UNIVERSITY OF TURKU

ENVIRONMENTAL AND LIFESTYLE FACTORS IN MULTIPLE SCLEROSIS

With Emphasis on Vitamin D, EBV Infection, Smoking and Cancer Risk

Kira Åkerlund

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ISBN 978-951-29-7962-2 (PRINT)
ISBN 978-951-29-7963-9 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Painosalama Oy, Turku, Finland 2020

To Rita, Viktor and Finn

UNIVERSITY OF TURKU
Faculty of Medicine
Department of Neurology
KIRA ÅKERLUND: Environmental and lifestyle factors in multiple sclerosis with emphasis on vitamin D, EBV infection, smoking and cancer risk
Doctoral Dissertation, 155 pp.
Doctoral Programme in Clinical Research
February 2020

ABSTRACT

Background: Multiple sclerosis (MS) is the most common chronic inflammatory and neurodegenerative autoimmune disease of the central nervous system. The cause of MS remains unknown, but both genetic and environmental predisposing factors have been identified. Vitamin D deficiency, smoking and Epstein-Barr virus (EBV) infection are the most prominent identified environmental risk factors for MS, but how early before MS onset these risk factors have impact is not known. EBV infection, smoking and vitamin D deficiency are risk factors also associated with cancer. The risk of cancer among Finnish MS patients has not been studied since 1990s.

Aims of the study: Finnish Maternity Cohort (FMC) is a serum bank collected from Finnish pregnant women since 1983. The main aims of this thesis were to study whether maternal smoking and vitamin D status during pregnancy are associated with later risk of MS among Finnish women, and to study whether maternal EBV antibodies affect the MS risk of the mother and her offspring. Further aims were to investigate the safety and efficacy of vitamin D supplementation in combination with fingolimod therapy in MS patients, and to study cancer risk and factors affecting it among MS patients in Southwest Finland from January 2004 to December 2012.

Results: Vitamin D deficiency during pregnancy was associated with an almost 2-fold risk of MS in both the Finnish mothers and their offspring. Smoking Finnish mothers had a 45% higher future MS risk compared to non-smokers. Highest titers of maternal EBV antibodies in comparison with the lowest tripled the MS risk in the mother and doubled it in her offspring independently of vitamin D and cotinine levels. Use of vitamin D supplements as an add-on to fingolimod showed beneficial effects on magnetic resonance image (MRI) outcomes and depression. Risk of cancer among MS patients in Southwest Finland was equal to controls, but age at breast cancer diagnosis was significantly higher among the MS patients. Smoking history of the patients was incompletely documented.

Conclusions: Correcting for vitamin D deficiency during pregnancy can be beneficial in MS prevention for both the mother and her child. Smoking is a risk factor for MS in Finnish women and should be a target for lifestyle intervention in young women. Prevention of EBV infection by vaccination could hold potential for decreasing the risk of both numerous cancers and MS, but the risk of increasing MS predisposition by postponing the timing of the EBV infection is a potential risk that must be taken into consideration.

Keywords: Multiple sclerosis, pregnancy, vitamin D, EBV infection, smoking, cancer

TURUN YLIOPISTO
Lääketieteellinen tiedekunta
Neurologia
KIRA ÅKERLUND: Ympäristö- ja elämäntapatekijät MS-taudissa painopiste D-vitamiinissa, EBV-infektiossa, tupakoinnissa ja syöpäriskissä
Väitöskirja, 155 s.
Turun kliininen tohtoriohjelma
February 2020

TIIVISTELMÄ

Taustaa: Pesäkekovettumatauti eli multippeliskleroosi (MS) on yleisin krooninen tulehduksellinen ja rappeuttava keskushermoston autoimmuunisairaus. Sen aiheuttaja on tuntematon, mutta useita altistavia geneettisiä ja ympäristötekijöitä on tunnistettu. D-vitamiinin puutos, tupakointi ja Epstein-Barr virus- (EBV) infektio ovat merkittävimmät ympäristöriskitekijät, mutta sitä kuinka varhain nämä riskitekijät vaikuttavat ei tunneta. Tupakointi ja EBV-infektio ovat riskitekijöitä paitsi MS-tautiin myös syöpään sairastumiseen. Suomalaisten MS-potilaiden syöpäriskiä ei ole tutkittu 1990-luvun jälkeen.

Tavoitteet: Väitöskirjatyön keskeisenä tavoitteena oli tutkia, onko suomalaisten äitien raskaudenaikaisella tupakoinnilla ja matalalla D-vitamiinipitoisuudella yhteys äidin ja lapsen myöhempään riskiin sairastua MS-tautiin, ja onko äidin EBV vasta-aineilla vaikutusta äidin ja lapsen myöhempään MS-riskiin. Lisäksi väitöskirjassa tutkittiin D-vitamiinilisän tehoa ja turvallisuutta MS-potilailla yhdistettynä fingolimodihoitoon ja selvitettiin MS-potilaiden syöpäriski Varsinais-Suomessa tammikuun 2004 ja joulukuun 2012 välisenä ajanjaksona.

Tulokset: Äidin raskaudenaikainen D-vitamiinipuutos kaksinkertaisti suomalaisen äitiyskohortin naisten ja raskauksista syntyneiden lasten riskin sairastua MS-tautiin. Äidin EBV vasta-aineet lisäsivät MS taudin riskiä kolminkertaiseksi naisilla ja 2.5-kertaiseksi heidän jälkeläisillään riippumatta seerumin D-vitamiini- ja kotiniinitasoista. Kohortin tupakoivien suomalaisten naisten riski sairastua MS tautiin tulevaisuudessa oli 45% korkeampi kuin tupakoimattomilla. D-vitamiinilisä fingolimodia käyttävillä MS potilailla oli turvallista ja sillä oli suotuisa vaikutus magneettikuvauksella todettaviin MS-tautimuutoksiin ja depressioon kahden vuoden seurannassa. Varsinais-Suomen MS potilaiden syöpäriski ei eronnut sairaanhoitopiirin kontrolliväestön syöpäriskistä, mutta MS-tautia sairastavilla rintasyöpä todettiin merkitsevästi myöhemmällä iällä kuin verrokeilla. Tupakointitausta oli puutteellisesti dokumentoitu.

Johtopäätökset: Raskaudenaikaisen D-vitamiinipuutoksen korjaamisesta saattaa olla hyötyä MS taudin ehkäisemisessä niin äideillä kuin heidän jälkeläisillään. Tupakointi on MS taudin riskitekijä myös suomalaisilla naisilla ja tupakoinnin ennaltaehkäisy tulisi olla keskeinen interventio nuorilla naisilla. EBV-infektion ennaltaehkäisy rokotteella saattaisi suojata MS taudilta, mutta EBV infektion lykkääntyminen rokotteella saattaisi jopa lisätä MS riskiä.

Avainsanat: MS-tauti, raskaus, D-vitamiini, EBV-infektio, tupakointi, syöpä

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Abbreviations

25(OH)D	25-hydroxyvitamin D
Ab	Antibody
AE	Adverse event
ARR	Annual relapse rate
BBB	Blood-brain barrier
BC	Breast cancer
BMI	Body mass index
CI	Confidence interval
CIS	Clinically isolated syndrome
CMV	Cytomegalovirus
CNS	Central nervous system
CSF	Cerebrospinal fluid
DIS	Dissemination in space
DIT	Dissemination in time
DMT	Disease modifying therapy
EA-D	Early antigen, diffuse
EA-R	Early antigen, restricted
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
EDSS	Expanded disability status scale
EP	Evoked potential
FMC	Finnish maternity cohort
FTY720	Fingolimod
GA	Glatiramer acetate
Gd	Gadolinium
HHV-4	Human herpesvirus 4
HLA	Human leucocyte antigen
ICD	International Classification of Diseases
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin

IM	Infectious mononucleosis
IS	Immunosuppressant
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NEDA	No evidence of disease activity
NF	Neurofilament
NK	Natural killer
OCB	Oligoclonal bands
OCT	Optic coherence tomography
ON	Optic neuritis
OR	Odds ratio
POMS	Pediatric onset MS
PPMS	Primary progressive multiple sclerosis
PBVC	Percentage brain volume change
RCT	Randomized controlled trial
RR	Risk ratio
RIS	Radiologically isolated syndrome
RRMS	Relapsing remitting multiple sclerosis
SHS	Secondhand smoking
SPMS	Secondary progressive multiple sclerosis
UV	Ultraviolet
UVB	Ultraviolet B
UVR	Ultraviolet radiation
VDR	Vitamin D receptor
VCA	Viral capsid antigen

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Munger KL, Åivo J, Hongell K, Soilu-Hänninen M, Surcel HM, Ascherio A. Vitamin D status during pregnancy and risk of multiple sclerosis in offspring of women in the Finnish Maternity Cohort. JAMA Neurology. 2016 May 1;73(5):515-9. doi: 10.1001/jamaneurol.2015.4800.

II Munger KL, Hongell K, Åivo J, Surcel HM, Soilu-Hänninen M, Ascherio A. 25-Hydroxyvitamin D deficiency and risk of MS among women in the Finnish Maternity Cohort. Neurology. 2017 Oct 10; 89(15):1578-1583. doi: 10.1212/WNL.0000000000004489. Epub 2017 Sep 13.

III Munger KL, Hongell K, Cortese M, Åivo J, Soilu-Hänninen M, Surcel HM, Ascherio A. Epstein-Barr virus and multiple sclerosis risk in the Finnish Maternity Cohort. Annals of Neurology. 2019 Sep;86(3):436-442. doi: 10.1002/ana.25532. Epub 2019 Jul 3.

IV Hongell K, Silva DG, Ritter S, Meier DP, Soilu-Hänninen M. Efficacy and safety outcomes in vitamin D supplement users in the fingolimod phase 3 trials. Journal of Neurology. 2018 Feb:265(2):348-355. doi: 10.1007/s00415-017-8697-3. Epub 2017 Dec 14.

V Hongell K, Kurki S, Sumelahti ML, Soilu-Hänninen M. Risk of cancer among Finnish multiple sclerosis patients. *Multiple Sclerosis and Related Disorders*. 2019 Aug 5; 35:221-227. doi: 10.1016/j.msard.2019.08.005. [Epub ahead of print]

1 Introduction

Multiple sclerosis (MS) is the most common chronic, inflammatory, demyelinating and neurodegenerative autoimmune disease affecting the central nervous system (CNS) in young adults. It is a leading cause of disability, early retirement and socioeconomic burden (Rotstein *et al.*, 2006; Fromont *et al.*, 2014; Hollenbach and Oksenberg, 2015; Filippi *et al.*, 2018). Women are affected more commonly than men and a steady global increase in MS incidence and prevalence in females has been observed in recent years (Koch-Henriksen and Sørensen, 2010; Trojano *et al.*, 2012; Sumelahti *et al.*, 2014; Leray *et al.*, 2016; Filippi *et al.*, 2018; Magyari and Sorensen, 2019). The rapidly increased female predominance of MS may reflect a possible role of environmental risk factors mainly affecting women (Orton *et al.*, 2006; Koch-Henriksen and Sørensen, 2010). Several studies support the presence of a clear genetic component, whereas the relatively low concordance among monozygotic (MZ) twins and an increasing concordance in Finnish dizygotic (DZ) twins during the past two decades indicate that environmental factors also play a central role in the increasing prevalence and risk of developing MS (Särkijärvi *et al.*, 2006; Kuusisto *et al.*, 2008; Hedström *et al.*, 2009). The cause of MS remains unknown, but a generally accepted hypothesis is that it is a predominantly immune-mediated inflammatory and neurodegenerative disease and is likely to be a product of complex associations between genetic susceptibility, infectious risk factors and environmental exposures (Leray *et al.*, 2016; Sospedra and Martin, 2016). Some of the most pertinent environmental factors consistently associated with an increased risk of developing MS are previous Epstein-Barr virus (EBV) infection, vitamin D deficiency, smoking and adolescent obesity (Hedström *et al.*, 2009; Leray *et al.*, 2016; Olsson, Barcellos and Alfredsson, 2016; Filippi *et al.*, 2018). Whether a potential future vaccine or antiviral treatment of EBV would be beneficial in MS prevention, or whether a vaccine with partial efficacy potentially leading to later timing of the primary EBV infection could in a worst case scenario indeed increase the MS risk are not known. Although the epidemiological evidence supporting the role of vitamin D deficiency as a risk factor for developing MS is comprehensive (Munger *et al.*, 2006), the optimal timing of vitamin D supplement initiation for MS susceptible subjects or to the general population is not known. Determining whether

this would ultimately lead to a reduction in MS occurrence would require decades of follow-up and taking into account changes in other known and unknown predisposing factors. Although the disease biomarkers, clinical and paraclinical measurements (including imaging) and MS treatments have evolved such that it is possible in most cases to stop the inflammatory phase, there is still no cure for MS. The highest unmet therapeutic need is the treatment of progressive disease and restoration of CNS damage. Despite the challenges, the ultimate goal of MS research needs to be prevention through more effective identification and modification of lifestyle and environmental risk factors.

2 Review of the Literature

2.1 Multiple sclerosis

2.1.1 Clinical features

MS has traditionally been classified largely based on its clinical characteristics into the phenotypes relapsing remitting (RRMS), secondary progressive (SPMS), primary progressive (PPMS) and progressive relapsing MS (PRMS) (Lublin and Reingold, 1996). Since the phenotypes are more complex and often clinically overlapping, refinements were proposed in 2013 in an attempt to add clinically relevant information to MS categorizations (Lublin *et al.*, 2014). The reclassified categories are descriptive of the level of inflammatory activity observed compared to the degenerative progression of the disease and include the inactive clinically isolated syndrome (CIS), and if active, eventually RRMS. The designation of progressive MS disease comprises both the former PPMS (progressive accumulation of disability from onset) and SPMS (progressive accumulation of disability after initial relapsing course) phenotypes. Progressive MS can be divided into the subtypes active and with progression, active without progression, inactive with progression and inactive without progression. Activity is determined by clinical relapses or magnetic resonance imaging (MRI) activity (contrast-enhancing lesions; new and enlarging T2 lesions), whereas progression is mainly clinically evaluated (Lublin *et al.*, 2014; Oh, Vidal-Jordana and Montalban, 2018).

In a majority of the MS population (~80-85%), the disease starts with an acute relapse and succeeds with a relapsing remitting course. RRMS is characterized by acute exacerbations and remissions during the early focal inflammation phase, followed by progression due to neurodegeneration (Weinshenker, Issa and Baskerville, 1996; Leray *et al.*, 2010; Confavreux and Vukusic, 2014). A relapse is considered to be a clinically expressed acute, focal or multifocal inflammation and demyelination in the CNS, characterized by occurrence, recurrence or worsening of neurological symptoms during at least 24 hours (Youl *et al.*, 1991; McDonald *et al.*, 2001).

The majority of RRMS cases eventually convert to SPMS over time (after approximately 10-20 years in untreated MS patients), defined by a steady disease

progression and disability accumulation (Confavreux, Aimard and Devic, 1980; WEINSHENKER *et al.*, 1989; Debouverie *et al.*, 2009; Tedeholm *et al.*, 2015). The median time to SPMS conversion has been about 19 years in several large studies (Tremlett *et al.*, 2008; Rzepiński *et al.*, 2019), whereas it is less clear when RRMS patients convert to SPMS in the modern treatment era (Oh, Vidal-Jordana and Montalban, 2018).

PPMS affects ~10-15% of the MS population and is characterized by neurodegeneration leading to disease progression and accumulated disability (Confavreux and Vukusic, 2014).

Pediatric onset MS (POMS) affects <10% of the MS population and is defined as MS debuted in childhood (<15-18-year-old subjects). In Finland, child cases are considered pediatric when <16 years old. 95-100% of all POMS are classified as RRMS. Progression to SPMS is slower than in the adult MS population (Boiko *et al.*, 2002; Krupp, Banwell and Tenembaum, 2007; Bar-or, 2008; Krupp *et al.*, 2013; Waldman *et al.*, 2014; Alroughani and Boyko, 2018).

If the criteria of MS are not readily met, the first relapse event is referred to as a CIS, which most often is monosymptomatic, and usually affects the optic nerve, spinal cord or brainstem. The overall probability of developing MS after an attack of optic neuritis (ON) is 50% in 15 years (Brodsky *et al.*, 2008). The risk of developing MS increases if there are paramedical findings of cerebrospinal fluid (CSF) oligoclonality or at least one clinically silent white matter lesion in the MRI (Brex *et al.*, 2002; Tintoré *et al.*, 2006, 2008; Fisniku *et al.*, 2008).

In 2009, Okuda et al introduced a formal description of the radiologically isolated syndrome (RIS), based on observations of incidental MRI findings suggestive of demyelinating disease in persons without typical clinical MS presentations (Okuda *et al.*, 2009). Later study observations suggest that a significant proportion of RIS cases have cognitive impairment similar to that seen in part of the MS population and approximately two-thirds of RIS cases develop radiological progression and 30-45% develop either acute or progressive neurological symptoms during a mean follow-up time of five years (Granberg *et al.*, 2013; Okuda *et al.*, 2014).

Life expectancy among MS patients is reduced by ~10 years compared to the general population and mortality is almost 3-fold higher than in the general population (Sumelahti *et al.*, 2010; E Kingwell *et al.*, 2012; Lunde *et al.*, 2017). Patients older at MS onset or with PPMS have shorter survival (E Kingwell *et al.*, 2012; Scalfari, Knappertz, *et al.*, 2013). In about 50% of the MS population, MS is the cause of death (Sumelahti *et al.*, 2010). Infection and suicide rates are higher (Scalfari, Knappertz, *et al.*, 2013) and excess mortality rates from accidents and other comorbidities, except from cancer (Brønnum-Hansen, Koch-Henriksen and Stenager, 2004; Koch-Henriksen *et al.*, 2017), are higher than among the general

population (Brønnum-Hansen, Koch-Henriksen and Stenager, 2004; Burkill *et al.*, 2017; Lunde *et al.*, 2017; Pirttisalo *et al.*, 2018). Stroke was observed to decrease the survival rate among MS patients in a population-based Finnish Northern Ostrobothnia cohort and infections in Southwest Finland (Krökki *et al.*, 2014; Murtonen *et al.*, 2018), whereas mortality from cancers in the MS population of higher age was increased in Finland (Sumelahti *et al.*, 2002).

2.1.2 Epidemiology

Approximately 2.3 million people have MS worldwide (Multiple Sclerosis International Federation, 2013), over 700,000 individuals in Europe (European Multiple Sclerosis Platform, 2013; Multiple Sclerosis International Federation, 2013; Browne *et al.*, 2014; Gitto, 2017), and the worldwide prevalence varies with geography and ethnicity (Pugliatti, Sotgiu and Rosati, 2002; Kingwell *et al.*, 2013). The disease is associated with a high economic burden of ~14.6 billion euros within Europe in 2010 (Gustavsson *et al.*, 2011). MS onset usually occurs at an age ranging from 20 to 40 years. The incidence and prevalence of the MS population are increasing globally with a female-to-male predominance of 2-3:1 (Bentzen *et al.*, 2010; Koch-Henriksen, Magyari and Laursen, 2015; Howard, Trevick and Younger, 2016; Leray *et al.*, 2016; Amato *et al.*, 2018; Filippi *et al.*, 2018; Magyari and Sorensen, 2019).

The epidemiological hallmark of MS is an uneven geographic distribution. The incidence and prevalence of MS vary significantly between countries, with a global median prevalence of 33 per 100,000 people (Sumelahti *et al.*, 2000, 2003; Tienari *et al.*, 2004; Simpson *et al.*, 2011, 2019; Trojano *et al.*, 2012; Oh, Vidal-Jordana and Montalban, 2018). In North America and Europe the prevalence rates are high (>100/100,000), whereas prevalence levels are low in Eastern Asia and Sub-Saharan Africa (2/100,000) (Leray *et al.*, 2016). A higher global risk of MS has been strongly associated with latitude, with an increasing MS occurrence following a longitudinal south-north gradient in northern parts of the globe and a north-south gradient in southern part of the world, and with a minimal prevalence at the equator (Alonso and Hernán, 2008; Koch-Henriksen and Sørensen, 2010; Simpson *et al.*, 2011, 2019).

A high-risk focus for MS in Scandinavia, Finland included, was identified in the 1960s (Kurtzke, 1968, 1974). Finland is a high-risk region for MS with large regional epidemiological differences, and prevalence varies from 100 to over 200 per 100,000 inhabitants in different areas (Sumelahti *et al.*, 2001, 2003; Sarasoja *et al.*, 2004; Tienari *et al.*, 2004; Krökki *et al.*, 2011; Holmberg *et al.*, 2013). National MS prevalence in Finland has not been studied, but an estimate of 10,000 to 11,000 patients at the end of 2018 has been presented based on Finnish MS register data (Laakso *et al.*, 2019). The female-to-male ratio in RRMS in western Finland has

increased from 2.2 to 2.7 since the 1990s and at the same time, the mean age of the MS population has increased from less than 40 years to over 50 years (Holmberg *et al.*, 2013; Sumelahti *et al.*, 2014; Pirttisalo, Soilu-Hänninen and Sipilä, 2019). The east-west gradient was recently confirmed by showing an age-standardized prevalence to European standard population of 280/100,000 in Southwest Finland versus 168/100,000 in North Karelia and an annual age-standardized incidence (5-year period from 2012 to 2016) of 12.1/100,000 versus 8.6/100,000 respectively (Pirttisalo, Soilu-Hänninen and Sipilä, 2019).

The prevalence of MS reported from other Nordic countries is also increasing. In Norway, MS prevalence was 208/100,000 inhabitants on December 31, 2013. This observation was based on the Norwegian Patient Registry, although without the evidence of a latitude gradient that had been observed in previous studies. The increase in Norwegian MS prevalence was speculated to have several causes, including improved and more accessible neurological health care services and diagnostics, with possible contributions of environmental risk factors such as smoking and vitamin D and changes in lifestyle behavior along with an increased survival in MS (Berg-Hansen *et al.*, 2014, 2015; Grytten, Torkildsen and Myhr, 2015). Similar factors may contribute to the prevalence changes observed in other Nordic countries.

In Sweden, nationwide prevalence on December 31, 2008, was 189/100,000 and significantly increased with increasing north latitude for both males and females. Incidence had increased to an average of 10.2/100,000 (Ahlgren, Odén and Lycke, 2014). In 2008, immigrants constituted 14% of the Swedish population. Prevalence increased in migrants from countries with a lower MS risk, and among Iranian migrants to a prevalence rate exceeding that in the general population, suggesting a genetic risk altered by changed environmental-lifestyle MS risk factors (Ahlgren *et al.*, 2010; Ahlgren, Odén and Lycke, 2011, 2012).

In Denmark, MS prevalence and incidence have been continuously updated with accumulated data from the Danish Multiple Sclerosis Registry established in 1956. Standardized prevalence increased to 232/100,000 inhabitants in 2013 (Bentzen *et al.*, 2010; Koch-Henriksen, Magyari and Laursen, 2015). Similar to other Nordic countries, MS prevalence in Iceland is 166.5/100,000 inhabitants (Elíasdóttir, Kjartansson and Olafsson, 2018).

The pooled prevalence of familial MS (FMS), defined as MS cases with at least one family member in the first, second, third degree or other relatives of probands that are affected by MS, has been calculated at 12.6% of the global total MS population, with a significant heterogeneity independent of latitude or ethnicity, that highlight the accumulation effects of genetics and environmental factors (Harirchian *et al.*, 2018).

2.1.3 Diagnosis and follow-up

2.1.3.1 Diagnostic criteria

There is no single specific test for MS, be it lab-based or otherwise, due to the unknown etiology and specific pathogenesis of the disease (Deisenhammer *et al.*, 2019). Diagnosis is based on the demonstration of dissemination of focal neurological deficits in space (DIS) and time (DIT), as well as exclusion of differential diagnoses (Aktas *et al.*, 2018). The first clinical diagnostic criteria for MS were established in 1965 (Schumacher *et al.*, 1965). Poser et al later added paraclinical evidence and laboratory findings to the diagnostic criteria of definite (Table 1) and probable MS in the 1980s (Poser *et al.*, 1983).

Table 1. Diagnostic criteria for multiple sclerosis by Poser et al., modified from Poser et al. 1983

Category	Attacks	Clinical evidence	Paraclinical evidence	CSF OCB/IgG
Clinically definite				
1. CDMS A1	2	2		
2. CDMS A2	2	1 and	1	
Laboratory supported				
1. LSDMS B1	2	1 or	1	+
2. LSDMS B2	1	2		+
3. LSDMS B3	1	1 and	1	+

OCB/IgG = oligoclonal bands or increased immunoglobulin G (IgG) specific to the cerebrospinal fluid (CSF). Paraclinical evidence of central nervous system (CNS) lesions = findings elicited by hyperthermia, evoked potential studies, computed tomography (CT) and nuclear magnetic resonance (NMR) scans or special urological studies. CDMS = clinically definite MS, LSDMS = laboratory supported definite MS. Modified table printed with permission (Poser *et al.*, 1983).

The McDonald diagnostic criteria for MS were issued in 2001 by the International Panel on the Diagnosis of Multiple Sclerosis (McDonald *et al.*, 2001) and have since been updated in 2005, 2010 and 2017 to facilitate diagnostic accuracy and exclusion of differential diagnoses. Diagnosis is made based on clinical and/or paraclinical (i.e. imaging and laboratory) findings of disseminated CNS lesions attributable to MS (Polman *et al.*, 2005; Brownlee *et al.*, 2017; Thompson *et al.*, 2018). The Barkhof-Tintore MRI criteria were used to demonstrate DIS in the two first versions and replaced by simplified MRI criteria for DIT contrived by Swanton et al in 2010, enabling diagnosis of MS based on a single MRI in some CIS patients (Swanton *et al.*, 2006). Revisions of MRI criteria for the diagnosis of MS proposed by the

European Magnetic Resonance Imaging in Multiple Sclerosis (MAGNIMS) network were incorporated in the 2017 revision (Rovira *et al.*, 2015; Filippi *et al.*, 2016).

Laboratory findings of cerebrospinal fluid (CSF)-specific oligoclonal bands (OCBs) were used as part of MS criteria in the 2001 and 2005 McDonald revisions, but were excluded in the 2010 revision. They were again included in the 2017 McDonald criteria, since CSF-specific OCBs in numerous studies were shown to be an independent predictor of the risk of a second attack in adult patients with a CIS (Thompson *et al.*, 2018).

CIS is defined as an episode of a symptom or symptoms attributable to a debut of MS, that lasts for 24 hours up to one month, but that does not yet meet the 2017 McDonald criteria for definite MS. Clinical evidence of at least one symptom episode attributable to focal CNS demyelination is compulsory for CIS and MS diagnosis. MRI of the CNS can demonstrate further DIS by one or more T2-hyperintense lesions (symptomatic or asymptomatic) that are characteristic for MS in two or more of four CNS areas: periventricular, cortical or juxtacortical, infratentorial brain regions and the spinal cord. MRI can confirm DIT by the simultaneous presence of gadolinium (Gd)-enhancing T1 lesions and non-enhancing lesions at any time or by a new T2-hyperintense or Gd-enhancing lesion on follow-up MRI, with reference to a baseline scan (Thompson *et al.*, 2018).

The Radiologically Isolated Syndrome Consortium presented their first retrospective cohort in 2014 with an observed five-year conversion rate to the first clinical event of 34% among RIS patients (mean age at RIS diagnosis of 37.2 years). Cervical lesions are central predictors of clinical conversion from RIS to CIS or MS (Granberg *et al.*, 2013; Lebrun, 2015), and infratentorial, spinal cord involvement and the total number of lesions are more relevant predictors of progression than Gd enhancement (Maia Jr. *et al.*, 2012; Okuda *et al.*, 2014).

Diagnosis of PPMS requires one year of disease progression independent of clinical relapse and two of the three following criteria must be met: A) One or more (symptomatic or asymptomatic) T2-hyperintense lesions characteristic of multiple sclerosis in at least one of the following brain regions: periventricular, cortical or juxtacortical, or infratentorial B) Two or more (symptomatic or asymptomatic) T2-hyperintense lesions in the spinal cord and C) Presence of CSF-specific OCBs (Thompson *et al.*, 2018). Definition of the time when RRMS converts to SPMS still poses a diagnostic challenge (Oh, Vidal-Jordana and Montalban, 2018). The 2017 McDonald criteria for MS in patients with an attack at onset demonstrated by DIS and DIT are shown in table 2.

For pediatric onset MS (POMS), many different diagnostic criteria have been proposed to meet the challenges of distinguishing MS from various acquired demyelinating syndromes that can occur in childhood. In most studies, the criteria

by the Pediatric International Study Group have been applied (Alroughani and Boyko, 2018).

The differential diagnosis of neuromyelitis optica spectrum disorders (NMOSD) should be considered in any patient being evaluated for MS, since MS treatments can exacerbate NMOSDs (Kimbrough *et al.*, 2012). Serological testing for antibodies to aquaporin 4 and myelin oligodendrocyte glycoprotein (MOG) should therefore be individually considered (Aktas *et al.*, 2018; Thompson *et al.*, 2018).

Table 2. The 2017 McDonald criteria for diagnosis of multiple sclerosis in patients with an attack at onset, modified from Thompson et al. 2018.

	Number of lesions with objective clinical evidence	**Additional data needed for a diagnosis of multiple sclerosis**
≥2 clinical attacks	≥2	None*
≥2 clinical attacks	1 (as well as clear-cut historical evidence of a previous attack involving a lesion in a distinct anatomical location)	None*
≥2 clinical attacks	1	Dissemination in space demonstrated by an additional clinical attack implicating a different CNS site or by MRI**
1 clinical attack	≥2	Dissemination in time demonstrated by an additional clinical attack or by MRI*** OR demonstration of CSF-specific oligoclonal bands****
1 clinical attack	1	Dissemination in space demonstrated by an additional clinical attack implicating a different CNS site or by MRI** AND Dissemination in time demonstrated by an additional clinical attack or by MRI*** OR demonstration of CSF-specific oligoclonal bands****

*No additional tests are required to demonstrate DIS and DIT. However, if possible, all patients in whom the diagnosis of MS is being considered should obtain a brain MRI. Spinal cord MRI or CSF examination is recommended in patients with scarce or atypical clinical findings lacking paraclinical supportive evidence of MS. If imaging and paraclinical tests are negative, an alternative diagnosis should be considered. **At least one attack must be supported by objective findings. Clinical diagnosis based on objective clinical findings for two attacks is most secure. Reasonable evidence for another previous inflammatory demyelinating attack is sufficient for diagnosis, but caution is needed in the absence of residual objective evidence. *** The MRI criteria for DIS and DIT are described in the text above. **** The presence of CSF-specific OCBs does not demonstrate DIT per se but can substitute for the requirements for demonstration of this measurement (Thompson et al 2018). CIS = clinically isolated syndrome; MS = multiple sclerosis; DIS = dissemination in space; DIT = dissemination in time; MRI = magnetic resonance imaging; OCB = oligoclonal bands; CSF = cerebrospinal fluid; CNS = central nervous system. Modified table printed with permission (Thompson *et al.*, 2018).

2.1.3.2 Follow-up

2.1.3.2.1 Clinical outcome measures

MS prognosis and intervention efficacy assessment are challenging, since the disease has a heterogenous nature that makes it difficult to capture disease activity and progression in a reliable, valid way. Many scorings reflecting the MS disease course have been introduced over time to meet the needs for objective and subjective evaluation tools in the clinic and for trial outcomes, for treatment and rehabilitation efficacy evaluation, and for assessment of the patients' need for assistance or capacity to work (Elovaara *et al.*, 2006).

The traditionally used primary clinical outcome measures in MS phase 3 trials are the relapse rate and the Expanded Disability Status Scale (EDSS) (Uitdehaag, 2014; van Munster and Uitdehaag, 2017). The American neurologist John Kurtzke created the Disability Status Scale (DSS) in the 1960s and later an expanded version (EDSS), as a method of qualifying MS disability. EDSS ranks the disease burden severity based on clinical symptoms on a scale from 0 (no clinical signs or subjective symptoms of MS in the neurological examination) to 10 (patient deceased) in 0.5-unit increments. EDSS scoring covers assessment of the functional systems: visual, brainstem, pyramidal, cerebellar, sensory, bladder and bowel, and cerebral, as well as consideration of gait and walking distance ability with or without aids (Kurtzke, 1983, 2015), and reflect fluctuations during relapses, remissions and disease progression useful in the evaluation of MS treatment efficacy (van Munster and Uitdehaag, 2017). Treatment of MS has been focused on reducing the number of relapses, regardless of the fact that the rate of relapses characteristic of RRMS shows no association with longtime disease prognosis (Confavreux and Vukusic, 2006; Scalfari, Neuhaus, *et al.*, 2013). The annual relapse rate (ARR) is, however, still used as a primary outcome measure in most clinical phase 3 trials (Lim and Constantinescu, 2010; van Munster and Uitdehaag, 2017) although it is becoming overly restricted in the era of new and emerging potent disease modifying treatments (DMTs) for MS that have led to a shift in treatment expectations from one of partial response to one of potent remission.

No evidence of disease activity (NEDA-3) has emerged as an outcome measure that consists of a composite of clinical relapses, EDSS disability and MRI outcomes (defined by the absence of clinical relapses, EDSS progression and radiological signs of disease, i.e. no new/enlarging T2 lesions or Gd-enhancing T1 lesions on MRI) (Rotstein, Healy, Malik, Chitnis, *et al.*, 2015; Lu *et al.*, 2017, 2018; Parks *et al.*, 2017).

Today, national MS patient registries have become widely used and provide insight about key observations including disability progression rate, predictors of

increased disability, and changes in lifespan of the natural history of MS (Glaser *et al.*, 2019). They provide a tool for treatment efficacy evaluation, both in MS research and clinics. Quality registers are valuable, as they provide data that cannot otherwise be captured, and particularly informative if multiple registries confirm similar findings that could also be utilized in conjunction with data from clinical trials to optimize treatment and improve long-term outcomes (Hurwitz, 2011a, 2011b; Koch-Henriksen, Magyari and Laursen, 2015). In Finland, a national MS register was launched in 2014 (Laakso *et al.*, 2019).

2.1.3.2.2 Radiological outcome measures

MRI technology and availability have improved since the 1990s and have become central in the diagnosis and follow-up of MS patients, possibly even contributing to the observations of increased MS prevalence in recent years by providing improved methods of case ascertainment (Howard, Trevick and Younger, 2016). In 2001, MRI was included in the MS diagnostic work-up recommendations of patients with CIS by an International Panel of experts, since it is more sensitive in detecting disease activity and new inflammatory lesions than clinical evaluation and monitoring of relapse numbers (McDonald *et al.*, 2001). Guidelines for MRI use in MS diagnostics and follow-up have been modified since then and continue to be updated by the MAGNIMS, allowing both earlier, more accurate diagnosis and initiation of treatment (Filippi *et al.*, 2016; Thompson *et al.*, 2018).

Relevant MRI findings are white matter focal lesions in the CNS, typical for MS in terms of distribution, morphology, evolution, and signal abnormalities on the following conventional MRI sequences; T2-weighted, T2-FLAIR (fluid attenuated inversion recovery) and T1-weighted scans before and after intravenous (IV) Gd contrast distribution (National Multiple Sclerosis Society, 2019). Gd enhancement is currently the reference standard to detect active inflammatory lesions in MS and demonstrates blood-brain barrier (BBB) breakdown in association with inflammation and demyelination. The disrupted BBB and glymphatic system allows the Gd molecule to traverse into the lesions where it can persist for two to three months and thus provide evidence of recent disease activity informative in MS treatment monitoring (Cotton *et al.*, 2003; Iliff, Goldman and Nedergaard, 2015; Jessen *et al.*, 2015; Saade *et al.*, 2018; National Multiple Sclerosis Society, 2019). T2 lesions are unspecific but can be used to measure disease activity by providing information about serial imaging when active lesions (new or enlarging T2 lesions) are present between two scans (Simon, 2014). Radiologically, the burden of MS disease is measured by the total volume of T2 lesions, and MRI lesion load in early disease is a prominent prognostic factor for disease accumulation as well as a

predictor of RIS conversion to CIS and MS (Maia Jr. *et al.*, 2012; Granberg *et al.*, 2013; Okuda *et al.*, 2014; Lebrun, 2015; Rovira *et al.*, 2015).

A more suitable MRI measure of irreversible pathology in progressive MS is brain and gray matter atrophy, which reflects demyelination, axonal loss and gliosis (Fisher *et al.*, 2008; Geurts *et al.*, 2012; Rocca, Absinta and Filippi, 2012; Nandoskar *et al.*, 2017). T1 hypointense lesions ("black holes") reflect focal neurodegeneration when chronic and not enhancing and correlate with MS-related disability (Sahraian *et al.*, 2010; Giorgio *et al.*, 2014). Annualized percentage brain volume change (PBVC) as a measure of brain atrophy is also used in clinical trials as an outcome measure, since MS patients are shown to lose more brain volume per year (0.7% brain volume/year demonstrated for a group consisting of patients with first-generation DMT or no treatment), well above rates associated with normal ageing (0.1-0.3% brain volume per year) (De Stefano *et al.*, 2014; Vollmer *et al.*, 2015).

2.1.3.2.3 Biomarkers as outcome measures

There is no specific test for MS due to the unknown etiology and specific pathogenesis of the disease (Deisenhammer *et al.*, 2019). However, there are biomarkers that can be used as helpful tools in diagnosis, prediction and follow-up. A biomarker is an objective and quantifiable measurement that reflects the activity of a disease process (Strimbu and Tavel, 2010). According to Amur et al, there are four different types of biomarkers: diagnostic, prognostic, predictive and response biomarkers (Amur *et al.*, 2015). Surrogate markers are defined as a subset of biomarkers characterized by specific criteria, making them suitable to use in trials as a substitute for a clinically meaningful endpoint as a direct measure of how a patient feels, functions or survives, and are expected to predict the effect of a therapy (Prentice, 1989; Hauser and Oksenberg, 2006).

Potential serum and CSF biomarkers have not yet led to consensus on a marker that fulfills the criteria for surrogacy, although CSF-specific OCBs are a hallmark of MS-specific changes found in the majority of MS patients and implemented in the last revision of the McDonald 2017 criteria to demonstrate DIT (Nandoskar *et al.*, 2017; Thompson *et al.*, 2018; Deisenhammer *et al.*, 2019). OCBs strongly predict conversion from CIS to definite MS and increase the risk of a relapse 1.7-fold independently of baseline MRI (Tintoré *et al.*, 2008; Dobson *et al.*, 2013). Lack of OCBs has a high negative predictive value of 88% in lumbar puncture (LP) of CIS patients (Tintoré *et al.*, 2001), indicating a red flag and consideration of alternative diagnoses in such patients during diagnostic work-up (Deisenhammer *et al.*, 2019). Differences in MS disease mechanisms between OCB positive and negative MS subjects in Scandinavia (Norway, Sweden and Denmark) have been suggested

related to genetic differences based on genome-wide association studies (GWAS) (Mero *et al.*, 2013).

Neurofilaments (NF) are components of the neuronal cytoskeleton released in CSF in association with CNS damage, and are promising as future biomarkers for MS activity, progression, and treatment response (Cairns, Lee and Trojanowski, 2004; Soelberg Sorensen and Sellebjerg, 2016; Varhaug *et al.*, 2018, 2019; Bridel *et al.*, 2019; Damasceno *et al.*, 2019; Martin *et al.*, 2019)(Teunissen and Khalil, 2012).

Optic coherence tomography (OCT) is an optical form of ultrasound imaging that has shown potential as a noninvasive documentation biomarker for cell loss in the retinal nerve fiber layer through serial measurements correlating with disability, whole-brain and gray matter atrophy (Francis, 2013; Narayanan *et al.*, 2014; Saidha *et al.*, 2015; Garcia-Martin *et al.*, 2017). OCT can be used to monitor macular edema in fingolimod-treated MS patients (Turaka and Bryan, 2012; Francis, 2013; Fragoso, 2017; Fruschelli *et al.*, 2019).

2.1.3.2.4 Electrophysiological outcome measures

The clinical use of function analyses by electro-neurophysiological measurements and evoked potentials (EPs) to confirm damage sites at different locations in the CNS has decreased in MS diagnostics (Paty *et al.*, 1988; Polman *et al.*, 2011; McGuigan, Fernández and Fernández, 2013). EPs, however, remain relevant, as they provide functional quantitative in vivo data on multimodal afferent and efferent pathways through cerebral and spinal long tracks and reveal subclinical lesions on the long sensory-motor pathways. Alteration of signal conduction is the main mechanism of signs and symptoms in MS, and multimodal EPs may thus serve as a representative measure of the functional impairment in the disease although they are insensitive to cerebellar, frontal and cognitive dysfunctions and have not yet been validated for evaluation of individual patients. Sensory EPs include brainstem auditory EP (BAEP, an auditive testing for abnormalities in hearing), visual EP (VEP) and somatosensory EP (SEP) (Hardmeier, Leocani and Fuhr, 2017). According to prospective and retrospective studies, EPs have good accuracy in monitoring and predicting short- and long-term functional evolution and disease progression and have a strong cross-correlation with EDSS (Kallmann *et al.*, 2006; Invernizzi *et al.*, 2011; Margaritella *et al.*, 2012; Ramanathan *et al.*, 2013; Schlaeger, D'Souza, *et al.*, 2014; Schlaeger, Schindler, *et al.*, 2014; Giffroy *et al.*, 2017; Martinelli *et al.*, 2017). In PPMS, VEP are abnormal in about 90% of the patients, adding diagnostic support (Leocani *et al.*, 2006). SEP can be used to objectively confirm abnormalities by stimulating sensation tracts with electrical signals registered in an arm or a leg, whereas motor evoked potentials (MEP) can be applied to demonstrate motor dysfunction in the CNS.

2.1.4 Treatment

2.1.4.1 Disease modifying therapies

DMTs have been available since the first publication of interferon (IFN) effect on MS disease in the mid-1990s. Since then, several DMTs have been approved, including treatments with oral bioavailability and IV distribution (Niiranen and Remes, 2017; Trojano *et al.*, 2017). The current DMTs reduce disease activity, seen as a reduction in ARR and MRI measures of disease burden, but their effect on disease progression and long-time disability accumulation is less clear (Wingerchuk and Weinshenker, 2016).

In Finland, interferons (IFNs) became available in the mid-1990s, glatiramer acetate (GA) in 2004, natalizumab in 2006, fingolimod (FTY) available since 2011 and reimbursed since 2012, alemtuzumab and teriflunomide (TRF) since 2013, dimethyl fumarate (DMF) since 2014 and cladribine since 2017. The current orally administered therapies include TRF, DMF, FTY and cladribine. Self-injectable therapies include IFN-beta, pegylated IFN, and GA. Monoclonal antibodies (Ab) natalizumab, alemtuzumab and ocrelizumab are administered IV (Multiple sclerosis: Current Care Guidelines Abstract, 2019). The chemotherapeutic agent mitoxantrone (MX) is rarely used due to the increased risk of malignancies and severe adverse events (SAEs), which are also consistently associated with the use of the traditional immunosuppressants (IS) azathioprine and cyclophosphamide (Achiron *et al.*, 2005; Lebrun *et al.*, 2008, 2011; Le Bouc *et al.*, 2012; Kingwell *et al.*, 2014; Wingerchuk and Weinshenker, 2016; Ragonese *et al.*, 2017; Li *et al.*, 2019). In the Finnish Current Care Guidelines for MS therapy, treatment initiation is guided by disease activity. MS is defined as active or highly active based on clinical relapses and MRI findings of new or enlarging T2 or Gd-enhancing lesions. Initiation of DMTs for RIS is still controversial and not recommended outside of clinical trials due to lack of beneficial evidence (Okuda *et al.*, 2009; Granberg *et al.*, 2013; Aktas *et al.*, 2018).

MS exacerbation treatment (mainly high-dose IV methyl prednisolone) has little or no effect on ARR or disease progression and is mainly aimed at alleviating relapses affecting mobility and function, including ON. Cessation of MS medication is recommended if there is no evidence of disease activity during 3 years of progression, and IFN-beta does not prevent permanent disability in SPMS (La Mantia *et al.*, 2013; Multiple sclerosis: Current Care Guidelines Abstract, 2019). Pharmacological treatments in Finland for RRMS and PPMS are shown in table 3.

Potential emerging immune modulating approaches (stem cells, DNA vaccines, nanoparticles, altered peptide ligands) for the treatment of MS, and therapeutic approaches for restoration of the damaged nervous system are also under

development (Dargahi *et al.*, 2017). One of the most pressing unmet needs is the development of DMTs that slow disability accumulation in progressive forms of MS.

Table 3. Drug treatments for active and highly active RRMS and active PPMS in Finland

MS type	Treatment
Active RRMS	Dimethylfumarate Glatiramer acetate Interferon beta Ocrelizumab Teriflunomomide
Highly active RRMS	Alemtuzumab Cladribine Fingolimod Mitoxantrone Natalizumab Ocrelizumab
Active PPMS	Ocrelizumab

Multiple sclerosis. Current Care Guidelines (referred September 7, 2019). www.kaypahoito.fi

2.1.4.1.1 Fingolimod

Fingolimod (FTY720, 2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol) became the first Food and Drug Administration approved oral drug for MS treatment in 2010 and has been available in Finland since 2011. It is an immunosuppressive drug derived from myriocin, a fungal metabolite that resembles sphingosine. Its mechanisms of action are related to binding four out of five sphingosine 1-phosphatase (S1P) receptors (S1P1 in particular, as well as S1P3, S1P4 and S1P5), and act on the peripheral immune system through modulation of S1P receptors, leading to internalization and down-regulation of sphingosine 1-phosphate receptors, inhibition of S1P activity and sequestration of T-lymphocytes in the lymph nodes, thereby preventing the release of lymphocytes from lymph nodes and thus leading to a reduction in T-cell trafficking into the CNS (Mandala *et al.*, 2002; Brinkmann, 2007; Aktas *et al.*, 2010). Interleukin-2 (IL-2) activated NK cells express S1P1,3,4,5, and it has been reported that S1P inhibits cell lysis of target cells including tumor cells and dendritic cells (DCs), an inhibitory activity reversed by FTY720 (Rolin *et al.*, 2010).

Efficacy of fingolimod treatment for RRMS has been demonstrated in 3 large Phase 3 clinical randomized controlled trials (RCT) ranging from 6 to 24 months, FREEDOMS I and II (FTY720 Research Evaluating Effects of Daily Oral Therapy in Multiple Sclerosis) and TRANSFORMS (Trial Assessing Injectable Interferon versus FTY720 Oral in Relapsing – Remitting Multiple Sclerosis) by showing decreased rates of relapse rates compared to placebo or weekly administered intramuscular interferon beta-1a, respectively. Compared to IFN-beta, fingolimod

(0.5mg daily orally administered) reduced relapse rates, MRI-activity (T2 and Gd-enhancing lesions) and in the 2 placebo-controlled trials fingolimod additionally slowed disease progression (Kappos *et al.*, 2006, 2010, 2015; Cohen *et al.*, 2010; Calabresi *et al.*, 2014; Sanford, 2014). Fingolimod also reduces brain volume loss compared to placebo but has safety issues warranting monitoring for infections, cancers, and certain transitory effects such as irregular cardiac function, decreased lymphocyte count and a higher level of liver enzymes (Guarnera, Bramanti and Mazzon, 2017). The drug is effective and relatively safe, with a well-described first-dose side effect of bradycardia and atrioventricular block (generally asymptomatic) related to S1P receptors in the heart during its first hours of use. Other relatively rare potential AEs include infections (herpes simplex, herpes zoster, Cryptococcus, EBV, hepatitis, Molluscum contagiosum, and leishmaniosis), lung and thyroid complications, refractory headaches, encephalopathy, vasculopathy, tumefactive lesions in MRI and ophthalmological disorders (Sanford, 2014; Fragoso, 2017). In the TRANSFORMS trial, 0.5% of patients receiving fingolimod developed macular edema, with a higher risk of 20% among patients with diabetes or uveitis (Cohen *et al.*, 2010). Therefore, a dilated fundus examination at baseline for diabetics and patients with a history of uveitis is recommended, and a 3- to 4-month follow-up macular OCT for MS patients undergoing fingolimod treatment (Lee, 2011; Multiple sclerosis: Current Care Guidelines Abstract, 2019). Overall, fingolimod did not show an increase in cancer risk in Phase 3 clinical trials (0.9% increase in fingolimod treated groups and 2.4% in placebo) (Kappos *et al.*, 2014). The majority of reported cancers in FREEDOMS were skin cancers, including basal cell carcinoma, melanoma and Bowen´s disease. Also a few breast cancers (BC) were reported (Kappos *et al.*, 2010; Calabresi *et al.*, 2014; Guarnera, Bramanti and Mazzon, 2017). Fingolimod has demonstrated efficacy in multiple in vitro and in vivo cancer models, suggesting a potential therapeutic role in cancer patients. A possible anticancer mechanism is through the inhibition of sphingosine kinase 1, a proto-oncogene with in vitro and clinical cancer association, and anticancer properties may be attributable to actions on several other molecular targets (White *et al.*, 2016).

Second-generation sphingosine-1-phosphate receptor 1 modulators (siponimod, RCP-1063, ONO-4641, ponesimod) show promise in reducing some of the side effects associated with fingolimod (bradycardia, liver function abnormalities, and pulmonary fibrosis) (Cree, 2014).

2.1.4.1.2 Vitamin D

The role of vitamin D as a modulator of calcium and phosphorous and in bone formation and maintenance is well known, but vitamin D receptor (VDR) is also

expressed in multiple cells and most organs in the human body and is involved in immune modulation (Marino and Misra, 2019).

Vitamin D is a prohormone and fat-soluble group of secosteroids available through diet, dietary supplementation and by the synthesis of cholecalciferol in the skin from cholesterol through a chemical reaction dependent on ultraviolet B (UVB) sun radiation (Holick *et al.*, 1980; Calvo, Whiting and Barton, 2005; MacDonald, 2019). Vitamin D3 (cholecalciferol, obtained from sunlight and diet) and D2 (ergocalciferol, obtained from diet) are the most important vitamin D precursors for humans (Holick, 2006; F. Holick and Holick, 2010; Ross *et al.*, 2011). Vitamin D action is expressed by the active vitamin D metabolite calcitriol (1,25-dihydroxycholecalciferol). Calcitriol promotes increased intestinal absorption and regulation of serum calcium, magnesium and phosphate, and has a regulatory role in development and maintenance of bone health alongside with multiple other biological effects on cell growth and neuromuscular functions (Holick, 2004; Norman, 2008; Ross *et al.*, 2011). The observation of VDR presence in human leucocytes paved the way for later discoveries of vitamin D immunomodulatory effects on immune functions and reduction of inflammation (Provvedini *et al.*, 1983; Holick, 2004).

Vitamin D (represents D2 or D3, collectively known as calciferol) is biologically inactive and is converted into its active form by enzyme hydroxylation in the liver and kidneys. D3 is converted in the liver to calcifediol (25-hydroxycholecalciferol) and D2 to 25-hydroxyergocalciferol. These two vitamin D metabolites are called 25-hydroxyvitamin D or 25(OH)D, which is the main circulating metabolite of vitamin D and measured from serum to determine a person´s vitamin D status (Hollis, 1996; DeLuca, 2004; Herrmann *et al.*, 2017). Calcifediol is hydroxylated by the kidneys to calcitriol, which is the biologically active form of vitamin D. Of the human requirement for vitamin D, 80-90% is provided by production in the skin induced by UVB action (Holick *et al.*, 2011; Wacker and Holick, 2013). Food provides only about 100 IU of vitamin D daily, and if fortified approximately 300-400 IU per day (Pierrot-Deseilligny and Souberbielle, 2017). Recommendations on target 25(OH)D serum levels vary across authorities (US labs generally report levels in ng/mL, other countries in nmol/L. 1 ng/mL equals ~2.5 nmol/L). The toxic levels of vitamin D, potentially generating hypercalcemia, appear to be located largely above serum levels of 375 nmol/L, corresponding to a daily intake much higher than 10,000 IU (Hathcock *et al.*, 2007). A review from 2014 concluded that the most beneficial serum levels of 25(OH)D for all outcomes is approximately 75 nmol/mL, although there is controversy due to differences between ethnic groups (Bischoff-Ferrari, 2014).

2.1.4.1.3 Role of vitamin D supplementation in MS

Vitamin D has been promoted for use in several non-skeletal diseases (including MS, cancer, type 1 diabetes) because of its immunomodulatory, anti-inflammatory, antioxidant and anti-fibrotic actions (Jamka *et al.*, 2015; Murdaca *et al.*, 2019). In the majority of MS patients, vitamin D deficiency is abundant at the time of diagnosis and can safely be targeted to a level of 75-150 nmol/L through vitamin D supplementation in doses of 50-100 ug daily (conversion 1 ug = 40 international units, IU) (Soilu-Hänninen *et al.*, 2008; Åivo, Lindsröm and Soilu-Hänninen, 2012; Pierrot Deseilligny and Souberbielle, 2013; Hoel *et al.*, 2016; Pierrot-Deseilligny and Souberbielle, 2017).

MS patients have lower vitamin D levels during relapse than remission (Soilu-Hänninen *et al.*, 2005, 2008; Correale, Ysrraelit and Gaitn, 2009), and an inverse association between relapse rate and 25(OH)D levels has been observed in MS patients with POMS and adult-onset disease (Mowry *et al.*, 2010; Simpson *et al.*, 2010; Runia *et al.*, 2012). Vitamin D association with MS progression is less clear, but low levels at the start of RRMS have been associated with earlier SPMS conversion in a retrospective longitudinal 3-year follow-up study, indicating an association between vitamin D deficiency and early SPMS conversion (Muris *et al.*, 2016). Higher vitamin D levels in early MS disease course have been shown to predict reduced disease activity, MRI brain atrophy and lesion load as well as reduced clinical disease progression (Ascherio *et al.*, 2014). Serum 25(OH)D levels inversely correlate with MRI activity demonstrated by Gd-enhancing lesions and new T2 lesions (Løken-Amsrud *et al.*, 2012; Mowry *et al.*, 2012).

However, accumulated data on vitamin D supplementation effect in MS treatment has been inconsistent (James *et al.*, 2013; Jagannath *et al.*, 2018; McLaughlin *et al.*, 2018; Zheng *et al.*, 2018). A systematic review and meta-analysis of 5 randomized clinical trials (RCTs), showed that vitamin D supplementation was not beneficial in controlling MS relapses (OR 0.98, 95% CI 0.45–2.16) (James *et al.,* 2013), nor did D_3 appear to have therapeutic effect on EDSS score (mean difference – 0 .01, 95% CI -0.34–0.33) or ARR (mean difference 0.05, 95% CI 0.01–0.1) in a meta-analysis evaluating these clinical outcome measures in RCTs performed prior to 2017 (Zheng *et al.,* 2018).

However, these previous systematic reviews on vitamin D for the clinical efficiency of MS have had some limitations that were addressed in a recent systematic review of RCTs which found disease outcome improvement (EDSS, ARR, serum 25(OH)D levels, quality of life, cytokine profile, mobility, MRI T2 lesion load and new T2 or Gd-enhancing T1 lesions, safety and AEs) in 3 out of 10 trials, pointing to a conclusion that vitamin D supplementation benefit may be most apparent in MS subjects with lower baseline vitamin D levels (Berezowska, Coe and Dawes, 2019). Also, another meta-analysis from 2018 concluded that vitamin D

supplementation might have a therapeutic role in MS, whereas there is uncertainty with regard to the appropriate dose (McLaughlin *et al.,* 2018), warranting future well-performed dose-ranging, placebo-controlled RCTs. If successful, trials will help answer the questions of when, how much, and what type of vitamin D the patients with MS should be supplemented with, as well as what level of serum vitamin D should be targeted for optimal benefit (Cree, 2014). Since September 2017, there is also an ongoing comprehensive systematic review and meta-analysis of RCTs on vitamin D supplementation in MS, aimed at assessing the effectiveness of vitamin D supplementation on clinical and para-clinical outcomes (ARRs, EDSS, Gd-enhancing lesions of MRI and cytokine levels of tumor necrosis factor and IL as the main outcomes) in patients with MS (Ghajarzadeh *et al.*, 2019).

Serum vitamin D levels may be a predictor of improvements in disease pathology from vitamin D supplementation, suggesting 25(OH)D levels in serum and initial calcemia should be screened for before initiation of supplementation and followed up six months after initiation and then annually to check for compliance. Until dose-ranging, placebo-controlled RCTs give conclusive answers, levels above the estimated beneficial plateau (100-150 nmol/L) should not be exceeded due to lack of beneficial evidence and an increased risk of AEs (Berezowska *et al.,* 2019; Pierrot-Deseilligny and Souberbielle, 2017).

In a randomized placebo-controlled trial in Finnish MS patients, cholecalciferol supplementation at a dose of 20,000 IU/week lead to serum 25(OH)D levels of approximately 100 nmol/L and significantly less Gd-enhancing T1 lesions with good safety and no treatment-related AEs (Soilu-Hänninen *et al.,* 2012). Vitamin D supplementation is already implemented in the national treatment guidelines for MS patients in Finland, and recommended at a daily oral dose of 50-100 ug to reach serum 25(OH)D levels of ≥100 nmol/L in particular among MS patients with low vitamin D levels (Multiple Sclerosis: Current Care Guidelines Abstract, 2019). Supplementation may have beneficial effects on the early inflammatory component of MS disease and should be continuous in temperate zones since vitamin D storage is short-lasting (up to 6 weeks) in the organism and risk of vitamin D deficiency is continuous at latitudes with low annual UVB radiation (Pierrot-Deseilligny and Souberbielle 2017; Berezowska *et al.,* 2019).

2.1.5 Pathogenesis of MS

2.1.5.1 Role of inflammation and neurodegeneration in MS

MS is a chronic autoimmune neurological disease associated with CNS inflammation and neurodegeneration mediated by the adaptive and innate arms of an unregulated immune response (Compston and Coles, 2008; Hollenbach and

Oksenberg, 2015; Murdaca *et al.*, 2019). Clinical disease expression is heterogenous, varying from mild "benign" MS to rapidly disabling disease. The primary trigger of the upregulated immune response in MS remains unknown, but in the early inflammatory cascade, a response is triggered against myelin antigens, such as myelin basic protein, proteolipid protein, myelin-associated glycoprotein, MOG, and gangliosides. In early disease, the myelin surrounding the nerve cell axons is affected and injured, causing various clinical symptoms and pathological white matter lesions visual to the eye (widespread scars) (Waxman, 1982; Compston and Coles, 2008; Howard, Trevick and Younger, 2016). The myelin sheaths surrounding the axons are built by oligodendrocytes, supportive cells under a family of glial cells, of which astrocytes are the most common. In CNS damage, astrocytes increase in number causing the visible lesions in imaging. MS pathology is characterized by focal inflammatory infiltrates, breakdown of myelin sheaths, microglia activation, proliferation of astrocytes, gliosis, as well as various grades of axonal degeneration associated with oxidative stress and mitochondrial injury (Lassmann, 2014; Simons, Misgeld and Kerschensteiner, 2014). Demyelinated lesions are disseminated in the CNS and MS involves both white and gray matter (Geurts *et al.*, 2012; Gh Popescu and Lucchinetti, 2012; Hollenbach and Oksenberg, 2015; Howard, Trevick and Younger, 2016). Circulating autoimmune T-cells are triggered to activation by unknown target antigens, possibly as a result of cross-reactivity, and adhere to blood vessel walls and migrate across the BBB into the CNS, where they recognize cells presenting myelin fragments on the human leukocyte antigen (HLA) complex, to which the T-cells connect and cause further inflammation through the release of signal substances (cytokines) that increase the inflammation and attract macrophages (Kawanokuchi *et al.*, 2008; Stinissen and Hellings, 2008; Bartholomäus *et al.*, 2009; Hemmer, Kerschensteiner and Korn, 2015)(Sospedra and Martin, 2016). CD4+ Th1 cells were considered to be the main effectors, but more recently Th17 cells were identified as the primary effectors (Langrish *et al.*, 2005; Korn *et al.*, 2009). Suppressor T-cells (T regulatory cells, Tregs) have an antagonistic role in this cascade of events, but their function is impaired in MS, contributing to the disease mechanisms (Viglietta *et al.*, 2004; Workman *et al.*, 2009). B-cells produce auto-antibodies against myelin and bind to the myelin, as do blood proteins of the complement system, facilitating the damage caused by tissue-consuming macrophages that release myelin degrading enzymes (Cepok *et al.*, 2005). B cells, plasma blasts and plasma cells, which produce immunoglobulins (Ig) detected as OCBs in the CSF, are adjacent in meninges, plaques and CSF of most MS patients (Esiri, 1980; Henderson *et al.*, 2009).

Histologically, CNS lesions are classified as acute, chronically active and inactive, with the acute lesions demonstrating marked perivascular inflammatory cell infiltrates, composed mainly of T cells, macrophages, mononuclear cells, B- and

plasma cells. Demyelination eventually ensues, with phagocytosis of myelin debris by microglial cells and macrophages. The myelin-producing oligodendrocytes are destroyed by inflammatory infiltration and gliosis, resulting in demyelination-associated reduced nerve conduction, conduction block and various clinical symptoms such as numbness, pain, burning, itching, motoric deficits, ON and balance disturbances. Cognitive symptoms include memory disturbances, decreased judgement, fatigue and inattention. Remyelination is activated by oligodendrocyte progenitor cells. Axonal injury is associated with the inflammation in active MS lesions, whereas the axonal loss found on histological examination is likely responsible for the chronic and progressive symptoms in MS. Later in the disease course, clinically silent acute lesions may contribute to axonal injury (Howard, Trevick and Younger, 2016).

2.1.5.2 Genetic risk factors

Clustering of MS-affected individuals in families and a high concordance rate in monozygotic twins (MZ) pose some evidence of a genetic component to the disease. MZ twins run a ~17% risk of developing MS disease, compared to 2% for dizygotic (DZ) twins and siblings in general, as well as for offspring of a parent with MS, whereas the risk is about 0.2% for the general population in northern Europe (Westerlind *et al.*, 2014, 2015). In a Finnish MS twin study from 2008, the concordance rate among twins had increased over 20 years, suggesting that the reported increase in MS incidence in Finland is predominantly caused by environmental factors. The pairwise concordance was 30% for MZ twins and 14.3% for DZ twins, and the corresponding proband-wise concordance rates were 46.2% and 25%. The genetic variance (heritability) was 15%, the common environmental variance 73.3% and the unique environmental variance 11.1% (Kuusisto *et al.*, 2008). There is broad consensus that MS disease is multifactorial and that the MS-prone genotype results from various independent and interacting polymorphic genes with risk alleles common in the population, each exerting a small impact on the overall risk in interaction with additional risk factors at an individual level (Anna Karin Hedström, Sundqvist, *et al.*, 2011; Hedström, Bomfim, *et al.*, 2014; Hollenbach and Oksenberg, 2015; Baranzini and Oksenberg, 2017; Hedström, 2019).

The association between specific HLA variants within the major histocompatibility complex (MHC) gene complex (chromosome 6p21) and MS was first established in the 1970s (Compston and Coles, 2008). Genes encoding antigen-presenting molecules within the MHC region seem to account for the majority of the genetic MS risk (Hollenbach and Oksenberg. 2015; Baranzini and Oksenberg, 2017). HLA genes are recognized to have a critical role in histocompatibility and transplant

outcome and are associated with many infectious, inflammatory, autoimmune and pharmacological disease phenotypes and cancers.

HLA-class I molecules HLA-A, -B, and –C primarily bind and present peptides from endogenous synthesized proteins (such as viral and tumor peptides) to CD8+ T-cells and serve as ligands for the killer immunoglobulin-like receptors on the surface of natural killer (NK) cells. The class II molecules, HLA-DR, -DQ, and -DP, are associated as heterodimers on the cell surface of antigen-presenting cells such as B cells, dendritic cells (DC) and macrophages, and serve as receptors for processed peptides derived from membrane and extracellular proteins (bacterial peptides) presented primarily to CD4+ T-lymphocytes (Sollid, Pos and Wucherpfennig, 2014; Hollenbach and Oksenberg, 2015).

HLA association with both RRMS and PPMS risk has been observed across all populations studied. The HLA-DRB1*15 allele contributes to increased risk of MS with an OR of approximately 3 in most populations, and accounts for approximately 11% of MS heritability. This gene has a vitamin D-responsive element (VDRE) zone in the promoter region, strongly suggesting an involvement in mechanisms linked to vitamin D (S. V Ramagopalan *et al.*, 2009; Laursen *et al.*, 2015). Other HLA-DRB1 alleles have also been independently associated with increased MS risk (Hollenbach and Oksenberg, 2015; Hedström, 2019)(Seboun *et al.*, 1999).

Genome-wide association studies (GWAS) and hypothesis-driven studies with extensive complementary genome coverage of selected regions have resulted in over 100 identified MS risk variants at 103 loci outside of the MHC (Beecham *et al.*, 2013; Baranzini and Oksenberg, 2017). A majority of these loci encode immune-response proteins involved in the main stages of MS pathogenesis (Bashinskaya *et al.*, 2015; Harirchian *et al.*, 2018)(Sawcer, Franklin and Ban, 2014). Despite a large number of gene loci associated with MS risk, they account for only half of the disease heritability (Patsopoulos, 2018).

The HLA-A*02:01 allele exerts a protective effect on MS risk (OR 0.7) (Brynedal *et al.*, 2007; Bergamaschi *et al.*, 2010; Sawcer, Franklin and Ban, 2014; Hedström, 2019).

2.1.5.3 Environmental risk factors

Findings from studies of genetic impact on MS disease strongly argue for environmental exposures playing an essential role in determining disease risk, and these are important to define since they can be modified and MS occurrence potentially preventable (Faridar *et al.*, 2012; Eskandari *et al.*, 2015; Mokry *et al.*, 2016; Correale, Farez and Gaitán, 2017; Alfredsson and Olsson, 2019; Simpson *et al.*, 2019).

Table 4. Established and possible lifestyle and environmental risk factors for MS

Factor	OR	HLA gene interaction	Combined OR (nongenetic factor + HLA allele)	Effect during adolescence	Immune system implied	Level of evidence
Smoking	~1.6	Yes	14	No	Yes	+++
EBV infection (seropositivity)	~3.6	Yes	~15	Yes	Yes	+++
Vitamin D level <50 nM	~1.4	No	NA	Probably	Yes	+++
Adolescent obesity (BMI >27 at age 20 years)	~2	Yes	~15	Yes	Yes	+++
CMV infection (seropositivity)	0.7	No	NA	Unknown	Yes	++
Night work	~1.7	No	NA	Yes	Yes	++
Low sun exposure	~2	No	NA	Probably	Yes	++
Infectious mononucleosis	~2	Yes	7	Yes	Yes	++
Passive smoking	~1.3	Yes	6	No	Yes	+
Organic solvent exposure	~1.5	Unknown	Unknown	Unknown	Unknown	+
Oral tobacco/nicotine	0.5	No	NA	Unknown	Yes	+
Alcohol	~0.6	No	NA	Unknown	Yes	+
Coffee	~0.7	No	NA	Unknown	Yes	+

The level of evidence for the role of a particular lifestyle or environmental factors in MS is not easy to define. Large prospective studies are, with a few exceptions, rare in MS. CMV, cytomegalovirus; EBV, Epstein-Barr virus; HLA, human leukocyte antigen; MS, multiple sclerosis; NA, not applicable; OR, odds ratio; +++, high level of evidence: drawn from large prospective studies or if case-control observation is supported by Mendelian randomization studies; ++, Case-control observations, if replicated and/or supported by independent methods; +, Non-replicated observations (included to enable further observations). Table reprinted with permission (Olsson, Barcellos and Alfredsson, 2016).

Genetic predisposition and female gender only explain a fraction of MS risk increase, and there is strong evidence for associations with environmental influence, such as EBV infection, exposure to tobacco smoke, limited sun exposure/low vitamin D, high latitude gradient and adolescent obesity (Munger, Chitnis and Ascherio, 2009; Simpson *et al.*, 2011, 2019; Ascherio, Munger and Lünemann, 2012; Munger *et al.*, 2013; Lucas *et al.*, 2015). The changes in risk that occur with migration cannot be explained by genetics but rather support the impact role of environmental factors (Isobe *et al.*, 2013; Ascherio and Munger, 2016). Accumulated evidence suggests that the risk of developing MS is determined by a combination of genetic and environmental factors (Olsson et al. 2017). The environmental risk factors for MS can influence both adaptive and innate immunity. Certain risk factors, including EBV infection, smoking and obesity, interact with HLA MS risk genes and argue for shared pathogenic pathways involving adaptive immunity (Olsson, Barcellos and Alfredsson, 2016).

2.1.5.3.1 Epstein-Barr virus and risk of MS

EBV or human herpesvirus 4 (HHV-4) is a γ-herpesvirus that infects up to 95% of the adult population (Saha and Robertson, 2019). Primary infection is orally transmitted and occurs in oropharyngeal epithelial cells. In early childhood, the primary infection is usually benign but can cause the syndrome of infectious mononucleosis (IM) if attracted in adolescence or early adulthood. EBV mainly infects B cells and epithelial cells of the oral mucosa, as well as T or NK lineage cells, and following resolution of the primary infection, the virus persists in circulating memory B cells (Lindsey, 2012). The EBV life cycle in humans can be divided into a lytic replication phase in epithelial cells and a lifelong latent phase, periodically reactivating (Murata and Tsurumi, 2014). Antibodies to some EBV antigens persistently remain detectable (indicating recurrent antigenic stimulation). In the vast majority of non-immunocompromised individuals, the virus causes no apparent disease, and is well adapted to co-existence with its human host. In immunosuppressed hosts, however, EBV can cause lymphoproliferative disorders and the virus has been associated with some neoplasms (various lymphomas, nasopharyngeal and gastric carcinomas), as well as some autoimmune diseases (Lindsey, 2012).

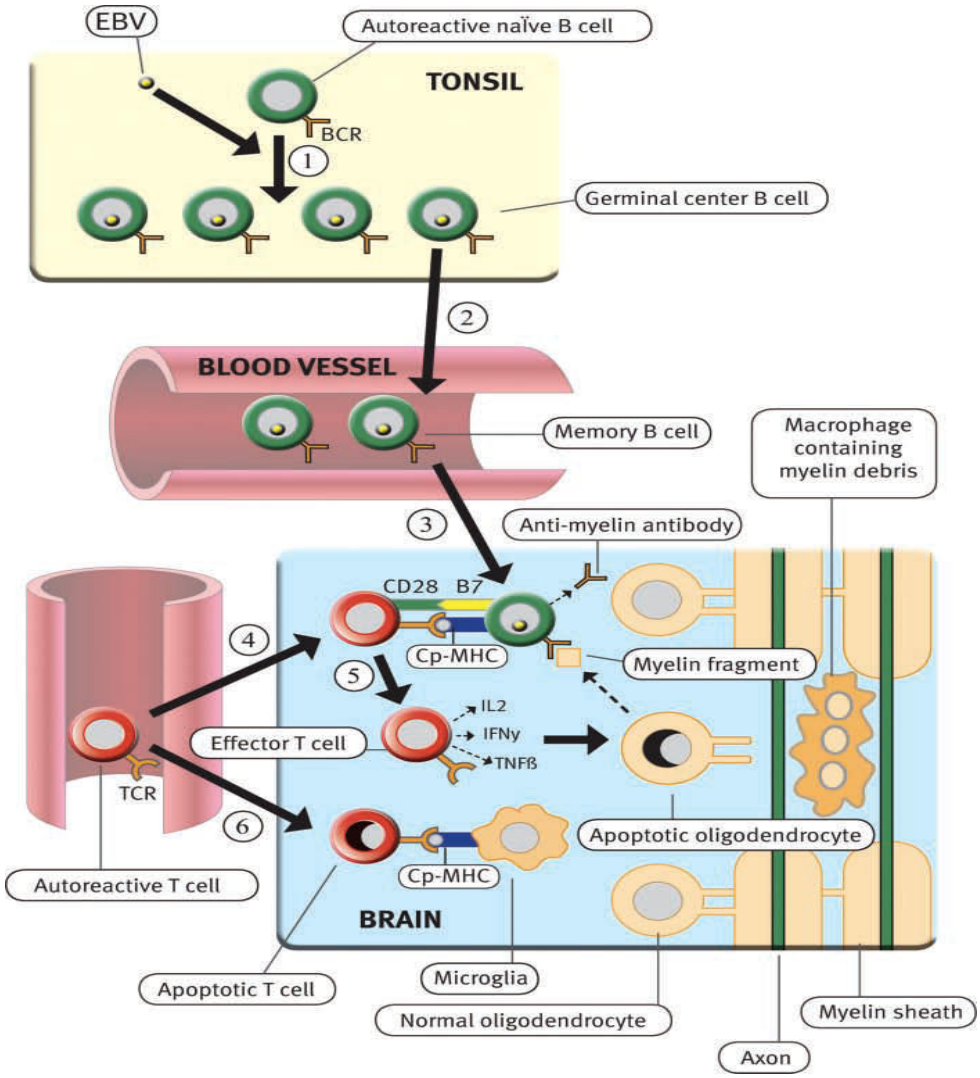
The association between EBV and MS was suggested for the first time almost five decades ago (Tourtellotte, 1971), and has been substantiated in numerous subsequent studies. EBV remains the most pertinent of the many infectious agents that have been suggested to play a role in MS development (Ascherio and Munger, 2007), and although causality is debated, collective circumstantial evidence is compelling (Ascherio and Munger, 2010; Alfredsson and Olsson, 2019). More than 99% of MS patients are seropositive for EBV, and the odds ratio (OR) for MS is 0.00 in seronegative individuals (Pakpoor, Disanto, *et al.*, 2013). EBV has been identified as a risk factor for MS in Caucasian, Afro-American and Latin American populations (Langer-Gould *et al.*, 2017).

In MS studies, EBV antibodies (Abs) of interest include those to the restricted and diffuse forms of early antigen (EA-R, EA-D), viral capsid antigen (VCA) and EBV nuclear antigen (EBNA, including six proteins EBNA-1, EBNA-2, etc.). Ab titers can be detected with various methods (e.g. immunofluorescence, IF; enzyme-linked immunosorbent assays, ELISA) (Henle and Henle, 1966; Reedman and Klein, 1973; Lindsey, 2012). Abs to VCA appear soon after primary infection (IgM) and later remain detectable (IgG) during latency, whereas Abs to EA are usually undetectable after the acute infection has resolved but may reappear during viral reactivation. EBNA Abs appear during resolution of primary infection and remain detectable during latency. Almost all EBV studies on MS risk association have measured mainly serum IgG Abs (as compared to IgM or IgA, and CSF Abs) (Lindsey, 2012).

Primary EBV infection is a strong risk factor for MS, and almost all case-control studies comparing the prevalence of EBV infection or EBV Ab titers in MS with controls have found a significant increase in the MS group. IgG Abs to EBNA and VCA are both increased, with higher significance for EBNA if tested in the same study (Lindsey, 2012). EBNA-1 and VCA Abs, at various degrees in separate studies, are increased also among CIS subjects (Lünemann *et al.*, 2010; Lucas, A.-L. Ponsonby, *et al.*, 2011). A previously conducted prospective nested case-control study showed that all primarily EBNA1-seronegative subjects had converted before MS onset (Levin *et al.*, 2010), suggesting EBV infection may be a prerequisite for development of adulthood MS. Increasing IgG Ab titers against EBNA have been associated with an increased MS risk in healthy adults (Ascherio and Munger, 2016). EBNA1 Ab titers positively correlate with MS occurrence and higher titers are found among MS subjects compared to controls (Sundström *et al.*, 2009; Sundqvist *et al.*, 2012; Zhou *et al.*, 2016), which can be interpreted as evidence for causality of EBV in MS. EBV infection during adolescence or later is associated with an increased risk for developing MS, whereas this does not seem to be the case for infection during childhood (Ascherio and Munger, 2015). Individuals who have incurred clinically overt IM have a >2- fold risk to develop MS (S. V. Ramagopalan *et al.*, 2009; Handel *et al.*, 2010). These findings suggest that there is a specific window of time for when EBV infection implies a higher risk for MS. A recent study on whether remote infection with EBV and other common herpesviruses affect susceptibility of pediatric MS strongly linked EBV-VCA seropositivity to associate with a 7.4-fold increased risk (Nourbakhsh *et al.*, 2018). Several studies suggest that prevalence of EBV infection is also increased in children with MS (Banwell *et al.*, 2007), although unlike the adult MS population, a significant minority of children are not EBV-infected. To test for the causality of EBV in MS, Abs should be tested years before disease onset. Previously conducted prospective studies have found that EBNA and EBNA-1 Abs were most predictive of MS risk in adults (Sundström *et al.*, 2004; Munger *et al.*, 2011). In studies on EBV correlation with disease activity, there is evidence suggestive of increased anti EA-Ab association with more active MS disease (Wandinger *et al.*, 2000; Buljevac *et al.*, 2005).

HLA risk genes and IM seem to interact synergistically in increasing the risk of MS. An interaction between anti-EBV titers and these genes has been observed leading to a 16-fold higher risk of MS associated with abundance of HLA-DRB1*15:01 and absence of HLA-A*02 in combination with high EBNA 385-420 IgG titers compared to individuals who did not carry any of these factors (Sundqvist *et al.*, 2012). An interaction between obesity and a history of IM during adolescence has been associated with an increased MS risk with an OR of approximately 14. This finding suggests that the HLA risk alleles that encode molecules regulating T cell adaptive immunity might show common pathogenic pathways triggering MS when

they interact with measures of EBV infection (Hedström *et al.*, 2015). One study that has not been replicated with similar findings detected EBV-related DNA in CNS lymphoid infiltrates (Serafini *et al.*, 2007). Anti-CD20 treatments that deplete B cells, in which EBV resides latent, are effective in MS (Hauser et al 2008), providing some support for the EBV hypothesis. Of many suggested theories, one possible explanation of the mechanism by which EBV might trigger MS is molecular mimicry, by sharing antigen epitopes with a brain antigen and thus, through cross-reactivity, provoking CNS autoimmunity associated with EBV reactivation (Esposito *et al.*, 1999; Gabibov *et al.*, 2011).



2.1.5.3.2 Smoking and risk of MS

Already a decade ago, at least 8 epidemiologic studies investigating smoking as an environmental risk factor for MS had found support for the supposition (Antonovsky *et al.*, 1965; Thorogood and Hannaford, 1998; Ghadirian *et al.*, 2001; Hernán, Olek and Ascherio, 2001; Zorzon *et al.*, 2003; Hernán *et al.*, 2005; Pekmezovic *et al.*, 2006). Since then, epidemiological evidence for an association between smoking and MS risk has rapidly increased (Hawkes, 2007; Hedström *et al.*, 2009; Handel and Ramagopalan, 2011; Handel *et al.*, 2011; Wingerchuk, 2012; Salzer *et al.*, 2013; O'Gorman and Broadley, 2014; Zhang *et al.*, 2016; Degelman and Herman, 2017; Poorolajal *et al.*, 2017; Hedström, 2019). Smoking also contributes to worse outcome in MS, earlier conversion from CIS to definite MS and from RRMS to SPMS and accelerates disease progression (Sundström and Nyström, 2008; Healy *et al.*, 2009; Correale and Farez, 2015; Ramanujam *et al.*, 2015)(Manouchehrinia *et al.*, 2013).

Prior study observations suggest that smoking is an independent risk factor for MS (Hedström *et al.*, 2009), and this risk may be modified by interaction with other genetic and environmental risk factors, whereas the role of smoking in MS progression is more controversial (Arruti *et al.*, 2015). Adult tobacco smokers of both sexes run an increased risk of developing MS compared to never-smokers (OR 1.5, 95% CI 1.3–1.8) and there is a dose-response correlation between MS risk and cumulative dose of smoking (OR 1.8 and 1.4 for female and male subjects, respectively), which remain up to 5 years after smoking cessation (Hedström *et al.*, 2009). Serum levels of cotinine, the metabolite of nicotine used as an objective marker for cigarette smoking, have also been associated with a 1.5 OR increase in

◄ **Figure 1.** Proposed role of EBV infection in the development of MS. EBV infects autoreactive naïve B cells in the tonsil, driving them to enter germinal centers where they proliferate intensely and differentiate into latently infected autoreactive memory B cells (Step 1), which then exit from the tonsil and circulate in the blood (Step 2). The number of EBV-infected B cells is normally controlled by EBV-specific cytotoxic CD8+ T cells, which kill proliferating and lytically infected B cells, but not if there is a defect in this defense mechanism. Surviving EBV-infected autoreactive memory B cells enter the CNS where they take up residence and produce oligoclonal IgG and pathogenic autoantibodies, which attack myelin and other components of the CNS (Step 3). Autoreactive T cells that have been activated in peripheral lymphoid organs by common systemic infections circulate in the blood and enter the CNS where they are reactivated by EBV-infected autoreactive B cells presenting CNS peptides (Cp) bound to major histocompatibility complex (MHC) molecules (Step 4). These EBV-infected B cells provide costimulatory survival signals (B7) to the CD28 receptor on the autoreactive T cells and thereby inhibit the activation induced T cell apoptosis, which normally occurs when autoreactive T cells enter the CNS and interact with non-professional antigen-presenting cells such as astrocytes and microglia, which do not express B7 costimulatory molecules132–134 (Step 6). After the autoreactive T cells have been reactivated by EBV-infected autoreactive B cells, they produce cytokines such as interleukin-2 (IL2), interferon-γ (IFNγ) and tumor necrosis factor (TNFβ), and orchestrate an autoimmune attack on the CNS with resultant oligodendrocyte and myelin destruction (Step 5). Reproduced (Pender and Burrows, 2014) with permission (Pender, 2011).

MS risk (Salzer *et al.*, 2013). Use of snuff over 15 years, on the other hand, decreases MS risk (OR 0.3, 95% CI 0.1–0.8) (Hedström *et al.*, 2009). Simultaneous use of snuff and smoked tobacco exert antagonistic effects on risk of developing MS, suggesting that nicotine is an unlikely compound to account for the association between smoking and increased MS risk (Hedström *et al.*, 2013). There is some evidence supporting a possible neuroprotective feature of nicotine in several conditions and possibly in MS (De Jonge and Ulloa, 2007; de Saussure *et al.*, 2007; Picciotto and Zoli, 2008). Nicotine may exert systemic effects on the immune system through inhibition of the production of proinflammatory cytokines from immune cells, such as macrophages, via the α7-subunit of the acetylcholine nicotinic receptor (De Jonge and Ulloa, 2007; Tracey, 2007). However, clinical evidence of beneficial nicotine use is still missing (Kaakkola and Tuominen, 2013).

Exposure to environmental tobacco smoking (secondhand smoking, SHS) is associated with increased risk for MS in non-smokers (A. K. Hedström *et al.*, 2011) and parental smoking has been associated with risk of MS in offspring (Mikaeloff *et al.*, 2007), whereas no risk increase in early onset MS among offspring exposed to parental smoking during pregnancy has been demonstrated (Montgomery *et al.*, 2008). The pathogenesis and biological mechanism linking tobacco and MS risk is unknown. A variety of mechanisms have been suggested, such as the potential of nicotine to increase microvascular bloodflow and to cause leakage in the BBB, which is a suggested initiating event of MS (McDonald, 1994). Another explanation is immunotoxicity by impairment of antigen-mediated signaling in T cells (Hans *et al.*, 1993; Sopori and Kozak, 1998; Kalra *et al.*, 2000) or an elevation in peripheral blood leucocyte counts and important inflammation markers (such as C-reactive protein and IL-6). Impairment of humoral- and cell-mediated immunity and abnormalities in T cell function have been observed in smokers (Hughes *et al.*, 1985; Petitti and Kipp, 1986; Moszczyński *et al.*, 2001; Bermudez *et al.*, 2002).

Smoking increases the risk of MS by approximately 50%, with considerable variations in different contexts, populations and subjects. A significant interaction between smoking and two genetic risk factors, carriage of HLA DRB1*15 and absence of HLA A*02, has been described, suggesting that the risk of MS disease associated with HLA genotypes may be strongly influenced by smoking (Anna Karin Hedström, Sundqvist, *et al.*, 2011). The strongest genetic associations with MS are located within the HLA complex and having the primary risk allele class II HLA-DRB*1501 increases the MS risk 3-fold in the Scandinavian populations. Protective genes include class I allele HLA-A*02 (OR 0.7). Having HLA-DRB*15:01 and lacking HLA-A02* results in a combined OR of 5 among non-smokers and an OR of 14 for smokers. The genetic component has been estimated at 50% of the contribution to MS (Hedström, 2019).

Mechanisms linking smoking to MS risk are speculated to involve lung inflammation with a proinflammatory profile, as the interaction with MS risk HLA genes argues for an action on adaptive immunity, possibly by activation of auto-aggressive cells resident in the lungs subsequently attacking the CNS (Hedström, 2019). Possible interactions between lung irritative agents and HLA genes with regard to MS risk have been described to be consistent with class II allele-specific recognition of autoantigenic peptides in the lungs, resulting in organ-specific inflammatory disease. For MS, absence of HLA-A*0201 may result in autoreactive T cells persisting and launching an immune response against the self-antigen. Epidemiologic and experimental studies support the hypothesis that different sources of lung irritation may contribute to induce an immune reaction against modified self-proteins or against potentially auto-aggressive cells resident in the lungs, and promote MS development in genetically susceptible subjects (Doyle and Mamula, 2002; Cloos and Christgau, 2004; Makrygiannakis *et al.*, 2008; Odoardi *et al.*, 2012; Hedström *et al.*, 2018).

2.1.5.3.3 Vitamin D and risk of MS

The first study to link vitamin D status with MS risk was a prospective study among nurses in US, which found that higher dietary intake of vitamin D was associated with lower MS risk (Munger *et al.*, 2004). Another study performed in US women found that gestational dietary vitamin D intake was associated with a decreased MS risk in the offspring (Mirzaei *et al.*, 2011). Supplementation during pregnancy, early childhood and adolescence can lower the risk of MS disease in the offspring (Chaudhuri, 2005), although study observations on vitamin D benefit during pregnancy as MS protective for the offspring have been inconsistent (Salzer *et al.*, 2012; Ueda *et al.*, 2014). The effect of maternal vitamin D deficiency during pregnancy and MS risk in offspring has not been studied previously.

Serum levels of 25(OH)D ≥100 nmol/L were associated with an approximately 60% decreased risk of developing MS in non-Hispanic Caucasian patients from a US war veterans' cohort (Munger *et al.*, 2006). In a Swedish study, women with serum levels of 25(OH)D ≥75 nmol/L from pregnancy gestational samples were associated with an odds ratio of 0.39 for developing MS (Salzer *et al.*, 2012). High sun exposure has been shown to be protective against MS and low sun exposure as an MS risk factor (Ascherio, Munger and Lünemann, 2012). A study from Tasmania from 2003, that used skin actinic damages as a measure UV radiation exposure, found that higher sun exposure during childhood and early adolescence was associated with a reduced risk of MS (Van Der Mei *et al.*, 2003). A significant association between infrequent summer outdoor activity and increased MS risk has been found in Norway and in Italy (Bjørnevik *et al.*, 2014). Another study performed in Australia showed that sun

exposure and vitamin D are independent risk factors for CNS demyelination (Lucas, A-L Ponsonby, *et al.*, 2011). Vitamin D production in the skin induced by UV radiation is the main source of vitamin D3 in humans (Holick *et al.*, 1980), but the independent effects on MS risk of both UV radiation and vitamin D deficiency have demonstrated how complexly UV radiation and vitamin D status interact with one another and the risk of developing MS (Ascherio and Munger, 2016).

Opinion about whether there is sufficient evidence to conclude that low sun exposure and vitamin D increase the risk of multiple sclerosis is divided. General public health advice to receive sufficient sun exposure to avoid vitamin D insufficiency (<50 nmol/L) should also ensure any benefits for multiple sclerosis, but must be tempered by the risk of skin cancers (Amato *et al.*, 2018).

2.1.5.3.4 Socioeconomic and lifestyle factors and risk of MS

Lifestyle and environmental factors may act years before the clinical onset of MS, and many individuals present with multiple CNS lesions on MRI at clinical onset of MS, suggesting a subclinical phase of several years (Marrie *et al.*, 2013) altered by predisposing risk factors years before MS onset. Most lifestyle and environmental factors appear to have the greatest impact during adolescence (Olsson, Barcellos and Alfredsson, 2016). Migration studies show that MS risk depends on the age of migration so that those migrating from a low-risk country to a high-risk country before adolescence show a MS risk much similar to that of the high-risk country (Gale and Martyn, 1995; Ahlgren *et al.*, 2010; Simpson *et al.*, 2011)(Berg-Hansen *et al.*, 2015). Whether this is related to changed environment or lifestyle changes is difficult to distinguish. The availability of functioning health care systems, neurologists, and diagnostic tools (e.g. MRI) that have improved in the last decades may add to the reported increase in worldwide MS prevalence and incidence (Howard, Trevick and Younger, 2016) but does not explain the rapid globally increased female predominance of RRMS. This observation may more likely be linked to risk factors affecting women (e.g. increased obesity, smoking, and reproductive behavior changes).

The first association between obesity and MS was found in a US study, where women with a body mass index (BMI) ≥30 kg/m^2 at age 18 had a 2.25-fold increased risk of developing MS compared to normal-range BMI subjects (18.5 to < 21 kg/m^2) after adjusting for age, latitude at age 15, ethnicity and smoking. There was no association between baseline BMI and MS risk (Munger, Chitnis and Ascherio, 2009). Confirming findings of a ~two-fold MS risk associated with various ages of adolescent obesity were found for both male and female subjects in studies on Swedish, Norwegian and Italian populations (Hedström, Olsson and Alfredsson, 2012; Wesnes *et al.*, 2015). Childhood obesity mainly among girls has also been associated with increased risk of POMS, CIS and adult-onset MS (Langer-Gould *et al.*, 2013; Munger

et al., 2013). Childhood obesity is strongly associated with adolescent and adulthood obesity (Deshmukh-Taskar *et al.*, 2006). Obesity interacts with genetic and environmental factors to increase the odds for MS, as shown in a study including data from case-control studies in the US and Sweden. A BMI of ≥27 kg/m^2 in young adulthood combined with HLA-DRB*15 risk alleles increased MS risk 7-fold compared to non-carriers of MS risk alleles with a BMI of <27. Similarly, for obese HLA-A*02 carriers, the risk for MS was significantly elevated (Hedström, Bomfim, *et al.*, 2014). A significant interaction between adolescent obesity and IM (EBV) associated with a 6-fold MS risk increase has also been reported, further increasing the obesity-associated MS risk (Hedström *et al.*, 2015). Biological mechanisms underlying the association between MS and obesity are unknown, but one plausible mechanism is linked to lower levels of circulating vitamin D among adults and children (Parikh *et al.*, 2004; Smotkin-Tangorra *et al.*, 2007). Smoking, EBV infection and obesity interact with HLA immune response risk genes such that MS risk increases, suggesting a pathogenic pathway of action involved in adaptive immunity, leading to autoimmune attack on the CNS (Olsson, Barcellos and Alfredsson, 2016).

Occupational exposures to organic solvents, including painting products and varnish, have been associated with MS proneness, increasing the risk (OR 1.5, 95% CI 1.2–1.8, P = 0.0004) among both never and ever smokers, and an increased risk for MS associated with carriage of HLA-DRB1*15 and absence of HLA-A*02 alleles (Hedström *et al.*, 2018).

In addition to ethnicity, socioeconomic status (SES) affects the risk of developing MS, with both high and low SES associated with MS risk in different studies. Those studies reporting an MS increase association with low SES came from more egalitarian countries, whereas association between increased MS risk and high SES were reported from more unequal countries (Goulden, Ibrahim and Wolfson, 2015). An adverse socioeconomic status in childhood and adulthood is associated with a proinflammatory phenotype and is therefore an important exposure to consider. In one Californian population-representative case-control study, the OR for MS, associated with the socioeconomic statuses of childhood and adulthood, were significantly higher for factors such as parents renting versus owning a home (at age 10 of the cohort child), lower versus higher educational level, low versus high life course and low versus high social mobility (Briggs *et al.*, 2014). In a Danish population-based case-control study evaluating socioeconomic and reproductive factors associated with MS risk (especially with the increased MS prevalence among women), found that having newborn children reduced MS risk in women but not in men, and childbirths reduced the risk of MS by about 46% during the following 5 years. Terminated pregnancies also seemed to have a protective effect on the risk of MS occurrence. Socioeconomic status, educational level and sanitary conditions in youth did not show an association with MS risk (Magyari, 2015).

In addition to EBV, many other viruses have been suggested to play a role in increasing MS risk, including two other herpes viruses, human herpes virus-6 (HHV-6, which infects T lymphocytes, neurons and oligodendroglia, among others) and varicella zoster virus (VZV). Some studies have shown that HHV-6 DNA and proteins can be detected in neurons and oligodendrocytes near MS plaques, virus reactivation in blood correlates with MS disease activity, and high antibody titers have been shown to predict an increased MS risk (Challoner *et al.*, 1995; Sundström *et al.*, 2004; Höllsberg *et al.*, 2005), whereas the evidence linking VZV and MS is even more controversial (Lindsey, 2012). On the other hand, human cytomegalovirus (CMV) may have a protective role in MS (Bray *et al.*, 1983).

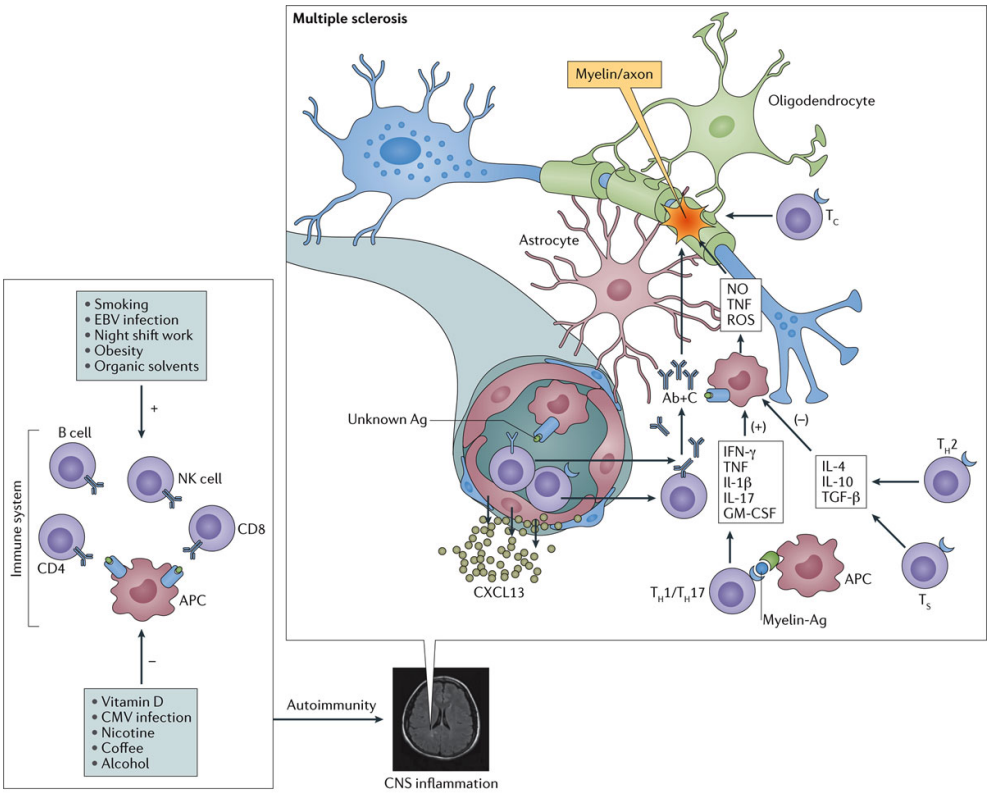


Figure 2. Lifestyle and environmental factors affect the immune system to trigger and/or perpetuate multiple sclerosis. Depicted here are lifestyle and environmental factors that can affect immune system function, which can lead to neuroinflammatory attack on the CNS and eventually lead to multiple sclerosis. APC, antigen-presenting cells; Ab, antibody; C, complement; CMV, cytomegalovirus; EBV, Epstein–Barr virus; GM-CSF, granulocyte monocyte colony stimulating factor; myelin-Ag, myelin antigens; NO, nitric oxide; ROS, reactive oxygen species; T_C, T cytotoxic cell; TGF-β, transforming growth factor β; T_H, T helper cell; TNF, tumor necrosis factor; T_S, T suppressor cell. Reprinted with permission (Olsson, Barcellos and Alfredsson, 2017).

Environmental and lifestyle factors associated with reduced MS risk include sufficient levels of vitamin D and sun exposure (Ascherio and Munger, 2016), serological evidence of CMV infection (Bray *et al.*, 1983; Sundqvist *et al.*, 2014), alcohol use (Hedström, Hillert, *et al.*, 2014), high coffee consumption (Hedström *et al.*, 2016) and nicotine exposure (snuff) (Alfredsson and Olsson, 2019). There is circumstantial evidence of organic solvent and night shift work association with increased MS risk (Hedström *et al.*, 2018). Shift work during adolescence may increase the MS risk 1.7-fold (Anna Karin Hedström, Akerstedt, *et al.*, 2011). Cesarean child delivery (Graves *et al.*, 2017) and breastfeeding have been associated with a reduced MS risk in offspring (Conradi *et al.*, 2013; Ragnedda *et al.*, 2015).

2.2 MS and cancer

2.2.1 Risk of cancer among MS patients

In the aging population with MS, comorbidities such as cancer play a growing role. In Finland, a total of 250,000 people had had cancer at some point in their lives in 2011 and there were 30,000 new diagnosed cancer cases. Cancer becomes more common with increasing age and the annual number of cancer deaths among Finns has remained stable around 11,700, with pulmonary cancer as the most common cause of cancer death. The most common cancer in women is BC (n = 4,900) and in men prostate cancer (n = 4,700) (Pukkala and Rautalahti, 2013; The Finnish Cancer Registry, 2019).

The immune system plays an important role both in MS and cancer, making it plausible that cancer risk is altered in MS. Studies of cancer risk in MS patients have, however, been inconsistent (Elaine Kingwell *et al.*, 2012; Tabarés-Seisdedos and Rubenstein, 2013; Capkun *et al.*, 2015; Marrie *et al.*, 2015; Roshanisefat *et al.*, 2015; Kyritsis, Boussios and Pavlidis, 2016; Thormann *et al.*, 2016). Most studies have suggested a reduced overall risk for cancer among MS patients and no effect of long-term exposure to the immunomodulatory (IM) DMTs GA and IFN. Some studies suggest a possible association of DMTs and increased BC risk (Achiron *et al.*, 2005; Kingwell *et al.*, 2014), and risk of BC seems to be elevated in the Danish MS population (Nielsen *et al.*, 2006). Several reports suggest an increase in cancer risk among MS patients treated with traditional IS therapies such as azathioprine, cyclophosphamide and MX (Achiron *et al.*, 2005; Lebrun *et al.*, 2008, 2011; Le Bouc *et al.*, 2012; Kingwell *et al.*, 2014; Ragonese *et al.*, 2017).

Because of their action on the immune system, and due to a lack of available long-term data, a special warning of the potential risk of cancer accompanies the use of recent IS such as cladribine, fingolimod, natalizumab, alemtuzumab, ocrelizumab, and possibly DMF and TRF (Bar-or, 2008; Tully, Barkley and Silber, 2015; Ajdacic-

Gross *et al.*, 2016; McGinley, Moss and Cohen, 2017; Montalban *et al.*, 2017; Sabol *et al.*, 2017; Clerico *et al.*, 2017; Coles *et al.*, 2017; Comi *et al.*, 2017; Giovannoni, 2017; Hauser *et al.*, 2017; Havrdova *et al.*, 2017; D'Amico *et al.*, 2018; Lebrun and Rocher, 2018).

Cancer risk among Finnish MS patients has previously been studied from an incidence cohort from 1964 to 1993 followed until year 1999. In a cohort of 1,597 MS patients, 85 cancers were identified. A small increased risk of hematological malignancies and CNS tumors was observed, but no association of overall cancer and MS (Sumelahti, Pukkala and Hakama, 2004). Cancer deaths have been overrepresented (35%) among MS patients in three different hospital districts, compared to the general population in a previous Finnish study (Sumelahti *et al.*, 2002). There are no previous studies assessing the risk of cancer among Finnish MS patients in the era of MS DMTs.

2.2.2 Smoking and the risk of cancer

Smoking is the most significant single environmental risk factor for cancer, and tobacco contains over 40 carcinogenic substances. Smoking is the cause of 40% of cancers in males and 9% in females. Approximately 90% of lung cancers are caused by smoking, and smokers run a 10- to 50-fold increased risk of developing lung cancer. Other cancers associated with smoking are oral and throat cancers, esophageal cancers, pancreas cancer, urinary bladder cancer, cervix cancer, gastric cancer, acute myeloid leukemia, mesothelioma and renal cancer, and smoking is linked to increased risk for liver, rectal and colon cancer. Smoking seems to increase the risk of aggressive prostate cancer, adrenal cancer, biliary cancer and thyroid cancer to some extent (Jyrkkiö, Boström and Minn, 2012). The association between smoking and BC has been less clear, but in recent well conducted cohort studies, the association has been found and is relatively consistent with a 5-32% higher risk for current smokers and a 5-18% higher risk for former smokers, compared to never smokers (Reynolds *et al.*, 2004; Gram *et al.*, 2005; Olson *et al.*, 2005; Cui, Miller and Rohan, 2006; Ha *et al.*, 2007; Xue *et al.*, 2011; Deroo, Cummings and Mueller, 2011; Luo *et al.*, 2011; Bjerkaas *et al.*, 2013; Rosenberg *et al.*, 2013; Gaudet *et al.*, 2013; Dossus *et al.*, 2014; Nyante *et al.*, 2014). Smoking has been modestly but significantly associated with BC particularly among women who initiated smoking at adolescent or peri-menarcheal ages in a UK study. The relative risk for BC associated with smoking was greater for patients with a family history of BC (Jones *et al.*, 2017). Smoking seems to be associated with BC also among Japanese and Americans (Nagata *et al.*, 2006; Gaudet *et al.*, 2013; Nyante *et al.*, 2014) and smoking for several years before first childbirth increases the risk of BC (Bjerkaas *et al.*, 2013).

The mechanisms underlying the development of tobacco-related cancers are only partly known. Tobacco products contain several carcinogens, of which the most well-known are nicotine derivatives N-nitrosamines, polycyclic aromatic hydrocarbon, aromatic amines, aldehydes, phenols, benzene, nitrometan, ethylenoxide ad polonium (Huang and Chen, 2011). The cancer-inducing mechanisms of tobacco smoke carcinogens are not mapped in detail, but the most well-known route of mechanism is mediated through cyclo-oxygenase (COX) and its derivates. Smoke carcinogens increase COX-2 expression and activity in many cells. The COX-2 derivates prostaglandine E2, thromboxane A2 and prosthacycline act in both cardiovascular and cancer pathogenesis. In many cancer cells, an over-expression of COX-2 has been described (Jyrkkiö, Boström and Minn, 2012).

2.2.3 EBV infection and the risk of cancer

EBV is ubiquitous and infects up to 95% of the worldwide population (Saha and Robertson, 2019). The virus remains latent in memory B cells and epithelial cells and is known to be oncogenic in immunocompromised conditions, and it may also cause severe acute diseases as a primary or reactivated infection. EBV can trigger malignancies of lymphoid and epithelial origin in humans, and is associated with B cell malignancies (Burkitt's lymphoma and other non-Hodgkin's lymphomas, Hodgkin's lymphoma, CNS lymphomas, post-transplant lymphoproliferative disorder, acquired immunodeficiency syndrome-associated lymphoma, NK lymphomas and T cell lymphomas, as well as non-keratinizing nasopharyngeal carcinomas and is sporadically linked to some gastrointestinal cancers). The oncogenicity of the virus is controlled in immunocompetent individuals but is associated in particular with malignancies reflecting primary infection sites, such as B cell lymphomas and nasopharyngeal carcinomas in immunosuppressed individuals (Andrei, Trompet and Snoeck, 2019). In vitro studies have elucidated the oncogenic potential and underlying mechanisms of EBV in B cell lymphomagenesis (Saha and Robertson, 2019). In an exploratory study from 2017, a non-random association of EBV and BC was seen and differed by age and race, supporting further studies with refined design and size (Glaser *et al.*, 2017).

Some EBV antivirals have been beneficial in vivo, but less successful in the clinic and to date no antiviral treatment for EBV has been approved. Interestingly, a recent study showed that leflunomide (a drug used to treat rheumatoid arthritis) and teriflunomide inhibit EBV-induced lymphoproliferative disease and lytic viral replication at a clinically relevant dose in vitro and may therefore be potentially useful in clinical prevention of EBV-induced lympho-proliferative disease in the near future (Bilger *et al.*, 2017). Novel molecules and strategies aimed at treating EBV-related diseases are under investigation (Andrei, Trompet and Snoeck, 2019).

2.2.4 Vitamin D and the risk of cancer

Vitamin D regulates cellular signaling networks and cascades of crucial roles in cancer biology and plays a role in processes regulating cell differentiation, proliferation, angiogenesis and apoptosis, a discovery that creates potential for several applications of vitamin D in cancer (AlMatar *et al.*, 2017; Skrajnowska and Bobrowska-Korczak, 2019). Higher levels of circulating 25(OH)D appear to be associated with reduced risk for and improved survivorship of certain malignancies (McNamara and Rosenberger, 2019). Accumulated data from clinical and preclinical investigations suggest that vitamin D deficiency may contribute to carcinogenesis risk, and that vitamin D supplementation could potentially contribute to reduction and progression of certain cancers, particularly when used in combination with existing therapies (AlMatar *et al.*, 2017). Vitamin D insufficiency has recently been associated with ovarian cancer (Toriola *et al.*, 2010). Laboratory and genetic studies demonstrate promising anticarcinogenic properties of vitamin D, whereas results of observational and human studies assessing vitamin D supplementation for the prevention of cancers have been inconsistent. Recently reported findings from major clinical trials have reported no cancer protection from vitamin D supplementation (Brown, 2019). A randomized, double-blind, placebo-controlled trial (the Vitamin D Assessment study, ViDA) evaluating the potential efficacy of monthly high-dose vitamin D supplementation in reducing the incidence and intermediate outcomes of several chronic and acute diseases showed no beneficial effect on cancers (Scragg, 2019). No official institutional guidelines recommend vitamin D supplementation for cancer prevention (McNamara and Rosenberger, 2019).

An UVB – vitamin D – cancer hypothesis proposing that reduced cancer incidence at lower geographical latitudes is related to high levels of vitamin D from UVB exposure is also controversial (Brown, 2019). There may be an association between BC risk, menopausal status and vitamin D levels according to a pooled analysis of two RCTs and one prospective cohort study that showed an 82% lower BC incidence rate with serum 25(OH)D levels above 150 nmol/L compared to <50 nmol/L (McDonnell *et al.*, 2018). Vitamin D receptor polymorphisms may affect the risk and mortality of BC, although the role of vitamin D receptors in cancer etiology is still equivocal (McNamara and Rosenberger, 2019), and claims of vitamin D health benefits are ahead of the evidence.

3 Aims

Vitamin D insufficiency, EBV infection and smoking have been identified as risk factors for MS, but it is not known when the risk begins nor when intervention with these risk factors would be beneficial. There is some evidence of beneficial effects of vitamin D supplementation in the treatment of MS, but with the exception of interferons, little is known about whether vitamin D supplementation can be useful as an add-on treatment to other DMTs such as fingolimod. Smoking is not only a risk factor for MS but also for cancers. Most studies so far investigating the association between cancer and MS have suggested a reduced risk of overall cancer that is not altered by long-time exposure to injectable DMTs. Cancer risk among MS patients in Finland has not been studied in the era of DMTs. The specific aims of this study were:

1. To assess whether serum 25(OH)D levels in early pregnancy are associated with future risk of MS in women in the Finnish Maternity Cohort (FMC) (Original article II) and their offspring (Original article I).
2. To determine whether smoking during pregnancy is associated with future risk of MS in women in the FMC (Original article III).
3. To study whether serum EBV antibodies in early pregnancy are associated with the risk of MS in women and their offspring in the FMC (Original article III).
4. To study whether vitamin D supplementation in addition to fingolimod has a beneficial impact on the clinical course, MRI and safety outcomes as an add-on treatment in MS patients in the Phase 3 fingolimod trials (Original article IV).
5. To assess the risk of cancer among MS patients from a prevalence cohort in the hospital district in southwestern Finland from year 2004 to 2012 compared to non-MS patients from the same hospital district (Original article V).

This work consists of three separate Studies (1-3). The Original articles I-III are based on Study 1, and the Original articles IV and V are based on Studies 2 and 3, respectively.

Study 1, Original article I-III. The FMC Study investigated the risk of MS association with serum 25(OH)D, EBV antibodies and cotinine levels among women and offspring to women in the cohort with a pregnancy serum sample in the FMC, which is a nationwide biorepository of serum samples collected during the first and early second trimester of pregnancy for routine prenatal testing since 1983.

Study 2, Original article IV. Pooled data from phase 3 FREEDOMS trials was analyzed post hoc to evaluate whether patients in the Phase 3 fingolimod trials using vitamin D supplements have better clinical, MRI and safety outcomes than non-users.

Study 3, Original article V. This observational nested case-control population based study assessed the cancer risk among Finnish MS patients in a hospital district cohort from southwestern Finland during the DMT era from January 1, 2004, to December 31, 2012, compared to a randomly chosen 10-fold control population of non-MS patients from the same hospital district matched by year of birth and gender by using hospital administrative data and chart review for case confirmation.

4 Materials and Methods

4.1 Setting

Finland is geographically located at latitudes of 60°N and 70°N, and is a high-risk region for MS. The prevalence differs from 100 to nearly 300 per 100,000 inhabitants in different areas, with foci of high prevalence in Ostrobothnia and southwestern Finland (Sumelahti *et al.*, 2001, 2003; Sarasoja *et al.*, 2004; Krökki *et al.*, 2011; Holmberg *et al.*, 2013; Pirttisalo, Soilu-Hänninen and Sipilä, 2019). In southwestern Finland, the age-standardized prevalence to European standard population is 280/100,000 and the incidence is 12.1/100,000, which are among the highest in Finland and globally very high. The female to male ratio in western Finland has increased from 2.2 to 2.7 since the 1990s and the mean age of the MS population has increased over the same period from less than 40 years to over 50 years (Sumelahti *et al.*, 2014; Pirttisalo, Soilu-Hänninen and Sipilä, 2019). In the aging population with MS, comorbidities such as cancer play a growing role. In Finland, a total of 250,000 people had had cancer at some point in their lives in 2011 and there were 30,000 new diagnosed cases. Cancer becomes more common with increasing age and the annual number of cancer deaths among Finns has remained stable around 11,700, with pulmonary cancer as the most common cause of cancer death. The most common cancer in women is BC (n = 4,900) and in men prostate cancer (n = 4,700) (Pukkala and Rautalahti, 2013; The Finnish Cancer Registry, 2019). A moderate to severe vitamin D deficiency is displayed in almost half of newly diagnosed Finnish MS patients, and a high rate of vitamin D insufficiency has previously been reported among pregnant, non-MS Finnish women (71%) regardless of their vitamin D intake meeting current Nordic recommendations (10 ug daily) (Viljakainen *et al.*, 2010).

Specialized medical care is organized by hospital district, distributed between 20 hospital districts in mainland Finland, each of which belongs to one of the five university catchment areas. The patient registers of Turku University Hospital, which is the central hospital of the Southwest Finland hospital district covering a population of 472,139 people on January 31, 2012, contains clinical data on all patients that have visited public hospitals in the district from 2004 onwards. This patient register covers practically all patients with MS and cancers requiring specialized medical care in the hospital district.

In Finland, there are various registers of public health care that ensure outstanding coverage of patients with MS. The hospital Discharge Register with its continuation, The Care Register for Health Care, contains data on all patients that have been admitted to specialized inpatient care, undergone day surgery, or visited specialized outpatient care in the country. Annually, data retrieved from the electronic client and patient record systems of health care units are submitted to the National Institute for Health and Welfare. Finnish residents are eligible for reimbursement of medical expenses under the Health Insurance Act. The Social Insurance Institutions registry tracks reimbursement recipients, including patients with self-administered disease modifying treatments for MS.

The Finnish Maternity Cohort (FMC) is a nationwide biorepository of serum samples established in 1983 and situated in Biobank Borealis at Oulu University Hospital since 2017. The biobank serum samples are collected from women during the first or early second trimester of pregnancy for routine prenatal testing and contains a collection of approximately 2.0 million samples from more than 850,000 women covering ~98% of all pregnancies since the FMC establishment.

4.2 Subjects and methods

4.2.1 Finnish Maternity Cohort study (Study 1, Original Articles I–III)

The FMC Study is a prospective, nested case-control study conducted in May 2011 that investigated the risk of MS among the women and offspring of the women with a serum sample (date of sample during first or early second trimester of pregnancy, 5^{th} to 95^{th} percentile: months 2-4 of pregnancy) in the FMC biobank. Samples were collected for routine prenatal testing at local maternity care units. Blood samples remaining after routine screenings are stored at -25°C in a protected biorepository at the Northern Finland Biobank Borealis in Oulu, where they are available for scientific research.

I Vitamin D Status During Pregnancy and Risk of Multiple Sclerosis in Offspring of Women in the Finnish Maternity Cohort

II 25-Hydroxyvitamin D deficiency and risk of MS among women in the Finnish Maternity Cohort

III Epstein-Barr virus and multiple sclerosis risk in the Finnish Maternity Cohort.

4.2.1.1 Subjects

Women with MS were identified by linking the FMC database to the Finnish Hospital Discharge Register (FHDR, or HILMO), which includes both inpatient (since 1967), outpatient neurologic visits (since 1998) and patients in longer-term care institutions (since 1994). Diagnostic codes for MS or related disorders (ICD-10 codes G35, G36, H46, ICD-9 and ICD-8 codes 340, 341, 367, 377) were searched for between 1983 and 2009. The majority of Finnish MS patients are diagnosed by neurologist evaluation at an inpatient clinic. To identify cases not in the HILMO, the FMC database was also linked with the registry of the Social Insurance Institution of Finland (SII, or Kela) to search for recipients of prescription drug reimbursements for MS DMTs that are admitted based on certificates issued by a doctor confirming the MS diagnosis. In the FMC, we identified 1,264 female MS cases listed in HILMO or Kela. For 1,252 of these, we had either a medical record or Kela confirmation of the diagnosis. For 612 women, medical records were available and reviewed and all but 5 of them were clinically confirmed, by Poser criteria for cases occurring prior to 2001 and by the McDonald criteria for cases from 2001 to 2009. The remaining 640 women without available medical records were listed by the Kela registry as DMT receivers. The earliest diagnosis date recorded in HILMO, Kela or the medical records was set as the date of MS diagnosis. The date of MS onset was available only for cases confirmed by medical record review and this date was used as the index date to identify serum samples in the FMC collected prior to MS onset, whereas the date of diagnosis was used for the Kela-confirmed cases. MS patients were matched to up to 3 controls on date of birth (±2 years) and area of residence (by postal code). From pregnancies occurring prior to diagnosis, from 1 to 3 serum samples were available, and a total of 6,200 serum samples from 1,092 cases and 2,123 controls. For 511 cases and 831 controls, ≥2 samples were available.

Cases of MS among children born to women in the FMC between January 1, 1983, and December 31, 1991, were identified by HILMO and Kela searches as described for the women. Mothers of individuals with a confirmed MS diagnosis were identified through over-generation linkage via the Population Census Register. Mothers were then linked to the FMC database by their personal identification number. Child-mother pairs with available serum samples from the pregnancy were included in the study. Medical charts of the children were then reviewed, and MS diagnosis was confirmed by study neurologists. MS was confirmed and a maternal serum sample available for 193 children (138 confirmed by medical record review and 55 by the reimbursement/prescription of MS DMTs) and up to 2 matched controls were found for 176 of them (for 17 cases a suitable control could not be identified). Controls were matched by region of birth (south, southwest, southeast, middle and north), date of maternal sample collection (±60 days), date of mother's birth (±6 months) and date of offspring birth (±2 months). The total number of

controls was 326. There were an additional 5 controls that were selected but ultimately not matched.

4.2.1.2 Laboratory analyses

FMC serum samples of 1-3 ml volume remaining after routine prenatal screenings, previously processed and stored in the Finnish National Institute for Health and Welfare in Oulu, are since 2017 stored at -25°C at the Borealis Biobank in Oulu.

25-hydroxyvitamin D (Original article I-III)

Serum 25(OH)D levels were measured in all pre-diagnostic case and control samples for the women and in maternal samples for offspring cases and controls in the FMC using a chemiluminescence microparticle immunoassay and an Architect i2000SR automatic analyzer (AbbottDiagnostics).

Cotinine (Original article III)

Serum cotinine, the biomarker for cigarette smoking, was measured using a commercially available quantitative immunoassay kit (OraSure Technologies, Bethlehem, PA, USA) (sensitivity=96-97%, specificity=99-100%). Intra- and inter-assay variation are 3.5-6.2%, and 6.0-9.6%, respectively. The limit of detection was 0.08 ng/ml. Women were considered non-smokers if their cotinine levels were <10 ng/ml and smokers if their cotinine levels were >25 ng/ml. Cotinine levels of ≥10 ng/ml and ≤25ng/ml were considered equivocal and were categorized separately.

EBV and CMV IgG measurement (Original article III).

For the offspring analysis, maternal IgG antibodies to the EBV antigens VCA, EBNA-1, EA-D and to CMV were measured using an enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (DiaMedix Corp., Miami, FL). For the analysis among the FMC women, only serum EBNA-1 IgG antibodies were measured because these antibodies were the strongest predictors of MS risk in previous prospective studies. Samples were positive for EBV IgG antibodies if the ELISA index value was ≥1.10 and negative if the index value was <0.90. Index values in between were equivocal. For CMV, index values of <8.0 were negative, ≥10 were positive, and 8.0-9.99 were equivocal. Quality control samples were included among the study samples and coefficients of variation ranged from 3.1% (VCA) to 8.2% (EA-D).

4.2.1.3 Statistics

Original article I. All analyses were done using SAS v9.3. (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 the 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 of 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 of less than 0.05 was considered statistically significant.

Original article II. Main analysis included 1,092 cases and 2,123 controls. For women with several 25(OH)D measurements, levels were averaged as an estimate of their longer term 25(OH)D exposure and modeled as described above. In addition to matching factors (age and residence), multivariate models were adjusted for time of sample collection (number of samples collected during or after the 2004 recommendation of vitamin D supplement for pregnant women), gravidity (1, 2, ≥3) and parity (0, 1, ≥2). The missing indicator method was used to model gravidity and parity for women missing information on these covariates in order to retain all observations in the analyses. Tests for linear trends across the categories and quintiles were conducted by assigning the median 25(OH)D value for each category/quintile to all cases/controls in that category and modeling the median 25(OH)D as a linear variable. In sensitivity analysis, we restricted to MS cases with clinical confirmation (i.e. medical record review) of MS (n = 604) and cases with multiple 25(OH)D measurements from multiple pre-diagnostic pregnancies (n = 511) and their matched controls. To evaluate seasonal variation of 25(OH)D and MS association, samples were categorized by month of collection into months of high UV light (May – October, n = 800 cases/1,740 controls) and low UV light (November – April, n = 764 cases/1,677 controls). Women with samples collected in both high and low UV months will be in both analyses, and we averaged 25(OH)D levels for women with several samples collected within the high or low UV month period. Unconditional regression adjusting for age, geographic location and other factors listed above, was used to estimate relative risks (RRs) and 95% CIs associated with 25(OH)D 50 nmol/L increases in the UV-specific analysis.

Original article III. SAS v9.4 was used to conduct the statistical analysis, and the approach was similar in women and offspring. EBV IgG antibody analyses were restricted to the offspring of EBV positive women with at least one matched EBV positive control (FMC women: 1,049 MS cases and 1,867 controls; offspring: 170

MS case mothers and 311 control mothers) and analyses of CMV IgG antibodies were restricted to CMV positive matched case/control groups (offspring: 116 MS case mothers and 177 control mothers). The index values for IgG antibodies against the EBV and CMV antigens were standardized to control values and modeled as continuous variables and quintiles determined based on the distribution of the standardized IgG index value for each antigen among the controls. Consistent with the matched design, conditional logistic regression was used to estimate the relative risks and 95% CIs. In the offspring analysis, models were adjusted for offspring gender, gestational age at sample collection, and maternal cotinine and 25(OH)D levels (<30 nmol/L, 30-50, >50) previously measured in these samples (*original article I*, data not shown). In the analysis among women, models were adjusted for parity and gravidity (as previously described), and previously measured 25(OH)D and cotinine levels (*original article II*, data not shown). Trend p-values for the quintile analyses were calculated by modeling the median IgG antibody index value for each quintile as a continuous variable. Sensitivity analyses were conducted by restricting to EBV positive cases of MS confirmed by medical record review (FMC women n = 588; offspring n = 138) and matched controls.

4.2.2 Fingolimod and vitamin D study (Study 2, Original article IV)

This study assessed the effect of vitamin D supplement use on clinical, MRI, and safety outcomes by a post hoc analysis of pooled data from the phase 3 FREEDOMS pivotal fingolimod trial patients compared to casual and no use of vitamin D.

4.2.2.1 Subjects

This was a post hoc analysis of pooled data from the Phase 3 FREEDOMS I and II trials (Kappos *et al.*, 2010; Calabresi *et al.*, 2014). Data on use of multivitamins and vitamin D were retrieved from the "Concomitant Medications" section of the electronic case report forms. Vitamin D users were defined as those on concomitant vitamin D supplementation during the core study period, whereas patients using multivitamins were not considered vitamin D users unless they also took vitamin D. Patients (n = 1,953) were categorized into 3 groups:

1) Daily users: daily supplemental vitamin D use 100% of the time during the study (n = 120)
2) Casual users: supplemental vitamin D use <100% of the study time (n = 170)
3) Non-users: no use of supplemental vitamin D (n = 1,663)

The analysis was limited to patients from countries where the use of vitamin D was prominent (USA, n = 731; Canada, n = 74; Australia n = 24)(daily users n =110, 80% female; casual users n = 157, 80.9% female; non-users n = 562, 74.7% female; total n = 829). Vitamin D supplement use in other countries was very limited.

4.2.2.2 Evaluated outcomes

Clinical outcomes were evaluated by aggregate ARR up to month 24 (M24), proportion of patients with confirmed relapse at month 12 (M12) and M24 and EDSS change from baseline to M24. Safety was evaluated by AE reporting, including depression (no scale or questionnaire or assessment tool for depression was used separately).

MRI outcomes were evaluated by numbers of new/newly enlarged T2 lesions from baseline to M12 and M24, proportion of patients free of these changes at M12 and M24, number of Gd-enhancing T1 lesions at M12 and M24 as well as proportion pf patients free of these findings at M12 and M24. Percent brain volume change (PBVC) was evaluated from baseline to M12 and 24. Safety was evaluated by incidence of infections and depression.

4.2.2.3 Statistics

Baseline characteristics were compared using the Wilcoxon test for quantitative variables and the Chi-square trend test for categorical variables (electronic case report forms, eCRF). Various generalized linear models were adjusted for treatment, vitamin D use, baseline, age, region and treatment - vitamin D use interaction. The ARR at month 24 by vitamin D use was studied using a negative binominal regression model adjusted for treatment, vitamin D use, number of relapses in the previous 3 years, baseline EDSS, and region. MRI outcomes by vitamin D use were calculated from a Rank ANCOVA or logistic regression model with the following covariates: treatment, vitamin D use, region, baseline volume or number of lesions, and treatment-vitamin D use interaction. In the evaluation of MRI outcomes by treatment within vitamin D subgroups, report was made at the group level (pair-wise comparisons) due to the small number in each of the vitamin D subgroups.

4.2.3 MS and cancer risk study (Study 3, Original article V)

The study on cancer risk among Finnish MS patients was a population-based nested case-control study assessing cancer risk among MS patients and a non-MS patient 10-fold control population in a hospital district cohort from southwestern Finland during a period from January 1, 2004, to December 31, 2012.

4.2.3.1 Subjects

Patients with ICD-9 (3400A) and ICD-10 (G35) codes for MS were searched for from the hospital administrative data in the Southwest Finland hospital district from January 1, 2004 to December 31, 2012, as described previously (Åivo *et al.*, 2017; Murtonen *et al.*, 2018). A total of 1,074 confirmed MS cases (70.7% female, n = 759; 29.3% male, n = 315) were treated in the hospital district, including the deceased cases (5.9%, n = 70) after January 1, 2004. Cancers in the MS cohort and control group were searched for by ICD-10 codes C00-C96. Cancers diagnosed both before and after the date of definite MS diagnosis were included. The control population was a 10-fold population (n = 10,740) with the same gender and year of birth, randomly selected from the Turku University Hospital patient register. Another separate control population from the same patient pool was used to verify the stability of the results. A total of 818 patients in the summoned MS and control cohorts were diagnosed with cancer (mean age 58.8 years). 68 cancer cases were found in the MS cohort and after adjusting for multiple cancers in part of the subjects, a total of 61 MS patients with cancer comorbidity and 757 controls with cancer remained.

ORs and 95% CIs were calculated for the overall risk of cancer and for the risk of specific cancer types in the cohorts. Patient documents of the confirmed MS cases with diagnoses of cancers were reviewed to assess disease type, smoking habits, alcohol use, profession, use of vitamin D supplements, BMI, parity, family history of cancers and MS, first symptom of MS, first symptom or sign of cancer, received cancer treatments (surgery, radiation and chemotherapy), other surgery, EDSS, the use of MS therapies, use of other medications including hormonal replacement therapies, type of cancer or cancers and the outcome of cancer. The causes of all deaths during the study period were obtained by review of the patients' charts as described previously (Murtonen *et al.*, 2018).

4.2.3.2 Statistics

The ORs were calculated with 95% CIs and p-values were calculated using Pearson's χ^2 test. Age at BC diagnosis was compared between groups using the ANOVA test. All statistical tests were two-tailed and p-values less than 0.05 were considered statistically significant. Statistical analyses were performed using R Statistics version 3.0.2 with standard packages. Kaplan-Meier (KM) analysis was performed for cumulative incidence proportion and age (years) at BC diagnosis for MS and year of birth- and gender-matched controls. Significance was assessed by log-rank test.

4.2.4 Ethical aspects

Study 1, Original articles I-III. 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). An informed consent system based on the opt-out principle has been in operation nationwide in Finland since 2001. The FMC study was approved by the data protection authorities at the National Institute for Health and Welfare (THL), the Regional Ethics Committee of the Northern Ostrobothnia Hospital District and by the Office of Human Research at the Harvard T.H. Chan School of Public Health. Before year 2001 no informed consent was needed, but since year 2001, informed consent of the women was obtained for serum sample storage at the biobank for research purposes.

Study 2, Original article IV. In the fingolimod and vitamin D supplement study no further written consents were required since there was no direct contact with the patients in the data mining pool. The previous FREEDOMS studies were performed in accordance with ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All persons included in these study trials had given their informed consent prior to inclusion in the studies.

Study 3, Original article V. This was a register study and no written consents were required since there was no direct contact with the patients. The study was performed in accordance with the code of ethics of the World Medical Association, i.e. ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. The study was registered and approved by the Turku Clinical Research Center (Turku CRC) and ethical committee approval was obtained from the joint Ethics Committee of Tampere University and the Pirkanmaa Hospital District.

5 Results

5.1 Finnish Maternity Cohort study (Study 1, Original publication I–III)

5.1.1 Vitamin D and risk of MS among offspring (I)

In this prospective, nested case-control study conducted in May 2011, mean age at offspring MS diagnosis was 19.8 years (SD 3.2), a low average age as a consequence of the study period since establishment of the FMC in 1983. The cases and controls did not differ in terms of the mother's age, gestational age, and the serum sample collection season. No cases and 2 controls had a mother with MS. Of the 193 offspring MS cases, 163 were female. There were more women in the offspring case group (84% versus 66% among controls). Serum samples were collected during the first trimester (70%) and early second trimester (99% prior to 28 weeks' gestation). Mean 25(OH)D levels did not differ according to the trimester of sample collection (first trimester 36.59 nmol/L, second trimester 36.19 nmol/L). The maternal serum 25(OH)D average level was 34.59 nmol/L (8.74 – 160.49 nmol/L) for offspring cases and 37.49 nmol/L in mothers of controls. Only 2 cases and 8 controls had maternal 25(OH)D levels of >75 nmol/L and no cases and 1 control had maternal 25(OH)D seral levels of >99.9 nmol/L. A 50 nmol/L increase in the maternal 25(OH)D level was associated with a 48% reduced risk of MS in offspring in the matched analysis (adjusted for gender, gestational age and season at time of sample collection), although this finding did not reach statistical significance (relative risk [RR] 0.52, 95% CI 0.22–1.19, p-value 0.12). Children of mothers with deficient 25(OH)D had an increased risk of developing MS compared to children of non-deficient mothers. In multivariate analyses using 25(OH)D a priori categories, clearly deficient maternal levels were associated with an almost 2-fold increased MS risk in the child (<30 nmol/L versus 30 to <50 nmol/L, RR 1.90, 95% CI 1.20–3.01, p-value 0.04). Maternal 25(OH)D levels in the bottom quintiles 1 and 2 (<31.50 nmol/L) were associated with a 20% to 90% increased offspring MS risk compared to maternal levels in the top quintile (median 56.21 nmol/L, p trend 0.09).

In the unmatched analysis, a 43% reduced risk of MS was associated with every 10.00 nmol/L increase in maternal 25(OH)D level (RR 0.57, 95% CI 0.28–1.18, p-

value 0.13) and a 59% increased risk of MS among offspring to vitamin D deficient mothers (<30 nmol/L versus 30 to <50 nmol/L, RR 1.59, 95% CI 1.04–2.42, p-value 0.03). In analyses stratified by child gender this association was only evident in female offspring (<30 nmol/L versus 30 to <50 nmol/L, RR 1.75, 95% CI 1.09–2.81, p-value 0.02).

MS risk was 90% higher (nearly 2-fold increased risk) in offspring of vitamin D deficient mothers (serum 25[OH]D <30 nmol/L) compared to offspring of mothers who were not deficient (RR 1.90, 95% CI 1.20–3.01, p-value 0.006), a statistically significant difference supporting the role of prenatal vitamin D in determining MS risk and suggesting that correction of vitamin D deficiency during pregnancy may reduce the risk of MS in offspring.

5.1.2 Vitamin D and risk of MS among women (II)

An expected seasonal distribution of 25(OH)D levels among the FMC women was exhibited (with increasing levels towards summer and decreasing levels towards winter months), but with remarkably stable averages from 1983 until 2004 (when Finland formally recommended that pregnant women take vitamin D supplements). The average pre-diagnostic 25(OH)D serum levels were 1.3 nmol/L lower among women who developed MS compared to controls. Among cases and controls >50% had deficient levels (<30 nmol/L) and in both groups over one third had insufficient levels (30 to <50 nmol/L). There were only 6 cases and 9 controls with serum 25(OH)D levels of ≥75 nmol/L, and of these only 1 case and 2 controls had levels of ≥100 nmol/L. There was an average of 9.5 years between first sample collection and the recorded date of MS diagnosis among 604 MS cases with medical record confirmation.

In multivariate-adjusted analyses including all cases and controls, a 25(OH)D 50 nmol/L increase was associated with a 39% reduced MS risk (adj RR 0.61, 95% CI 0.44–0.85, p-value 0.003). Deficient women had a 43% increased MS risk compared to women with 25(OH)D serum levels of ≥50 nmol/L, and a 27% increased risk compared to women with insufficient levels of 30 to <50 nmol/L (adj RR 1.27, 95% CI 1.07–1.50, p-value 0.005).

In quintile analyses, women with extreme 25(OH)D deficiency (bottom 2 quintiles <26.8 nmol/L) had a 53-66% increased MS risk compared to women in the 25(OH)D top quintile (≥41 nmol/L), and there was a statistically significant overall trend of increasing MS risk with decreasing 25(OH)D (p-value <0.0001). In the analyses stratified by season of sample collection, similar inverse associations between serum 25(OH)D and MS risk were observed. The RR associated with a 50 nmol/L increase when using serum samples collected in high UV months May – October was 0.71 (95% CI 0.52–0.91, p-value 0.02) and for serum samples collected

in low UV months November – April, RR was 0.65 (95% CI 0.43–0.98, p-value 0.04). In analyses restricted to 511 MS cases and controls with ≥2 samples prior to diagnosis, risk estimates were stronger with a 50 nmol/L 25(OH)D increase associated with a 41% reduction of MS risk (adj RR 0.59, 95% CI 0.34–1.03, p-value 0.07) and levels of <30 nmol/L with a 2-fold increase in MS risk as compared to levels of ≥50 nmol/L. In quintile analyses, women in the 2 bottom quintiles (Q1-Q2) with serum 25(OH)D levels of <26.8 nmol/L had a 37% - 87% increased risk of MS compared to women in the top quintile of ≥41 nmol/L (Q1 versus Q5: RR 1.37, 95% CI 0.89–2.12; Q2 versus Q5: RR 1.87, 95% CI 1.25–2.79, p trend 0.03), with similar results among women with MS confirmed by medical record review and their matched controls.

5.1.3 EBV and risk of MS in women and offspring (III)

In offspring analyses, 70% of maternal samples were collected during the first trimester of pregnancy and 98.3% and 97.8% of mothers of cases and controls, respectively, were positive for EBV infection, whereas similarly 79.6% and 73.4% of case-control mothers were positive for CMV infection. Maternal seropositivity to EBV and CMV was not associated with offspring MS risk increase (EBV: RR 1.28, 95% CI 0.32–5.21, p-value 0.73; CMV: RR 1.39, 95% CI 0.87–2.22, p-value 0.17). No statistically significant associations were observed after adjusting for offspring gender, gestational age at sample collection, cotinine and 25(OH)D levels (EBV: RR 0.74, 95% CI 0.17–3.19, p-value 0.68; CMV: RR 1.23, 95% CI 0.74–2.03, p-value 0.43).

A one standard deviation increase in maternal VCA IgG index value was associated with a 34% increased offspring MS risk (RR 1.34, 95% CI 1.07–1.67, p-value 0.01). After adjusting for the factors described, the results remained mainly unchanged (RR 1.41, 95% CI 1.11–1.80, p-value 0.006). In quintile analyses, children of mothers in the highest VCA IgG index value quintile had a 2-fold increased risk of MS occurrence (RR 2.01, 95% CI 1.05–3.86, p trend 0.01), a strong association that remained after adjusting for offspring gender, gestational age at sample collection, maternal 25(OH)D and cotinine levels (RR 2.44, 95% CI 1.20–5.00, p trend 0.004). No associations were seen with a one standard deviation increase in maternal EBNA-1-, EA-D- or CMV IgG index values and offspring MS risk either without or with adjustment for offspring gender, gestational age at sample collection and maternal 25(OH)D and cotinine levels or in quintile analyses.

The Pearson correlation coefficients between maternal EBV IgG and 25(OH)D levels were: VCA 0.01; EBNA-1 -0.04; EA-D 0.05; CMV 0.08. After adjustment for EBV IgG levels and cotinine, maternal deficient 25(OH)D levels <30 nmol/L, as compared to levels of 30-50 nmol/L, remained statistically significantly associated

with a nearly 2-fold increased MS risk in the offspring (adjusted for VCA IgG: RR 1.95, 95% CI 1.20–3.17, p-value 0.007; adjusted for EBNA-1 IgG: RR 1.98, 95% CI 1.23 –3.19, p-value 0.005; adjusted for EA-D: RR 1.97, 95% CI 1.23–3.16, p-value 0.005). There was no statistical interaction between the maternal EBV IgG and 25(OH)D or cotinine levels and MS risk in the offspring, and the results remained materially unchanged after restricting the analysis to the 124 cases with medical record review confirmation of MS and 225 matched controls.

Among the FMC women, there were 1,049 MS cases and 1,867 controls that were EBV seropositive prior to MS diagnosis. The mean age at MS diagnosis was 37 (SD 7.1) and the mean age at sample collection was 27.6 (5.1) for cases and 27.6 (5.1) for controls. The mean EBNA-1 IgG index among cases and controls were 4.60 (0.50) and 4.37 (0.70), respectively. The mean levels for 25(OH)D among cases and controls were 29.6 (12.2) and 31.0 (13.3), whereas the mean levels for cotinine (ng/ml) for cases and controls were 35.8 (79.8) and 28.0 (69.6), respectively. Serum samples were collected an average of 9.4 years prior to MS diagnosis, and 10.4 years prior to diagnosis for clinically confirmed cases, for whom the date of MS onset was available.

Notably, women who developed MS had higher mean pre-onset serum cotinine levels than the controls (35.8 ng/ml versus 28.0 ng/ml, and cotinine levels indicative of recent smoking were observed in 24.5% of cases versus 19.7% of controls). Women with cotinine levels of >25 ng/ml (smokers) had a 45% increased risk of MS as compared to women with cotinine levels of <10 ng/ml (non-smokers) (RR 1.45, 95% CI 1.19 – 1.17, p-value 0.0002), the results were mainly unchanged after adjusting for EBNA-1 index values, parity, gravidity, and 25(OH)D levels (RR 1.50, 95% CI 1.21 – 1.85, p-value 0.0002).

In the available pre-onset samples, 99.3% of the 1,053 women who developed MS and 97.7% of the 1,892 controls were seropositive for EBV, yielding a nearly 3.5-fold increased MS risk associated with EBV seropositivity (RR 3.44, 95% CI 1.55–7.66, p-value 0.003). After adjusting for parity, gravidity, serum 25(OH)D and cotinine levels, RR was 3.54 (95% CI 1.56–8.02, p-value 0.0024).

A one standard deviation increase in EBNA-1 index value levels in pre-onset samples was associated with an increased risk (RR 1.61, 95% CI 1.44–1.80, p-value 1.11e-16) with similar results after adjusting for parity, gravidity, 25(OH)D and cotinine levels (RR 1.57, 95% CI 1.40–1.76, p-value 2.22e-15).

In quintile analyses as EBNA-1 IgG index levels increased, there was an increase in MS risk (RR top versus bottom quintile 3.42, 95% CI 2.56–4.56, p trend <1.11e-16), an association that persisted after adjusting for factors previously described.

The previously observed (*original article II*) increased risk of MS among women with deficient 25(OH)D levels as compared to women with sufficient levels persisted after adjusting for EBNA-1 IgG and cotinine (RR1.41, 95% CI 0.99–2.01, p-value

0.06) and results were not changed by restriction to cases with medical review confirmation of MS. Serum EBNA-1 IgG index value and 25(OH)D levels were not correlated (r -0.003).

Among women in the FMC, those with the highest versus lowest quintile of EBNA-1 IgG levels had a 3-fold higher risk of MS (RR 3.21, 95% CI 2.37–4.35, p trend <1.11e-16). In conclusion, smoking increases the risk for MS in the smoking subject and offspring of mothers with high VCA IgG during pregnancy appear to have an increased risk of MS, and the increase in MS risk among women with elevated pre-diagnostic EBNA-1 IgG levels is consistent with previous results. The EBV-associated MS risk in mothers and offspring was not confounded or modified by 25(OH)D or cotinine levels.

5.2 Fingolimod and vitamin D supplement (Study 2, Original publication IV)

Within the study population, ARR after two years was significantly lower in the 0.5 mg fingolimod group compared to placebo (0.188 and 0.370, respectively; p-value 0.0004). No difference in ARR was observed between the vitamin D supplement user groups, nor did the proportion of patients with relapses differ between vitamin D ´daily´, ´casual´ or ´non-users´ at M12 and M24. The EDSS change from baseline for two years was minimal and did not differ between the vitamin D ´daily´ and ´non-user´ groups (p-value 0.262). The fingolimod treatment effect compared to placebo was seen in all subgroups, but the small sample size makes it impossible to conclude a treatment effect between the vitamin D use subgroups.

Vitamin D supplement as an add-on therapy to fingolimod had beneficial effects on MRI outcomes, such that the proportion of patients free of new/enlarging T2 lesions significantly favored vitamin D daily versus non-users (M12, p-value 0.038; M24, p-value 0.009). The mean numbers of new/enlarging T2 lesions from baseline to M12 and M24 were lower in the vitamin D daily user groups compared to non-users but did not reach statistical significance.

The proportion of patients free of Gd+ T1 lesions was higher among daily vitamin D users compared to non-users at M12 and M24 (whereas there was no difference between casual and non-users) and the mean number of Gd+ T1 lesions was lower between daily users and non-users at M12 and M24, and also lower for casual users at M12 compared to non-users (p-value 0.04). PBVC at M12 was significantly lower in the vitamin D daily than non-user group (p-value 0.018) and remained low (non-significant) at M24.

There was a trend of less depression reported as an AE in the daily vitamin D supplement user group (5.5%) compared to casual (10.8%) and non-users (11.9%) among patients in the FREEDOMS trials. The incidence of infections was similar in

all groups, with nasopharyngitis and upper respiratory tract infection being the most common. No severe adverse events (SAEs) where seen and safety outcomes did not differ from the control group. Our study suggests that there is a basis for recommendation of vitamin D supplement not only for patients treated with IFN, but vitamin D supplements can also be beneficial when combined with fingolimod treatment in MS subjects.

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

In the cohort of 1,074 MS patients (70.7% female, 29.3% male), 70 patients (5.9%) deceased after January 1, 2004. A total of 818 patients in the summoned MS and control cohorts were diagnosed with cancer (mean age 58.8 years). We found 68 diagnoses of cancer in the MS cohort, and after adjusting for multiple cancers in part of the subjects, there were 61 MS patients with cancer comorbidity (5.7%, mean age 57.3 years) and in the 10-fold control population matched for gender and date of birth we found 757 cancer cases (7.0%, mean age 58.9 years)(ANOVA p-value 0.317). Among the 70 MS patients that deceased during the study period, there were a total of 11 cancer cases (5 cases of BC, 3 intestinal cancers, 2 pulmonary cancers and 1 thyroid cancer). In only 4.3% of the deceased patients, cancer was the cause of death (2 cases of BC and 1 intestinal cancer). Other causes of death were respiratory infection (n = 2), unlocalized infections (n = 2) and respiratory insufficiency (n = 4).

The overall risk of cancer among MS patients in our study population of southwestern Finland did not differ from the control population (OR 0.80, 95% CI 0.6–1.0, p-value 0.092). The mean age at cancer diagnosis did not statistically differ between the MS and control cohorts (two-tailed t-test p-value 0.317). All BC cases were female and age at BC diagnosis was significantly higher among the MS patients (n = 18) compared to controls (n = 207) (61.7 vs. 55.7 years, ANOVA test p-value 0.010) and the risk of BC was slightly lower in the MS group (OR 0.9, 95% CI 0.5–1.4, p-value 0.566). Among MS patients with BC, 7 subjects had no DMT, 5 had received DMT (IFN, of which 3 patients switched to GA) and for 6 data was not available (3 cases were defined as PPMS). Only 1 (5.6%) patient had a history of smoking, while 8 (44.4%) were self-reported non-smokers and for 50% no data on smoking was available. Data on lifestyle and environmental confounding factors (e.g. alcohol use, gravidity and parity, BMI, family history of cancer, hormonal replacement therapies and vitamin D use) was scarce or missing and no conclusions of DMT impact on cancer risk could be made due to the small patient numbers.

In the MS cohort, we observed a decreased risk for prostate cancer and increased risk for oral cavity cancers, colon cancer, lung cancer, renal cancer, brain cancer and

thyroid cancer. Among the MS patients with high-odd cancers, DMTs used were IFN, GA, natalizumab (n = 1), and one case had participated in a study to receive either TRF or placebo, but patient numbers were too small to draw any conclusions of statistical significance, neither for cancer risk, nor for DMT risk impact.

Table 5. Summary of study findings

Study 1, original publications I-III		
Maternal serum status impact on MS risk	mother	offspring
Vitamin D deficiency (<30 nmol/l)	yes♣	yes♦
Cotinine (>25 ng/ml, smoker)	yes♥	
CMV IgG seropositivity		no♠
EBV seropositivity	yes♠	no♠
VCA IgG index value ↑		yes♠
EBNA-1 IgG index value ↑	yes♠	no♠
EA-D IgG index value ↑		no♠
Study 2, original publication IV		
MS and vitamin D beneficial effect at M12/M24	users M12 (vs non-users)	users M24 (vs non-users)
Clinical outcome measures (ARR, EDSS)	no	no
Radiological outcome measures (proportion of patients free of new/ enlarging T2 lesions, proportion of patients free of Gd+ T1 lesions, lower mean number of Gd+ T1 lesions)	yes	yes
Study 3, original publication V		
Cancer risk	MS population vs control population	
Overall cancer risk	OR 0.80 (95% CI 0.6–1.0)	p-value 0.092

ARR = annual relapse rate; EDSS = expanded disability status scale; OR = odds ratio; CI = confidence interval; EBV = Epstein-Barr virus; EBNA-1 = EBV nuclear antigen; IgG = immunoglobulin G; CMV = cytomegalovirus; VCA = viral capsid antigen; EA-D = early antigen D; M = Month. P-value less than 0.05 was considered statistically significant. ♣Unaffected by EBNA-1 IgG and cotinine levels; ♦Unaffected by EBV IgG and cotinine levels; ♥Unaffected by EBNA-1 index values and 25(OH)D levels; ♠Unaffected by cotinine and 25(OH)D levels

6 Discussion

6.1 The Finnish Maternity Cohort study

6.1.1 Vitamin D levels and risk of MS in women and their offspring

In the FMC study, we found that women with low serum 25(OH)D levels of <30 nmol/L had a 43% higher MS risk, and for women with ≥2 samples available prior to MS diagnosis (511 cases), MS risk was 2-fold higher in those with 25(OH)D levels of <30 nmol/L as compared to women with levels of ≥50 nmol/L (repetitive findings are less affected by random variations than those based on a single measurement). A 50 nmol/L increase in 25(OH)D was associated with a 39% reduced risk of MS.

For the offspring, we showed that maternal vitamin D deficiency in early pregnancy was associated with a nearly 2-fold increase in risk of MS in their children compared to vitamin D non-deficient mothers.

It has previously been unclear whether in utero exposure to vitamin D deficiency exerts an effect on risk of MS in offspring, with no former published data on the risk in the Finnish population, although previous evidence hints this could be the case in other populations. A higher intake of vitamin D during pregnancy has been associated with a lower risk of MS in offspring (Mirzaei et al. 2011). In the northern hemisphere, MS patients are more likely to be born in spring after winter months of low sunlight and consequent low levels of 25(OH)D during gestation (Willer *et al.*, 2005; Torkildsen *et al.*, 2012), whereas the pattern is reversed in the southern hemisphere with excess MS births in November and December (Staples, Ponsonby et al. 2010). Previous Swedish studies investigating the association between 25(OH)D serum levels and MS risk found no significant association between gestational vitamin D levels and MS in a small cohort of 37 cases (Salzer *et al.*, 2012), and there was no association between future MS risk and 25(OH)D levels in dried blood spot sample levels collected from neonates in another study (Ueda *et al.*, 2014). The findings may have been altered by degradation of 25(OH)D in older samples and lower participation among controls, perhaps whereby the controls might not be truly representative of the general population. On the other hand, a large case-control study including over 500 MS patients and 1,000 controls that assessed

25(OH)D levels in neonate dried blood spot samples from the Danish Newborn Screening biobank found that a 25 nmol/L increase reduced the risk of MS by 30% (Nielsen *et al.*, 2017), which is also in line with our study findings. Previous studies have shown that neonatal umbilical cord 25(OH)D levels correlate with maternal 25(OH)D levels (Godang *et al.*, 2014), suggesting that maternal vitamin D levels are an adequate indicator of 25(OH)D levels to which the fetus is exposed.

The strength of our FMC study is its large size and coverage, including samples from about 98% of all pregnancies in Finland since 1983. The risk of selection bias is minimized by the excellent coverage of MS patients in Finland for the Hospital Discharge Register and Social Insurance Institution register for reimbursement used in case selection. Because the FMC was established in 1983, offspring cases of mothers in this cohort were young with an average age of 19.8 years at MS diagnosis, accompanied by the possibility that the association between gestational vitamin D levels and MS risk decreases at older ages. The majority of women in our study had deficient levels of vitamin D and further studies will be needed to estimate the possible dose-response effect in populations with higher variation in vitamin D levels. For the offspring, we did not have information on risk factors for MS such as HLA DRB1*1501 status, EBV infection, cigarette smoking, vitamin D levels or obesity during childhood or adolescence, but previous work suggests these are not major confounders of the vitamin D and MS association, with perhaps obesity being an exception (Munger *et al.*, 2011; Mokry *et al.*, 2015, 2016; Rhead *et al.*, 2016). Since 25(OH)D levels are likely to correlate between mothers and their children also later in life due to shared behavioral factors, e.g. sun exposure and use of vitamin D supplementation in addition to shared genetic risk factors, it is possible that the increased risk of MS in children born to vitamin D-deficient mothers is a consequence of low levels of vitamin D during childhood and adolescence, and reverse causation can be debated.

We found that women from the cohort who were vitamin D-deficient had an up to 2-fold increased risk of developing MS, when comparing one or repetitive samples with 25(OH)D samples from vitamin D-sufficient mothers. Since previous studies suggest historically low vitamin D levels during pregnancy in Finnish pregnant women (Viljakainen *et al.*, 2010), we specifically sought to examine whether deficient and insufficient 25(OH)D levels during the first and early second trimester of pregnancy are associated with an increased MS risk in the mothers. There is much evidence supporting a role for adequate vitamin D nutrition in reducing MS risk (Ascherio and Munger, 2016). Two previous prospective studies examining whether 25(OH)D levels in healthy subjects predict future MS risk have found that elevated levels of 25(OH)D of ≥75 nmol/L (Salzer *et al.*, 2012) and ≥100 nmol/L (Munger et al. 2006) were associated with an approximately 60% decreased risk of later developing MS. Both studies included only a few non-Hispanic Caucasian MS

subjects and one was not able to directly examine whether deficient vitamin D levels were associated with an increased risk of MS, and only 5% of individuals had 25(OH)D levels below 50 nmol/L (Munger et al. 2006), while the other reported no associations with 25(OH)D lower than 75 nmol/L (Salzer *et al.*, 2012).

Our study of reproductive age Finnish women is the largest longitudinal investigation to date to directly assess whether levels of vitamin D in healthy individuals predict the risk of MS occurrence. We included 1,092 women with MS diagnosed between 1983 and 2009 with at least 1 serum sample collected prior to date of MS diagnosis and a 3-fold control population from the cohort. Among the 604 women with MS confirmed by medical record review, there was an average of 9.5 years between first sample collection and recorded date of MS diagnosis, reducing reverse causation as an explanation of our results.

Over half of the patients and controls had deficient serum 25(OH)D levels of <30 nmol/L, and over one third of patients and controls had insufficient levels (30 to <50 nmol/L). Less than 10 subjects among cases and controls, respectively, had 25(OH)D levels of ≥75 nmol/L. Given the low numbers of patients with sufficient and high 25(OH)D levels, we were not able to assess MS risk associated with elevated vitamin D levels, but our findings are consistent with linear association of 25(OH)D and MS risk with decreasing risk as vitamin D levels rise. Our findings therefore complement previous prospective studies of similar design conducted in the US and Sweden that observed decreased MS risk in association to elevated levels of 25(OH)D (Munger *et al.*, 2006; Salzer *et al.*, 2012).

In our study, deficient levels were mainly found in samples obtained prior to 2004, which is the year when Finland formally recommended that pregnant women take vitamin D supplements. However, our findings of deficient and insufficient vitamin D levels during pregnancy in Finnish women support previous study observations (Viljakainen *et al.*, 2010).

Serum samples were collected from the mothers at ~10-14 weeks gestation. Longitudinal studies of 25(OH)D in pregnant women and comparison of levels in pregnant and nonpregnant women suggest that 25(OH)D levels during the first trimester are reflective of non-pregnancy levels (More *et al.*, 2003; Viljakainen *et al.*, 2010). Since the majority of women in the FMC are Caucasian, our results may not be generalizable to females of other racial groups. We did not have specific information on demographic variables such as ethnicity or education level. Vitamin D deficiency may also increase the risk of MS in men, since previous findings support decreased MS risk with increasing 25(OH)D levels also in males (Munger et al. 2006; Salzer et al. 2012), but a separate study would be required to verify such speculations.

Our findings directly support vitamin D deficiency during pregnancy as a risk factor for MS in both mothers and offspring and extend on previous prospective

study observations of 25(OH)D levels in young adults, suggesting that many individuals are exposed to an increased MS risk, which strengthens the rationale for broad public health interventions to prevent and correct deficient vitamin D levels. It is less clear what specific recommendation (if any) with regards to timing of vitamin D supplementation can be made. The timing of vitamin D effect may be as early as during the fetal period. A previous study has shown that 71% of women and 15% of newborns are vitamin D-deficient during pregnancy although a mean total intake of vitamin D among mothers met current Nordic recommendations during the study period (Viljakainen et al. 2010). This observation is also supported by our study findings and justifies follow-up of vitamin D levels in vitamin D-deficient subjects and pregnant women after initiation of vitamin D supplementation. More research on the benefits, timing, optimal dose and serum target level of vitamin D supplementation on MS and comorbidity risk is needed but striving to achieve vitamin D sufficiency over the lifespan is likely to have multiple benefits. Since studies support vitamin D levels in adolescence as an important modulator of MS, vitamin D supplementation during this period, or earlier, might mitigate MS risk.

6.1.2 Vitamin D levels, EBV and smoking

We observed that offspring of mothers with high VCA IgG during pregnancy have an increased risk of MS that has not been previously observed. The risk of MS was 2.5-fold increased among offspring of women with the highest levels (top quintile) versus the bottom quintile. No elevated risk for MS in the offspring was associated with maternal IgG levels against EBNA-1, EA-D or CMV. Among the women with increased pre-diagnostic EBNA-1 IgG levels we observed a 3-fold higher MS risk in the highest versus lowest quintile, a finding that is consistent with previous study results. These associations were not confounded or modified by maternal serum 25(OH)D or cotinine levels, which has not been demonstrated previously. Women who developed MS had higher mean pre-onset serum cotinine levels than the controls, and those with cotinine levels indicative of smoking had a 45% increased risk of MS compared to non-smokers, which is consistent with previous study observations of smoking as a risk factor for MS. The results were not altered by EBNA-1 index value, 25(OH)D levels, parity or gravidity. The previous observed increased risk of MS among vitamin D-deficient women was not confounded by EBNA-1 IgG and cotinine, and in the offspring, the 2-fold risk of MS associated with maternal vitamin D deficiency persisted after adjustment for EBV IgG levels and cotinine.

It has not previously been known whether in utero exposure to maternal EBV IgG Abs is associated with MS risk in the offspring, nor if there is an interaction with serum 25(OH)D and cotinine levels altering the risk. The association of EBV and

MS has been substantiated in multiple studies. Vitamin D status and many other factors continuously modulate the global MS risk, from pregnancy to MS triggering in adulthood and subsequent disease progression, whereas smoking is known to increase the risk of MS occurrence and disease progression in smoke-exposed subjects. These risk factors are also known to complexly interact with genes by reducing or adding to the risk of MS. HLA risk genes and IM seem to interact synergistically in increasing the risk of MS. An interaction between anti-EBV titers and the genes has been observed leading to a 16-fold higher risk for MS associated with abundance of HLA-DRB1*15 and absence of HLA-A*02 in combination with high EBNA 385-420 IgG titers compared to individuals who did not carry any of these factors (Sundqvist et al. 2012). An interaction between obesity and a history of IM during adolescence has been associated with an increased MS risk with an OR of approximately 14. This finding suggests that HLA risk alleles that encode molecules regulating T cell adaptive immunity might show common pathogenic pathways triggering MS when they interact with measures of EBV infection (Hedström *et al.*, 2015). A significant interaction between smoking and two genetic risk factors, carriage of human leucocyte antigen (HLA) DRB1*15 and absence of HLA A*02, has been described, suggesting that risk of MS disease associated with HLA genotypes may be strongly influenced by smoking (Anna Karin Hedström, Sundqvist, *et al.*, 2011).

In addition to evaluating a possible risk association between MS and EBV Abs during pregnancy affecting both mothers and offspring, we sought to examine whether our findings would be further confounded by maternal serum 25(OH)D and cotinine levels. We did not, however, evaluate Abs against CMV and possible impact on MS risk among the mothers. Only risk-association with EBNA1 was conducted since these Abs have been identified as a predictive marker among adults at the time of our study.

Prior study observations suggest that smoking is an independent risk factor for MS. Adult tobacco smokers of both sexes run an increased risk of developing MS compared to never-smokers and there is a dose-response correlation between MS risk and cumulative dose of smoking, which remains up to 5 years after smoking cessation (Hedström *et al.*, 2009). Serum cotinine has also been associated with a 1.5 OR increase in MS risk (Salzer *et al.*, 2013). Use of snuff over 15 years, on the other hand, decreases MS risk (Hedström *et al.*, 2009). Exposure to SHS is associated with an increased MS risk in non-smokers (A. K. Hedström *et al.*, 2011) and parental smoking has been associated with risk of MS in offspring (Mikaeloff *et al.*, 2007), whereas no risk increase of early onset MS among offspring exposed to parental smoking during pregnancy has been demonstrated (Montgomery et al. 2008). Mechanisms linking smoking to MS risk are speculated to involve lung inflammation with a proinflammatory profile, as the interaction with MS risk HLA genes argues

for an action on adaptive immunity, possibly by activation of auto-aggressive cells resident in the lungs subsequently attacking the CNS. Possible interactions between lung irritative agents and HLA genes with regard to MS risk have been described to be consistent with class II allele-specific recognition of autoantigenic peptides in the lungs, resulting in organ specific inflammatory disease. For MS, absence of A*0201 may result in autoreactive T cells persisting and launching an immune response against the self-antigen (Hedström *et al.*, 2018).

There are no previous studies on smoking prevalence among the Finnish MS population. Smoking habits and prevalence, demographics and perinatal and pregnancy outcomes among pregnant women in the FMC have been studied for the years 1987-2011 in 9,627 randomly selected pregnancies based on measured serum cotinine levels (≥4.73 ng/mL deemed high) and self-reported smoking status. Of the women, 7.7% undisclosed smoking but had high cotinine levels and 4.5% were inactive cigarette smokers, while 71.6% were non-smoker and 16.2% were active cigarette smokers. The prevalence of active cigarette smokers decreased from the mid-1990s onwards among women aged ≥30 years, presumably due to the ban on cigarette smoking in most workplaces (Männistö *et al.*, 2016). In a US study from 2006, smoking was not higher among MS patients than controls, whereas other and more recent studies have reported a higher prevalence of smoking among MS patients in Sweden, the USA and Norway (Nortvedt, Riise and Mæland, 2005; Friend *et al.*, 2006; Turner *et al.*, 2007; Hedström *et al.*, 2009; Hedström, Olsson and Alfredsson, 2016). Smoking is underrepresented, but also underreported, among pregnant women, making cotinine levels a more reliable study tool for evaluation of tobacco exposure (Dietz *et al.*, 2011; Jain, 2017). We did not have data on self-reported smoking, neither for women nor offspring in our study cohort.

Cotinine ($C_{10}H_{12}N_2O$), a by-product of nicotine ($C_{10}H_{14}N_2$) metabolism, is the most sensitive and specific biomarker for cigarette smoking and tobacco exposure widely used to distinguish smokers from non-smokers in epidemiologic studies (Jarvis *et al.*, 1987; Benowitz *et al.*, 2009). Serum cotinine levels do not distinguish smoking from other means of nicotine exposure, nor do they exclude simultaneous use of snuff and smoked tobacco that may have antagonistic effects on risk of developing MS (Hedström *et al.*, 2013). Presumably, snuffing is rare in the FMC cohort and unlikely to exert a significant impact on the MS risk estimation (0-1% of Finnish women are snuffers according to THL tobacco statistics from 2017). The magnitude of SHS exposure has declined in recent years due to proportionally fewer smokers and more clean indoor air regulations. In Finland, since 1994, the legal age to buy tobacco products was raised from 16 to 18 years, and the indirect advertisement of tobacco products and cigarette smoking indoors at workplaces (excluding restaurants), in governmental buildings and in public transportation was

banned. In 2004, indoor smoking was banned in restaurants, as well. The public display of cigarette products at shops was banned in 2010 (Männistö *et al.*, 2016).

In our study cohort, we could not evaluate the number of mothers who ceased to smoke during early pregnancy (an estimate of 49.2% in the general Finnish population in 2017, but half as high in 2007) (THL Tobacco Statistics, 2017). Smoking during pregnancy is associated with adverse birth outcomes, such as low birth weight, pre-maturity, neonatal mortality, childhood respiratory illnesses and abnormal nervous tissue development (Vyhlidal *et al.*, 2013). Breastfeeding has been associated with a decreased risk of MS in children in some studies (Conradi *et al.*, 2013; Ragnedda *et al.*, 2015). Cotinine is secreted in breast milk, and milk cotinine is positively associated with cigarette consumption, as well as inversely associated with the time since the last cigarette (Jacob, Golmard and Berlin, 2015). Maternal exposure to tobacco while breastfeeding has not been associated with MS risk in offspring (Graves *et al.*, 2017). In our study, we did not have data on breastfeeding, but given the previous study findings, offspring exposure to cotinine in milk is unlikely to have an impact on the results, while breast feeding regardless of cotinine levels may have had an impact on MS risk among offspring. Smoking cessation may have had an impact on MS risk in women not accounted for in this study, but given our study findings, this would not alter the MS risk association with EBV antibodies or 25(OH)D levels.

Racial and ethnic differences in cigarette smoking and in the rate of metabolism of nicotine and cotinine have been described previously. At the same daily level of cigarette smoking, lower serum cotinine concentrations are observed in whites than in blacks (Wagenknecht *et al.*, 1990; Caraballo *et al.*, 1998; Benowitz *et al.*, 2009). Since the majority of women in the FMC are Caucasian, our results may not be generalizable to females of other racial groups. However, results of previous studies in more diverse populations suggest that our findings can be extrapolated to Finnish men (DeLorenze *et al.*, 2006; Levin *et al.*, 2010) and possibly to other racial groups (A. K. Hedström *et al.*, 2011; Langer-Gould *et al.*, 2017). The cotinine cut points generally used are presumably highly applicable with the same reliability to the majority or all the subjects in our cohort of mainly Caucasian subjects.

In our study, there are some limitations to consider. EBV IgG antibodies were measured in serum samples collected mainly during the first trimester of pregnancy (70% of the samples). As cotinine is a short-term biomarker of smoking, we cannot rule out residual confounding or misclassification of women who were past smokers or light smokers as non-smokers. We did not have information on other MS risk factors, including obesity in early life, HLA status, breastfeeding, and a variety of demographic variables, including ethnicity or education level for either the offspring or mothers, though previous studies suggest none of these are confounders of the EBV-MS association (Levin *et al.*, 2005; Gianfrancesco *et al.*, 2014). Because

breastfeeding is unlikely to be associated with maternal antibody levels in early pregnancy, differences in breast feeding behavior do not provide a plausible explanation for the higher MS risk among offspring of mothers with higher VCA IgG levels.

6.1.3 EBV infection and risk of MS

In our study, a novel finding was the 2.5-fold increased risk for MS among offspring of mothers with high EBV VCA IgG Abs during pregnancy. Our observation that high EBNA-1 IgG levels were associated with MS risk in Finnish mothers confirms previous reports of increasing IgG Ab titers against EBNA being associated with an increased risk of MS in healthy adults (Ascherio and Munger, 2016). The association of EBV and MS was first suggested over 40 years ago (Tourtellotte, 1971) and has been substantiated in multiple subsequent studies (Ascherio and Munger, 2010; Owens and Bennett, 2012). Virtually all (99%) MS patients are seropositive for EBV (Pakpoor, Pakpoor, *et al.*, 2013), but EBV seropositivity is high (95%) also in non-MS subjects (Luzuriaga and Sullivan, 2010). In our study, 98.3% of offspring case mothers and 97.8% of control mothers were positive for EBV infection. For the mother cases and controls, 99.3% and 97.7% respectively, were seropositive for EBV in their pre-onset samples, yielding a nearly 3.5-fold increased MS risk associated with EBV seropositivity. Maternal Ab titers were measured an average of 9 years prior to their MS diagnosis, greatly reducing the probability of reverse causation.

Primary infection with EBV is a strong risk factor for MS (Levin *et al.*, 2010), and a history of IM more than doubles the risk of developing MS (Handel *et al.*, 2010). We did not have data on a history of IM (typically a manifestation of primary EBV infection during adolescence or young adulthood), neither for the mothers, nor for the offspring in the cohort. The strengths of our study include the utilization of a population-based cohort; the FMC includes 98% of all pregnancies in Finland since 1983. Identifying MS cases via the nationwide hospital and prescription registries also minimized selection bias. The prospective nature of the study is a considerable strength, as exposure to maternal EBV IgG Abs necessarily occurred prior to the offspring developing MS.

6.1.4 Could higher maternal EBV antibodies postpone the EBV infection in the offspring

Primary infection with EBV is a strong risk factor for MS (Levin et al. 2010). In healthy adults, increasing IgG Ab titers against the EBNA have been associated with an increased MS risk (Ascherio and Munger, 2016). Increased Ab titers could be

speculated to be the consequence of a poor elimination of the pathogen through cell-mediated immunity (analogous to observations in JC-virus carriage wherein high antibody titers show a poor control over viral replication) (Sundqvist *et al.*, 2014).

Since viral Abs, including those against EBV, are known to cross the placenta during pregnancy (Gotlieb-Stematsky *et al.*, 1983), they may potentially delay the age at primary EBV infection in the child by conferring temporary protection. This could possibly alter the child's immune response to EBV infection and thus MS risk. EBV has oncogenic properties increasing the risk of certain cancers, in particular among immunocompromised individuals. A prior FMC study showed an association between higher maternal VCA IgG Ab levels and increased risk of testicular cancer in their male offspring (Holl *et al.*, 2008). Maternal EBV Abs may potentially affect the risk of other long-term diseases in offspring. One prior study found that among 66 infants with maternal EBV IgG Abs at birth, 12% continued to have detectable Abs after 4 months, and half of these infants became infected between 20-24 months old while half remained uninfected by EBV through their second year (Chan *et al.*, 2001). In our FMC study on EBV and MS risk, we showed that maternal VCA IgG levels during pregnancy were associated with MS risk among offspring. A one standard deviation increase in maternal VCA IgG index value was associated with a 34% increased MS risk in the offspring, and in quintile analyses, children of mothers in the highest quintile of VCA IgG index value had a 2-fold increased risk of MS occurrence. No association was observed with EBNA-1 IgG, EA-D IgG or CMV IgG index values.

The biological mechanism behind this observation is uncertain. One possibility is that the child's own immune response to a primary EBV infection is delayed if the offspring of mothers with high VCA IgG are exposed to higher maternal VCA IgG during pregnancy. One hypothesis is that an "older" child may be prone to a more aberrant immune response to primary EBV infection than a "younger" child once the maternal protection wanes, and this may predispose them to MS. An alternative explanatory hypothesis is that shared genetics (e.g. HLA haplotypes) between the mother and child influence their immune response to EBV such that the mother's elevated VCA IgG levels may be an indicator of an elevated Ab response to EBV in the offspring.

Although current study findings suggest an association, little is still known about how maternal Abs may influence the child's immune response to EBV, and how this affects the risk of chronic diseases in the offspring.

6.1.5 Potential for EBV vaccination

EBV infection has been associated with morbid conditions such as MS and cancer. The absence of an anti-EBV drug might be partly explained by the difficulty in

diagnosing IM (long incubation time of 4-6 weeks), difficulty in achieving high antiviral concentrations in the oropharynx where the EBV is released at high titers, and the fact that symptoms and signs of the disease are not the consequence of viral replication but the immunological response to circulating EBV-infected B cells and infiltrating tissues of different organs. Potential antiviral therapeutics, including vaccines, to treat or prevent EBV related diseases are under evaluation, but are still not approved in the clinic for treatment of EBV infections (Andrei, Trompet and Snoeck, 2019).

There are few studies examining whether maternal EBV Abs are associated with longer-term chronic diseases in the offspring. In prior FMC studies, higher maternal VCA IgG Ab levels showed an association with a 2.5-fold increased risk of testicular cancer among male offspring (Holl et al. 2008), whereas maternal EBV Abs were not associated with acute lymphoblastic leukemia in the offspring (Tedeschi et al. 2009). It is possible that a future EBV vaccine with excellent coverage providing lifelong protection against EBV infection could prevent chronic diseases such as MS and cancers, presumably if targeted at a very early age. This kind of vaccine would hold the potential of being beneficial in subjects genetically susceptible to MS.

However, EBV prevention is hard to achieve, since a safe and efficient vaccine is difficult to create. A vaccine with only partial efficacy in prevention of this large group of highly pertinent herpesvirus that latently hides in circulating B lymphocytes is accompanied with risks. Even if EBV seems to be critically involved in MS development, a future EBV vaccine of modest effectiveness might only postpone the time of primary EBV infection and thus potentially increase the MS risk, since EBV infection in adolescence appears to be associated with a higher risk of developing MS. Treatment and prevention of chronic diseases associated with the globally highly abundant EBV virus that is well adapted to coexistence with its human host, causing no apparent disease in the vast majority of individuals with normal immunity, still calls for rigorous research clarifying the role of EBV in MS. Means to identify target groups that could benefit from a vaccine would need to be developed, and extensive research is warranted on how and when a possible vaccine should be targeted and timed to prevent the development of MS without compromising safety.

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

In our 24 months follow-up study on efficacy and safety outcomes in vitamin D supplement users among RRMS patients in the pivotal fingolimod phase 3 trials, we showed that vitamin D is safe, and vitamin D ´daily users´ had significantly better

MRI outcomes compared to ´non-users´. Among daily users a higher proportion of patients were free of new/enlarging T2 lesions compared to non-users. The mean number of MRI lesions was lower, and the proportion of patients free of Gd-enhancing lesions was higher in daily users. PBVC was significantly lower at month 12 and remained non-significantly lower at month 24 for the daily vitamin D users compared to non-users. The incidence of depression as an AE was lower among daily vitamin D supplement users (5.5%) compared to non-users (11.9%), although this finding did not reach statistical significance. Other AEs (incidence of infections) were equally frequent in the groups, supporting previous findings of vitamin D safety. The clinical outcome measures EDSS change from baseline, ARR and proportion of patients with relapses were similar across the vitamin D user groups.

The effect of vitamin D in MS patients treated with fingolimod has not been studied previously, and we found only one previous study assessing the effect of serum vitamin D levels on MS activity by DMT class including fingolimod, which showed a reduction in new inflammatory events and in relapses alone per 25(OH)D tertile increase among patients treated with fingolimod (Rotstein, Healy, Malik, Carruthers, *et al.*, 2015). Studies on beneficial effects of vitamin D supplementation in established MS have been controversial, but previous studies on vitamin D supplementation as an add-on treatment to IFN have yielded some positive results. In a phase 2 placebo-controlled randomized trial with vitamin D as an add-on therapy to IFN-beta, beneficial effect was observed on MRI outcomes (significantly less Gd-enhancing T1 lesions), but not on relapses or EDSS progression for patients randomized to receive vitamin D at a dose of 20,000 IU/week (Åivo, Lindsröm and Soilu-Hänninen, 2012). A large vitamin D add-on to IFN-beta trial, the SOLAR study, met the primary MRI endpoints, and showed a 30% reduction in relapse rate, but the efficacy of vitamin D on relapses and other clinical endpoints was not statistically significant (Grimaldi *et al.*, 2012). In the Benefit trial, low serum 25(OH)D levels were associated with worse clinical and MRI outcomes in MS patients treated with IFN-beta 1b (Ascherio *et al.*, 2014). The BEYOND-trial, in which a higher dose of 500 ug was compared to the standard dose (250 ug) of IFN-beta 1b in 1482 patients, higher 25(OH)D levels were associated with lower rates of MS activity observed on MRI such that a 50 nmol/L increase in serum 25(OH)D levels was associated with a 30% lower rate of new lesions and the lowest rate of new lesions was observed among patients with 25(OH)D levels greater than 100 nmol/L. Results for brain atrophy and clinical progression were equivocal (Fitzgerald *et al.*, 2015). There is less evidence of vitamin D effect as an add-on to other first line DMTs than IFN and for second line DMTs in particular (James *et al.*, 2013; Berezowska, Coe and Dawes, 2019). It has been suggested that IFNs and vitamin D may have synergistic effect and observations of vitamin D efficacy in MS patients using IFN-beta would therefore not be generalizable to other DMTs (Stewart

et al., 2012). In support of this, the same study that showed beneficial effect of vitamin D serum levels on MS activity in the DMT subgroups for fingolimod (77 patients) and IFN-beta, did not find any effect of vitamin D in the subgroup treated with GA (Rotstein, Healy, Malik, Carruthers, *et al.*, 2015). The VIDAMS trial is assessing the efficacy of vitamin D as an add-on to GA and will hopefully provide more conclusive information (Bhargava *et al.*, 2014).

A recent publication has showed that in patients treated with fingolimod, those with the highest baseline 25(OH)D levels had a significantly lower number of active lesions at baseline MRI and the same effect, although weaker, was observed also at a 2-year follow-up when adjusting for baseline disease activity. No linear correlation between baseline 25(OH)D levels and ARR or time to first relapse was found, but patients with vitamin D levels of ≥100 nmol/L showed a lower number of Gd-enhancing and combined unique activity (CUA) lesions at baseline compared to patients with the lowest 25(OH)D levels (< 50 nmol/L, p-value <0.05), as well as fewer CUA lesions at 2-year follow-up also when accounting for baseline disease activity level (Ferré *et al.*, 2018). MRI is highly sensitive for detecting disease activity and new T2 and Gd enhancing T1 lesions may occur sub-clinically. These are seen more frequently than clinical relapses (Barkhof *et al.*, 1992; Barkhof, 2002) and since there are previous observations of a strong correlation between therapy effect on active lesions and relapse rate (Sormani *et al.*, 2011), MRI may serve as a surrogate marker for relapses.

In a study that assessed the mechanistic rationale that may explain potential clinical effects of vitamin D in MS, vitamin D was found to regulate expression dynamics of a large gene-gene interaction system which primarily regulates immune modulatory processes modulating MS activity. Targets of IFN-beta and a regulator of sphingosine-1-phosphate bioavailability were found among the vitamin D regulated genes. More specifically, vitamin D regulated SGPP1, a gene that encodes for an enzyme that catalyzes the degradation of S1P. Degradation of the S1P receptor decreases receptor availability to lymphocytes and thus acts in the same direction as fingolimod, providing a potential beneficial mechanism of action of vitamin D alongside fingolimod (Munger *et al.*, 2014).

Vitamin D supplementation may be beneficial in MS comorbid conditions, such as depression (Shaffer *et al.*, 2014), diabetes mellitus (Nakashima *et al.*, 2016), infections (Hewison, 2011), respiratory tract infections in particular (Bergman *et al.*, 2013; Martineau *et al.*, 2019) and cancer (Bjelakovic *et al.*, 2014). Potentials for fingolimod and vitamin D in cancer treatment have been described. Fingolimod reverses the inhibitory effect of S1P on NK cell lysis of tumor target cells or dendritic cells (DCs) attributed to by binding S1P1 on NK cell surface (Rolin *et al.*, 2010; Pyne and Pyne, 2013). Vitamin D3 activates NK cells to kill melanoma cells, an activity related to induction of apoptosis (Lee *et al.*, 2011). In vitro studies for

fingolimod, the biologically active D3 metabolite and calcitripotriol, showed that they augment IL-2-activated NK cell lysis of certain tumor cell lines (K562 and RAJI), as well as immature and mature DCs, observations that may be potentially used for treating MS or other autoimmune diseases in the future (Al-Jaderi and Maghazachi, 2013). Since fingolimod has an immunosuppressive effect due to the reduction of circulating lymphocytes, intrinsic cancer surveillance may also be impaired. Fingolimod AEs include increased rates of viral infections such as VZV, and also chronic, treatment refractory human papilloma virus (HPV) warts have been described. Thus, risk of cancers associated with HPV may be increased in fingolimod treated patients (Triplett *et al.*, 2018).

Depression is the most common co-morbidity with the greatest impact on quality of life in people with MS (D'Alisa *et al.*, 2006; Taylor *et al.*, 2014). An inverse association between serum 25(OH)D levels and depression risk has been shown in a meta-analysis comprising 11 cross-sectional studies in over 400,000 mostly elderly non-MS patients (Ju, Lee and Jeong, 2013), whereas an association of depression with vitamin D intake and serum 25(OH)D levels is less established in MS patients (Taylor et al. 2015). One objective of our study was to assess the reported depression among vitamin D supplement users and non-users. Depression as an AE was lower among the fingolimod treated patients who were vitamin D users compared to non-users.

A major caveat of our study was that we did not know serum levels of 25(OH)D in the study subjects. Confounding factors, such as smoking (a risk factor for worse clinical outcomes in MS), genetic factors, ethnicity, socioeconomic status and differences in lifestyle, precise latitude and previous DMT were not evaluated. Age, gender, BMI, MS duration since diagnosis, and EDSS and relapse rate at baseline, were included in the baseline characters. BMI and T2 lesion volume were significantly lower for baseline characters in the vitamin D daily user subgroup compared to non-users. Some of these confounding factors may have altered our results and our findings might not be generalizable to all ethnic groups. We did not evaluate cancer incidence in this short-term follow-up. Vitamin D supplementation RCTs are mandatory for future vitamin D recommendations in MS, and due to vitamin safety and potential mechanisms of action in demyelinating disease (Lucas, A-L Ponsonby, *et al.*, 2011), it could in particular be interesting in a target group of RIS subjects (a patient group known to be affected with subclinical symptoms of cognitive impairment), and since there is still no evidence-based support for benefit or safety for initiation of DMTs used for MS. In future Phase 3 studies, considering the accumulated evidence pointing to the benefits of vitamin D with low risks and costs, it may become difficult to randomize patients to not receive any vitamin D supplementation in a placebo arm.

6.3 Environmental factors and risk of cancer among MS patients

In this nested case-control study we assessed cancer risk among Finnish MS patients in the hospital district of Southwest Finland during the DMT era from 2004 to 2013. We confirmed similar findings as in the pre-DMT era for the same population (Sumelahti, Pukkala and Hakama, 2004). The overall cancer risk in the MS cohort did not significantly differ from the controls in the same hospital district. The age at BC diagnosis was statistically significantly higher among MS patients than controls, but BC risk did not significantly differ between the groups. Numbers in other cancer subgroups were small, preventing us from drawing further conclusions.

Previous studies on cancer risk among the MS population have been inconsistent, which might reflect the variability of different populations, time of analysis and variability in the MS pharmacotherapies used.

In Finland, the most common cancer in women is BC and in men prostate cancer (Pukkala and Rautalahti, 2013; The Finnish Cancer Registry, 2019). Risk for prostate cancer in our MS cohort was also decreased compared to controls and is in line with a majority of previous study observations (Kyritsis, Boussios and Pavlidis, 2016), although numbers in our study were small.

Our findings of higher age at BC diagnosis among MS patients compared to controls could speculatively be linked to a protective effect of a more active immune system. An alternative explanation is a negative surveillance bias caused by neglect of the patients' symptoms and complaints other than the MS-related ones (there was no data on the BC tumor size at diagnosis to support this speculation), or reluctance of patients to attend the screening mammography in addition to the follow-up burden caused by frequent MS clinic visits. The difference at age of BC was as long as six years, making the latter explanation less likely.

Increased BC risk has been associated with smoking in several recent studies (Olson *et al.*, 2005; Cui, Miller and Rohan, 2006; Nagata *et al.*, 2006; Ha *et al.*, 2007; Xue *et al.*, 2011; Deroo, Cummings and Mueller, 2011; Luo *et al.*, 2011; Bjerkaas *et al.*, 2013; Rosenberg *et al.*, 2013; Gaudet *et al.*, 2013; Dossus *et al.*, 2014; Jones *et al.*, 2017). Data on smoking was scarce, but 44.5% of the BC patients in our MS cohort were non-smokers and for 50% data was not available. The percentage of smokers (5.6%) was not lower than reported in the elder nationwide Finnish female population (5%), but lower than for smoking Finnish females aged 20-64 years (13%) (THL tobacco statistics 2017). The reported low rate of smoking among MS BC patients could contribute to the slightly lower BC rate as well as the higher age at diagnosis. The MS patients in our cohort had mostly used platform MS therapies, and BC patients in our MS cohort received only IFN or GA, but the patient numbers were too small to draw any conclusion on the possible impact of DMTs on cancer risk in our cohort.

In the ocrelizumab Phase 3 studies, an equal or increased risk of BC among the RRMS population and a signal for an increased risk among the PPMS patients were observed (Hauser *et al.*, 2017; Montalban *et al.*, 2017). If the non-significant trend of a lower baseline BC risk among MS patients observed in our study is true, then even an equal risk for BC among patients receiving ocrelizumab, as compared to IFN and placebo, could suggest that there is an increased BC risk associated with ocrelizumab exposure. Evaluation of cancer risk in the MS population remains relevant in the era of new and emerging DMTs and in the evaluation and management of their potential long-term safety profiles.

We found trends of increased risk for brain cancer, oral cavity cancer, renal cancer, lung cancer, colon cancer in our MS cohort. The increased risk of brain cancer, in line with observations prior to the DMT era (Sumelahti et al. 2004), may reflect a surveillance bias from a more frequent routine MRI screening of MS patients (Kingwell et al 2012). Patient numbers in cancer subgroups that displayed increased risk in our MS cohort were very small. The increased rate of renal and colon cancer in our MS cohort might reflect a more common referral policy to urologists and gastroenterologists for MS-related symptoms and dysfunctions. Increased risk for oral cavity cancer, lung and thyroid cancer could reflect a shared risk factor for MS and cancer such as smoking, or possibly reflect a more frequent routine of X-ray imaging related to disease exacerbations and screenings for underlying infections. Among the small number of MS patients diagnosed with brain cancer, renal cancer, lung cancer and oral cancer in our cohort we found that 40% were smokers, 30% non-smokers and for 30% data was not available. This percentage of smokers was higher than the percentage of smokers in the Finnish population in general, and it could therefore be speculated that smoking rather than MS itself had an impact on the increased rates of these cancer subtypes (except for brain cancer, which has not been associated with smoking), although data on smoking for the control cohort was not available.

Our MS cohort was reasonably large, representing about 15% of the total Finnish MS population, but for the detection of rare and slowly developing diseases such as cancers, large population-based studies spanning decades of follow-up are necessary. The number of cancer patients in the MS cohort was small (n = 61) and for specific cancer subgroups even smaller, making statistical conclusions uncertain. Data on ethnicity was not available, but the southwestern Finnish population is mainly Caucasian and study findings may not apply to other ethnicities and geographical areas. Our results were not confounded by complicated histories of different DMTs, but the numbers were also too small to make any conclusion on impact of DMTs on cancer risk possible. Another caveat of our study was also the scarce information on possible confounding factors that may alter the risk of both MS and cancer, such as parity, smoking, vitamin D status, hormonal replacement

therapy and other medications, alcohol abuse, BMI, family history of MS and cancers and socioeconomic status.

Cancer deaths are overrepresented in at least part of the Finnish MS population (Sumelahti *et al.*, 2002), which might be attributable to the longer life expectancy of MS patients. Cancer diagnosis in our cohort was preceded by MS diagnosis in the majority of our cohort, which is in line with the known fact that MS is predominantly a disease of young adults, whereas the risk of cancer occurrence increases over time. Risk factors such as obesity, low parity and smoking need to be documented and taken into account in population studies. There are studies suggesting a possible anti-cancer activity of TFR on aggressive subtype tripe-negative BC and non-small cell lung cancer (Huang *et al.*, 2015; Jiang *et al.*, 2018). DMTs with possible cancer protective properties, including fingolimod, also warrant further studies on this potential.

7 Conclusions

The purpose of this thesis was to investigate whether pre-diagnostic serum 25(OH)D levels during pregnancy are associated with later MS risk in the mother and her offspring. We investigated whether maternal serum EBV antibodies during pregnancy are likewise associated with MS risk in either mother or child, and whether there is an association independent of serum 25(OH)D levels and cotinine. Furthermore, we performed datamining and post hoc analysis of pooled data from the Phase 3 FREEDOMS trials to assess whether vitamin D supplementation is beneficial and safe combined with fingolimod in the treatment of RRMS patients. We also updated data on cancer risk among MS patients in southwestern Finland in the era of DMTs, compared to controls from the same hospital district. The conclusions based on the results presented in this thesis are as follows.

I. We showed that maternal vitamin D deficiency during early pregnancy doubled the risk of MS risk in the offspring. Correcting the mother's vitamin D deficiency prior to or during pregnancy is likely to have a beneficial effect on the risk of MS in her child.

   We further showed that maternal vitamin D deficiency during pregnancy was associated with a 43% higher risk of MS when compared to women with sufficient levels in the cohort, whereas a MS risk reduction of 39% was associated with a 50 nmol/L increase in 25(OH)D levels. Vitamin D-deficient women with multiple pre-diagnostic samples available had a 2-fold higher MS risk as compared to women with sufficient 25(OH)D levels. The results support vitamin D deficiency as a risk factor for MS and strengthen the rationale for broad public health interventions to improve vitamin D levels, especially in pregnant women and subjects with manifest MS or disease susceptibility. Correcting vitamin D deficiency and insufficiency to sufficient levels is likely to be safe, cost-effective and to have multiple potential health benefits.

II. Women with levels of cotinine >25 ng/ml (smokers) had a 45% increased risk of MS as compared to women with cotinine levels of <10 ng/ml (non-

smokers) confirming smoking as a risk factor for MS also in the Finnish population.

III. We observed that offspring of mothers with high Epstein-Barr VCA IgG during pregnancy have an increased risk of MS that has not been previously observed. The risk of MS was 2.5-fold increased among offspring of women with the highest levels (top quintile) versus the bottom quintile. No elevated risk for MS in the offspring was associated with maternal IgG levels against EBNA-1, EA-D or cytomegalovirus. Among the women with increased pre-diagnostic EBNA-1 IgG levels, we observed a 3-fold higher MS risk in the highest versus lowest quintile, a finding that is consistent with previous study results. These associations were not confounded or modified by maternal serum 25(OH)D or cotinine levels, which has not been demonstrated previously.

IV. We found that Vitamin D supplementation as an add-on therapy to fingolimod for MS was safe in the FREEDOMS study population pool, showing beneficial outcomes on MRI T2 lesions and PBVC of statistical significance in vitamin D users compared to non-users during a 2-year follow-up. Depression as an AE was non-significantly lower among supplement users compared to non-users. These findings support a beneficial role of D vitamin supplementation in RRMS patients treated with fingolimod. Supplementation may also be beneficial in depressive co-morbidity.

V. We observed that the overall risk of cancer among MS patients in southwestern Finland was equal to controls from the same hospital district. Age at BC diagnosis was significantly higher and there was a slight trend of lower BC risk among MS patients compared to the control patients. Impact of smoking and DMTs on cancer risk could not be evaluated due to small patient numbers in cancer subgroups and incomplete data on lifestyle factors, including BMI, smoking and vitamin D supplementation.

As an overall conclusion and recommendation based on our results, we propose correcting vitamin D deficiency during pregnancy at the population level and in particular in pregnancy planning for MS patients. In all MS patients, we recommend analyzing 25(OH)D levels after MS diagnosis and initiation of vitamin D supplementation targeting sufficient levels. Based on previous study findings regarding safety and detectable effects on MS disease activity in the Finnish population, a vitamin D dose of 50 to 100 ug/day, targeting serum 25(OH)D levels above 100 nmol/L could be beneficial for MS patients. Serum levels should preferably be re-evaluated after initiation of vitamin D supplementation to confirm compliance and achievement of targeted levels. Correcting for vitamin D deficiency

is likely to be beneficial in both pregnant women and their offspring also from a broader health perspective. Maternity care units could be suitable for screening, initiation of supplementation as well as for follow-up of vitamin D-deficient pregnant women in this aspect. Elevated maternal viral Abs in serum may traverse the placenta and protect the child from early EBV infection. This could possibly delay primary EBV infection in the child such that MS risk is altered. Rigorous future studies are needed before a potential EBV vaccine could be targeted at MS prevention. Whether or not smoking during pregnancy affects the long-time risk of offspring developing MS, as evaluated by exposure to elevated cotinine levels in utero should be further assessed. Future studies clarifying whether different forms of lung irritation, such as air pollution triggers, contribute to MS occurrence, as well as studies clarifying the mechanisms of the smoking related MS risk are warranted.

Furthermore, given the vitamin D supplementation safety profile, MS patients treated with fingolimod could probably benefit from 25(OH)D levels above those currently considered to be sufficient for bone health, also if a causal effect has not yet been shown. Our findings suggest a possible beneficial impact of vitamin D supplementation, not only in MS disease activity, but also on depression and consequently, quality of life.

MS patients in southwestern Finland do not have an increased, nor decreased, risk for cancers compared to non-MS patients. Further population-based studies spanning longer follow-up periods are needed to confirm the MS patient's cancer risk during the era of evolving MS treatments. Risk factors shared by MS and other comorbidities, such as cancer, remain highly relevant, and smoking should be actively screened for and cessation encouraged for the population in general and MS patients in particular.

Acknowledgements

This study was carried out at the Division of Clinical Neurosciences in the University of Turku and Turku University Hospital during 2011-2019.

I wish to express my sincere gratitude to the following people:

Professor Risto O. Roine, the head of the Division of Clinical Neurosciences, for the possibility to conduct this study in combination with clinical work at Turku University Hospital.

Docent Merja Soilu-Hänninen, my supervisor, who has not only taught me the principles of research work, but who has been a true role model in so many ways. You have encouraged and inspired me tremendously in my career. Thank you for efficiently providing visions and ideas, instant response and feedback. Thank you so much for helping me focus on my work. Without your patience and excellent guidance, finishing this project would not have happened.

Docent Matias Röyttä and Docent Juha-Pekka Erälinna, members of the follow-up committee, thank you for all your support. A special thank you to Docent Juha-Pekka Erälinna for proposing a brilliant supervisor to me. My deep and sincere gratitude goes to Merja for not saying no.

Professor Markus Färkkilä and Docent Mikko Kallela, the official reviewers of this thesis, thank you for all your time and constructive feedback. I also want to thank Gail Eriksson Hallberg for English revision of this thesis.

Members of the FMC study group Professor Alberto Ascherio, Docent Heljä-Marja Surcel, Research Scientist Kassandra Munger, MD, PhD, ScD, Julia Åivo, MD, PhD, and Marianna Cortese, MD, PhD, thank you for your skilled work.

Docent Marja-Liisa Sumelahti, MD, PhD, Samu Kurki, PhD, thank you for all your expertise and patience. A special thank you to Samu for getting back at the statistics ever again. Diego G. Silva, Shannon Ritter, Daniela Piani Meier, thank you for your collaboration. I am so privileged to have had all of you as my co-authors. A special thank you to Katariina Hänninen, MD, for review of patient charts.

Thank you to all the patients who participated in this study.

I have had the privilege to work with splendid colleagues and friends at the Department of Neurology in Turku University Hospital, Åland Central Hospital and in Skåne Region in Sweden. Thank you each and every one. Thank you to Christer

Nilsson for welcoming me at Skåne University Hospital. Thank you to the "MS-team" for inspiration and support, especially to my friend Jessika Nystedt.

Julia Åivo, Riina Katainen, Henry Karlsson, Anna Östberg and executive secretary Leila Laakso-Kantonen, thank you for your help and assistance in the dissertation process.

The original publications were supported by grants from The Finnish Medical Foundation, The Maire Taponen Foundation, The Perklén Foundation and The Cultural Foundation of Åland, thank you for your help.

I especially thank my dearest friends and family for all your support. My mother Rita Hongell, most of my love and respect for living beings and my will to be and do my best comes from you. Thank you for everything you have done and do. My father Harri Joensuu, thank you for all your love, support, acceptance, humor and just being there. My sisters Petra and Mikaela, l don´t even know where to begin nor where to end. If laughter lengthens life, then all the laughter we have shared will give me endlessly of time to figure out the words I wish to express. Thank you BFF Camilla Holmén, you are my rock.

Last but not the least, I am endlessly thankful to my husband Viktor and for our family. Thank you for your love, patience, support, courage, versatility and for not running out of solutions when I do. And how could I in words ever express the gratitude I feel for the most precious treasure and teacher in this life, our son Finn. With you I cherish every second.

Malmö, January 2020
Kira Åkerlund

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UNIVERSITY OF TURKU

Painosalama Oy, Turku, Finland 2020

ISBN 978-951-29-7962-2 (PRINT)
ISBN 978-951-29-7963-9 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)