Brex et al. 2008) and especially the presence of infratentorial lesions (Tintore, Rovira et al. 2010, Swanton, Fernando et al. 2009) have been shown to correlate with higher disability accumulation.
Brain atrophy reflects axonal loss, demyelination and gliosis in patients with MS and is considered a measure of irreversible pathology (Simon 2006). Global atrophy can be present early in the disease course (Simon 2012) and it correlates generally better than other MRI measures with clinical disability (Fisher, Lee et al. 2008). Focal neurodegeneration is reflected by T1 hypointense lesions, “T1 black holes”, when chronic and not enhancing (Sahraian, Radue et al. 2010).

2.1.4 Comorbidity in MS

2.1.4.1 Comorbidity and secondary conditions

Comorbidity refers to the entire burden of illness other than the condition of interest, whereas secondary conditions are disorders that are direct or indirect consequences of the primary condition (Marrie, Hanwell 2013). Comorbidity is common in patients with MS, with the most common comorbidities being depression (23.7%), anxiety (21.9%), hypertension (18.6%) and hyperlipidemia (10.9%) (Marrie, Cohen et al. 2015). Osteoporosis is a key secondary condition in MS (Marrie, Hanwell 2013).

Physical and mental comorbidities have diverse effects and may lead to diagnostic delay, accelerate disability progression, increase mortality and exert a negative impact on the health-related quality of life (Marrie, Horwitz 2010).

2.1.4.2 MS and fracture risk

Many neurological conditions and treatments used in neurology clinics, e.g. corticosteroid pulses and antiepileptic drugs, predispose patients to osteoporosis (Dobson, Yarnall et al. 2013). In the Global Longitudinal Study of Osteoporosis in Women (GLOW), MS and Parkinson's disease were the neurological conditions which displayed a significantly increased risk of osteoporosis (Dennison, Compston et al. 2012) and several studies have shown that osteopenia or osteoporosis are more common in patients with MS than in the general population (Cosman, Nieves et al. 1998, Weinstock-Guttman, Gallagher et al. 2004, Marrie, Cutter et al. 2009, Hearn, Silber 2010). Even patients with newly diagnosed MS or CIS have been shown to have lower bone mineral density (BMD) than healthy controls (Moen, Celius et al. 2011b), suggesting there are common etiological factors for both MS and osteoporosis. Patients with MS have lower vitamin D levels than controls (Ascherio, Munger et al. 2010) and the effects of vitamin D on bone metabolism are well known (DeLuca 2004, Holick 2006). Smoking is another common risk factor for both MS and osteoporosis (Handel, Williamson et al. 2011, Watanabe, Inoue 2016). The bone health seems to be further compromised in patients with longstanding disease (Hearn, Silber 2010, Marrie, Cutter et al. 2009). The level of disability seems to be a major contributor to the pathogenesis of osteoporosis in patients with MS (Olsson, Oturai et al. 2015, Tyblova, Kalincik et al. 2015, Weinstock-Guttman, Gallagher et al. 2004). The increased risk of epilepsy in MS patients (Marrie, Reider et al. 2015) and the consequent use of anticonvulsants decrease BMD (Farhat, Yamout et al. 2002). High-dose corticosteroids may be used during relapses and these agents may also reduce the MS patients' BMD values (Weinstock-Guttman, Gallagher et al. 2004, Tyblova, Kalincik et al. 2015). Patients with MS also fall frequently and over 50% of patients report at least one fall during the last 3 to 6 months (Finlayson, Peterson et al. 2006, Peterson, Cho et al. 2008, Moen, Celius et al. 2011a, Nilsagård, Gunn et al. 2015). This increased risk of falls in patients with MS is due to impairments in gait, balance, coordination and cognition and in cerebellar, sensory and pyramidal functions (Gunn, Newell et al. 2013, Gunn, Creanor et al. 2013, Sosnoff, Socie et al. 2011). The reduced BMD and the tendency to fall both

contribute to the increased risk of fractures in MS patients. Studies from Denmark, Netherlands and UK have reported 1.4 - 1.99-fold risk of fractures in MS patients compared with controls (Bazelier, de Vries et al. 2012, Bazelier, van Staa et al. 2012, Bazelier, Bentzen et al. 2012, Bazelier, van Staa et al. 2011, Ramagopalan, Seminog et al. 2012). A previous study from northern Finland suggested a high frequency of fractures in the early stage of MS (Krökki, Bloigu et al. 2014).

2.1.5 Treatment

2.1.5.1 Current disease modifying therapies

The current disease modifying therapies (DMTs) reduce disease activity; this is seen as a reduction in the ARR and MRI measures of disease burden in patients with relapsing-remitting MS. Some reduce the disability accumulation in randomized controlled trials (RCTs) but their effects on disability over longer periods are less clear (Wingerchuk, Weinshenker 2016).

The current orally administered therapies include teriflunomide, dimethylfumarate and fingolimod. Self-injectable therapies include interferon beta (IFN-beta), glatiramere acetate (GA) and daclizumab. Monoclonal antibodies natalizumab and alemtuzumab are administered intravenously. The chemotherapeutic agent, mitoxanthrone, is only rarely used due to the increased risk of malignancies (Wingerchuk, Weinshenker 2016).

2.1.5.2 Interferon beta

IFN-beta became available 20 years ago and was the first immunomodulatory therapy approved for MS. Today, two IFN-beta 1b preparations and three IFN-beta 1a preparations have been licensed. IFN-beta preparations are administered by either subcutaneous or intramuscular injection, with treatment intervals varying from alternate days to bi-weekly administration (La Mantia, Di Pietrantonj et al. 2016).

The mechanisms of action of IFN-beta are not fully understood but potentially include reducing T cell activation, induction of anti-inflammatory cytokine shift, prevention of T cell trafficking across the BBB and induction of T regulatory cells (Tregs). IFN-beta inhibits T cell activation by downregulating the expression of major histocompatibility

complex (MHC) class II and costimulatory molecules, thus interfering with antigen presentation, and preventing the interaction of costimulatory molecules B7/CD28 and CD40/CD40L. IFN-beta reduces the levels of T helper (Th) 1 induced cytokines such as IFN-γ and interleukin (IL) 12 while it induces anti-inflammatory Th2 responses. It can significantly alter the IL-12/IL-10 ratio into a neuroprotective direction while non-responders show decrease in IL-10/IL-12p70 ratio. Several mechanisms by which IFN-beta prevents lymphocyte migration have also been proposed (Compston, Coles 2008, Dhib-Jalbut, Marks 2010).

IFN-beta is considered safe; its most common adverse effects are flu-like symptoms and injection site reactions (Francis, Grumser et al. 2003). During IFN-beta treatment, neutralizing antibodies (NABs) may develop, abrogating the treatment effect. The presence of NABs is detected by using IFN-beta induced gene products, namely myxovirus A (MxA) protein, low levels of MxA indicating presence of NABs (Sorensen 2008).

2.1.6 Etiology

The cause of MS is still unknown. Based on intensive epidemiological, genetic and immunological studies, the most popular hypothesis is that it is an autoimmune disease precipitated in genetically susceptible individuals as a consequence of a complex interplay between the genetic risk factors, environmental exposures and infectious agents (Sospedra, Martin 2016).

2.1.6.1 Immunopathogenesis of MS

2.1.6.1.1 Autoimmunity in MS

The major hypothesis in MS pathology is that myelin specific autoreactive T cells are activated outside the CNS and immune activation is then transferred to CNS (Hemmer, Kerschensteiner et al. 2015). An activation of autoreactive T cells in the periphery might result from cross-reactivity, a bystander effect or molecular mimicry (Sospedra, Martin 2005, Stinissen, Hellings 2008). Activated T cells cross the BBB in a process mediated by adhesion molecules, chemokines and matrix metalloproteinases (MPP). Once in the CNS, they recognize their target antigen presented by local antigen presenting cells (APCs) and are activated again (Bartholomäus, Kawakami et al. 2009). This leads to the

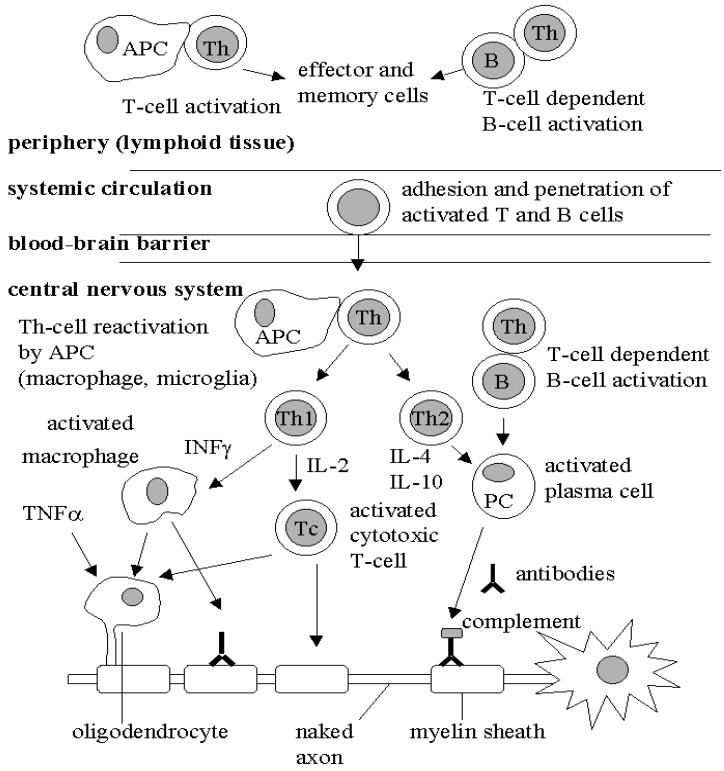
secretion of pro-inflammatory cytokines and recruitment of other immune cells to the CNS and activation of macrophage and microglia, leading to demyelination (Kawanokuchi, Shimizu et al. 2008, Murphy, Lalor et al. 2010). Antibodies secreted by plasma cells also target the myelin sheets and glial cells (Cepok, Rosche et al. 2005).

CD4+Th1 cells were regarded as the main effector cells but more recently Th17 cells have been identified as the primary effectors of MS pathogenesis (Langrish, Chen et al. 2005, Korn, Bettelli et al. 2009). In addition to CD4+Th cells, cytotoxic CD8+ cells are assumed to have a role as inductors or effectors in MS. The vast majority of lymphocytes in MS plaques are CD8+ T cells and the majority of CD8+ T cells isolated from MS plaques or CSF have been shown to contain clonal expansions indicating responses against selected antigens and an active role in plaque formation (Huseby, Huseby et al. 2012).

B cells, plasma blasts and plasma cells are also detected in plaques, meninges and CSF of most MS patients (Henderson, Barnett et al. 2009). Plasma cells produce immunoglobulins within the lesions (Esiri 1980) which is detected as oligoclonal bands in CSF. An enrichment of B cells in the CSF is associated with a more active disease course (Cepok, Rosche et al. 2005) and CSF oligoclonal bands in patients with CIS have been associated with an increased risk of conversion to MS (Dobson, Ramagopalan et al. 2013) suggesting antibody mediated tissue damage in MS. Nonetheless, the target antigens in MS are not known despite intensive searching (Hohlfeld, Dornmair et al. 2015, Hohlfeld, Dornmair et al. 2016).

T regulatory cells are the key mechanism in limiting excessive immune responses. Tregs are either naturally generated in the thymus (nTregs) or derived from naïve CD4+cells in the periphery (iTregs). Forkhead box P3 (Foxp3) is the most specific marker of Tregs, expressed by nTregs and some populations of iTregs. Tregs inhibit effector T cells by secreting anti-inflammatory cytokines and by direct cell-cell contacts and are mandatory for maintaining peripheral tolerance (Workman, Szymczak-Workman et al. 2009). Tregs from MS patients have lower suppressive capabilities which may contribute to MS pathogenesis (Viglietta, Baecher-Allan et al. 2004).

Figure 1. Immunopathogenesis in MS. By Kuusisto 2008, reprinted with permission.



APC-antigen presenting cell; IL-interleukin; INF-interferon; PC-plasma cell; Tc-cytotoxic T cell; Th-T helper cell; TNF-tumor necrosis factor

2.1.6.1.2 Autoimmunity vs degeneration

Studies on autopsal material have shown areas of demyelination and oligodendrocyte loss with activation of microglia but no major lymphocyte infiltration (Barnett, Prineas 2004, Henderson, Barnett et al. 2009). In areas of normal-appearing white matter (NAWM), oligodendrocyte loss and microglial activation are also seen with only marginal lymphocyte activity (Henderson, Barnett et al. 2009). This has convinced some authors to propose that MS is primarily a neurodegenerative disorder with inflammation being only a secondary response to tissue degeneration (Stys, Zamponi et al. 2012, Trapp, Nave 2008). In this model, cytodegeneration of oligodendrocytes and myelin is the primary event and leakage of autoantigens out of the CNS triggers the adaptive immune responses (Stys, Zamponi et al. 2012). This is supported by the clinical observations that disease progression is usually independent of relapses (Scalfari, Neuhaus et al. 2013) and established

immunomodulatory therapies have little effect in the disability progression even though they significantly affect inflammatory activity (Filippini, Del Giovane et al. 2013).
Arguments not supporting the degeneration hypothesis are the facts that inflammation is invariably present at every disease stage (Frischer, Bramow et al. 2009). Initial lesions are also associated with inflammation even though the inflammation may be confined to the perivascular spaces or meninges (Kutzelnigg, Lassmann 2014). There is also genetic evidence supporting the autoimmune nature of MS. HLA class II alleles expressed on cells of innate immunity are associated with an increased risk of MS and most risk genes identified by GWAS are associated with adaptive immune system (Sawcer, Franklin et al. 2014).

2.1.6.1.3 Role of cytokines

Cytokines are low-molecular mass proteins that act as intercellular messengers at the site of inflammation (Kothur, Wienholt et al. 2016). In MS, pro-inflammatory cytokines are mainly produced by CNS invading leukocytes. These cytokines induce CNS resident cells to produce a wider range of cytokines, which in turn helps to recruit more leukocytes and influence the behaviour of the tissue invading leukocytes themselves (Becher, Spath et al. 2017).
T cells are categorized into different subsets based on their production of specific cytokines. The original Th subsets included Th1 and Th2 cells but new Th subsets have since been described, including Th17, Th22, Th9 and Treg cells (Raphael, Nalawade et al. 2015). Polarizing and signature cytokines of different Th subsets are shown in table 2.
In MS, Th1 and Th17 related cytokines are typically more elevated in the CSF (Kothur, Wienholt et al. 2016). Th1 cytokines include IFN-γ and tumour necrosis factor alpha (TNFα) which are potent pro-inflammatory cytokines associated with increased MHC I and MHC II antigen presentation, macrophage activation, oligodendrocyte apoptosis and expression of adhesion molecules (Rosenman, Shrikant et al. 1995, Schroder, Hertzog et al. 2004, Hövelmeyer, Hao et al. 2005). IL-17 accounts for most of the pathogenic functions of Th17 cells, such as impairing BBB integrity and recruiting neutrophils (Kebir, Kreymborg et al. 2007, Simmons, Liggitt et al. 2014).
Th2 cells are involved in allergies and atopic illnesses and in the defence against parasites. They were initially considered anti-inflammatory as they are able to suppress Th1 mediated autoimmunity (Berger 2000). Subsequently, it has been found that Th2 cell can promote several autoimmune diseases, especially those with humoral immune responses.

However, Th2 cytokines IL-4 and IL-13 can mediate protection by suppressing Th1/Th17 development and inhibiting production of pro-inflammatory cytokines (Raphael, Nalawade et al. 2015).

Transforming growth factor beta (TGF-β) and IL-10 are inhibitory cytokines considered to be the major mechanism of suppression used by Tregs (Workman, Szymczak-Workman et al. 2009). TGF- β is produced by both nTregs and iTregs expressing Foxp3 and is important in maintaining the homeostasis of nTregs in the periphery. TGF-β is also required for the generation of Foxp3+ iTregs, regarded as Th3 cells (Marie, Letterio et al. 2005). Another subset of iTregs, Type 1 regulatory T cells (Tr1), not expressing Foxp3, require IL-10 for induction. These cells are able to produce high levels of IL-10 and TGF- β (Roncarolo, Gregori et al. 2006).

Table 2. Main polarizing and effector cytokines of different Th cell subpopulations. Modified from Raphael et al. 2015

Main polarizing cytokine	T cell subset	Main effector cytokines	Suspected actions
IL-12	Th1	IFN-γ, TNF	Cellular immunity Clearance of intracellular pathogens Autoimmunity
IL-4	Th2	IL-4, IL-5, IL-13	Humoral immunity Clearance of extracellular pathogens
IL-1, IL-6, IL-23, TGF-β	Th17	IL-17, IL-22, IL-21, IL-25, IL-26	Tissue inflammation Clearance of extracellular pathogens Autoimmunity
TGF-beta, IL-4	Th9	IL-9	Antihelminth activity Antitumor immunity Tissue inflammation
IL-6, TNF	Th22	IL-22	Tissue Inflammation
TGF-beta, IL-10	Treg	IL-10, TGF-beta	Immune regulation and tolerance

IFN-interferon; IL-interleukin; TGF-Transforming growth factor; Th–T helper; TNF - tumor necrosis factor; Treg-T regulatory cell

Cytokines are pleiotropic in nature i.e. their effect depends on the target cell. They act in complex synergistic networks and the action of a certain cytokine is affected also by the cytokine milieu (Becher, Spath et al. 2017). Thus, dividing cytokines into pro- and anti-inflammatory is an oversimplification and most of the Th cell subsets and cytokines considered pathogenic can also promote immunoprotection under certain conditions (Raphael, Nalawade et al. 2015).

2.1.6.2 Genetics

Family studies suggest that the familial clustering seen in MS is determined mainly genetically (Ebers, Sadovnick et al. 1995). The MS risk is increased to 2-6% in first degree relatives of MS patients (Sadovnick, Baird et al. 1988) and concordance is approximately 25% in monozygotic (MZ) twins, 5% in dizygotic (DZ) twins and 3% in non-twin siblings (Willer, Dyment et al. 2003). In a Finnish MS twin study, the pairwise concordance for MZ twins was 30% and for DZ twins 14.3% (Kuusisto, Kaprio et al. 2008). There is no increased risk in adoptive relatives of MS patients (Ebers, Sadovnick et al. 1995) and in step-siblings (Dyment, Yee et al. 2006) whereas the risk is intermediate in the offspring of conjugal pairs (Robertson, O'Riordan et al. 1997).

2.1.6.2.1. Genetics of MS

Association between human leukocyte antigen (HLA) genes and multiple sclerosis was first established in the 1970s (Compston, Coles 2008). HLA-DRB1 genes encode the MHC II class molecules that present peptide antigens to CD4+ T cells (Sollid, Pos et al. 2014). A large genome-wide association study conducted as part of the Wellcome Trust Case Control Consortium 2 (WTCCC2) project confirmed HLA-DRB1*15:01 as the major risk allele for MS ((imsgc), The International Multiple Sclerosis Genetics Consortium, (wtccc2), Wellcome Trust Case Control Consortium 2 et al.). In addition to HLA-DRB1*15:01 alleles HLA-DRB1*03:01, HLA-DRB1*13:03 and HLA-DPB1*03:01 are associated with an increased MS risk whereas HLA-A02:01 allele exerts a protective effect (Sawcer, Franklin et al. 2014).

Outside MHC more than 100 susceptibility genes have been found ((imsgc), International Multiple Sclerosis Genetics Consortium, Beecham et al. 2013, (imsgc), The International Multiple Sclerosis Genetics Consortium, (wtccc2), Wellcome Trust Case Control Consortium 2 et al.). Most are located in regulatory regions and are immunologically relevant (Sawcer, Franklin et al. 2014).

2.1.6.2.2. Vitamin D related genes in MS

Supporting a role for vitamin D in MS etiopathogenesis, variants of CYP27B1 and CYP24A1 were found to be associated with an increased risk of MS in the genome wide association studies. Both genes are involved in vitamin D metabolism and affect the serum levels of 25-hydroxyvitamin D (25(OH)D) and 1,25-dihydroxyvitamin D (1,25(OH)2D). CYP27B1 encodes an 1α-hydroxylase that converts 25(OH)D

into its active form 1,25(OH)2D and CYP24A1 encodes 24-hydroxylase that hydroxylates both 1,25(OH)2D and 25(OH)D (Christakos, Ajibade et al. 2012). In addition to the CYP27B1 variant found in the Wellcome Trust Genome-wide association study, rare loss-of-function variants of CYP27B1 were associated with increased risk of multiple sclerosis in the study of Ramagopalan et al. supporting a causative role of vitamin D deficiency in the etiology of MS (Ramagopalan, Dyment et al. 2011). Recently, a study published in Nature Genetics further supported a causative effect of 3 single nucleotide variants of the CYP27B1 and CYP24A by excluding the effect of environmental or lifestyle factors or reverse causality using a Mendelian randomization method (Rhead, Bäärnhielm et al. 2016).

2.1.6.3 Environmental risk factors

Genetic burden scores cannot alone predict MS susceptibility (Isobe, Damotte et al. 2013) underlining the importance of non-genetic risk factors in MS etiology. The latitudinal gradient in MS incidence and prevalence (Simpson, Blizzard et al. 2011), migration studies (Gale, Martyn 1995) and rapid changes in the MS incidence and female-male ratio (Koch-Henriksen, Sørensen 2010) support the role of environmental risk factors in the pathogenesis of MS. Vitamin D deficiency, smoking and Epstein-Barr virus (EBV) infection are the strongest contenders for environmental risk factors (Ebers 2013, Hayes, Hubler et al. 2015).

2.1.6.3.1 Epstein-Barr virus

Several lines of evidence link EBV infection to MS risk (Pakpoor, Giovannoni et al. 2013, Owens, Bennett 2012, Ascherio, Munger 2010). A history of infectious mononucleosis (IM) more than doubles the risk of developing MS (Handel, Williamson et al. 2010). More than 99% of patients with MS are seropositive for EBV (Pakpoor, Disanto et al. 2013) although there is a high rate (95%) of seropositivity also in the general population (Luzuriaga, Sullivan 2010). In a recent meta-analysis, the odds ratio (OR) for MS was 0.00 in seronegative individuals when only studies that used two independent methods for EBV detection were included (Pakpoor, Disanto et al. 2013). In a large prospective study, Levin et al. found a total absence of incident MS in individuals seronegative for EBV. After seroconversion, which was detected in about every third member of the study

population during the follow-up, a similar risk for MS was reached with those who were EBV positive at the baseline (Levin, Munger et al. 2010).

Different mechanisms to explain the role of EBV in triggering MS have been proposed, such as molecular mimicry; in susceptible individuals, immune response to EVB infection may cross-react with myelin antigens involving both T-cells and B-cells (Höllsberg, Hansen et al. 2003, Rand, Houck et al. 2000). The presence of EBV-infected B cells in MS lesions and activation of CD8+ T cells at sites of greater EBV-infected cell density was noted in one study (Serafini, Rosicarelli et al. 2007) but other studies have been able to reproduce these findings only partially (Lassmann, Niedobitek et al. 2011). Higher EBV-specific CD8+ T-cell responses have been reported in patients with MS than in healthy volunteers (Jilek, Schluep et al. 2008).

2.1.6.3.2 Smoking

Cigarette smoking has been consistently associated with an increased risk of MS with an OR of 1.5 (Hedström, Bäärnhielm et al. 2009a, Handel, Williamson et al. 2010). In addition to smoking history, serum levels of cotinine, a metabolite of nicotine used as an objective marker for cigarette smoking, have been associated with an increased risk of multiple sclerosis with a similar OR of 1.5 (Salzer, Hallmans et al. 2013). Smoking also has a negative effect on the disease course in MS. It has been reported that smoking speeds up the conversion from CIS to definite MS (Di Pauli, Reindl et al. 2008, Correale, Farez 2015) and increases the risk of conversion from relapsing-remitting to progressive disease and accelerates disability progression (Manouchehrinia, Tench et al. 2013, Sundström, Nyström 2008, Healy, Ali et al. 2009). Beneficial effects of smoking cessation on disease progression have been described (Manouchehrinia, Tench et al. 2013, Ramanujam, Hedström et al. 2015).

Passive smoke exposure has been associated with increased MS risk (Hedström, Bäärnhielm et al. 2011, Sundström, Nyström et al. 2008) whereas snuff use does not appear to increase the MS risk, suggesting that nicotine is unlikely to account for the association between smoking and increased MS risk (Hedström, Bäärnhielm et al. 2009b). In the EAE model, nicotine even ameliorates the disease severity and thus it must be the other components of cigarette smoke that cause more severe disease (Gao, Nissen et al. 2014). Odoardi et al demonstrated that autoreactive T cells may be primed in the lung tissue to gain the capacity to enter the CNS. Environmental factors, including smoking, might elicit a pathogenic response of these cells (Odoardi, Sie et al. 2012).

2.1.6.3.3 Latitude

Early studies established latitudinal variation in MS frequency, incidence and prevalence increasing with the distance from the equator (Acheson, Bachrach et al. 1960, Kurtzke, Beebe et al. 1979, Miller, Hammond et al. 1990). However, not all studies have found an association between latitude and MS prevalence (Poppe, Wolfson et al. 2008, Melcon, Gold et al. 2008) and the latitude gradient has been questioned recently (Koch-Henriksen, Sørensen 2010, Zivadinov, Iona et al. 2003). Nonetheless, two comprehensive meta-analyses found a significant positive latitude effect on MS incidence (Alonso, Hernán 2008) and prevalence (Simpson, Blizzard et al. 2011) in global terms. An inverse prevalence gradient was found for Italy, Scandinavia and North Atlantic regions. The inverse gradient in Italy was reversed, yielding a positive gradient effect similar to the rest of Europe, after adjustment with HLA-DRB1 frequencies (Simpson, Blizzard et al. 2011). The inverse gradient in the Scandinavian region may be explained by nutritional factors (Kampman, Wilsgaard et al. 2007). Thus it seems that there is indeed a latitudinal gradient in MS incidence but it seems to becoming less marked (Alonso, Hernán 2008, Wallin, Page et al. 2004, Hernán, Olek et al. 1999), probably as a result of increased incidence of MS in former low-risk areas (Alonso, Hernán 2008, Ascherio, Munger 2007a). A common genetic background is likely to contribute to the geographic variation in MS incidence (Compston, Sawcer 2002) but migration studies (Kurtzke, Beebe et al. 1985, Gale, Martyn 1995) and change in the latitude gradient suggest that there must be an environmental factor acting on the MS risk.

2.1.6.3.4 UV radiation

Many environmental and social factors correlate with latitude, the strongest correlate being the intensity of UVR. Satellite-derived data on UV intensity (UVI) revealed a strong association with MS frequency in studies conducted in France (Orton, Herrera et al. 2006), North America (Beretich, Beretich 2009) and globally (Sloka, Silva et al. 2011) which further reinforces the sunshine hypothesis. Several studies have also examined the risk of MS among people living in the same area but exposed to different levels of UVR. In a study based on death certificates in the USA, outdoor work and residence in a high sunlight area were both associated with lower MS mortality (Freedman, Dosemeci et al. 2000). Similar results were found in a Swedish study (Westberg, Feychting et al. 2009). Case-control studies from Norway (Kampman, Wilsgaard et al. 2007), and Tasmania (van

der Mei, I a F, Ponsonby et al. 2003) found that increased outdoor activities in childhood and adolescence were associated with a decreased MS risk. A study based on 81 pairs of monozygotic twins discordant for MS showed that early sun avoidance increased the risk of MS (Islam, Gauderman et al. 2007). Recall bias is a potential concern in these case-control studies (Ascherio, Munger et al. 2010). Nonetheless in the aforementioned Tasmanian study, actinic damage, which is an objective measure of past sun exposure, was independently associated with decreased risk of MS (van der Mei, I a F, Ponsonby et al. 2003).

Exposure to UVB radiation is the major source of vitamin D for most people (Holick 2010) and vitamin D is considered the signal transducer of UVB radiation in MS (Hayes, Cantorna et al. 1997). However, possible mechanisms for immunosuppressive effects of UVB radiation independent of vitamin D have also been described (Becklund, Severson et al. 2010). As 25(OH)D levels reflect past UVB exposure, it is possible that the association between UVB, vitamin D and risk of MS is caused by UVB or vitamin D alone or by combination of both factors. The Ausimmune study found that both UVB exposure (measured as self-reported sun exposure, skin phenotype and actinic damage) and serum 25(OH)D levels were independently associated with risk of a first demyelinating event (FDE) (Lucas, Ponsonby et al. 2011). However, UVB exposure is a better measure of lifetime 25(OH)D levels than a single measure of 25(OH)D at the time of FDE and these results do not contradict the hypothesis that 25(OH)D levels alone affect the MS risk (Ascherio, Munger et al. 2011).

2.1.6.3.5 Vitamin D

In a prospective study investigating about 200 000 women in the USA, vitamin D intake was measured by a food frequency questionnaire. Women in the top quintile of vitamin D intake had a 33% decreased risk of MS during the 30-year follow-up compared with those in the lowest quintile. In addition, women taking 400 IU vitamin D per day or more from supplements had a 41% lower MS incidence compared with non-users. These results were not confounded by latitude or UVB exposure (Munger, Zhang et al. 2004). In Norway, a lower MS risk was reported in individuals who ate fish three times or more per week compared with those with low consumption. Supplementation with cod-liver oil was associated with less MS in the subgroup with less summer outdoor activities (Kampman, Wilsgaard et al. 2007).

Two prospective studies have examined the effect of 25(OH)D concentrations on the future MS risk in healthy individuals. A study conducted on US military personnel found that elevated levels of 25(OH)D >100nmol/l were associated with a 60% decreased risk of later developing MS (Munger, Levin et al. 2006). In a study using two population based biobanks in Sweden, 25(OH)D levels >75nmol/l were associated with a similar 60% decrease in MS risk (Salzer, Hallmans et al. 2012). In a Canadian study of children with a FDE, a reduced serum 25(OH)D level was associated with an increased risk of definite MS (HR per 10 nmol/l decrease 1.11) in the subsequent three years (Banwell, Bar-Or et al. 2011).

Whether the inverse association between vitamin D exposure and risk of MS extends to early life is still unclear. One Swedish prospective study measuring maternal 25(OH)D levels during pregnancy found no association with the MS risk of the offspring (Salzer, Hallmans et al. 2012), neither did a study using dried blood spot samples (DBSS) collected from neonates (Ueda, Rafatnia et al. 2014). At odds with these reports, it has been claimed that a higher dietary vitamin D intake during pregnancy is associated with a lower MS risk in the offspring (Mirzaei, Michels et al. 2011). The month of birth has been associated with the MS risk i.e. there are a higher number of spring births, thus lower gestational UVB exposure and maternal vitamin D levels, among MS patients (Willer, Dyment et al. 2005, Staples, Ponsonby et al. 2010).

2.2 Vitamin D

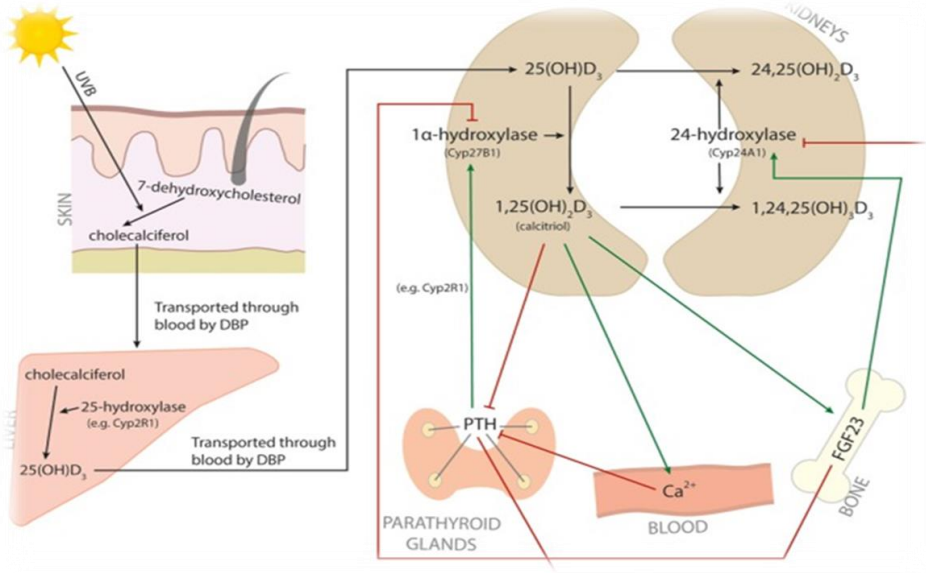
2.2.1 Sources and Metabolism

Humans obtain vitamin D from their diet, dietary supplements and exposure to UVB (Holick 2007b). Vitamin D has two precursors, ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3). Vitamin D2 is solely obtained from diet but its natural sources are scarce. Vitamin D3 is also obtained from diet, mainly from oily fish or fish liver oil, but the major source of vitamin D3 is exposure of the skin to sunlight (Holick 2010). Skin contains 7-dehydroxycholesterol which is converted into pre-vitamin D3 by UVB. Pre-vitamin D3 is then rapidly isomerized to vitamin D3 (Holick 2007b). Vitamin D (D represents D2 or D3) obtained from diet or sun exposure is biologically inert and must be converted into its active form in order to exert its effects on calcium metabolism and other

functions (DeLuca 2004). Vitamin D is converted to 25(OH)D in the liver by one or more cytochrome P450 vitamin D 25-hydroxylases, of which CYP2R1 has been suggested to be the key enzyme (Christakos, Ajibade et al. 2012). 25(OH)D is the main circulating metabolite of vitamin D and serum levels of 25(OH)D are used to determine the patient's vitamin D status (DeLuca 2004). In the kidneys, 25(OH)D is further hydroxylated by 1-α hydroxylase (CYP27B, 1α(OH)ase) to 1,25(OH)2 D which acts as a steroid hormone and is the most important active metabolite of vitamin D (Christakos, Ajibade et al. 2012). The effects of 1,25(OH)2D are mediated through the intracellular vitamin D receptor (VDR). The VDR is a transcription factor which works together with other transcription factors; of these, the retinoid X receptor (RXR) is the most important. The interaction between the VDR with vitamin D regulates the expression of genes which contain the vitamin D response element (VDRE) (Pludowski, Holick et al. 2013, Rosen, Adams et al. 2012).

Since 1,25(OH)D has widespread effects, its production in the kidneys is tightly regulated by serum calcium and phosphorus levels, parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF-23) (Holick 2010, Pludowski, Holick et al. 2013). Hypocalcemia leads to elevated levels of PTH which stimulate the transcription of CYP27B, thus increasing levels of 1,25(OH)2D. 1,25(OH)2D in turn suppresses the production of PTH and also downregulates its own production by negatively regulating the CYP27B gene. Both 25(OH)D and 1,25(OH)2D are hydroxylated by 25-hydroxyvitamin D 24-hydroxylase (CYP24A1, 24(OH)ase). Thus, CYP24A1 decreases the serum levels of 1,25(OH)2D by catabolizing 1,25(OH)2D and decreasing the pool of 25(OH)D. CYP24A1 is reciprocally regulated when compared with CYP27B1 (Christakos, Ajibade et al. 2012).

Figure 2. The metabolic pathway of vitamin D. Red arrows indicate inhibition, and green arrows indicate induction. From Dankers et al, 2017. Printed with permission.



DBP-vitamin D binding protein; FGF-fibroblast growth factor; PTH-parathyroid hormone

2.2.2 Skeletal effects of vitamin D

The major function of vitamin D is to regulate serum calcium homeostasis (DeLuca 2004). Vitamin D acts on intestine, bone and kidneys to increase serum concentrations of calcium (Suda, Ueno et al. 2003). 1,25(OH)2D enhances intestinal calcium absorption by promoting the expression of an epithelial calcium channel and a calcium binding protein (Christakos, Dhawan et al. 2011, Christakos 2012). If the dietary intake of calcium is insufficient, then 1,25(OH)2D mobilizes calcium from bone. 1,25(OH)2D stimulates osteoblasts to produce receptor activator nuclear factor kappa ligand (RANKL). After binding with its receptor, RANK, in preosteoclasts, RANKL induces the maturation of preosteoclasts into osteoclasts. These osteoclasts then destroy the bone matrix to mobilize calcium and phosphorus from the skeleton. PTH is required for this bone-resorbing effect of vitamin D (Suda, Ueno et al. 2003, DeLuca 2004). In conjunction with PTH, 1,25(OH)2D also increases the tubular reabsorption of calcium (DeLuca 2004).

Dietary calcium intake is the preferred way to maintain serum calcium concentrations under normal conditions (DeLuca 2004). Decreased intestinal calcium absorption due to

vitamin D deficiency leads to a transient fall in serum calcium concentrations which induces the secretion of PTH. PTH induces osteoclastogenesis which in turn leads to a decrease in the bone mineral density. Thus, by causing secondary hyperparathryroidism, vitamin D deficiency precipitates and worsens osteoporosis and osteopenia (Holick 2007a). A chronic vitamin D deficiency leads to insufficient calcium-phosphorus product in the serum. This causes a bone mineralization defect, resulting in rickets in children and osteomalacia in adults (Holick 2006).

Vitamin D seems to affect musculoskeletal health in more ways than simply by influencing bone metabolism. There is evidence that vitamin D has beneficial effects on muscle strength (Pludowski, Holick et al. 2013) and a comprehensive meta-analysis revealed that vitamin D supplementation also reduces the falling tendency (Murad, Elamin et al. 2011). There is still an on-going debate about what represent optimal serum levels of vitamin D. Some authors consider levels of 50 nmol/l sufficient for bone health, whereas some argue that levels over 75 nmol/l are needed, based on the finding that calcium transport was increased from 45 to 65% when 25(OH)D levels increased from 50 to 80 nmol/l (Heaney, Dowell et al. 2003, Ross 2011, Holick 2007a). In biochemical studies on 25(OH)D hydroxylase kinetics, the low end of optimal 25(OH)D status is 80 nmol/l (Heaney, Armas et al. 2008). Traditionally living populations in East Africa have a mean serum 25-hydroxyvitamin D concentration of 115 nmol/l (Luxwolda, Kuipers et al. 2012) and the physiological range of 25(OH)D appears to be 80-115 nmol/l (Heaney 2014).

2.2.3 Immunological effects of vitamin D

In addition to its important role in bone mineralization, vitamin D controls the growth and metabolism of many cell types, including cells of the adaptive and innate immune systems (Jurutka, Whitfield et al. 2001). The presence of VDR in human lymphocytes was one of the first observations of the possible immunomodulatory effects of vitamin D (Provvedini, Tsoukas et al. 1983). Subsequently, VDR has been found in all cells of the immune system and it has been shown that cells of the immune system are also able to metabolize 1,25(OH)2D (Veldman, Cantorna et al. 2000, Chen, Sims et al. 2007, Correale, Ysrraelit et al. 2009, Peelen, Knippenberg et al. 2011).

1,25(OH)2D has been demonstrated to prevent the onset and ameliorate the course of experimental autoimmune encephalitis (EAE), which is an animal model of MS

(Lemire, Archer 1991). In animals with active disease 1,25(OH)2D halted disease progression and even resolved an established disability (Cantorna, Hayes et al. 1996). Colecalciferol inhibits EAE in female but not male mice (Spach, Hayes 2005).

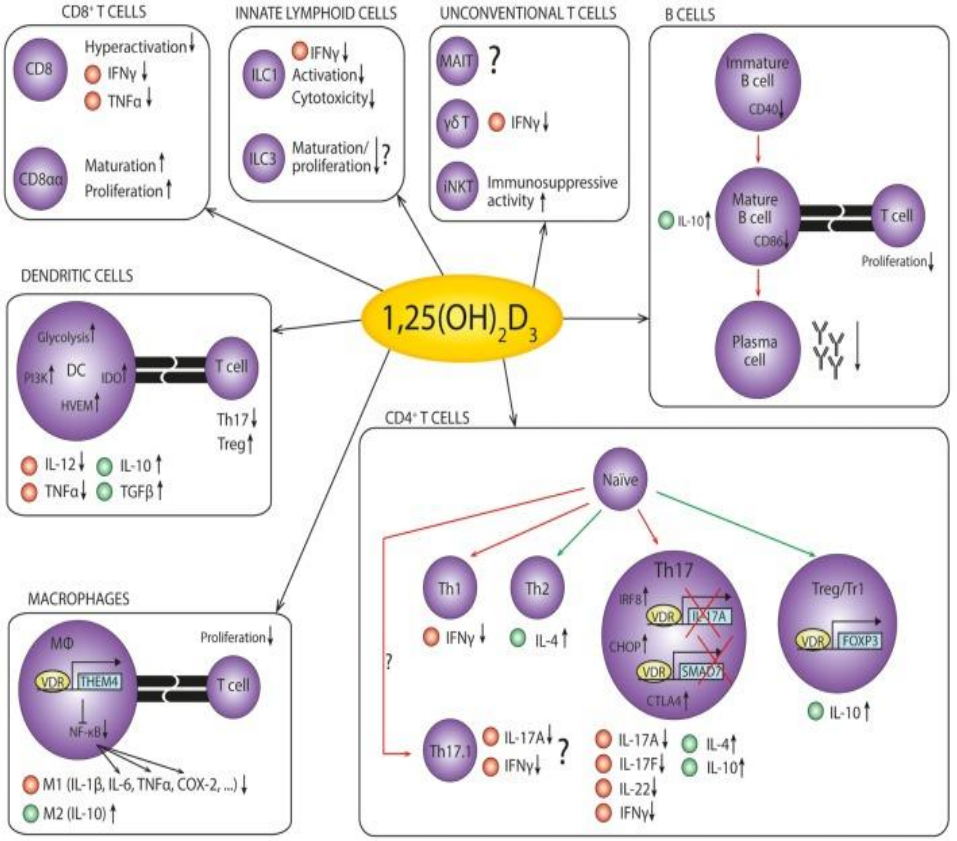
APCs, especially dendritic cells (DCs), are suggested to be the key targets for the immunomodulatory properties of 1,25(OH)2D (Adorini, Penna et al. 2004). 1,25(OH)2D inhibits the proliferation and maturation of DCs and decreases the expression of costimulatory molecules CD40, CD80 and CD86 (Penna, Adorini 2000, Adorini, Penna et al. 2004). 1,25(OH)2D modulates maturing DCs, leading to abrogation of IL-12 and enhanced production of IL-10 (Penna, Adorini 2000, Griffin, Lutz et al. 2001). These 1,25(OH)2D conditioned tolerogenic DCs are able to promote T-cell differentiation into Treg cells and suppress the proliferation of pro-inflammatory T-cells (Dankers, Colin et al. 2016).

1,25(OH)2D has also direct effects on T cells. It has been shown to directly inhibit Th1 and Th17 differentiation and the production of IL-17 (Takeuchi, Reddy et al. 1998, Ikeda, Wakita et al. 2010, Joshi, Pantalena et al. 2011) and increase effector CD4+T-cell sensitivity to extrinsic apoptotic signals (Spach, Pedersen et al. 2004). In contrast to this 1,25(OH)2D promotes the induction of Foxp3+ Tregs (Urry, Chambers et al. 2012) and IL-10 producing Tregs (Correale, Ysrraelit et al. 2009).

In vivo vitamin D levels have been associated with improved Treg function (Smolders, Thewissen et al. 2009), decreased production of the pro-inflammatory cytokines (Muris, Damoiseaux et al. 2013) and shifting the Th1/Th2 balance towards Th2 (Smolders, Thewissen et al. 2009). Seasonal variation in thymic output has also been shown; May born infants had lower 25(OH)D levels and higher thymic output than November born infants and it is hypothesized that vitamin D has positive influence in thymic negative selection (Disanto, Watson et al. 2013).

The effect of vitamin D on B cells is less clear. The VDR is expressed in B cells at rather low levels but is up-regulated with B cell stimulation (Chen, Sims et al. 2007). In vitro, vitamin D can inhibit plasma cell generation and induce apoptosis (Chen, Sims et al. 2007) and reduce antibody production (Lemire, Adams et al. 1984, Heine, Anton et al. 2002). 1,25(OH)2D also inhibits T-cell co-stimulation (Drozdenko, Scheel et al. 2014) and enhances regulatory B-cell activity (Heine, Niesner et al. 2008) but in vivo data have not confirmed these findings (Rolf, Muris et al. 2016).

Figure 3. An overview of the anti-inflammatory effects of $1,25(OH)_2D_3$ on cells of the immune system. Red dots represent pro-inflammatory cytokines, while green dots represent anti-inflammatory cytokines. Red arrows indicate decreased differentiation, and green arrows indicate increased differentiation. From Dankers et al. 2017. Printed with permission



2.2.4 Effect of vitamin D levels on disease course

Vitamin D status has been associated with MS disease activity in several studies. MS patients have lower vitamin D levels during relapse than remission (Soilu-Hänninen, Airas et al. 2005, Soilu-Hänninen, Laaksonen et al. 2008, Correale, Ysrraelit et al. 2009). A retrospective cohort study found that higher 25(OH)D levels were associated with a better possibility to remain relapse-free in the previous 24 months (Smolders, Menheere et al. 2008). An inverse association between relapse rate and 25(OH)D levels has been shown in patients with pediatric and adult-onset MS (Mowry, Krupp et al. 2010, Simpson, Taylor et al. 2010, Runia, Hop et al. 2012). In addition, MRI activity, seen as new T2 and

Gd enhancing lesions, has been shown to inversely correlate with levels of 25(OH)D (Løken-Amsrud, Holmøy et al. 2012, Mowry, Waubant et al. 2012). The association between disease progression and 25(OH)D levels is less clear. Muris et al showed that low vitamin D levels at the start of RRMS were associated with early conversion to SPMS (Muris, Rolf et al. 2016). In cross-sectional studies, patients with lower vitamin D levels have been shown to exhibit a higher disability level (van der Mei, I a F, Ponsonby et al. 2007, Smolders, Menheere et al. 2008) but in a large retrospective follow-up study, no association was observed between 25(OH)D status and disability progression (Muris, Smolders et al. 2016a). This study had some limitations i.e. a heterogeneous study population and a relatively short follow-up of three years. In a study among participants of the BENEFIT trial, Ascherio et al. showed that higher vitamin D levels early in disease course predicted reduced disease activity, MRI lesion load and brain atrophy and a lower level of clinical progression during five years' follow up (Ascherio, Munger et al. 2014).

2.2.5 Vitamin D supplementation in patients with MS

Safety data suggests that high dose vitamin D supplementation (short-term use of up to 40 000 IU/d) is well tolerated and no serious adverse events have been reported (Kimball, Ursell et al. 2007, Burton, Kimball et al. 2010).

The effect of high dose vitamin D supplementation on the relapse rate has been rather disappointing. A meta-analysis conducted in 2013 found no effect of high-dose vitamin D supplementation on relapse risk (James, Dobson et al. 2013) and two recent reviews came to similar conclusions (Pozuelo-Moyano, Benito-León et al. 2013, Canadian Agency for Drugs and Technologies in Health 2016). However, sample sizes were small and there was heterogeneity between studies with respect to the doses and formulations used and the duration of vitamin D treatment (James, Dobson et al. 2013). An open label study with 156 patients with RRMS detected a strong inverse relationship between relapse rate and serum 25(OH)D levels after supplementation (Pierrot-Deseilligny, Rivaud-Péchoux et al. 2012) and in natalizumab treated patients with hypovitaminosis D, correcting 25(OH)D levels with oral vitamin D supplementation was associated with a decreased ARR (Laursen, Søndergaard et al. 2016).

A few studies have addressed the effect of vitamin D supplementation on MRI outcomes. Kimball et al observed a decreased number of T1 Gd+ lesions after escalating vitamin D3 doses (Kimball, Ursell et al. 2007) and a lower incidence of new T1Gd+, T2 and black

holes was found in the vitamin D3 treated group in CIS patients (Derakhshandi, Etemadifar et al. 2013). Other studies have not detected any effect on MRI outcomes (Mosayebi, Ghazavi et al. 2011, Stein, Liu et al. 2011).

The SOLAR study (NCT01285401) is the largest randomized controlled trial (RCT) of vitamin D supplementation as add-on therapy; it enrolled 229 MS patients already receiving IFN-beta. The results were presented at the ECTRIMS 2016 congress in London in September 2016. No effect was demonstrated on the NEDA (no evidence of disease activity) primary outcome, but high dose vitamin D significantly improved MRI outcomes. The ARR was reduced by 30%, which was not statistically significant although this was probably attributable to the sample size, i.e. there was some evidence of a beneficial effect of high-dose vitamin D on clinical disease activity (Smolders, Hupperts et al. 2016).

Several groups have investigated the effect of vitamin D treatment on immunological outcome measures in healthy controls and patients with MS and other autoimmune diseases. In patients with MS, vitamin D supplementation has been shown to increase the levels of TGF-β (Mahon, Gordon et al. 2003) and lower the concentrations of MMP-9 (Kimball, Vieth et al. 2011). Increased serum levels of IL-10 have been shown in one study (Ashtari, Toghianifar et al. 2015). The effects of the supplementation on IL-17 are conflicting (Golan, Halhal et al. 2013, Toghianifar, Ashtari et al. 2015) but otherwise no effect on serum levels of cytokines or chemokines has been observed (Ganesh, Apel et al. 2013, Muris, Smolders et al. 2016b). Kimball et al. detected reduced proliferative responses of T cells against CNS autoantigens after vitamin D3 supplementation (Kimball, Vieth et al. 2011) and in line with this result, Mosayebi et al described decreased T cell proliferation and increased TGF beta and IL-10 expression in the supernatant of PBMC of vitamin D supplemented patients (Mosayebi, Ghazavi et al. 2011). In the study of Smolders et al., T cell proliferation was not affected but an increased proportion of IL10+CD4+ T cells and a decrease in the ratio of IFN-γ/IL4+ CD4+ T cells were observed after vitamin D3 supplementation (Smolders, Peelen et al. 2010). In a recent study high-dose colecalciferol reduced the proportion of IL-17+CD4+ T-cells (Sotirchos, Bhargava et al. 2016a). SOLARIUM, a sub-study of SOLAR, investigated the immunoregulatory effects of high dose vitamin D3 supplementation in MS patients treated with IFN-beta. Vitamin D supplementation did not affect the proportion of lymphocytes with a regulatory phenotype or pro-inflammatory Th-cells but overall data pointed towards the prevention of an immunological imbalance (Muris, Smolders et al. 2016b). An overview of vitamin D supplementation studies in MS is presented in table 3.

Table 3. Studies assessing the effect of vitamin D supplementation on immunological and clinical parameters in patients with MS.

Author, Year	Study design	Vitamin D supplement	Results	Serum levels of 25(OH)D
Goldberg 1986	Open, 24 months, 16 RRMS	D3 5000 IU/d	Relapses ↓	37-200 nmol/l
Achiron 2003	Open, 6 months, 5 RRMS	Alfacalcidol 1,5ug/d	3 stable, 1 improved, 1 relapsed	-
Mahon 2003	RCT, 6 months, 39 MS (17 treatment, 22 placebo)	D3 1000 IU/d	TGF-β↑ TNF-α, INFγ, IL-13↔ IL-2mRNA↓	70±20 nmol/l
Wingerchuk 2005	Open, 48 weeks, 15 RRMS	Calcitriol escalating to 0,5-2,5 ug/d	ARR↓	-
Kimball 2007	Open, 28 weeks, 12 MS	D3 escalating from 28 000 to 280 000 IU/week	Gd+ lesions ↓ ARR; EDSS↔	386 ±157 nmol/l
Burton 2010	Open label RCT, 12 months, 49 MS (25 treatment, 24 control)	D3 escalating from 4000 to 40 000 IU/day	T-cell proliferation↓ Relapses↓(trend) EDSS↔	mean peak 413 nmol/l
Kimball 2011	Open label RCT, 12 months, 49 MS (25 treatment, 24 control)	D3 escalating from 4000 to 40 000 IU/d	Mononuclear cell proliferative responses to CNS autoantigens↓	179 ±76 nmol/l
Smolders 2010	Open, 12 weeks, 15 RRMS on IFN-beta	D3 20 000 IU/d	IL-10+CD4+ proportion↑ INF-γ/IL4+CD4+ ratio ↓	380 (151-535) nmol/l
Knippenberg 2011	Open, 12 weeks, 15 RRMS on IFN-beta	D3 20 000 IU/d	B-cell differentiation↔ B cell isotype switching↔ BAFF levels↔	380 (151-535) nmol/l
Mosayebi 2011	RCT, 6 months, 62 MS (28 treatment, 34 placebo), on IFN-beta	D3 300 000 IU/month i.m.	EDSS↔ Gd+ lesions ↔ T-cell prolif↓ TGF-β, IL-10 expression↑	140 nmol/l

Stein 2011	RCT, 6 months, 23 RRMS (12 low dose, 11 high dose),	D2 low dose 1000 IU/d high dose 7000 IU/d	T1 Gd+ lesions, T2 lesions ↔ High dose EDSS↑ High dose Relapses↑	Ld 69 nmol/l Hd 120 nmol/l
Steffensen 2011	RCT, 96 weeks, 68 RRMS (35 treatment, 33 placebo)	D3 20 000 IU/week	BMD↔	123 (113-133) nmol/l
Kampman 2012	RCT, 96 weeks, 68 RRMS (35 treatment, 33 placebo)	D3 20 000 IU/week	Relapses ↔ EDSS ↔ MSFC ↔	123 (113-133) nmol/l
Pierrot-Deseilligny 2012	Open, Three years, 156 RRMS	D3 average 3010 IU/d	Inverse relapse rate with 25(OH)D levels	-
Shaygannejad 2012	RCT, 12 months, 50 RRMS (25 treatment, 25 placebo)	Calcitriol up to 0,5ug/d	EDSS↔ Relapse rate↔	-
Derakshandi 2013	RCT, 30 optic neuritis (15 treatment, 15 placebo)	D3 50 000 IU week	Black holes, new T1 Gd+ lesions, new T2 lesions ↓	-
Golan 2013	RCT, 12 months, 45 RRMS (21 low dose, 24 high dose) on IFN-beta	D3 low dose 800 IU/d high dose 4370 IU/d	Low dose IL-17↑ High dose IL-17↔ IL-10, IFNγ↔ EDSS↔ RR↔ FLS↔	Low dose 68 ±11 nmol/l High dose 123 ±32 nmol/l
Bhargava 2015	Open, 90 days, 27 MS, 30 HC	D3 5000 IU/d	Increase in 25(OH)D lower in MS patients	Change in HC 82.4 ± 5.2 nmol/l MS 65.9 ± 5.9 nmol/l
Etemadifar 2015	Open label RCT, 15 pregnant RRMS (treatment 6, controls 9) from 12-16 weeks of gestation until delivery	D3 50 000 IU/week	EDSS progression↓ RR↓	85± 37 nmol/l
Rosjo 2015	RCT, 96 weeks,	D3 20 000 IU/week	ALCAM, CCL21, CXCL16,IL-1Ra,	123±34 nmol/l

	68 RRMS (35 treatment, 33 placebo)		MMP-9, OPG, OPN, PTX3, FRP3, TNF-R1, TGF-β1↔	
Ashtari 2015	RCT, 12 weeks, 94 RRMS (44 treatment, 45 placebo) on IFN-beta	D3 50 000 IU every five days	IL-10↑	212±107 nmol/l
Toghianifar 2015	RCT, 12 weeks, 94 RRMS (44 treatment, 45 placebo) on IFN-beta	D3 50 000 IU every five days	IL-17↓	212±107 nmol/l
Muris 2016	RCT, 48 weeks, 53 RRMS (30 treatment, 23 placebo) on IFN-beta	7000 IU/d 4weeks 14000 IU/d up to week 48	Total amount of lymphocytes↔ Proportion of Treg and Breg↔	231 (162-250) nmol/l
Sotirchos 2016	RCT, 6 months, 40 RRMS (21 low dose, 19 high dose)	Low dose 800 IU/d High dose 10 400 IU/d	Low dose: no effect High dose: IL-17+CD4+↓ CD161+CD4+↓ Effector memory CD4+↓ Central memory CD4+↑ Naive CD4+↑	Mean change low dose: 17 nmol/l high dose 87 nmol/l

ALCAM-activated leukocyte cell adhesion molecule; ARR-annual relapse rate; Breg-B regulatory cell; CCL- chemocine (C-C motif) ligand; CXCL -chemokine (C-X-C motif) ligand; EDSS-expanded disability status scale; FLS-flu like symptoms; IFN-interferon; IL-interleukin; MMP-matrix metalloproteinase; OPG-osteopontegrin; OPN-osteopontin, PTX-pentraxin; RCT-randomized controlled trial; RRMS-relapsing-remitting multiple sclerosis; sFRP-secreted frizzled related protein; sTNF-R-soluble tumor necrosis factor receptor; TGF-transforming growth factor; Treg- T regulatory cell

3 AIMS OF THE STUDY

Vitamin D deficiency has been identified as a risk factor for MS but it is not known when the risk begins. Much less is known on whether vitamin D has any effect on established MS and what would be the mechanism of action. The specific aims of this study were:

1. To study the association between maternal vitamin D status during early pregnancy and the risk of MS in the offspring in the Finnish Maternity Cohort. (Study 1, Original article I)

2. To evaluate the safety and efficacy of vitamin D supplementation in MS patients receiving IFN-beta therapy. (Study 2, Original articles II&III)

3. To investigate the mechanism of action of vitamin D supplementation in MS patients receiving IFN-beta therapy. (Study 2, Original article IV)

4. To update the MS prevalence and to study the fracture risk and the role of vitamin D in the risk of fractures in MS patients in southwest Finland. (Study 3, Original article V)

This work consists of three separate Studies (1-3), on which the five Original articles (I-V) are based. In the following text, the Studies are referred to with Arabic numerals (1-3) and Original articles with Roman numerals (I-V).

Study 1, Original article I. The Finnish Maternity Cohort (FMC) Study investigated the risk of MS among children born to women with a pregnancy serum sample in the FMC, a nationwide biorepository of serum samples collected during the first trimester of pregnancy for routine prenatal testing since 1983.

Study 2, Original articles II-IV. The Finnish Vitamin D Study was a randomized, placebo-controlled multi-center clinical trial assessing safety and clinical and radiological efficacy of vitamin D supplementation as an add-on therapy in MS patients receiving IFN-beta therapy.

Study 3, Original article V. The MS prevalence and fracture risk study aimed to identify patients with definite MS diagnosis and comorbid fractures in the hospital district of southwest Finland from January 1, 2004 to December 31, 2012 using hospital administrative data and chart review for case confirmation.

4 MATERIALS AND METHODS

4.1 Setting

Finland is located between latitudes 60°N and 70°N and belongs to a high risk region for multiple sclerosis, with a prevalence of 100-200 per 100 000 inhabitants in different areas. Moderate vitamin D deficiency is very common in healthy Finnish men (Välimäki, Alfthan et al. 2004) and medical patients (Kauppinen-Mäkelin, Tähtelä et al. 2001) in winter time. Almost half of newly diagnosed Finnish MS patients display a moderate to severe vitamin D deficiency.

In Finland, specialized medical care is organized by hospital districts. There are 20 hospital districts in mainland Finland, each of them belonging to one of the five university hospital catchment areas. Turku University Hospital is the central hospital of the southwest Finland hospital district. The patient register of Turku University Hospital contains clinical data on all patients that have visited public hospitals in the hospital district of southwest Finland from 2004 onwards, thus covering practically all patients with MS and comorbidities requiring specialized medical care in the hospital district. In 31.12.2012, the prevalence date for MS prevalence and fracture study, the population of southwest Finland was 472 139.

The Hospital Discharge Register and its continuation, The Care Register for Health Care, contain data on all patients that have been admitted to specialized inpatient care, have undergone a day surgery or have visited specialized outpatient care in Finland. The data are retrieved from electronic client and patient record systems of health care units and submitted to the National Institute for Health and Welfare once a year. All permanent residents of Finland are eligible for reimbursement of medical expenses under the Health Insurance Act. The Social Insurance Institutions registry tracks recipients of reimbursement, including patients with self-injectable and orally administered disease modifying drugs for MS. These various registers of public health care ensure an excellent coverage of patients with MS in Finland.

The Finnish Maternity Cohort is a nationwide biorepository of serum samples collected during the first trimester of pregnancy for routine prenatal testing. It was established in

1983 and contains over 1.9 million serum samples from more than 850 000 women, covering approximately 98% of all pregnancies since 1983.

4.2 Subjects and methods

4.2.1 Finnish Maternity Cohort study (Study 1)

Finnish Maternity Cohort (FMC) Study investigated the risk of MS among children born to women with a pregnancy serum sample in the FMC, a nationwide biorepository of serum samples collected during the first trimester of pregnancy for routine prenatal testing since 1983.

4.2.1.1 Subjects

Cases of MS among children born to women in the FMC between January 1, 1983 and December 31, 1991 were identified by searching through the Finnish Hospital Discharge Register for diagnostic codes for MS and related disorders (ICD-10 codes G35, G36 and H46 and ICD-9 and ICD-8 codes 340, 341, 367 and 377). To identify cases not in the Finnish Hospital Discharge register, the register of the Social Insurance Institution for medical reimbursements was searched for recipients of reimbursement for disease modifying therapy, including glatiramere acetate, IFN beta-1a and IFN beta-1b. Mothers of those individuals with confirmed MS diagnosis were identified by over-generation linkage step via the Population Census Register. Mothers were then linked to the FMC database by their personal identification number. Child-mother pairs with available serum sample of the pregnancy were included in the study. Medical charts of the children were then reviewed and MS diagnosis confirmed by study neurologists. Multiple sclerosis was confirmed and a maternal serum sample available for 193 children. Matched control was found for 176 of them. Controls were matched with region of birth, date of maternal sample collection, date of mother's birth and date of children's birth. Total number of controls was 326. There were additional 5 controls that were selected, but ultimately not matched.

4.2.1.2 Laboratory Analyses

Samples of the FMC are processed and stored at −25°C in the Finnish National Institute for Health and Welfare in Oulu. In our study, maternal 25(OH)D levels were measured using a chemiluminescence microparticle immunoassay and an Architect i2000SR automatic analyzer (Abbott Diagnostics).

4.2.1.3 Statistics

All analyses were done using SAS, V 9.3. (SAS Institute Inc). The 25(OH)D levels were modeled (1) as a continuous variable, (2) as quintiles based on the distribution of maternal 25(OH)D levels in the controls, and (3) as a priori categories consistent with deficient (<30 nmol/l), insufficient(30 to <50 nmol/l) and sufficient (≥50 nmol/l) levels. Conditional logistic regression was used in the main analysis to estimate the rate ratios and 95% CIs and included 176 cases with 326 matched controls. Analyses were further adjusted for sex of the child, gestational age at sample collection and season (summer, winter, or spring/fall) of sample collection. In secondary analyses, an unconditional logistic regression adjustment was performed for all the matching factors in all 193 cases and 331 controls and stratified by sex of the child (female: 163 cases and 218 controls; male: 30 cases and 113 controls). A *P* value less than 0.05 was considered statistically significant.

4.2.2 Finnish Vitamin D study (Study 2)

The Finnish Vitamin D Study was a randomized, placebo-controlled multi-center clinical trial assessing the safety and clinical and radiological efficacy of vitamin D supplementation as an add-on therapy in MS patients receiving interferon-beta therapy.

4.2.2.1 Subjects

Patients for the Finnish Vitamin D study were recruited from the outpatient polyclinics of Turku, Helsinki, Tampere, Oulu and Kuopio University Hospitals and the Central Hospitals of Central Finland and Ostrobothnia. Inclusion criteria were age between 18-55 years, RRMS according to the McDonald criteria, treatment with IFN-beta 1b for at least

1 month, no neutralizing antibodies to IFN-beta as measured by the indirect MxA test, an EDSS score ≤ 5.0, appropriate contraceptive methods for women of childbearing potential and signed written informed consent. Exclusion criteria were serum calcium >2.6 mmol/l, serum 25(OH)D >85 nmol/l, primary hyperparathyroidism, pregnancy or unwillingness to use contraception, alcohol or drug abuse, use of immunomodulatory therapy other than IFN-beta 1b, known allergy to cholecalciferol or peanuts, therapy with digitalis, calcitonin, vitamin D3 analogues or vitamin D, any condition predisposing to hypercalcemia, sarcoidosis, nephrolithiasis or renal insufficiency, significant hypertension (blood pressure >180/110 mm Hg), hyperthyroidism or hypothyroidism in the year before the study began, a history of kidney stones in the previous 5 years, cardiac insufficiency or significant cardiac dysrhythmia, unstable ischemic heart disease, depression or inability to be subjected to serial MRI scans.

The subgroup study on patients with baseline disease activity consisted of patients who had either at least one Gd-enhancing T1 lesion as detected with brain MRI or at least one relapse within the year preceding the study baseline. The immunological substudy consisted of patients who had given written consent also for this substudy and had baseline and month 12 serum samples available.

At the study baseline, there were 34 patients in the vitamin D group and 32 patients in the placebo group. In the vitamin D group, 3 patients discontinued the study, two of them due to discontinuation of IFN-beta. One patient discontinued the study due to personal reasons on day 203. The month 12 MRI and laboratory tests were performed and this patient was included in the analysis. In the placebo group, one patient was lost to follow up and one discontinued due to personal reasons. Thus, 32 patients in the vitamin D group and 30 in the placebo group were included in the analysis. In the subgroup of patients with baseline disease activity, there were 15 patients in the vitamin D group and 15 patients in the placebo group. In this subgroup, 13 patients in the vitamin D treatment arm and 14 patients in the placebo arm completed the study. In the immunological substudy, there were 30 patients in the vitamin D group and 29 patients in the placebo group.

4.2.2.2 Study product and protocol

The study product was cholecalciferol (Dekristol), corresponding to 20 000 IU of vitamin D3, in arachis oil inside a refined gelatin capsule or identically appearing matching placebo capsules (Swiss-Caps, Switzerland). The study product was dosed perorally once a

week. A private company (Joutsen Apteekki, Turku, Finland) organized the importing, packaging and labelling of the study product. Patients were randomized 1:1 to treatment with either Dekristol or matching placebo capsules. Patients and study personnel directly involved in the conduct of the study were blinded to the treatment code.

There were six study visits arranged over 12 months. At the screening visit, concurrent illnesses and concomitant medication were recorded. The clinical examination included a physical examination, EDSS, height, weight, heart rate, blood pressure, ECG, timed 10 foot tandem walk (TTW10) and timed 25 foot walk (T25FW) tests. Laboratory analyses conducted at screening included pregnancy test, MxA, serum calcium, creatinine, phosphate, alkaline phosphatase, albumin, magnesium, iPTH and 25(OH)D.

Serum levels of calcium, creatinine and phosphate were measured at 1, 2, 3, 6, 9 and 12 months. Plasma levels of alkaline phosphatase, albumin, magnesium, iPTH and 25(OH)D were measured at 6 and 12 months, ECG at 2, 6 and 12 months and MxA at 12 months. EDSS and timed walk tests were measured again at 12 months. Adverse events were assessed at every visit. Cytokine analyses were performed from serum samples taken at screening and at 12 months.

In the case of a relapse, the patients contacted the study centers for unscheduled visits within 7 days of relapse onset. At the unscheduled visit, an EDSS was performed and the investigator defined whether a relapse had occurred. If needed, relapses were treated with methylprednisolone 1 g daily for 3 days.

The primary endpoints were T2 BOD, proportion of patients with serum levels of 25(OH)>85 nmol/l or iPTH <20 ng/l at 6 and 12 months, and safety and tolerability (number of adverse events). Secondary endpoints included change in EDSS, relapse rate, time to first relapse, change in TTW10 and T25FW, number of T1 enhancing lesions, T1 enhancing lesion volume (mm3), number of new or enlarging T2/PD lesions and MRI activity (defined as the presence of Gd and/or new/enlarging T2 lesions).

4.2.2.3 MRI evaluation

A standardized MRI study was performed within 2 weeks before or at the randomization visit and within 2 weeks before or at the 12 month visit. A 1.5T scanner was used in each center. The scanning protocol included a dual echo T2/PD and a post-contrast T1 weighted sequence covering the whole brain with continuous 3 mm slices. Central analyses were performed at the Neuroimaging Research Unit, Vita-Salute University, Milan,

Italy. The total number of Gd enhancing lesions, number of new/enlarging T2/PD lesions and new Gd enhancing lesions, T2 lesion volume (BOD) (mm3) and T1 enhancing lesion volume (mm3) were evaluated. Lesion volumes were quantified by experienced observers using a local thresholding segmentation technique (Jim 5, Xinapse Systems Ltd, Northants, UK).

4.2.2.4 Laboratory analyses

Serum samples were freshly frozen and kept at −70°C. Levels of 25(OH)D were measured using a commercially available assay, 25-hydroxyvitamin D 125I RIA Kit (DiaSorin Catalogue No 68100E, Stillwater, Minnesota, USA). Two quality control samples were included in each assay series, and the specimens and controls were assayed in duplicate. The sensitivity of this assay is 4.0 nmol/l and the intra-assay coefficient of variation is <10%.

Concentrations of IFN-γ, IL-17A, IL-2, IL-10, IL-9, IL-22, IL-6, IL-13, IL-4, IL-5, IL-1β and TNF-α were determined using a commercial fluorescent bead immunoassay kit (Human Th1/Th2/Th9/Th17/Th22 13plex Kit FlowCytomix BMS817FF, eBioscience, USA) and concentrations of latency associated peptide (LAP) (TGF-β) by fluorescent bead immunoassay kit (Human LAP FlowCytomix Simplex Kit, eBioscience, USA). The sensitivities were: LAP 0.69 ng/ml, IFN-γ 1.6 pg/ml, IL-17A 2.5 pg/ml, IL-2 16.4 pg/ml, IL-10 1.9 pg/ml, IL-9 1.5 pg/ml, IL-22 43.3 pg/ml, IL-6 1.2 pg/ml, IL-13 4.5 pg/ml, IL-4 20.8 pg/ml, IL-5 1.6 pg/ml, IL-1β 4.2 pg/ml and TNF-α 1.6 pg/ml. All cytokine analyses were performed in the Department of Clinical Microbiology and Immunology, University of Turku, Turku, Finland.

HLA typing of major DR–DQ haplotypes was performed in the Immunogenetics Laboratory of the University of Turku with a PCR-based, lanthanide-labeled hybridization method using time-resolved fluorometry for detection.

4.2.2.5 Statistics

SAS V. 9.2. was used in the analyses in the main study and the substudy on patients with baseline activity. The sample size calculation was performed such that with 40 patients

in each treatment arm, a difference of 1000 mm3 in MRI T2 BOD (SD 1700) and a difference of 30% in the proportion of patients with 25(OH)D>85 nmol/l would be detected (two sided x2 test, a=0.05, power=80%). Logistic regression was used to analyze vitamin D status by visit and to analyze MRI activity. Non-parametric rank analysis of covariance (ANCOVA) was used in analyzing MRI T2 BOD and EDSS at 12 months with baseline values as covariates, controlling for center. ANCOVA was used to analyze the change in TTW10 or T25FW (after log transformation), with TTW10 or T25FW at baseline and center as covariates. The numbers of Gd enhancing lesions on T1 and new/enlarging lesions on T2 scans were analyzed using a generalized linear mixed model based on a Poisson distribution. T1 enhancing lesion volume was analyzed using Fisher's exact test at each time point. Correlations between EDSS and MRI T2 BOD were calculated by Spearman's rank correlation coefficient. A p value <0.05 was considered statistically significant.

In the immunological substudy, the statistical analysis was performed with SAS version 10.0. Changes in cytokine concentrations were evaluated using paired t-testing. P-values less than 0.05 were considered significant.

4.2.3 MS prevalence and fracture risk study (Study 3)

The MS prevalence and fracture risk study aimed to identify patients with definite MS diagnosis and comorbid fractures in the hospital district of southwest Finland from January 1, 2004 to December 31, 2012 using hospital administrative data and chart review for case confirmation.

4.2.3.1 Subjects

To identify patients with definite MS diagnosis and comorbid fractures, we performed a computerized search for ICD codes (ICD-9and ICD-10 codes 3400A and G35 for MS and ICD-9 and ICD-10 codes for all fractures) from January 1, 2004 to December 31, 2012 from hospital administrative data in Turku University Hospital (TYKS) in southwest Finland. Case ascertainment was performed by review of the medical records. Dates of definite MS diagnosis and fracture were retrieved and patients with fracture

event after the definite MS diagnosis were included in the study. Fractures were identified as high- or low-energy fractures using the same definitions as Morrison and coworkers (Morrison, Fran et al. 2013). High-energy injuries, such as those occurring in motor vehicle or bicycle accidents, sports or a fall from higher than one meter, were excluded from the study. A low-energy fall was defined as falling from standing height on a flat surface or on stairs. Other low-energy fractures were also identified. A clinical osteoporotic fracture was defined as a low-energy fracture of the vertebrae, rib, collar bone, pelvis, hip, femur, tibia, humerus, or ulna/radius. Low-energy fractures of facial bone, hand, ankle, or foot were considered other types of fractures. EDSS was assessed from medical records at the time of the fracture if possible, or from the previous visit to the neurology outpatient clinic. Medical records were also searched for information concerning body mass index (BMI), smoking status, vitamin D levels, corticosteroid treatment, and use of vitamin D and calcium supplements. Some patients had undergone BMD testing by dual-energy X-ray absorptiometry scanning (DXA) as part of their clinical investigation after the fracture.

For each patient with MS, ten control patients of the same gender and with the same year of birth were randomly selected from the Turku University Hospital patient register.
In the prevalence calculation, patients with MS living in December 31, 2012 were included. Diagnosis was confirmed by review of the medical records. Information of population in 10-year age groups and date of death before the prevalence date was searched via the personal identification number from Population Register center of Finland. Age-adjusted total and gender-specific prevalences for definite MS per 100 000 were calculated in December 31, 2012 in the population of University Hospital District of southwest Finland with 95% confidence intervals.

4.2.3.2 Statistics

Statistical analyses were performed using R Statistics version 3.0.2 (Free Software Foundation, Boston, MA, USA). The RRs and confidence intervals (95% CI) for each diagnosis were calculated using Pearson's chi-squared test. Another randomly chosen individually matched 10-fold control population was used to verify the similarity of the results. Kaplan-Meier analysis was used to study the fracture-free survival time from the diagnosis of MS to the observed fracture event.

4.2.4 Ethical aspects

There is a legal basis for the collection and scientific use of the FMC (The law of the National Institute for Health and Welfare 828/1981, 327/2001 and 668/2008668/2008). Since 2001 a nationwide informed consent system based on the opt-out principle has been in operation. The FMC study was approved by the data protection authorities at the National Institute for Health and Welfare, the Regional Ethics Committee of the Northern Ostrobothnia Hospital District, and the Office of Human Research Administration at the Harvard T.H. Chan School of Public Health.

In the Finnish Vitamin D study, vitamin D supplementation or placebo was administered as an add-on treatment to recombinant IFN-beta-1b, which is approved for the treatment of patients with relapsing-remitting MS. Thus no patients participating in the study were prevented from receiving active therapy. Vitamin D has many health benefits e.g. prevention of osteoporosis in addition to the investigated putative beneficial effect on MS activity. Vitamin D, at the doses given, is safe with a low risk of serious adverse events. All patients who received the study medication had the opportunity at all times to withdraw from the study. Patients provided written informed consent before initiating study procedures. The study protocol was approved by the ethics committee of Turku University and Turku University Hospital and the National Agency of Medicines, Helsinki, Finland. The study was undertaken in accordance with the Declaration of Helsinki and the European Medicines Agency Note for Guidance on Good Clinical Practice.

MS prevalence and fracture risk study was a register study and as there was no direct contact with the patients, no written consents were required. The study was registered and approved by the Turku Clinical Research Center and ethical committee approval was obtained from the joint Ethics Committee of Tampere University and Pirkanmaa Hospital District. Permission to use hospital administrative data was obtained from the Turku University Hospital.

5 RESULTS

5.1 Finnish Maternity Cohort (Study 1, Original article I)

There were no differences between cases and controls in terms of mother's age, gestational age, and season at the time of serum sample collection. The mean age at MS diagnosis was 19.8 (3.2) years, the young average age being due to the fact that the source population comprises only individuals born after 1983. There were more women in the case group. Seventy percent of serum samples were collected at or before 12 weeks' gestation and 99% prior to 28 weeks. Maternal 25(OH)D levels ranged from 3.50 ng/ml to 64.30 ng/ml (8.74 to 160.49 nmol/l, respectively) with an average of 13.86 ng/ml (34.59 nmol/l) in mothers of cases and 15.02 ng/ml (37.49 nmol/l) in mothers of controls. Only 2 cases with MS and 8 controls had maternal 25(OH)D levels of more than 30.05 ng/ml (75.00 nmol/l), and no MS cases and only 1 control had maternal 25(OH)D levels of more than 40.06 ng/ml (99.99 nmol/l). Mean 25(OH)D levels did not differ according to the trimester of serum collection (first trimester, 14.66 ng/ml [36.59 nmol/l] and second trimester, 14.50 ng/ml [36.19 nmol/l]).

In the matched analysis adjusted for sex, gestational age, and season at time of sample collection, a 20.03 ng/ml (50.00 nmol/l) increase in maternal 25(OH)D level was associated with a 48% reduced risk of MS in the offspring although this did not quite reach statistical significance (relative risk [RR], 0.52; 95% CI, 0.22-1.19; $P = 0.12$). Children of mothers with deficient gestational levels of 25(OH)D had an increased risk of developing MS compared with children born to mothers with non-deficient levels. When using a priori categories of 25(OH)D levels, clearly deficient maternal 25(OH)D levels during pregnancy were associated with a nearly doubled risk of MS in the child (<12.02 ng/ml vs 12.02 to <20.03 ng/ml [<30 nmol/l vs 30 to <50 nmol/l]) : RR, 1.90; 95% CI, 1.20-3.01; p = 0.04). Maternal 25(OH)D levels in the two lowest quintiles (less than 12.62 ng/ml [31.50 nmol/l]) were associated with a 20% to 90% increased risk of MS among the offspring compared with maternal 25(OH)D levels in the top quintile (median 25[OH]D, 22.52 ng/ml [56.21 nmol/l]; *P* trend = 0.09)

In the unmatched analysis, a 43% reduced risk of MS was associated with every 4.01-ng/ml (10.00 nmol/l) increase in maternal 25 (OH)D level (RR, 0.57; 95% CI, 0.28-1.18; $P = 0.13$), and a 59% increased risk of MS among children born to vitamin D deficient mothers (<12.02 ng/ml vs 12.02 to <20.03ng/ml; RR, 1.59, 95%CI, 1.04-2.42; $P = .03$).

In analyses stratified by sex of the child, this association was only evident in female children (<12.02 ng/mL vs 12.02 to <20.03 ng/ml: RR, 1.75; 95%CI, 1.09-2.81; *P* = .02).

5.2 Finnish vitamin D study (Study 2, Original articles II and III)

5.2.1 Primary outcomes

5.2.1.1 Serum levels of vitamin D and PTH (II)

Characteristics of the patients in the Finnish vitamin D study are shown in table 4. In the vitamin D group, mean serum 25(OH)D increased from 54 (19-82) nmol/l at baseline to 110 (67-163) nmol/l at 12 months. In the placebo group, the mean serum 25(OH)D level was 56 (range 16-81) nmol/l at baseline and 50 (17-94) nmol/l at 12 months. The difference between the vitamin D and placebo groups at month 12 was statistically significant (p<0.0001). In the vitamin D treated group, the percentage of patients with serum levels of 25(OH)D >85 nmol/l was significantly higher compared with placebo group at 6 and 12 months (6 months: 76% vs 3%, p<0.001; 12 months: 84% vs 3%, p<0.001). PTH suppression to a level <20 pg/ml was not obtained in either treatment arm. In the vitamin D treated patients, the median iPTH concentration at baseline was 37 (1-77) pg/ml and at 12 months 36 (20-67) pg/ml. In the placebo group, the median serum level of iPTH at baseline was 41 (15-69) pg/ml and at 12 months 42 (18-125) pg/ml.
In the subgroup of patients with baseline activity, serum 25(OH)D levels increased from 55 (35–82) nmol/l at baseline to 115 (range 78–163) nmol/l at month 12 in the vitamin D treated patients but the corresponding level remained unchanged in the placebo group i.e. 50 nmol/l (24–81) at baseline and 48 nmol/l (30–68) at 12 months.

5.2.1.2 Safety (II)

There was one severe adverse event in the vitamin D group (erysipelas in the IFN-beta injection site) and two in the placebo group (elbow fracture, hip surgery). Diarrhea was a side effect in five patients in the vitamin D group and two patients in the placebo group and fever in five patients in the placebo group and two in the vitamin D group. Otherwise

there were no differences in the amount of adverse events between treatment groups. There was no hypercalcemia in the vitamin D treated patients. The lack of a MxA response (MxA <50 μg/l) was detected in three patients in both treatment arms at 12 months.

Table 4. Characteristics of the patients in the Finnish vitamin D study

	Main study		Patients with baseline activity	
	Vitamin D	Placebo	Vitamin D	Placebo
No. of patients	34	32	15	15
Gender (F/M)	21/13	20/12	9/6	9/6
Age (years) (median, range)	39 (22-53)	35 (24-53)	37 (25-53)	32 (24-47)
BMI (kg/m^2) (median, range)	24 (18-40)	24 (19-38)	24 (20-31)	26 (19-32)
EDSS score (median, range)	2.0 (0-5.0)	1.5 (0-4.0)	2.0 (0-3.5)	2.0 (0-4.0)
Disease duration (years) (median, range)	3.0 (0.5-21.3)	2.4 (0.2-15.2)	3.0 (0.6-15)	1.5 (0.3-4.7)
ARR (mean, SD)	0.49 (0.51)	0.52 (0.49)	0.67 (0.38)	0.83(0.37)
Duration of IFN-beta therapy (months) (median, range)	24 (2-149)	17 (1-110)	23 (4-82)	10 (2-53)

ARR-annual relapse rate; BMI-Body mass index; EDSS-Expanded disability status scale; IFN-interferon

5.2.1.3 MRI T2 burden of disease (II, III)

T2 BOD detected in the brain MRI was the primary MRI outcome measure. T2 BOD increased more in the placebo group (median change 287 mm3) compared with the vitamin D treated patients (median change 83 mm3) but the difference was not statistically significant (p=0.105). When patients with a lack of an MxA response were excluded from the analysis, the p-value became close to achieving statistical significance (0.055). Also in the subgroup of patients with disease activity at baseline, T2 BOD increased more in the placebo group (median change 570 mm3) compared to the vitamin D treated group patients (median change 104 mm3) but the difference was not statistically significant either (P = 0.105). MRI parameters are shown in Table 5.

5.2.2. Secondary outcomes

5.2.2.1. MRI parameters (II, III)

The total number of T1 Gd enhancing lesions decreased significantly in both groups (p=0.002) but this change was significantly higher in the vitamin D group (p=0.004). In the subgroup of patients with baseline disease activity, the number of T1 Gd enhancing lesions also decreased significantly more in the vitamin D treated group (p=0.027). The number of new/enlarging T2 lesions at 12 months was higher in the placebo group but the difference was not statistically significant (p=0.286). In the subgroup of patients with baseline activity, the difference in the number of new/enlarging lesions was more pronounced in favor of the vitamin D group but did not reach statistical significance (p= 0.132). The percentage of patients with MRI activity at 12 months was lower in vitamin D treated patients but the difference was not statistically significant (p=0.322). In the subgroup with baseline disease activity, the number of patients with MRI activity at 12 months was lower in the vitamin D group and this almost reached statistical significance (p=0.08). MRI parameters are shown in Table 5.

5.2.2.2. Relapses, EDSS, and T25FW and TTW10 (II, III)

The annual relapse rate decreased during the study in both treatment arms in the main study and in the substudy with patients with baseline activity, but the difference between treatment groups was not statistically significant in either study. There was no significant difference in the time to first relapse between groups in main study (HR 1.12, 95% CI 0.41 to 3.1) or the subgroup of patients with baseline disease activity (0.84, 95% CI 0.23 to 3.1). The mean EDSS score decreased in the vitamin D group and remained the same in the placebo group during the main study (p=0.071). In the whole group of patients, EDSS was significantly correlated with T2 BOD. In the subgroup of active patients, there was no change in median EDSS in either treatment group (P = 0.274). When the walk test results at 12 months were compared with baseline values, there was a statistically significant decrease in TTW10 in the vitamin D group (p=0.001) and a non-significant decrease in the placebo group (p=0.291). The difference between the groups between baseline and 12 months was not statistically significant (p=0.076). There were no statistically significant differences between the treatment groups in T25FW (p=0.907).

Table 5. MRI parameters in vitamin D and placebo treated patients

	Main study					Patients with baseline activity				
	n	Vitamin D	n	Placebo	p	n	Vitamin D	n	Placebo	p
T2 BOD (mm3) (median, SE)										
Baseline	34	4494(1754)	32	5893(1891)		15	4391(2305)	15	9930(2375)	
Change from baseline	32	83(128)	30	287(283)	0.105	13	104(240)	14	570(3259)	0.105
No. of T1 Gd+ lesions (mean, SD)										
Baseline	34	0.6 (2.4)	32	0.7 (2.1)		15	1.5 (3.6)	15	1.5 (2.9)	
Month 12	32	0.1 (0.2)	30	0.7 (3.5)	0.004	13	0.1 (0.3)	14	1.5 (5.0)	0.027
Patients with T1 Gd+ lesions (n (%))										
Baseline	34	6 (18)	32	6 (19)		15	6 (40)	15	6 (40)	
Month 12	32	2 (6)	30	4 (13)		13	1 (8)	14	3 (21)	
No. of new/enlarging T2 lesions (mean, SD)	32	0.5 (1.0)	30	1.1 (2.2)	0.286	13	0.61 (1.26)	14	1.85 (2.44)	0.132
Patients with new/enlarging T2 (n (%))	32	8 (25)	30	11 (37)		13	3 (23)	14	8 (57)	
MRI activity (n (%))										
Baseline	34	6 (18)	32	6 (19)		15	6 (40)	15	6 (40)	
Month 12	32	8 (25)	30	11 (37)	0.322	13	3 (23)	14	8 (57)	0.080

BOD-burden of disease; Gd+ gadolinium enhancing; MRI-magnetic resonance imaging

5.2.3. Serum levels of 25(OH)D and cytokines in the immunological substudy (IV)

Characteristics of the patients in the immunological substudy are shown in Table 6. Serum levels of 25(OH)D increased from 54 (19-82) nmol/l at baseline to 109 (67-163) nmol/l at month 12 in the vitamin D treated group. In the placebo group, mean serum 25(OH)D was 55 (16-81) nmol/l at baseline and 51 (17-94) nmol/l at month 12. The treatment

groups did not differ statistically significantly according to the DQB1*0602 status, neither did the groups differ according to their smoking status. None of the patients had received corticosteroids within 30 days prior to serum samples.

Table 6. Characteristics of the patients in the immunological substudy

	Vitamin D	**Placebo**
Number of patients	30	29
Sex F/M	18/12	19/10
Age (median, range)	38 (22-53)	35 (24-53)
DQBI*0602 positive/negative	20/10	13/15*
BMI (kg/m2) (median, range)	24 (18-40)	24 (19-38)
EDSS score (median, range)	2.0 (0-4.5)	1.5 (0-4.0)
Disease duration (years) (median, range)	3 (0.5-21)	2 (0.2-15)
ARR (median, 95% CI)	0.5 (0.3-0.6)	0.5 (0.3-0.7)
Smokers/Non-smokers	10/20	9/20

BMI-Body mass index EDSS-Expanded disability status scale ARR-annual relapse rate

*data missing for 1 patient

Serum levels of LAP (TGF-β) increased significantly in the vitamin D treated group from a mean of 47 (SE 11) ng/ml at baseline to 55 (SE 14) ng/ml in 12 months ($p = 0.0249$). In the placebo group, serum levels of LAP also increased, but this increase did not reach statistical significance ($p = 0.173$). Serum levels of IFN-gamma ($p = 0.0519$), IL-17A ($p = 0.0666$) and in IL-9 ($p = 0.0679$) increased in the vitamin D treated patients but these elevations did not achieve statistical significance. The levels of IL-2, IL-10, IL-22, IL-6, IL-13, IL-4, IL-5, IL-1β and TNF-α did not change statistically significantly in either group. Changes in the cytokine levels are shown in Table 7.

Cytokine levels were assessed also in the DQB1*0602 positive and negative subgroups within the vitamin D3 and placebo treatment arms. The levels of LAP, IFN-γ, IL-17A, IL-2, IL-10, IL-9, IL-22, IL-6, IL-13, IL-4, IL-5, IL-1β and TNF-α did not change statistically significantly in any of these subgroups but there was a trend in LAP in DQB1*0602 positive subgroup of vitamin D3 treated patients, whose LAP levels increased from 45 (SE 15) ng/ml to 55 (SE 19) ng/ml ($p = 0.0512$).

Table 7. Changes in cytokine levels in vitamin D and placebo treated patients

Cytokine	Vitamin D			Placebo		
	Baseline	Month 12	p	Baseline	Month 12	p
LAP	46.6 (10.8)	54.8 (13.6)	0.0249	39.1 (7.4)	49.5 (9.3)	0.173
IFN gamma	565.9 (258.7)	767.0 (338.4)	0.0519	332.0 (160.7)	334.8 (121.4)	0.9654
IL-17A	326.8 (99.6)	387.5 (114.1)	0.0666	307.2 (109.2)	329.8 (104.0)	0.5243
IL-9	117.0 (36.5)	133.3 (39.4)	0.0679	187.9 (77.7)	181.7 (59.6)	0.8661
IL-2	239.2 (82.0)	288.5 (117.0)	0.2420	164.5 (43.5)	155.9 (40.9)	0.7934
IL-10	25.1 (7.9)	43.6 (17.9)	0.1466	34.8 (18.3)	22.6 (9.6)	0.2503
IL-22	521.2 (67.0)	498.8 (75.5)	0.5138	464.7 (63.2)	449.4 (58.0)	0.7688
IL-6	9.7 (5.7)	11.4 (7.2)	0.4068	9.1 (6.4)	7.00 (4.8)	0.2723
IL-13	166.2 (64.6)	173.3 (68.9)	0.4282	111.9 (23.7)	133.4 (33.2)	0.2736
IL-4	92.0 (37.9)	100.9 (28.6)	0.2089	52.6 (20.1)	61.4 (19.8)	0.2979
IL-5	147.0 (71.1)	183.5 (91.8)	0.2437	139.0 (69.8)	113.4 (63.9)	0.6166
IL-1beta	106.6 (24.7)	120.5 (30.0)	0.1064	83.4 (23.5)	111.8 (28.5)	0.1815
TNF-alpha	22.9 (6.8)	29.2 (9.6)	0.1028	13.6 (4.0)	18.1 (5.8)	0.1911

Values shown are mean (SD) ng/ml for LAP and pg/ml for other cytokines. IFN-interferon; IL-interleukin; LAP-latency associated peptide; TNF- tumor necrosis factor

5.3 MS prevalence and fracture study (Study 3, Original publication V)

5.3.1 Prevalence (V)

A total of 1114 MS cases were identified and alive on December 31, 2012. A review of patient charts led to exclusion of 9.8% (n=110) of MS cases due to false ICD coding or misclassification. The final MS cohort included 1004 cases of MS, 279 men and 725 women. This means that in a regional population of 472 139, the total prevalence was 212.6 per 100 000 (95% CI 199.5–225.8); when calculated according to gender, it was 121.4 per 100 000 (114.5–128.4) for men and 258.3 per 100 000 (247.9–268.6) for women.

5.3.2 Fractures (V)

A total of 100 patients with MS experienced at least one fracture during the study period. In 58 patients, the fracture or the fractures were considered as being osteoporotic. In 46 of these patients, the fracture event was single, and in 11 patients, two fracture events occurred. One of the patients experienced four fracture events. A total of 42 patients experienced a fracture that was considered non-osteoporotic. The injury mechanism was a low-energy fall in 59% of osteoporotic fractures and in 65% of hip fractures. In non-osteoporotic fractures, the injury mechanism was falling in 64% of cases. The median time between diagnosis and first fracture was 16 years in the osteoporotic fracture group and 8 years in the non-osteoporotic fracture group. Median EDSS was 6.0 in the osteoporotic group and 4.5 in the non-osteoporotic fracture group. BMD measurement was performed in 12 of 58 MS patients with an osteoporotic fracture. The T scores in 10 patients were osteoporotic and in two patients, they were osteopenic with the hip T scores ranging from −0.1 to 4.5 and the lumbar spine T scores from −0.8 to −4.5. The vitamin D supplement was used by 43 of all patients and a calcium supplement by 35 patients. Mean vitamin D levels were 89 nmol/l in the osteoporotic group and 81 nmol/l in the non-osteoporotic group. Most of the values had been analyzed after the first fracture event and after the initiation of vitamin D supplementation. Data of BMI or smoking status was not available. Data on corticosteroid pulses before the fracture event was not available for all patients with diagnosis before the year 2001 and the use of corticosteroids could not be included in the statistical analyses.

Relative risks were 1.33 (95% CI 1.10–1.60) for any fracture and 1.50 (95% CI 1.18–1.90) for osteoporotic fractures and were significantly higher in the patients with MS compared with the controls. In particular, RRs for hip fractures (5.00, 95% CI 2.96–8.43) and fractures of the humerus (2.3, 95% CI 1.32–4.42) and femur (2.11, 95% 0.73-6.03) were elevated in the patients with MS in comparison with the controls.

6 DISCUSSION

6.1 Maternal vitamin D levels and risk of MS in the offspring

In the Finnish Maternity Cohort study, we showed that a maternal vitamin D deficiency in early pregnancy was associated with a 2-fold increase in the risk of MS in the offspring. It has not been clear previously whether in utero exposure to vitamin D deficiency is relevant with respect to the MS risk, but there is evidence hinting that this might be the case. In the northern hemisphere, MS patients are more likely to be born in spring, especially April or May, after winter months of low sunlight and the consequent low gestational serum levels of 25(OH)D (Torkildsen, Grytten et al. 2012). In the southern hemisphere, the pattern is reversed, with excess of MS births in November and December (Staples, Ponsonby et al. 2010). A higher vitamin D intake during pregnancy has also been associated with lower risk of MS in the offspring (Mirzaei, Michels et al. 2011).

There are two previous studies which have investigated the association between the gestational or neonatal levels of 25(OH)D and the risk of MS. A Swedish case-control study found no significant association between gestational vitamin D levels and risk of MS in the offspring, but their number of cases was small - only 37 (Salzer, Hallmans et al. 2012). Another Swedish study using DBSS collected from neonates also found no association between levels of 25(OH)D and future MS risk (Ueda, Rafatnia et al. 2014). There was some evidence of degradation of 25(OH)D in the older samples which may have contributed to the null finding, together with low overall levels of 25(OH)D in the study. Participation was lower among controls than cases (44% vs 88%) which makes it possible that the controls did not truly represent the vitamin D levels of general population. In contrast with these previous studies, and in line with our findings, a more recent study using DBSS belonging to the Danish Newborn Screening biobank, showed that a 25 nmol/l increase in the neonatal 25(OH)D resulted in a 30% reduced risk of MS. This large case-control study included over 500 MS patients and 1000 controls (Nielsen, Munger et al. 2017).

The strength of our study is its large size and population based setting of the Finnish Maternity Cohort, which includes samples of about 98% of all pregnancies in Finland since 1983. The registers used in case selection, Hospital Discharge Register and Social Insurance Institution register for reimbursements, have excellent coverage of patients with MS in Finland thus minimizing the risk of selection bias.

There are some limitations to be considered. Gestational 25(OH)D levels do not directly measure the levels in the developing fetus but neonatal umbilical cord 25(OH)D levels have been shown to correlate with maternal levels of 25(OH) D (Godang, Frøslie et al. 2014) and maternal vitamin D levels also affect fetal growth parameters and bone growth (Mahon, Harvey et al. 2010, Ioannou, Javaid et al. 2012); this is interpreted that maternal 25(OH)D levels are an adequate indicator of 25(OH)D levels to which the fetus is exposed. As the FMC was set up in 1983, our cases were young with an average age at diagnosis 19.8 years, possibly meaning that the association between gestational vitamin D levels and MS risk decreases at older ages. Most women in our study had deficient or insufficient levels of vitamin D and further studies in populations with higher variation in vitamin D levels will be needed to estimate the possible dose-response effect.

We did not have information on other risk factors for MS, e.g. EBV infection, cigarette smoking, high body mass during childhood or adolescence, HLA DRB1*1501status or vitamin D levels.

25(OH)D levels of mother and child are likely to correlate also later in childhood and adolescence due to shared behavioral factors, e.g. outdoor activities and/or use of vitamin D supplementation, as well as shared genetic factors. Thus, we cannot rule out that the increased risk of MS in children born to vitamin D deficient mothers is due to low levels of vitamin D during childhood or adolescence. Furthermore, association does not mean causation, and reverse causation remains an issue.

However, vitamin D deficiency is common in pregnant women, and correcting vitamin D deficiency would likely reduce the MS risk of the mother as well as in her offspring and have other health benefits in addition to decreasing MS risk. Thus there is a rationale for measuring serum vitamin D levels and correcting vitamin D deficiency in pregnant women.

6.2 Effect of vitamin D supplementation on MRI activity and clinical outcomes

Our study was the first randomized, double blinded, placebo controlled clinical trial examining the therapeutic effects of vitamin D3 supplementation in MS. The most notable result of our study was that after 12 months patients in the vitamin D group showed a significantly lower number of T1 enhancing brain MRI lesions compared with patients in

the placebo group (p=0.004). In a previous open trial involving 12 MS patients supplemented with 1000 ug/day of vitamin D for 28 weeks, a decline in the mean number of Gd enhancing brain MRI lesions per patient was also seen (Kimball, Ursell et al. 2007). In an Iranian study, 30 patients with opticus neuritis were randomized to receive either a weekly dose of 50 000 IU vitamin D or placebo. There were significantly fewer cases of second demyelinating attack in the treatment group. The incidences of black holes, cortical, juxtacortical, corpus callosal, new gadolinium-enhanced and new T2 lesions were also significantly lower in the vitamin D group (Derakhshandi, Etemadifar et al. 2013). The SOLAR study is the largest RCT of vitamin D supplementation as add-on therapy so far with 229 MS patients. The results of the SOLAR study were released in the ECTRIMS congress in October 2016, but have not yet been published. The SOLAR results indicated that a very high dose vitamin D (14 000 IU (350 µg) daily) significantly improved the primary MRI outcome, i.e. the number of combined unique activity MRI lesions at week 48 (Smolders, Hupperts et al. 2016). The ARR was reduced by 30%, which was not statistically significant likely due to the relatively small sample size, however suggesting a beneficial effect of high-dose vitamin D on disease activity. No effect was seen on EDSS progression. The results of this important trial are very much in line with the results from our study and support a role for vitamin D in MS care.

In our trial, a trend was seen favoring the vitamin D group in EDSS scores. In support of this finding, there was a statistical trend in MRI BOD, and a significant correlation between the EDSS values and MRI BOD in our patients. All patients were treated with IFN-beta and showed a decreased annual relapse rate from 0.51 to 0.26 and 0.28 in the placebo and vitamin D groups, respectively. Conceivably, vitamin D did not exert any additional effect on the relapse rate.

Mixed results have been obtained in previous small supplementation studies. In the study published by Burton et al, a high dose (10 000 IU/day) of vitamin D3 was found to be safe with evidence of immunomodulatory effects (Burton, Kimball et al. 2010). This trial was not powered nor blinded to properly address clinical outcomes. The control group was allowed to take up to 4000 IU/day of vitamin D and supplementary calcium if desired. However, clinical outcomes appeared to favor the high dose vitamin D group. In a study performed in Norway, the vitamin D3 product and placebo were identical to those used in our trial. Baseline 25(OH)D levels were similar (approximately 55 nmol/l in the Norwegian study as in ours) and a mean serum 25(OH)D concentration of 123 nmol/l was achieved with the 20 000 IU/week dose of cholecalciferol. Participants were allowed to

continue to consume the low dose vitamin D supplement they were using at study baseline. No effect of vitamin D was found on ARR, EDSS or MSFC (Kampman, Steffensen et al. 2012). In the study of Golan et al., a total of 45 patients were randomized to high (4370 IU/d) or low (800 IU/d) vitamin D3 for 12 months. There was no difference in the EDSS between groups (Golan, Halhal et al. 2013). In contradiction with this finding, in an Iranian study, EDSS scores were significantly lower in pregnant patients supplemented with vitamin D (50 000 IU per week) and they experienced fewer relapses during pregnancy and within 6 months after delivery in comparison with patients who received routine care, but this study investigated only 15 patients (Etemadifar, Janghorbani 2015). A study based on patient reported previous sun exposure, vitamin D intake and age at disability milestones from 219 veterans with progressive forms of MS found that a low average sun exposure before disease onset increased the risk to disease progression whereas the use of cod liver oil during childhood delayed progression (McDowell, Amr et al. 2011). In an Australian randomized controlled trial in 23 individuals with clinically active RRMS taking 1000 IU of vitamin D2 per day, no therapeutic advantage from additional high dose vitamin D2 of 6000 IU/day was found. However, the trial was limited by its small and selected patient sample (Stein, Liu et al. 2011). In a trial with 62 Iranian MS patients randomized to once monthly intramuscular 300 000 IU vitamin D injections (n=28) or placebo intramuscular injections (n=34), no significant differences were found in clinical or MRI parameters but there was decreased lymphocyte proliferation in the treated patients. Serum levels of 25(OH)D of approximately 150 moll/l were achieved in that study (Mosayebi, Ghazavi et al. 2011).

Our trial was properly blinded and the patients in the placebo group did not take any vitamin D, but due to the small sample size, the trial was not powered to address clinical outcomes. We did not measure brain atrophy and followed disability accumulation for only 1 year. Therefore, it is possible that there are alternative explanations for our results favoring the vitamin D group in disability accumulation and timed tandem walk other than reduced neurodegeneration. There is some evidence that vitamin D has beneficial effects on muscle strength (Pludowski, Holick et al. 2013). Doses of 17.5-25 mg/day (700-1000 IU/day) of vitamin D have been shown to significantly lower the risk of fall in older adults (Dawson-Hughes 2012) with 60 nmol/l assessed as the minimum serum level to obtain a benefit. Thus, the effect of vitamin D on muscle performance and balance could account for the trend towards improved timed tandem walk and EDSS scores that

we observed in the vitamin D3 treated MS patients in comparison with the placebo treated patients.

In our study, the effects on MRI activity and MRI T2 BOD were more pronounced in the subgroup of patients with baseline disease activity than in the overall study population. In this subgroup, 23% of vitamin D3 treated and 57% of placebo treated patients exhibited MRI activity (new or enlarging T2 lesions or Gd-enhancing lesions) at 12 months (P = 0.08). MRI T2 BOD increased by a median of 570 mm3 in the placebo patients but by only 104 mm3 in the vitamin D3 treated patients. The differences in MRI T2 BOD between the groups were not statistically significant but the mean of 466 mm3 smaller lesion volume growth in the vitamin D-treated subgroup appears to be clinically significant. MS patients with high baseline disease activity seem to benefit more from DMTs than patients with less disease activity. Patients who start the treatment later in the disease course do not gain the same benefits as those who begin treatment early in the course of multiple sclerosis (Miller 2004). DMTs have also been postulated to have greater benefits in younger patients because of their higher rate of relapsing activity (Tremlett, Zhao et al. 2008). Natalizumab has been shown to exert its optimal effect in patients with more disease activity, reflected as relapses or Gd enhancing lesions, before treatment (Miller, Khan et al. 2003, O'Connor, Miller et al. 2005). This was confirmed in the post hoc analysis of AFFIRM and SENTINEL, where it was shown that natalizumab was effective in reducing the risk of disability progression and relapses particularly in patients with highly active disease (Hutchinson, Kappos et al. 2009). The efficacy of fingolimod on ARR was more pronounced in patients with high baseline activity (Devonshire, Havrdova et al. 2012) and also with cladribine, a greater effect on disease activity has been described in the patient subgroups with either a high relapse tendency or high lesion activity at the baseline (Giovannoni, Cook et al. 2011). In 15 patients undergoing monthly MRI scans, a trend was visible that IFN-beta responders had a higher total number of Gd-enhancing lesions during the pretreatment period (Chiu, Richert et al. 2009). Our finding that the therapeutic effect of vitamin D was more pronounced in the active subgroup of MS patients supports an immunological mechanism of action for vitamin D in MS.

Similarly to our trial, all other clinical trials performed with vitamin D in MS so far, irrespective of the doses used, have failed to demonstrate efficacy on clinical endpoints, but have suggested an effect on disease activity as assessed by MRI. MRI is highly sensitive for detecting disease activity. The formation of new T2 lesions and Gd enhancing T1 lesions may occur sub-clinically; these are more frequently seen than clinical relapses

(Barkhof, Scheltens et al. 1992, Barkhof 2002). Thus MRI can serve as a surrogate marker for relapses; this proposal is supported by a study showing a strong correlation between the effect of therapy on active lesions and its effect on the relapse rate (Sormani, Stubinski et al. 2011). The demonstration of clinical efficacy of vitamin D as an add-on therapy with effective DMTs in MS would need very large numbers of patients and a long follow-up time to demonstrate any effect of disease progression. It may well become difficult to randomize patients to receive no vitamin D supplementation in a placebo-arm in future Phase III trials with vitamin D, considering the present evidence already points to the benefits of add-on use of vitamin D with very little risk of harmful side effects and low cost.

6.3 Immunomodulatory effects of vitamin D supplementation

We assessed the effect of vitamin D3 supplementation on the serum levels of multiple cytokines in RRMS patients treated with IFN-beta-1b. A duration of 12 months' supplementation with vitamin D3 significantly increased serum levels of 25(OH)D from a mean of 54 nmol/l to 109 nmol/l. Serum levels of LAP, the specific latency-associated peptide of TGF-β, significantly increased in the vitamin D3 treated, but not in the placebo group. TGF-β is one of the immune regulatory cytokines produced by T regulatory cells. TGF-β signaling in peripheral nTregs is critical in restraining Th1 cell, CD8+ cytotoxic cell and NK cell differentiation (Marie, Liggitt et al. 2006). TGF-β induces conversion of naïve CD4+ Tcells into iTregs (Workman, Szymczak-Workman et al. 2009) and it is critical in maintaining Foxp3 expression and maintenance and homeostasis of nTreg cells in the periphery (Li, Sanjabi et al. 2006, Marie, Letterio et al. 2005). In vitro treatment of cultured human skin Langerhans cells with 1,25(OH2)D3 evoked the expression of TGF-β and induction of Fox3+Tregs by these dendritic cells (van der Aar, Angelic M G, Sibiryak et al. 2011). Vitamin D treatment of mice has been shown to induce TGF-β1 production (Cantorna, Woodward et al. 1998).

We used LAP as a surrogate for bioactive TGF-β. TGF-β is synthetized as pre-pro-TGF-β which consists of a signal peptide, LAP and mature TGF-β. In the endoplasmic reticulum, TGF-β is cleaved from LAP and LAP non-covalently binds TGF-β to form latent TGF-β (LTGF-β) (Tran 2012). This complex is further linked to latent TGF-β binding protein (LTBP) for secretion to extracellular matrix (Hyytiäinen, Penttinen et al. 2004). After cleavage in extracellular space, bioactive TGF-β is released from LAP and LTBP.

Thus, LAP is synthetized and released in equal amounts with TGF-β and thus a LAP measurement should reflect TGF-β levels reliably and provide a means of monitoring TGF-β activity.

In our study, the concentrations of IFN-γ, IL-17A, IL-2, IL-10, IL-9, IL-22, IL-6, IL-13, IL-4, IL-5, IL-1β and TNF-α did not change statistically significantly in either the vitamin D3 treatment or placebo group, but there was a notable, although not statistically significant, increase in the pro-inflammatory cytokines IFN-gamma and IL-17, as well as IL-9 in the vitamin D treated patients. There are only a few RCTs which have examined the effects of vitamin D supplementation on serum cytokine levels in patients with MS. In line with our results, in the study conducted by Mahon et al., serum levels of TGF-β increased in MS patients treated with vitamin D3 (Mahon, Gordon et al. 2003). Results considering IL-17 have been conflicting (Golan, Halhal et al. 2013, Toghianifar, Ashtari et al. 2015) and IL-10 was increased in vitamin D treated MS patients in one study (Ashtati, Toghianifar 2015) but otherwise no effect of vitamin D on serum levels of cytokines have been found (Muris 2013, Sotirchos, Bhargava et al. 2016). At present, most of the information on the immunomodulating effects of vitamin D comes from in vitro studies. In vivo effects of vitamin D do not result from the induction of a single cell type or excretion of single cytokine but from a complex interplay between multiple vitamin D sensitive cells of innate and adaptive immune system (Peelen, Knippenberg et al. 2011). The action of a certain cytokine is also affected by the whole cytokine milieu (Becher, Spath et al. 2017). Increases in the serum levels of IFN-γ, IL-17 and IL-9 in the vitamin D group may be attributed to an elevation in the TGF-β activity, since it has been shown that in combination with IL-4 TGF-β can induce Th9 cells producing IL-9 and in combination with IL-6, lead to the production of Th17 cells producing IL-17, IFN-γ and IL-9 (Stassen, Schmitt et al. 2012). All the patients in our trial were treated by IFN-beta which may have modified the cytokine responses to vitamin D. It has been shown previously that vitamin D supplementation at a dose of 800 IU/day increases serum IL- 17 levels, whereas supplementation at a higher dose of 4370 IU/day was reported to display a trend to decreased IL-17 secretion in patients using IFN-beta (Golan, Halhal et al. 2013).

The strengths of this study are the blinded placebo-controlled design and analysis of large number of cytokines. Paired serum samples were collected at the same time of consecutive years, thus excluding seasonal effects. In addition, clinical and MRI data were available for the study cohort. Unfortunately, living cells for the determination of cytokines directly from stimulated lymphocytes instead of serum were not available in our study.

We showed that immunomodulatory effects of vitamin D can be seen at 25(OH)D serum levels of 110 nmol/l. The immunoregulatory effects of TGF-β may play a role in the improved MRI outcomes observed in the vitamin D treated group.

6.4 MS prevalence and risk of fractures in MS patients in southwest Finland

In this study, we estimated the prevalence of MS of 212 per 100 000 in southwest Finland in 2012. This is among the highest reported prevalence rates of MS globally and even high in the known high-risk area of western Finland (Krökki, Bloigu et al. 2011). The Finnish healthcare system with its publicly funded healthcare is ideal for generating health administrative data which can be exploited in epidemiological surveys such as this study. In Finland, patients with MS are diagnosed in referral centers in central and university hospitals i.e. virtually all patients with MS are diagnosed in public hospitals. Turku University Hospital has administrative data on all patients who have visited public hospitals in the hospital district of southwest Finland and therefore we are convinced that we have a complete coverage of all cases who fulfill the diagnostic criteria. The mortality data were also complete.

We assessed the risk of comorbid low-energy fractures in a cohort of 1004 patients with MS in the university hospital district in southwest Finland. We found a 1.3-fold overall risk of low-energy fractures and a 1.5-fold risk of osteoporotic fractures in patients with MS compared with a 10-fold age-and gender-matched control population from the same area. These results are in line with other studies assessing the fracture risk in patients with MS. Bazelier et al. reported a 1.4-fold risk of osteoporotic fractures in MS patients in the UK General Practice Research Database (Bazelier, van Staa et al. 2011). In a Dutch PHARMO record linkage study, patients with MS had a 1.7-fold risk for osteoporotic fractures (Bazelier, van Staa et al. 2012), and in a large Danish registry study, the risk for all fractures was elevated by 1.4-fold in patients with MS (Bazelier, de Vries et al. 2012). In a recent meta-analysis by Dong et al., the RR for fractures in MS patients was 1.58 (Dong, Zhang et al. 2015).

In our study, the risk ratio was increased, in particular for fractures of hip (RR 5.00) and humerus (RR 2.36), which are predominantly osteoporotic fractures (Court-Brown, Caesar 2006). Patients with MS are at increased risk of osteoporosis compared with the general population due to several reasons. The most important factor seems to be increasing

disability and consequent immobility (Olsson, Oturai et al. 2015, Tyblova, Kalincik et al. 2015, Weinstock-Guttman, Gallagher et al. 2004). In our cohort, the median disease duration from diagnosis of MS was 16 years in patients with osteoporotic fractures and 8 years in patients with other type of fractures. According to the results in Kaplan-Meier model, the risk of osteoporotic fracture increased steadily with disease duration, and the risk of some other type of fractures was greatest during the first 10 years after diagnosis. The median EDSS was 6 in the osteoporotic fracture group and 4.5 in the non-osteoporotic fracture group. A longer disease duration has been shown to correlate with risk of osteoporotic fractures (Bazelier, van Staa et al. 2012). The bone deficit is more severe in patients with long-standing disease and poor mobility (Marrie, Cutter et al. 2009, Hearn, Silber 2010) which is likely among the reasons of high osteoporotic fracture risk observed in our cohort.

The use of antiepileptic medications (Farhat, Yamout et al. 2002), low vitamin D levels (Holick 2007a), smoking (Handel, Williamson et al. 2011, Watanabe, Inoue 2016) and, to a lesser degree, the use of corticosteroids (Weinstock-Guttman, Gallagher et al. 2004, Tyblova, Kalincik et al. 2015) may contribute to risk of osteoporosis. Precise data about these confounding factors were not available, nor did we have any data referring to BMI or smoking habits. However, information on mean vitamin D level analyzed after the first fracture event was available, and no difference was observed (89 nmol/L in the osteoporotic group and 81 mmol/L in the non-osteoporotic group). A vitamin D level higher than 75 nmol/L is considered sufficient for bone health (Holick 2007a). In a study conducted by Soilu-Hänninen et al. in the hospital district of southwest Finland, the mean serum levels of 25(OH)D in newly diagnosed MS patients was 50 nmol/l. In our study, most of the vitamin D values had been analyzed after the initiation of vitamin D supplementation, explaining the relatively high mean levels of 25(OH)D. The exact starting dates of vitamin D supplementation were not available but it is probable that, in most cases, supplementation had been initiated after the first fracture event.

Our dataset has some limitations. Our data are based on prevalent MS cases and are not a follow-up from first date of diagnosis to the fracture event. Thus, we lack the information about the number of falls and data of BMI and smoking status, which may have a confounding effect on the risk of fractures. However, our cohort was reasonably large in size, representing about 15% of the total Finnish MS population. Case ascertainment was made by review of medical records, and our study data include the time interval between verified MS diagnosis and fracture event as well as the disease course and disability level

by EDSS before the fracture event. Each fracture site and injury mechanism could be investigated, and we had access to radiological and laboratory reports. Information gained from administrative data was thus sufficient for the purposes in this study.

7 CONCLUSIONS

The purpose of this thesis was to investigate whether serum 25(OH)D levels during pregnancy would be associated with the risk of MS in the offspring. Further, we performed a clinical trial assessing the efficacy of vitamin D supplementation on clinical and MRI outcomes in MS patients receiving IFN-beta therapy, as well as investigating the mechanism of the immune modulating effect of vitamin D in patients participating in this trial. We also updated the MS prevalence and studied the fracture risk and the role of vitamin D in the risk of fractures in MS patients in southwest Finland. The conclusions based on the results presented in this thesis are as follows.

1. We showed that a maternal vitamin D deficiency during early pregnancy doubled the risk of MS in her child. Correcting the mother's vitamin D deficiency during pregnancy may have a beneficial effect on the risk of MS in her offspring.

2. Vitamin D3 supplementation with a weekly dose of 20 000 IU (500 ug) for 12 months decreased the number of T1 enhancing brain MRI lesions in comparison with placebo in RRMS patients receiving IFN-beta therapy. Positive trends were seen in MRI burden of disease and EDSS favoring vitamin D3 supplementation.

3. Vitamin D3 supplementation but not placebo increased serum levels of LAP (TGF-beta) in RRMS patients receiving IFN-beta. The immunoregulatory effects of TGF-beta may play a role in the improved MRI outcomes that we observed in the vitamin D treated group. We also conclude that detectable immunological effects can already be seen at a mean serum level of 110 nmol/l of 25(OH)D.

4. We observed an MS prevalence of 212 per 100 000 in southwest Finland in 2012. This is one of the highest reported prevalence rates of MS anywhere in the world.

5. The increased risk of fractures was 1.3-fold for any fractures and 1.5-fold for osteoporotic fractures in patients with MS compared with healthy controls. In particular, the risk of fractures of hip and humerus was elevated.

As an overall conclusion and recommendation based on our results, we propose correcting vitamin D deficiency during pregnancy at the population level but especially in MS patients planning to become pregnant. In all MS patients, we recommend analyzing vitamin D levels after the diagnosis of MS and initiating vitamin D supplementation at a dose of 50 to 100 ug/day, targeting serum levels above 100 nmol/l. This dose and target serum level is recommended based on demonstrated safety and detectable effects on MS disease activity as well as our finding in Finnish MS patients of immunological effects with a dose of 20 000 IU/week (70 ug/day) of vitamin D3, that increased the mean serum vitamin D level from a mean of 54 nmol/l to 110 nmol/l.

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