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# MULTIPLE SCLEROSIS AND PREGNANCY: CLINICAL AND IMMUNOLOGICAL ASPECTS

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# ABSTRACT

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**Multiple sclerosis and pregnancy: clinical and immunological aspects**
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**Background:** Multiple Sclerosis (MS) is an autoimmune disease of the central nervous system that affects most commonly young women in their childbearing age. Previous studies have shown that MS relapse rate usually reduces during pregnancy and increases again after delivery. Patients with MS and their treating physicians are interested to know more about the risks the disease can cause to pregnancy and how pregnancy affects the disease. The reasons for increased relapse rate after delivery are not entirely clear, but loss of pregnancy related immune tolerance and changes in the hormonal status at the time of delivery seem to be of relevance.

**Aims and methods:** The aims of this study were to follow the natural course of MS during and after pregnancy, evaluate pregnancy related risks among MS patients, follow the inflammatory response of MS patients during and after pregnancy and clarify the risk of relevant co-morbidities known to affect other autoimmune diseases after pregnancy and compare these results to healthy controls.

This study was a part of a prospective nation-wide follow-up study of 60 Finnish MS patients. All eligible MS patients were enrolled in the study during the years 2003-2005. A prospective follow-up continued from early pregnancy until six months postpartum. MS relapses, EDSS scores and obstetric details were recorded. Blood samples were obtained from the patients at early, middle, and late pregnancy, after delivery and one month, three months and six months postpartum.

**Results:** MS patients were no more likely to experience pregnancy or delivery complications than the Finnish mothers in general. The need of instrumental assistance, however, was higher among mothers with MS. Disease activity followed the course seen in previous studies. The majority of mothers (90.2%) breastfed their babies. Contrary to previous results, breastfeeding did not protect MS patients from disease worsening after delivery in present study. Mothers with active pre-pregnancy disease chose to breastfeed less frequently and started medication instead.

MS patients presented with higher prevalence of elevated thyroid autoantibodies postpartum than healthy controls, but the rate of thyroid hormonal dysfunction was similar as that of healthy controls. The mode of delivery nor the higher rate of tissue damage assessed with C-reactive protein concentration were not predictive of postpartum relapses. The prevalence of gestational diabetes was slightly higher among mothers with MS compared to Finnish mothers in general, but postpartum depression was observed in similar rates. MS patients presented with significantly lower serum concentrations of vitamin D during pregnancy and postpartum than healthy controls.

**Conclusions:** Childbearing can be regarded as safe for mothers with MS as it is for healthy mothers in general. Breastfeeding can be recommended, but it should be done only after careful evaluation of the individual risk for postpartum disease activation. Considering MS patients tend to develop thyroid antibody positivity after delivery more often than healthy controls and that certain treatments can predispose MS patients to thyroid hormonal dysfunction, we recommend MS mothers to be screened for thyroid abnormalities during pregnancy and after delivery. Increased risk for gestational diabetes should be kept in mind when following MS mothers and glucose tolerance test in early pregnancy should be considered. Adequate vitamin D supplementation is essential for MS mothers also during pregnancy and postpartum period.

**Keywords:** Multiple sclerosis, pregnancy, relapse rate, breastfeeding, autoimmune thyroiditis, vitamin D

# TIIVISTELMÄ

Anna Jalkanen
**Raskaudenaikainen MS-tauti**
Neurologia, kliininen laitos, Turun yliopisto

**Tausta:** Multippeliskleroosi eli MS-tauti on yleisin nuorten aikuisten rappeuttava neurologinen sairaus, jonka oireet aiheutuvat demyelinaation ja aksonivaurion aiheuttamasta keskushermoston vioittumisesta. MS-tauti diagnosoidaan yleisimmin 20-45 -vuotiaana ja naisilla se on yli kaksi kertaa yleisempi kuin miehillä. Suurelle osalle MS-tautipotilaista perheenlisäysasiat ovat ajankohtaisia, ja potilailla ja heitä hoitavilla neurologeilla ja obstetrikoilla on tarve tietää miten sairaus tulee käyttäytymään raskauden aikana ja sen jälkeen ja miten sairaus mahdollisesti vaikuttaa raskauden kulkuun ja tulevaan jälkeläiseen. Aiempien tutkimusten perusteella tiedetään, että MS-taudin pahenemisvaiheet vähenevät raskauden aikana ja lisääntyvät synnytyksen jälkeen. Syitä tähän MS-taudille tyypilliseen taudinkulkuun ei täysin tunneta, mutta muutoksilla äidin immuunijärjestelmässä ja hormonipitoisuuksissa raskauden aikana ja synnytyksen yhteydessä ajatellaan olevan vaikutusta.

**Tavoitteet ja menetelmät:** Tämän tutkimuksen tavoitteena oli tutkia MS-taudin luonnollista kulkua raskauden aikana ja sen jälkeen, arvioida MS-tautia sairastavien äitien raskausaikaan ja synnytykseen liittyviä riskejä ja selvittää muissa autoimmuunisairauksissa esiintyvien raskauteen liittyvien liitännäissairauksien riskiä MS-potilailla verrattuna terveisiin äiteihin.

Tämä tutkimus oli osa suomalaista valtakunnallista raskaana olevien MS-potilaiden seurantatutkimusta, johon osallistui 60 raskaana olevaa MS-tautia sairastavaa äitiä vuosina 2003- 2005. Tutkimuspotilaita seurattiin kliinisesti raskauden alusta puoli vuotta synnytyksen jälkeen. Tautiaktiivisuutta seurattiin pahenemisvaiheiden ja EDSS-pisteiden avulla ja obstetriset muuttujat rekisteröitiin. Tutkimuspotilaista otettiin laboratorionäytteitä raskauden alussa, keskivaiheilla ja lopussa, 1-3 päivää synnytyksen jälkeen sekä yksi, kolme ja kuusi kuukautta synnytyksen jälkeen.

**Tulokset:** MS-tautia sairastavilla äideillä ei esiintynyt raskauteen tai synnytykseen liittyviä komplikaatiota sen enempää kuin suomalaisilla äideillä keskimäärin, mutta imukuppiavustuksen tarve synnytyksen yhteydessä oli heillä keskimääräistä suurempi. MS-taudin aktiivisuus muuttui samalla tavalla kuin aiemmissakin tutkimuksissa on osoitettu. Suurin osa (92 %) MS-tautia sairastavista äideistä imetti. Äidit, joiden MS-tauti oli ollut aktiivisempi ennen raskautta, joutuivat luopumaan imetyksestä lääkityksen takia muita useammin.

Kilpirauhasvasta-ainepositiivisuus oli MS-tautia sairastavilla äideillä synnytyksen jälkeen yleisempää kuin terveillä, mutta varsinaista kilpirauhasen hormonaalista toiminnanhäiriötä esiintyi yhtä paljon kuin terveillä äideillä. Synnytykseen liittyvää kudosvauriota arvioitiin mittaamalla C-reaktiivista proteiinipitoisuutta (CRP) pian synnytyksen jälkeen. Korkeamman CRP:n ei kuitenkaan todettu altistavan MS-potilaita synnytyksen jälkeisille pahenemisvaiheille. Raskausdiabetesta esiintyi MS-äideillä hiukan useammin kuin terveillä. MS-potilailla ei todettu suurentunutta riskiä synnytyksen jälkeiseen masennukseen. MS-potilaiden d-vitamiini–pitoisuudet olivat raskauden aikana merkittävästi matalampia kuin terveillä kontrolleilla ja 80 %:lla MS-potilaista d-vitamiinipitoisuus oli seuranta-aikana alle suositusten.

**Johtopäätökset:** MS-tautia sairastavien äitien raskauteen tai synnytykseen ei liity sen isompia riskejä kuin terveilläkään äideillä. Imetystä kannattaa suositella myös MS-äideille, mutta se ei saa olla esteenä asianmukaiselle immunomoduloivalle hoidolle sitä tarvittaessa. MS-tautia sairastavilla äideillä näyttää olevan taipumusta kehittää kilpirauhasen autoimmuunivasta-aineita synnytyksen jälkeen herkemmin kuin terveillä äideillä. Koska myös tietyt MS-tautiin käytettävät hoidot lisäävät riskiä kilpirauhasen toimintahäiriöille, MS-tautia sairastavia äitejä olisi hyvä seurata kilpirauhasen toiminnan suhteen raskauden alussa ja synnytyksen jälkeen. CRP-tasolla synnytyksen jälkeen ei ole ennusteellista arvoa MS-taudin aktiivisuuden mittarina. Hieman suurentuneen raskausdiabeteksen riskin vuoksi MS-äideille olisi hyvä tehdä sokerirasitustesti raskauden aikana. Riittävään d-vitamiinin saantiin on syytä kiinnittää erityistä huomiota MS-potilaan raskauden aikana ja synnytyksen jälkeen.

**Avainsanat:** MS-tauti, pesäkekovettumatauti, raskaus, imetys, autoimmuuni kilpirauhastulehdus, d-vitamiini

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# ABBREVIATIONS

| | |
|---|---|
| Ab | antibody |
| APC | antigen presenting cell |
| ARR | annual relapse rate |
| CES-D | center for epidemiologic studies depression scale |
| CMIA | chemiluminescent microparticle immunoassay |
| CNS | central nervous system |
| CSF | cerebrospinal fluid |
| DMF | dimethyl fumarate |
| DMT | disease modifying therapy |
| DM | diabetes mellitus |
| EAE | experimental autoimmune encephalomyelitis |
| EBV | Eppstein-Bar virus |
| EDSS | expanded disability status scale |
| ELISA | enzyme-linked immunosorbent assay |
| FN | fibronectin |
| GDM | gestational diabetes mellitus |
| HLA | human leucocyte antigen |
| HPLC | high-performance liquid chromatography |
| FSS | fatigue severity scale |
| $FT_4$ | free thyroxine |
| HHV-6 | human herpesvirus 6 |
| HPLC | high-performance liquid chromatography |
| IFN | interferon |
| IgG | immunoglobulin G |
| IvIg | intravenous immunoglobulin |
| JCV | John Cunningham virus |
| GA | glatiramer acetate |
| gw | gestational week |
| MRI | magnetic resonance imaging |
| MS | multiple sclerosis |
| MX | mitoxantrone |
| PML | progressive multifocal leucoencephalopathy |
| PP | postpartum |
| PPT | postpartum thyroiditis |
| OCB | oligoclonal bands |
| 25(OH)D | 25-hydroxyvitamin D |
| $1.25(OH)_2D$ | 1.25-dihydroxyvitamin D |

| | |
|---|---|
| PPMS | primary progressive multiple sclerosis |
| PRIMS | Pregnancy in MS Study |
| PRMS | progressive relapsing multiple sclerosis |
| RA | rheumatoid arthritis |
| RR | relapse rate |
| RRMS | relapsing-remitting multiple sclerosis |
| SD | standard deviation |
| SEM | standard error of mean |
| SLE | systemic lupus erythematosus |
| SPMS | secondary progressive multiple sclerosis |
| TPO-Abs | thyroid peroxidise antibodies |
| TG-Abs | thyreoglobulin antibodies |
| $T_{REG}$ | regulatory T cells |
| TSH | thyroid stimulating hormone |
| VCAM-1 | vascular cell adhesion molecule 1 |

# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by the Roman numerals I-IV.

I Jalkanen A, Alanen A, Airas L, and Finnish Multiple Sclerosis and Pregnancy Study Group. Pregnancy outcome in women with multiple sclerosis - results from a prospective nationwide study in Finland. Mult Scler 2010; 16: 950-955.

II Airas L, Jalkanen A, Alanen A, Pirttila T, Marttila RJ. Breast-feeding, postpartum and prepregnancy disease activity in multiple sclerosis. Neurology 2010; 75: 474-476.

III Jalkanen A, Saraste M, Gfeller A, Surcel H, Airas L. Increased thyroid autoimmunity among women with multiple sclerosis in the postpartum setting. Mult Scler J 2013; 19: 1734-42.

IV Jalkanen A, Kauko T, Koskinen JO, Waris ME, Airas L. Elevated high sensitivity C-reactive protein is associated with pregnancy-related co-morbidities but not with relapse activity in Multiple Sclerosis. Neurol Sci 2014 Oct 12. doi: 10.1007/s10072-014-1980-5.

V Jalkanen A, Kauko T, Turpeinen U, Hämäläinen E, Airas L. Multiple sclerosis patients and vitamin D during pregnancy and lactation. Acta Neurol Scand 2014 Sep 12. doi: 10.1111/ane.12306.

# 1. INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system. The autoimmune based aetiology of MS is not yet thoroughly known, as its pathogenesis appears complex and multifactorial involving T- and B-cell mediated reactions against self-molecules leading to tissue-specific destruction of the central nervous system (Disanto, et al. 2012). MS affects about 1% of the population worldwide (Compston and Coles. 2002). MS is the most common disabling neurological disease affecting young adults. MS prevalence is higher in areas distant from the equator and the influence of vitamin D or UV-radiation exposure on MS susceptibility is under enthusiastic investigation. MS mainly occurs in young people and more often women.(Orton, et al. 2006) This is why the reproduction and family planning themes are topical in this patient group. The relapse rate is typically reduced during late pregnancy but increases again in postpartum period (Birk, et al. 1990, Confavreux, et al. 1998, Korn-Lubetzki, et al. 1984, Roullet, et al. 1993, Saraste, et al. 2007b). The reasons for the increased postpartum activity are not entirely clear, but factors such as the abrupt decrease in oestrogen levels immediately after the delivery and the loss of immunosuppressive state of pregnancy are likely to be of importance (Airas, et al. 2007, Airas, et al. 2008, Whitacre, et al. 1999).

# 2. REVIEW OF LITERATURE

## 2.1 Autoimmunity

The role of the immune system is to protect us from the disease causing pathogens, such as viruses and bacteria, to control cancerous cells and promote healing of tissue injuries. In autoimmune diseases this sensitive balance and regulation is disturbed resulting in over-activation of the inflammatory cells and their attack against the body's own cells. Dysregulation of these self-reactant immune cells results in aimless inflammatory activity and eruption of the autoimmune disease, in which the disease course typically takes turns in flares and quiescent periods. Typical systemic autoimmune diseases with chronic inflammation are for example rheumatoid arthritis (RA), autoimmune thyroiditis and systemic lupus erythematosus (SLE). The target organ of the autoimmune process varies between different diseases with the exception of SLE, in which the target for autoimmune attack can be almost any tissue.

Specific molecular and cellular mechanisms behind these autoimmune diseases remain poorly understood. Genetic factors explain these diseases only partly, as concordance rates in monozygotic twins are 20-30% (Bogdanos, et al. 2012). Thus genetically predisposed individual needs to encounter certain environmental factors to develop the disease (Wahren-Herlenius and Dorner. 2013, Dasgupta and Saxena. 2012). Molecular mimicry is the leading hypothesis for pathogen-induced autoimmunity and has been postulated to be the primary cause of autoimmune diseases such as rheumatoid arthritis, diabetes and multiple sclerosis (MS) (Chastain and Miller. 2012). In molecular mimicry, T cells bearing spesific receptors for epitopes derived from foreign pathogens, such as viruses, are activated during an infection and cross-react with self-antigens inducing autoimmune disease (Wucherpfennig and Strominger. 1995, Chastain and Miller. 2012).

Both innate and adaptive immune systems seem to be involved in autoimmunity. Recent hypotheses emphasise the role of positive feed-forward loop, in which the innate immune cells produce type 1 interferon, which activates T- and B-cells. B-cells in turn produce autoantibodies, which stimulate dendritic cells of the innate system to produce more type 1 interferon. In addition to this, the impaired reactivity of adaptive immunity to the activated auto-reactive lymphocytes seems to result in and maintain persistent inflammation (Wahren-Herlenius and Dorner. 2013). In normal conditions, regulatory T cells ($T_{REG}$s) regulate ongoing immune responses and prevent autoimmunity. It has been suggested that the imbalanced number or function of these cells might explain the immune system dysregulation in autoimmune diseases (Dasgupta and Saxena. 2012).

### 2.1.1 Autoimmunity in the central nervous system; Multiple sclerosis

Multiple sclerosis (MS) is a prototype inflammatory autoimmune disease of the central nervous system (CNS) (Compston and Coles. 2002). MS disease process is believed to start with increased migration of the auto-reactive lymphocytes across the blood-brain barrier. As in the systemic autoimmunity, regulatory defects allow these cells to transit from physiological surveillance mode to auto-reactive pathological cascade attacking neurons, which results in local immune response in the CNS (Compston and Coles. 2008). It has been demonstrated that regulatory lymphocytes fail to suppress effector cells in MS patients, but not in healthy controls (Viglietta, et al. 2004). Abnormal immune response to a myelin antigen results in a formation of local inflammatory sites, which typically cluster around the lateral ventricles and corpus callosum, in the cortex and subcortical white matter, the optic nerves and brainstem, and throughout the spinal cord forming perivascular CD8+ infiltrated plaques. Inflammation is thought to seek to these particular sites due to the failure of the local regulatory function (Compston and Coles. 2008). This inflammation can result in demyelination and axonal degeneration if the re-myelination process fails to achieve full recovery.

## 2.2 Multiple sclerosis disease

### 2.2.1 Clinical features

MS is characterized by the presence of disseminated demyelinating lesions in the CNS. There are four distinct clinical disease patterns in MS: relapsing–remitting (RRMS); secondary progressive (SPMS); primary progressive (PPMS); and progressive relapsing (PRMS) (Lublin and Reingold. 1996). The relapsing –remitting form of MS makes up to 80% of the total number of MS cases. It is characterised by intermittent episodes of relapses and prolonged remissions (Compston and Coles. 2008). A relapse is considered as clearly defined attack of a new or recurrent objective neurological sign or symptom with full or partial recovery (Milo and Miller. 2014). Patients encounter acute episodes of neurological dysfunction during relapses. Neurological dysfunction appears in different grades of loss of motor or cognitive function depending on the aggression of the disease. A relapse is followed by a recovery and a symptom-free interval until the next relapse. Eventually these recurrent episodes lead to more permanent disabilities, which limit the patient's ability to function and work. (Compston and Coles. 2008)

### 2.2.2 Fatigue

Fatigue is the most common symptom among patients with MS as it has been reported to affect 50% to 80% of MS patients (Induruwa, et al. 2012). Fatigue has been defined as "an overwhelming sense of tiredness for no apparent reason" by the United Kingdom

Multiple Sclerosis society. Fatigue can be further divided into peripheral neuromuscular or central physical and mental fatigue. In MS fatigue is thought to be more that of a central origin resulting from the accumulating damage in the brain. (Chaudhuri and Behan. 2000) The pathophysiology of fatigue is yet not well known, but hypotheses based on inflammation, cerebral lesions or cortical atrophy exist (Induruwa, et al. 2012). Previous studies of Dimsdale et al. and Cho et al. support the role of low-grade inflammation in inducing symptoms of fatigue (Cho, et al. 2013, Dimsdale and Dantzer. 2007). The results have been inconclusive, as some studies have not been able to demonstrate relation between inflammation and fatigue. A study of Giovannoni et al. with 38 MS patients, of which 16 were relapsing-remitting, found no direct relation between systemic markers of inflammation and fatigue (Giovannoni, et al. 2001a). They observed, however, that patients with primary progressive disease were less fatigued than relapsing-remitting patients, which could be explained by the more significant role of inflammation in the pathogenesis of relapsing-remitting disease form and also fatigue.

### 2.2.3 Factors influencing relapse rate

Relapses in relapsing-remitting MS seem to reflect underlying inflammatory activity ('McDonald, I', 'Compston, A', 'Ebers, G', 'Lassmann, H', 'Matthews, B' and 'Wekerle, H'. 1998). Inflammatory mediators during acute infections are suggested to precede the onset of relapse in about 20% -30% of cases and the annual relapse rate in at-risk weeks has demonstrated to be 2.9 compared to 1.16 in weeks not at risk (Panitch. 1994, Vollmer. 2007). Accordingly, MS relapses have been shown to be associated with elevated CRP values (Soilu-Hanninen, et al. 2005, Giovannoni, et al. 2001b). Inflammatory factors can also exacerbate the previous neurological dysfunction by exaggerating the conduction block in demyelinated areas, a phenomenon called pseudo relapse (Smith and McDonald. 1999). There is substantial evidence for increased MS disease activation during the weeks around viral or bacterial infectious episode (D'hooghe, et al. 2010b, Correale, et al. 2006, Edwards, et al. 1998, Rapp, et al. 1995, Rutschmann, et al. 2002, Sibley, et al. 1985). The period of increased relapse rate associated with infection starts one week before the symptoms of the infection appear and continues until five weeks after the infection has terminated (Panitch. 1994). The possible mechanism underlying the infection driven relapses is suggested to be of non-antigen specific type of activation of the immune system promoting the migration of immune cells and also auto-reactive T cells into CNS (Vollmer. 2007). Non-traumatic, stressful life events were associated with increased risk of disease exacerbations in a meta-analysis consisting of 14 studies (Mohr, et al. 2004). Although a modest association has been suggested, further clarification has been requested on this issue, as consistent tools for defining and measuring stress in MS are lacking (D'hooghe, et al. 2010b). General anaesthesia and epidural anaesthesia

with low concentration local anaesthetics seem not to promote exacerbations and are considered safe for MS patients (Dorotta and Schubert. 2002, Bader, et al. 1988).

Furthermore, a negative correlation between traumatic or surgical episodes and MS exacerbations has been demonstrated (Sibley, et al. 1991). No association was found between caesarean section and postpartum relapses in an Italian cohort of MS patients (Pasto, et al. 2012). Still, strong evidence exists on the increased relapse rate after childbirth (Birk, et al. 1990, Confavreux, et al. 1998, Korn-Lubetzki, et al. 1984, Roullet, et al. 1993, Saraste, et al. 2007b). Relapse rate has been observed to peak particularly during the first three months after delivery (Confavreux, et al. 1998).

### 2.2.4 Epidemiology

MS is the most common demyelinating disease of the CNS occurring at an incidence of 2.5 million worldwide (Compston and Coles. 2002). The female to male sex bias in MS has increased from around 2:1 to almost 3.5:1 within half a century due to an increased incidence of MS in females, and not a decreased incidence in males (Orton, et al. 2006). This shift in sex ratio has been so rapid that purely genetic causes seem unlikely. Instead, gene-environment interactions seem possible and the changes in lifestyle factors in women, such as increased smoking, use of oral contraceptives, outdoor activity, timing of childbearing years and dietary habits have been suggested to be involved. (Orton, et. al. 2006) The overall incidence rate of MS is about 7 per 100 000 every year. The prevalence of the disease is around 120 per 100 000, and lifetime risk of one in 400 (Compston and Coles. 2002). MS typically starts at about 30 years of age, usually in the third or fourth decade (Compston and Coles. 2002). The majority of individuals diagnosed with MS are women in childbearing age and hence family planning and pregnancy-related questions are a common concern (Orton, et al. 2006).

### 2.2.5 Genetics

The familial recurrence rate of MS is about 20%. The reduction in risk changes from 3% in first-degree relatives (siblings, 5%; parents, 2%; and children, 2%), to 1% in second-degree and third-degree relatives (Compston and Coles. 2008). Population based twin studies from Canada and UK have shown higher clinical concordance rates in monozygotic than in dizygotic pairs (25% *vs* 5%) (Mumford, et al. 1994, Willer, et al. 2003). The results of familial studies, however, have been questioned, as the increased familial frequency might reflect common environment more than common genes (Kurtzke. 1993). At the same time studies of half-siblings and adoptees support the concept that genetic, and not environmental factors, are primarily responsible for familial aggregation (Sadovnick, et al. 1996, Ebers, et al. 1995, Oksenberg, et al. 2001). MS susceptibility has been identified to associate with two human leucocyte antigen (HLA) class II-haplotypes, HLA-DW2 and –DR2 spesificities (Olerup and Hillert. 1991).

The function of these molecules in the normal immune response is antigen binding and presentation and T cell repertoire determination (Oksenberg, et al. 2001). However, much of the genetic effect in MS remains yet unknown. There might be genes that are involved in the initial pathogenic events, while others could influence the development and progression of the disease (Oksenberg, et al. 2001).

### 2.2.6 Pathogenesis

MS pathogenesis is believed to be based on autoimmunity. Activated immune cells cross the blood-brain barrier and produce inflammatory plaques and axonal loss in the brain, spinal cord and optic nerves. This results in gliosis and demyelinated areas in CNS.

#### *2.2.6.1 Role of inflammation*

Inflammation is the first step in the disease process of MS. Inflammatory plaque formation requires presence of blood brain barrier (BBB) leakage and entrance of immune cells in the CNS. The autoimmune cascade starts with activation of myelin-reactive T lymphocytes by antigen presenting cells (APCs) and the development of membrane attack complexes in the CNS resulting in inflammatory lesions surrounded by infiltrating T lymphocytes, monocytes, and macrophages, as well as activated microglia and reactive astrocytes (Hernandez-Pedro, et al. 2013). Local inflammation with activation of macrophages, antibodies and complement and loss of function or death of myelin-producing oligodendrocytes leads to demyelination and possible sustainable neuronal damage if the process of re-myelination cannot repair the structural damage (Sriram. 2011).

#### *2.2.6.2 Role of neuro-degeneration and axonal damage*

Destruction of myelin sheaths in result of inflammation, as well as axonal damage and loss, proceed to glial scar formation. The disease progression in MS depends on the accumulation of the axonal damage (Compston and Coles. 2008). With time, recovery from each episode is incomplete and persistent symptoms accumulate. Progression of the disease starts around 40 years of age (Confavreux and Vukusic. 2006).

### 2.2.7 Environmental factors

The causes of MS are presumed to be heterogeneous, complex and multifactorial (Figure 1). Genetic and environmental interactions are likely to be of importance in causative origin of the disease. Genetic susceptibility added with environmental trigger are both proposed to be required to induce the disease (Oksenberg, et al. 2001). Environmental co-determinant factors such as viruses, bacteria, geographical latitude, and the endocrine system have been considered (Cermelli and Jacobson. 2000, Virtanen and Jacobson. 2012, Yao, et al. 2001, Kurtzke. 1993, Gomez, et al. 2013). According to the hypothesis of

Kurtzke, there is an infectious agent involved in the process of the disease and MS would be the rare late outcome of a specific but yet unknown infectious disease in adolescence and young adulthood (Kurtzke. 1993). Due to the heterogeneity of the disease, it seems plausible that MS might not be triggered by a single virus, but a complex set of viral infections (Krone and Grange. 2010). The risk of developing MS changes with migration from high-prevalence areas to low-prevalence areas and vice versa (Weinshenker. 1996). Higher prevalence of MS in northern hemisphere has been discussed to relate in geographical, genetic, environmental, cultural and behavioral differences (Harbo, et al. 2013). Furthermore, the potential contribution of solar ultraviolet (UV) radiation exposure in the risk of MS has also been suggested to depend on the vitamin D biological activity and sex of the patient (Kampman, et al. 2013, McDowell, et al. 2011, Orton, et al. 2011, Ramagopalan, et al. 2011).

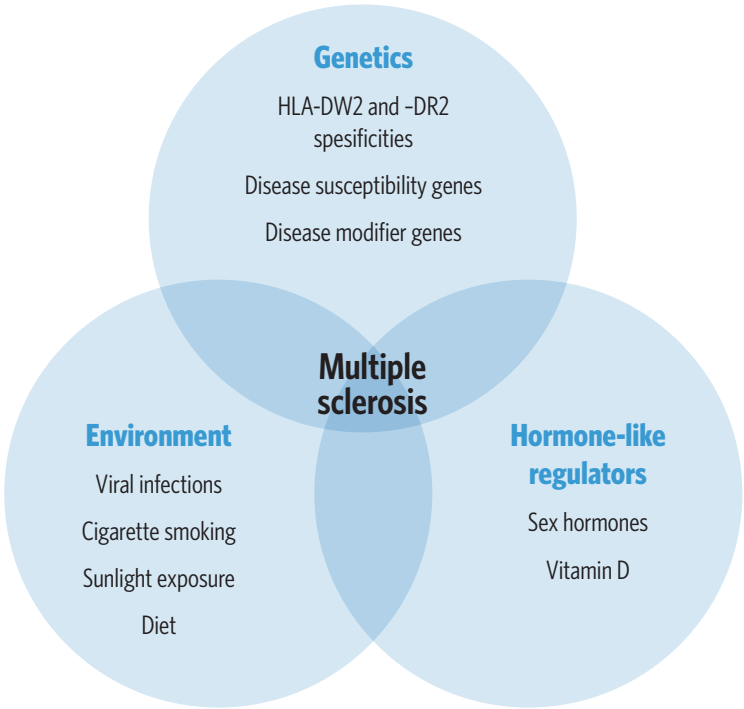


**Figure 1.** The suspected causative origin of Multiple sclerosis disease is multifactorial.

#### *2.2.7.1 Viruses and susceptibility to Multiple sclerosis*

Common herpes viruses like Epstein-Barr virus (EBV) and Human Herpes Virus 6 (HHV-6), have several properties that would make them capable of triggering MS pathological process. These viruses infect almost all individuals in early years of life and remain in an inactive latent form in the body retaining the ability to reactivate later. In addition, these viruses can infect CNS cells and also cause CNS disease. (Virtanen and Jacobson. 2012)

The most important finding associating EBV to MS is that MS patients present with higher sero-prevalence and higher titers of EBV antibodies than age-matched controls (Virtanen and Jacobson. 2012). Antibody titers against EBV have been observed to be significantly elevated already five or more years before the onset of MS among patients diagnosed later with MS compared with controls (Levin, et al. 2005). Increased titers of EBV-antibodies have been proposed to come from an early event in the pathological process of MS development (Levin, et al. 2005). Migration studies have suggested that MS susceptibility is acquired during infancy or childhood, which favors the role of a ubiquitous virus as a trigger (Orton, et al. 2006, Virtanen and Jacobson. 2012).

Another virus with higher antibody titers and prevalence in MS population is HHV-6. The DNA, protein and RNA of this virus have been found from different body fluids and brain tissue more often in MS patients than healthy controls (Virtanen and Jacobson. 2012). In addition, HHV-6 has been found in MS plaques significantly more frequently than in the normal appearing white matter of MS patients (Challoner, et al. 1995, Goodman, et al. 2003). It is not known, whether HHV-6 is a cause or consequence of MS plaque development.

#### *2.2.7.2 Sex hormones*

The incidence of MS is higher and peripheral immune responses more robust in women. However, disease progression and neuro-degeneration have been demonstrated to be either faster in men or are at best, no difference between the sexes has been observed. (Voskuhl and Gold. 2012) Protective role of oestrogen hormones in conjunction with vitamin D has been demonstrated on disease course and T-lymphocyte reactivity (Harbo, et al. 2013). On the other hand, protective effect of physiological testosterone has been suggested to be responsible, at least in part, for the decreased male susceptibility (Voskuhl and Gold. 2012). Effects of sex hormones have mostly been studied in experimental autoimmune encephalomyelitis (EAE), the animal model of MS induced in mice (Voskuhl and Palaszynski. 2001). These studies have demonstrated that oestrogens have anti-inflammatory effects in the peripheral immune system, including modulation of cytokine balance, downregulation of chemokines in the CNS, modulation of dendritic cell function, regulation of $T_{REG}$ cell population, and decreasing the expression of matrix metallo-proteinases (Kipp and Beyer. 2009).

#### *2.2.7.3 Vitamin D*

As the prevalence of MS increases with higher latitude, the possible link between sunlight exposure, vitamin D deficiency and MS has been eagerly studied for recent years (Hewer, et al. 2013, Weinstock-Guttman, et al. 2012). Increased relapse rate has been demonstrated to associate with low vitamin D levels (Tremlett, et al. 2008, Weinstock-Guttman, et al.

2012). Accordingly, vitamin D levels lagged by two months have been demonstrated to inversely relate to gadolinium enhanced lesion load of MS patients (Embry, et al. 2000). Higher vitamin D levels have been observed to boost innate immunity and down-regulate adaptive immune system (Zasloff. 2006, van Halteren, et al. 2002, Chen, et al. 2007). UV-radiation and vitamin D have been demonstrated to independently stimulate $T_{REG}$ cells and secretion of IL-10, reduce levels of the pro-inflammatory cytokine IL-17, and dampen T-helper (Th)-1 immune function, cascades that would account for the reduced MS risk (Lucas, et al. 2011). Furthermore, higher concentrations of vitamin D have been observed with improvement in immunological and radiological parameters of MS in clinical trials of vitamin D supplementation (Hewer, et al. 2013). Moreover, it has been suggested that the effect of certain genes on MS may be present only if vitamin D levels are low and the protective effect of vitamin D on MS might be present only in patients with particular genotype (Hewer, et al. 2013).

#### *2.2.7.4 Cigarette smoking*

Smoking cigarettes has been confirmed to be associated with MS susceptibility by a large meta-analysis of 14 papers and over 3000 MS patients (Handel, et al. 2011). Effect of smoking on disease progression is less certain. The mechanism of smoking affecting MS susceptibility is not completely known, but it seems more likely to be related to the neurotoxic and immune-modulatory components of tobacco smoke, as also passive smoking has been demonstrated to increase the risk for MS (Sundstrom, et al. 2008). Smoking has been estimated to associate with a 40 % average increase in risk of MS (Palacios, et al. 2011). Increasing female to male ratio of MS during the last decades has also been suggested to relate to the increased prevalence of smoking among women, as the two trends have shown a significant correlation (Palacios, et al. 2011).

### 2.2.8 Diagnosis

#### *2.2.8.1 Radiological diagnosis*

Brain MRI is the primary examination when MS is suspected (Fazekas, et al. 1999, Optic Neuritis Study Group. 1997, Paty, et al. 1988, Morrissey, et al. 1993, O'Riordan, et al. 1998). The radiological diagnostic criteria of MS are based on typical demyelinating plaques of white matter in brain MRI. According to the McDonalds criteria, disease activity needs to be demonstrated by evolving changes in time and space (Table 1) (Polman, et al. 2011, Milo and Miller 2014).

**Table 1.** 2010 McDonald diagnostic criteria adapted from Milo and Miller 2014.

| Clinical presentation | Additional data needed for MS diagnosis |
|---|---|
| ≥ 2 attacks; objective clinical evidence of ≥ 2 lesions or objective clinical evidence of 1 lesion with reasonable historical evidence of a prior attack | None |
| ≥ 2 attacks; objective clinical evidence of 1 lesion | Dissemination in space, demonstrated by:<br>• ≥ 1 T2 lesion in at least 2 of 4 MS-typical regions of the CNS (periventricular, juxtacortical, infratentorial, or spinal cord)<br>Or<br>• Await a further clinical attack implicating a different CNS site |
| 1 attack; objective clinical evidence of ≥ 2 lesions | Dissemination in time, demonstrated by:<br>• Simultaneous presence of asymptomatic gadolinium-enhancing and nonenhancing lesions at any time<br>Or<br>• A new T2 and/or gadolinium-enhancing lesion(s) on follow-up MRI, irrespective of its timing with reference to a baseline scan;<br>Or<br>• Second clinical attack |
| 1 attack; objective clinical evidence of 1 lesion (monosymptomatic presentation; clinically isolated syndrome) | Dissemination in space and time, demonstrated by:<br>For DIS:<br>• ≥ 1 T2 lesion in at least 2 of 4 MS-typical regions of the CNS (periventricular, juxtacortical, infratentorial, or spinal cord)<br>Or<br>• Await a second clinical attack implicating a different CNS site;<br>And<br>For DIT:<br>• Simultaneous presence of asymptomatic gadolinium-enhancing and nonenhancing lesions at any time<br>Or<br>• A new T2 and/or gadolinium-enhancing lesion(s) on follow-up MRI, irrespective of its timing with reference to a baseline scan;<br>Or<br>• Await a second clinical attack |
| Insidious neurologic progression suggestive of MS (primary progressive MS) | 1 year of disease progression (retrospectively or prospectively determined) plus 2 of 3 of the following criteria:<br>1. Evidence for DIS in the brain based on ≥ 1 T2 lesions in the MS-characteristic (periventricular, juxtacortical, or infratentorial) regions<br>2. Evidence for DIS in the spinal cord based on ≥ 2 T2 lesions in the cord<br>3. Positive CSF (isoelectric focusing evidence of oligoclonal bands and/or elevated IgG index |

#### *2.2.8.2 Laboratory diagnosis*

The radiological diagnosis is further supported by the detection of the intra-thecal inflammation in the cerebrospinal fluid (CSF). Presence of oligoclonal bands (OCBs) of IgG in the CSF but not in the serum reflect the compartmentalised central nervous system (CNS) humoral immune activation present in MS. CSF analysis, however, is not mandatory to make the diagnosis (Dobson, et al. 2013).

## 2.3 Treatment of Multiple sclerosis

Aim of the treatment of relapsing-remitting MS is the reduction of the frequency of relapses (Oreja-Guevara, et al. 2014).

### 2.3.1 Treatment of Multiple sclerosis relapses

Considerable relapse symptoms can be treated with three to five day high dose intravenous or oral corticosteroid pulse. Corticosteroid relieves the symptoms by shortening their duration, but it has not been convincingly demonstrated to improve patient's prognosis or prevent long-term disability. For serious relapses, the second line treatment is plasmapheresis. Therapeutic plasma exchange has proved to be beneficial in case of a fulminant demyelinating attack (Keegan, et al. 2005).

### 2.3.2 Disease modifying therapy

There is no curing therapy for MS. Immune-modulatory and immune-suppressive treatments have proved effective in preventing relapses in MS especially when performed early in the course of the disease.

**Table 2.** Disease modifying treatment choices in relapsing-remitting multiple sclerosis

| | ***Traditional*** | ***New*** | ***Highly active disease*** |
|---|---|---|---|
| First line | Interferon-β<br>Glatiramer acetate | Teriflunomide<br>Dimethyl fumarate | Natalizumab<br>Fingolimod<br>Alemtuzumab |
| Second line | Natalizumab<br>Fingolimod<br>Alemtuzumab | | |
| Third line | Mitoxantrone | | |
| Fourth line | Azathioprine | | |

#### *2.3.2.1 Traditional first line immune-modulatory treatments*

MS with moderate disease activity is usually first treated with interferon-β (INF-β) or glatiramer acetate (GA). Immune-modulatory INF-β has been demonstrated with effects on antigen presentation, co-stimulatory molecule expression, T-cell proliferation, and leukocyte migration (Vosoughi and Freedman. 2010). INF-β can disturb the thyroid function of the patient, usually within the first year of treatment (Caraccio, et al. 2005).

The clinical efficacy of GA is presumably mediated by induction of regulatory T cells, inhibitory and suppressive functions on T cell response and migration through the BBB and increasing the production of neuro-protective agents (Vosoughi and Freedman. 2010).

#### *2.3.2.2 New first line immune-modulatory treatments*

Teriflunomide is a new first line oral immune-modulatory treatment for MS. It is an active metabolite of leflunomide, which has been used for treatment of RA in adults (Osiri, et al. 2003). Its therapeutic mode of action is not fully elucidated, but it is an inhibitor of dihydroorotate-dehydrogenase and it has been shown to selectively reduce the activity of proliferating T- and B-cells in animal models and patients. It seems to have cytostatic immune-modulatory effects in limiting new inflammatory disease activity in MS, while not substantially limiting the treated patients' protective immune response. (Bar-Or. 2014)

Another new oral first line treatment option for MS is dimethyl fumarate (DMF), a dimethylester of fumaric acid. Fumaric acid esters were initially used in the treatment of psoriasis, an immunological skin disorder (Mrowietz, et al. 1999). DMF seems to have both anti-inflammatory and anti-oxidative neuroprotective effects. The safety profile of DMF is favourable, but gastrointestinal side effects and flushing diminish the tolerability of this drug. (Salmen and Gold 2014)

#### *2.3.2.3 Second line immune-modulatory treatments*

More aggressive MS can be treated with Natalizumab, the first monoclonal antibody treatment against MS. This antibody is a directed against very late activating antigen-4 (VLA-4) on the leucocyte, but not on the neutrophil, cell surface. Natalizumab inhibits the binding of leukocytes to vascular cell adhesion molecules (VCAM)-1 and fibronectin (FN) by antagonising the VLA-4 on leucocyte surface and thus preventing them from entering the CNS. As the efficacy of the treatments on MS disease activity improves, the risk for serious complications also increases. The most disabling complication of Natalizumab is progressive multi-focal encephalopathy (PML), a fatal, opportunistic infection of brain caused by John Cunningham virus (JCV). The risk of PML starts to increase after two years of treatment and is influenced by the JCV antibody status of the patient (Vosoughi and Freedman. 2010).

Fingolimod is the first oral second line disease-modifying treatment for MS. It modulates the sphingosine 1-phosphate receptor on lymphocytes and selectively retains autoreactive lymphocytes in lymph nodes to reduce damaging infiltration into the CNS (Groves, et al. 2013). The most common adverse effect of fingolimod has been a transient, mostly asymptomatic decrease in heart rate during the initiation of the drug (Gold, et al. 2013). Also macular oedema, elevations of liver enzymes and possible increased risk for infections have been reported.

Alemtuzumab is the second humanized monoclonal antibody treatment for MS causing rapid and prolonged lymphocyte depletion possibly by antibody-depended, cell-mediated

cytotoxicity. Alemtuzumab induces homeostatic reconstitution of the lymphocytes with relative increase in regulatory T cells and expansion of auto-reactive T cells. Alemtuzumab binds to a 12-amino acid cell surface protein, CD52, the function of which is unknown, but which is expressed at high levels on T cells and B cells leaving cells of the innate immune system unaffected. Alemtuzumab is administered at first time daily for five consecutive days. Subsequent cycle is given only at the time the disease activates again. Usually the efficacy sustains at least 12 months (Coles. 2013). The most common adverse effect of this treatment is autoimmunity towards the thyroid gland affecting up to 20 -30% of the treated MS patients. Approximately 1% of the patients acquire immune thrombocytopenia (Coles. 2013). Reconstitution of lymphopenia is known to induce autoimmunity also in other conditions, for example after allogeneic bone marrow transplantation (Hsiao, et al. 2001).

These three drugs Natalizumab, Fingolimod and Alemtuzumab can be used also as first line treatments in highly active MS.

#### *2.3.2.4 Third and fourth line immune-modulatory treatments*

Mitoxantrone (MX) is a systemic immunosuppressive cytotoxic agent widely used for treatment of breast cancer and leukaemia. MX is thought to act via a wide range of mechanisms, which include inhibition of T-cell activation, suppression of T-cell, B-cell and macrophage proliferation, impaired antigen presentation, prevention of macrophage-mediated demyelination and reduction of pro-inflammatory cytokine (Lim and Constantinescu. 2010). MX has been observed moderately effective in reducing the risk of disease progression and the frequency of relapses in patients affected by aggressive RRMS, PRMS and SPMS. Data from studies with longer follow-up have raised concerns about cardiotoxicity and acute leukaemias, occurring in about 12% and 0.8% of MX-treated patients respectively. For these reasons, MX treatment has been recommended to be limited to patients with worsening RRMS and SPMS after a careful assessment of the individual patients' risk and benefit profiles. (Martinelli Boneschi, et al. 2013) Use of MX in MS is decreasing due to the new more specific and better tolerated treatments.

If other MS treatments do not have an effect, Azathioprine can be tried as a fourth line treatment. It has been demonstrated to reduce MS relapses and slow disease progression. (Casetta, et al. 2007) Gastrointestinal problems, suppression of bone marrow and liver toxicity limit the use of this drug.

#### *2.3.2.5 Treatment during pregnancy*

According to present recommendations, all disease modifying treatments for MS should be discontinued before conception (Lu, et al. 2012). However, exposure

to IFN- β or GA during first trimester of pregnancy does not seem to increase the risk for spontaneous abortion, caesarean delivery, lower gestational age nor baby's malformations (Houtchens and Kolb. 2013). Thus IFN- β and GA treatments could be considered safe to be continued until first trimester of pregnancy especially in case of patient with high risk disease reactivation (Ruuskanen, et al. 2013, Ghezzi, et al. 2013, Tsui and Lee. 2011). Teriflunomide has caused teratogenicity in animal models and is thus contraindicated in pregnant women. Reliable contraception is necessary for women with childbearing potential using teriflunomide (Cree. 2013). As for DMF, there is not enough information on reproductive safety and results in available animal and human studies are conflicting (Cree.2013). Data on the use of natalizumab in pregnant women are currently inadequate, which is why natalizumab should not be used during pregnancy unless the clinical condition of the woman requires (Houtchens and Kolb. 2013). Fingolimod has been proved teratogenic in animal models and effective contraception is recommended for patients using it. Fingolimod has been recommended to be discontinued at least two months before conception. (Houtchens and Kolb. 2013) Animal studies on Alemtuzumab have shown increase in fetal deaths, but not malformations. The recommended wash-out period is four months. (Coles. 2013) Third or fourth line treatments are teratogenic and can impair fertility, which is why they cannot be used during pregnancy or for patients planning a pregnancy. (Martinelli Boneschi, et al. 2013, Casetta, et al. 2007)

Treatment of relapses with high dose corticosteroid or IV-immunoglobulin (IVIg) is generally accepted but rarely needed during pregnancy (Tsui and Lee. 2011).

#### *2.3.2.6 Treatment after delivery and breastfeeding*

There is sufficient evidence for INF-β and GA to have a delayed onset of efficacy, which is why treatment decisions regarding these drugs on patients with high MS activity should be done soon after delivery or even in advance (Comi, et al. 2001, Li and Paty. 1999). Breastfeeding mothers have been recommended not to start DMT after birth, as the available data on drug transfer into milk and their effects on newborns have been limited (Coyle, et al. 2004). According to current conception, however, transfer of GA into breast milk seems unlikely and IFN-β has been reported to be present in breast milk only in extremely small quantities (Ilett and Kristensen. 2005, Hale, et al. 2012). In addition, the absorption of these drugs thorough the intestine of the newborn is likely quite low (Ruuskanen, et al. 2013). Natalizumab, on the other hand, has also been detected in human breast milk and is not considered safe to use during breastfeeding because of potential injurious effects (Wehner, et al. 2009). Teriflunomide, DMF, Fingolimod and MX should be avoided during breastfeeding (Cree,B.A. 2013, Houtchens and Kolb. 2013). Use of Azathioprine during breastfeeding seems moderately safe (Sau. 2007).

Use of IvIg is safe and corticosteroids moderately safe for women experiencing an acute relapse while breastfeeding (Achiron, et al. 2004, Houtchens and Kolb. 2013). However, a washout period of four hours after oral administration and 24-48 hours after intravenous administration of high dose corticosteroid should be applied (Ruuskanen, et al. 2013, Houtchens and Kolb. 2013).

## 2.4 Multiple sclerosis during pregnancy and postpartum

### 2.4.1 Multiple sclerosis disease activity during pregnancy and after delivery

The first large prospective study assessing pregnancy related issues in MS was the Pregnancy in MS (PRIMS) study of 254 MS patients in 1993-1995 (Confavreux, et al. 1998). In this study, the annual relapse rate (ARR) was reported to decrease significantly during pregnancy compared to baseline, which was considered the year before pregnancy. The mean ARR was observed to decrease from pre-pregnancy 0.7 (SD 0.9) by two-thirds to 0.2 (SD 1.0) by the third trimester. During the three months postpartum, the ARR increased up to 1.20 (SD 2.0). After this it was observed to stabilize towards the baseline (Confavreux, et al. 1998).

Several other studies on this issue have been conducted afterwards confirming that the relapse rate is typically reduced during late pregnancy but increases in the postpartum period (Birk, et al. 1990, Confavreux, et al. 1998, Korn-Lubetzki, et al. 1984, Roullet, et al. 1993, Saraste, et al. 2007b, Finkelsztejn, et al. 2011). The reasons for the increased postpartum activity are not entirely clear, but factors such as the abrupt decrease in hormonal levels and changes in mother's immune function at delivery have been suggested (Airas, et al. 2007, Airas, et al. 2008, Whitacre, et al. 1999).

### 2.4.2 Effect of pregnancy on the long term outcome of Multiple sclerosis

Results of how pregnancy influences MS disease course in the long term have been inconsistent. Several studies have found no association (Roullet, et al. 1993, Thompson, et al. 1986, Weinshenker, et al. 1989). Favourable, delaying effect of pregnancy on MS disability progression has also been reported (D'hooghe, et al. 2010a, Runmarker and Andersen. 1995, Verdru, et al. 1994). However, some of these findings might be explained by the more active disease of the childless women and changes in lifestyle related to childbirth (D'hooghe, et al. 2010a).

### 2.4.3 Immunological changes of Multiple sclerosis patients during pregnancy

According to the Th1/Th2 hypothesis, fetus avoids maternal T-cell rejection through a bias towards T-helper (Th) 2 cytokine production. This tilt from the pro-inflammatory type 1 domination towards- type 2 cytokine prominence has previously been hypothesized

to account also for the pregnancy related amelioration of the disease activity in MS (Al-Shammri, et al. 2004, Lopez, et al. 2006, Gilli, et al. 2010, Gilmore, et al. 2004). During pregnancy, changes in levels of oestriol, progesterone and prolactin take place. Pregnancy associated high levels of oestrogens promote T helper type 2 deviation. However, according to the recent research, Th1/Th2 paradigm seems too simplistic and does not reflect the complex immunomodulation that occurs both locally at the feto-maternal interface and systemically in the mother (Chaouat. 2007, Sargent, et al. 2006). Endogenous modulation of the maternal immune system during pregnancy might be instead, at least in part, antigen specific (Patas, et al. 2013). Target of interest in clarifying the pregnancy related paradigm has lately turned towards the shift in $T_{REG}$ cell, Th17 cell and NK cell subpopulations during pregnancy (Patas, et al. 2013). During first trimester, the most abundant population of immune cells in the human decidua consists almost exclusively of $CD56^{bright}CD16^{-}$ cells (Karimi and Arck. 2010). MS patients have shown a decrease in this subpopulation in their peripheral blood in comparison to healthy individuals (De Jager, et al. 2008). However, $CD56^{bright}$ NK subpopulation was found to be increased in the third trimester of pregnant MS patients compared to postpartum period (Karimi and Arck. 2010 , Airas, et al. 2008). This immune cell subpopulation could be relevant in terms of the MS disease activity. It has also been possible to therapeutically increase the $CD56^{bright}$ cell numbers of MS patients by treating them with daclizumab or IFN-β (Bielekova, et al. 2006, Saraste, et al. 2007a, Patas, et al. 2013, Saraste, et al. 2007b).

### 2.4.4 Predictors of postpartum disease activity

Pre-pregnancy- and pregnancy-associated disease activity are among the strongest predictors of postpartum disease activity (McCombe and Greer. 2013, Vukusic, et al. 2004, Hughes, et al. 2013, Confavreux, et al. 1998). In the Pregnancy in MS (PRIMS) study also higher EDSS score in the beginning of pregnancy predicted postpartum relapses (Confavreux, et al. 1998). Elevated concentrations of pro-inflammatory cytokines during late pregnancy have also been shown to be associated with higher relapse rate postpartum (Langer-Gould, et al. 2010). Results in a recent study of 893 MS pregnancies showed that exposure to disease modifying therapy (DMT) prior to conception was associated with lower relapse rate postpartum (Hughes, et al. 2013). Either DMT might have remote effect on disease activity or this effect was caused by a selection bias of women with milder disease, who were able to stop treatment and become pregnant.

### 2.4.5 Multiple sclerosis and pregnancy outcome

It is generally held that MS does not affect the course or outcome of pregnancy (Fernandez Liguori, et al. 2009). There are limitations in all original studies addressing these questions, as they are either retrospective or register-based, or conclusions have

been drawn from very small patient numbers (Ferrero, et al. 2004, Chen, et al. 2009, Dahl, et al. 2005, Fernandez Liguori, et al. 2009, Worthington, et al. 1994, Mueller, et al. 2002, Orvieto, et al. 1999). Some inconsistencies in results, however, exist. A register-based Norwegian study of 649 MS mothers reported higher proportions in the neonates small for gestational age, the need for induced delivery, the use of forceps assistance and planned caesarean delivery compared to the 2.1 million control births (Dahl, et al. 2005). A register based study in Taiwan reported that MS mothers were more likely to give pre-term birth, have neonates small for gestational age and undergo caesarean section compared to 1392 healthy control mothers (Chen, et al. 2009). The European PRIMS study of 241 women with MS stated that pregnancy outcomes were not affected, but this study lacked a control group (Confavreux, et al. 1998). Register-based study of 198 American MS mothers found no significant difference in the pregnancy and delivery outcomes or rate of gestational diabetes compared to 1584 control women, but reported increased rates of maternal anemia among MS mothers, who were also observed to be re-hospitalized twice as likely during the three month postpartum period (Mueller, et al. 2002). A prospective study of 15 MS mothers before, during and up to three years following pregnancy reported no significant differences in the birth outcome or mean birth weight of the baby (Worthington, et al. 1994). Similar results were reported from the prospective Canadian study of Sadovnick et al. (Sadovnick, et al. 1994). In prospective follow-up of 15 MS mothers in Israel, obstetric complications or operative deliveries could not be related to the coexistence of MS (Orvieto, et al. 1999). Nevertheless, a meta-analysis performed by Finkelsztegn et al. reported that 10% of MS patients delivered prematurely with slightly decreased birth weights (Finkelsztejn, et al. 2011).

## 2.5 Immunological changes during pregnancy

During pregnancy mother's immune system is required to undergo complex immunological changes to tolerate the foreign father derived tissue of the fetus. The exact mechanisms behind this tolerance promoting process are not completely understood. The benign course of MS during pregnancy might be a by-product of these regulatory changes. Regulatory T ($T_{REG}$) cells have been suggested to play an important role in the suppressive activity of immune system during pregnancy (Schober, et al. 2012). Increase in circulating $T_{REG}$s in early pregnancy, peaking during the second trimester and declining postpartum has been observed (Somerset, et al. 2004). More recently it has been proposed that it might not be the number of $T_{REG}$s in peripheral blood that accounts for tolerance enhancement and successful pregnancy, but a selective migration of these cells into desidua, where the allogeneic cells of the fetus are encountered in the first place (Tilburgs, et al. 2008). Moreover, suppressive activity of $T_{REG}$ cells is probably regulated by the distinct changes in the $T_{REG}$ subset compositions. $T_{REG}$ cells

might have a key role in maintaining the subtle balance between inflammatory and anti-inflammatory activity during pregnancy (Schober, et al. 2012). The same changes in the $T_{REG}$ subset compositions that precede term labour seem to take place only earlier in cases of preterm delivery and pre-eclampsia (Schober, et al. 2012). This finding supports the hypothesis that adverse pregnancy outcomes may result from inadequate maternal tolerance (Saito, et al. 2007). In addition, pregnant women are more susceptible to certain infectious agents, such as respiratory viruses, which indicates that there are also systemic immunological changes taking place during pregnancy (Longman and Johnson. 2007). There is also evidence of heightened systemic inflammatory response in pregnant women, for example, increased leucocyte count and increased levels of C-reactive protein (Miller. 2009). Also sex hormones likely have an influence on the immunological changes during pregnancy, as significantly higher levels of oestrogen, oestriol and progesterone are measured during pregnancy. Of these, oestriol is detectable only during pregnancy (Voskuhl and Palaszynski. 2001).

## 2.6 Effects of pregnancy on autoimmune diseases

Pregnancy suppresses the inflammatory activity of many cell-mediated autoimmune diseases including RA, uveitis, psoriasis and MS. On the contrary, SLE worsens during pregnancy. Thus pregnancy seems to induce beneficial effects in diseases primarily driven by cell-mediated immunity including MS, but not in primarily antibody-mediated diseases such as SLE. The spesific biological mechanisms regulating these phenomena are still poorly understood. Recent evidence suggest that this may be occurring in an antigen-specific fashion as part of the feto-maternal tolerance rather than as a consequence of general “immunosuppression” during pregnancy (Patas, et al. 2013).

## 2.7 Postpartum thyroiditis

Postpartum thyroiditis is a very common form of autoimmune activation of the thyroid gland with incidence of 5.4% in general population. In certain autoimmune diseases such as diabetes mellitus (DM) and RA, the incidence is increased (Stagnaro-Green. 2012). This is suggestive of a genetically shared predisposition among these autoimmune diseases (Cotsapas, et al. 2011). The co-existence rates between MS and other autoimmune diseases, however, are much weaker (Broadley, et al. 2000). Small increase in the risk of autoimmune thyroiditis has been noticed for female patients with MS (Somers, et al. 2009). Certain MS treatments, such as Interferon-β (IFN- β) and Alemtuzumab, seem to increase the risk of MS patients to develop thyroid autoimmunity (Coles, et al. 1999, Jones. 2009, Monzani, et al. 2004). Disease course of MS during and after pregnancy is very similar to that seen in autoimmune thyroid disease as both

ameliorate during pregnancy and worsen again after delivery (Gaberscek and Zaletel. 2011). Shared susceptibility of these autoimmune diseases to develop postpartum immune reconstitution syndrome suggests there are common autoimmune induced mechanisms behind the disease activation after delivery. The hypothesis of autoimmune related imbalance in the number or function of the $T_{REG}$s fits well in this scenario (Weetman AP. 2010). The exact mechanisms behind this process remain yet unclear.

## 2.8 Thyroid peroxidase and thyroglobulin antibodies during pregnancy

The first thyroid auto-antigen discovered and shown to play a role in Hashimoto's thyroiditis was thyroglobulin antibody (TG-Ab) in 1956. Thyroid peroxidase antibodies (TPO-Ab) were discovered in the 1980's (Muller, et al. 2001). Antibodies to TPO appeared to be much more prevalent than antibodies to TG, exclusive presence of which are rare (Muller, et al. 2001). Interestingly, when bot antigens are present, the titers of TPO-Abs tend to be higher (Beever, et al. 1989). TPO-Abs are a more specific marker of autoimmune thyroid disease than TG-Abs (Sinclair. 2006). Thus for routine detection of thyroid antibodies, the TPO-Abs alone are sufficient (Sinclair. 2006). In addition, TPO-Abs, but not TG-Abs, can fix complement (Chiovato, et al. 1993).

One third to half of women who are positive for TPO- or TG-Abs in the first trimester will develop postpartum thyroiditis (Stagnaro-Green, et al. 2011). At least one long-term epidemiological study has shown that thyroid antibodies are a risk factor for the development of clinical hypothyroidism later in life (Männistö, et al. 2010).

The pathological significance of thyroid- and other auto-antibodies in MS is controversial (Barned, et al. 1995). Nevertheless, presence of TPO-Abs has been linked to several pregnancy complications: miscarriage, preterm delivery and placental abruption (Glinoer, et al. 1991, Stagnaro-Green, et al. 1990, Casey. 2006, Stagnaro-Green, et al. 2005, Abbassi-Ghanavati, et al. 2010, Haddow, et al. 2011). However, these associations have not been confirmed statistically significant in larger trials (Abbassi-Ghanavati. 2011). Even minor perturbations, such as subclinical hypothyroidism and thyroid antibody positivity, have also been linked to postpartum depression (Kuijpens, et al. 2001, Lazarus. 1999). There is evidence that mother's hypothyroidism during pregnancy can potentially damage the fetal neural development (Haddow, et al. 1999, Pop, et al. 1999, Zoeller and Rovet. 2004).

## 2.9 Vitamin D deficiency during pregnancy

Vitamin D insufficiency is common among healthy pregnant women worldwide (Hollis and Wagner. 2004, Wagner, et al. 2012). In Finnish cohort of 125 healthy pregnant women, 71% of patients were vitamin D deficient (< 50 nmol/l) even though 80%

of them used vitamin $D_3$ supplementation of 400 IU daily (Viljakainen, et al. 2010). Vitamin D deficiency is very common in Finland during the winter months due to the insufficient production of vitamin D in the skin (Savolainen, et al. 1980, Kauppi, et al. 2009). In addition to the classical functions of vitamin D in the calcium homeostasis, it has been suggested to influence also many non-skeletal health outcomes (Holick, et al. 2011). Low vitamin D concentration during pregnancy increases the mother's risk for pre-eclampsia, gestational diabetes mellitus, infection and pre-term delivery (Baker, et al. 2010, Bodnar, et al. 2007, Hensel, et al. 2011, Poel, et al. 2012, Wei, et al. 2013, Zhang, et al. 2008).

In addition to the adverse health effects on the mother, vitamin D deficiency during pregnancy has an impact on the fetal growth and bone health and possibly also to the fetal brain development and it may increase the risk of childhood infections, atopy and later MS in the offspring (Wagner, et al. 2012, Camargo, et al. 2011, Jones, et al. 2012, Chaudhuri. 2005). Vitamin D readily crosses placenta, which is why fetal vitamin D status is almost entirely dependent on the mother (Salle, et al. 2000).

### 2.9.1 25-hydroxyvitamin $D_3$ during pregnancy

The serum concentration of 25-hydroxyvitamin $D_3$ [$25(OH)D_3$] is the most reliable marker of vitamin D nutritional status (Dawodu and Akinbi. 2013). In the kidney 25(OH) D is converted to 1,25-dihydroxyvitamin D [$1,25(OH)_2D$], the most biologically active metabolite, the classical function of which is to maintain calcium homeostasis (Holick. 2004). Serum concentrations of 25(OH)D are the rate-limiting factor in the synthesis of 1,25-dihydroxyvitamin (Salle, et al. 2000). The 25(OH)D concentration is dependent on the nutritional intake and ambient sunlight exposure in the non-pregnant state (Ginde, et al. 2010). However, sunlight exposure may be more strongly associated with 25(OH) D levels than oral vitamin D intake (Perampalam, et al. 2011). During pregnancy, circulating concentrations of $1,25(OH)_2D$ gradually increase during the first and second trimesters, owing to an increase in vitamin D-binding protein concentrations in the maternal circulation (Holick, et al. 2011). During third trimester fetus begins to calcify the skeleton and this increased demand of calcium is met by increased production of $1,25(OH)_2D$ by the mother's kidneys and placenta (Holick, et al. 2011). Whether 25(OH) D level increases during pregnancy, is not well documented, but it could be assumed to be dependent on the oral supplementation duration and dosage and ambient sunlight exposure as in the non-pregnant state (Ginde, et al. 2010).

The effect of sunlight exposure on 25(OH)D concentration during pregnancy has thus far not been assessed, but significant seasonal variation in vitamin D levels has been observed also during pregnancy (Kokkonen, et al. 1983, Lamberg-Allardt, et al. 1984).

# 3. AIMS OF THE STUDY

The period of pregnancy in Multiple sclerosis disease offers researchers a unique chance to follow the changes resulting in natural disease amelioration. The aim of this thesis was to add a piece of knowledge in the field of pregnancy related Multiple sclerosis disease course by trying to elucidate the protective factors during pregnancy and distinguish some of the possible causes leading to disease activation after delivery.

The specific aims of this study were:

1. Prospectively follow the natural course of MS during and after pregnancy and evaluate the incidence of pregnancy complications and delivery risks of MS patients compared with the Finnish mothers in general

2. Study the breastfeeding manners of MS mothers with relation to their disease activity

3. Clarify the risk of MS patients to postpartum thyroiditis known to affect patients with other autoimmune diseases after pregnancy

4. Evaluate whether high sensitivity C-reactive protein (hsCRP) measurement can be used as a predictor of MS disease activity in the postpartum setting and whether CRP is useful in predicting pregnancy-related co-morbidities in MS

5. Evaluate the pregnancy and postpartum related changes in vitamin D levels among patients with MS and compare the results with healthy controls

# 4. PATIENTS AND METHODS

Details of materials and methods are presented in the original publications. Summary of participants, methods and main results is in Table 3.

## 4.1 Participants and study design

This study was a part of the Finnish Multiple Sclerosis and Pregnancy study. Study participants were gathered from neurological and obstetric units in the 15 central hospitals in Finland. The pregnant patients with MS encountered by the investigators were asked to participate in the study during the enrolment period of three years during 2003–2005. This resulted in 60 consenting participants with 61 pregnancies from 12/15 central hospitals around Finland; all participants had MS diagnosed according to Poser's or McDonald's criteria (McDonald, et al. 2001, Poser, et al. 1983). The capture rate was approximately 60%. Mainly geographical and investigator-related factors were responsible for the capture rate, which were unlikely to have created any bias in terms of the outcome measures. The majority of the participants (44/61; 72.1%) joined the study during early pregnancy at 10–12 gestational weeks (gw).

Patients were seen by a neurologist at 10-12 and 26-28 gw and at one month and six months postpartum. An obstetrical evaluation was performed at 10-12 gw and 35-37 gw and three months postpartum. Demographic data and data on the course of MS prior to the entry into the study were recorded. The outcome measures were: 1) mean annualized relapse rate (ARR) before, during and after pregnancy; 2) Expanded Disability Status Scale (EDSS) during and after pregnancy; 3) pregnancy-related variables: artificial insemination, gestational diabetes mellitus (GDM), preeclampsia, preterm and post-term infants; 4) Delivery-related variables: induction of labour, instrumental delivery, type of analgesia during vaginal delivery, frequency of caesarean sections, manual separation of the placenta; and 5) infant-related variables: mean gestational age, mean birth weight, low birth weight, high birth weight, infant deaths and malformations.

The following prospective data were collected: disability was assessed with EDSS at 10–12 and 26–28 gw and at one month and six months postpartum (KURTZKE. 1961). Each new relapse was recorded; a new relapse was confirmed by a neurological examination in 65% of cases (a relapse was defined as an appearance of a new objective neurological dysfunction lasting longer than 24 h and treatment was prescribed if the relapse caused major disability). Information on medication was collected at each visit by interviewing the patient; obstetrical data was obtained by interviewing the patients (they filled in

inquiry forms concerning earlier pregnancies and deliveries, methods of contraception, possible artificial insemination, and possible complications and need of hospital care during pregnancy) and by reviewing the delivery record. Timing and method of delivery, possible delivery complications, method of anesthesia used and weight of the newborn were recorded. The gathered data was analyzed in Turku.

**Table 3.** Summary of participants, methods and main results of the studies

| Study | Participants and materials | Methods | Main results |
|---|---|---|---|
| I | Relapse rates, EDSS scores, and obstetric outcome measures of 60 Finnish pregnant MS mothers | Prospective follow-up during pregnancy and six months postpartum | Mothers with MS are no more likely to experience pregnancy complications than are women generally |
| II | Relapse rates, use of DMTs and breastfeeding duration of 60 pregnant MS mothers | Prospective follow-up during pregnancy and six months postpartum | Mothers with stable pre-pregnancy disease were prone to breastfeed longer than mothers with active pre-pregnancy disease |
| III | Serum samples, FSS- and CES-D scores from 46 pregnant MS mothers and 35 healthy control mothers | S-TGAbs and S-TPOAbs: fluoroimmunoassay (AutoDELFIA)<br>S-TSH and S-FT4: CMIA | MS patients were observed with increased prevalence of elevated thyroid antibodies, but the prevalence of thyroid hormonal dysfunction was similar as among the healthy control mothers |
| IV | Relapse rates, serum samples and FSS-scores from 41 pregnant MS mothers and serum samples from 19 healthy pregnant control mothers | S-CRP: a separation-free immunometric assay (ArcDiaTM TPX) | Delivery related CRP did not predict postpartum disease activity, but elevated levels were associated with gestational diabetes and fatigue among MS patients. The CRP concentrations of MS mothers during pregnancy and postpartum did not differ from those of healthy controls. |
| V | Serum samples from 15 pregnant MS mothers and 6 healthy pregnant control mothers | S-25(OH)D3: HPLC | MS mothers were observed with significantly lower vitamin D concentrations than healthy control mothers. Majority of MS mothers were vitamin D deficient during pregnancy and postpartum |

EDSS= expanded disability status scale, DMT= disease modifying therapy, FSS= fatigue severity scale, CES-D= Center for Epidemiologic Studies Depression score, TGAbs= thyroglobulin antibodies, TPOAbs= thyroid peroxidase antibodies, TSH= thyrois stimulating hormone, FT4 =free thyroxine, CMIA=Chemiluminescent Microparticle Immunoassay, CRP= c-reactive protein, 25(OH)D3= 25-hydroxyvitamin-D3, HPLC= high-performance liquid chromatography

## 4.2 Control patients

### 4.2.1 Finnish Medical Birth Register

Pregnancy and delivery related variables of study patients were compared with the Finnish Medical Birth Register which is an official register collecting data from all births in Finland. The information in the Medical Birth Register has good validity, making it suitable for research use (Gissler and Shelley. 2002).

### 4.2.2 Thyroid antibody control patients

Thirty-five healthy volunteer control mothers were picked randomly from the cohort of mothers who had given birth between November 2009 and March 2010 in the Turku University Hospital. Control subjects were recruited by phone calls and they visited laboratory at six to nine months postpartum. The serum samples of these healthy controls taken in the beginning of pregnancy were obtained from the Finnish Maternity Cohort, which is a blood bank supported by National Institute for Health and Welfare. Samples of this blood bank are gathered in context of systematic screening of all Finnish pregnant mothers for certain infectious diseases in the beginning of pregnancy.

### 4.2.3 C-reactive protein control patients

Control samples were obtained from 19 healthy control mothers at 10-12, 26-28 and 35-37 gw respectively and 1-3 days, one month, three months and six months postpartum.

## 4.3 Ethics

Written informed consent was obtained from the participating MS patients and verbal informed consent from the control patients recruited by phone calls. The study was approved by institutional review board of the Hospital District of Southwest Finland initially in 2002 and the change concerning the healthy control patients in 2009.

## 4.4 Assessment of fatigue and depression

Study patients completed standardized questionnaires including Fatigue Severity Scale (FSS) and Center for Epidemiologic Studies Depression scale (CES-D) at 10-12 and 26-28 gw, one month and six months postpartum. Patients with FSS scores $\geq 4$ were considered to suffer from significant fatigue (Valko, et al. 2008). CES-D score $\geq 16$ was considered as a cutoff for clinical depression.

## 4.5 Laboratory assessments

Serum samples of study participants were obtained 10-12, 26-28 and 35-37 gestational weeks (gw) respectively and 1-3 days, 4-5 weeks (one month), 10-12 weeks (three months) and six months postpartum. Serum samples were centrifuged and stored at -40° Celsius.

### 4.5.1 Thyroid antibodies and hormones

Serum thyroid peroxidase antibodies (TPO-Abs) and thyroglobulin antibodies (TG-Abs) levels of 46 study MS patients were serially measured during pregnancy and six months postpartum. Thyroid-stimulating hormone (TSH) and free thyroxine ($FT_4$) measurements were performed on MS patient (n = 29) and healthy control (n = 35) serum samples taken at six months postpartum.

TG-Abs and TPO-Abs were measured using solid-phase, two-step, time-resolved fluoro-immunoassays (AutoDELFIA, PerkinElmer Corporation, Turku, Finland) in all available serum samples from MS patients (time points 10–12 gw n = 37; 26–28 gw n = 40, 35–37 gw n = 37, one month postpartum n = 43; three months postpartum n = 32 and six months postpartum n = 34) as well as controls (time point 10–12 gw n = 34 and time point six months postpartum n = 35). Samples were run in duplicates. According to PerkinElmer, values above 60 U/ml were regarded as elevated for TPO-Abs as well as for TG-Abs (Hansen, et al. 2006). Subjects were considered to present with thyroid autoimmunity if either of the antibodies were elevated. The most important performance characteristics of the assays have been described previously (Jensen, et al. 2004, Jensen, et al. 2005).

TSH and $FT_4$ measurements were performed using Chemi-luminescent Microparticle Immunoassay (CMIA; Abbott Diagnostics, Abbott Park, IL, USA). Reference values for normal thyroid function were 0.35–4.9 mU/l for TSH and 9–19 pmol/l for $FT_4$.

### 4.5.2 High-sensitivity C-reactive protein

The high-sensitivity C-reactive protein (hsCRP) was measured using a separation-free immunometric assay method based on ArcDiaTM TPX technology (ArcDia International Oy Ltd, Turku, Finland) as previously described (Koskinen, et al. 2004) in all available serum samples from MS patients (time points 10–12 gw *n* =35; 26–28 gw *n* =37; 35–37 gw *n* =35; 1-3 days postpartum *n*=31; one month postpartum *n* =38; three months postpartum *n* =32 and six months postpartum *n* =26) as well as controls (time points 10–12 gw *n* =14; 26-28 gw *n* =19; 35-37 gw *n* =18; 1-3 days postpartum *n* =11; one month postpartum *n*=14; three months postpartum *n* =14 and six months postpartum *n* =15.)

The one-step method applies microspheres (Ø 3.2µm) as solid-phase reaction carries and fluorescent nanoparticulate reporters (Ø 75nm) as tracer. As a result of the immunoassay

reaction, the fluorescent tracer concentrates on the microsphere surface. The signal measurement of the Plate Reader instrument from 384-well format plates relies on two-photon excitation fluorometry of individual microspheres. All samples were measured in quadruplicate (two 1:500 sample predilutions assayed as duplicates), and all samples from a single subject were measured in the same run. In addition, a standard curve against which of the samples were plotted was measured in each run. The reference intervals for CRP during pregnancy were considered 0.32-11.9mg/l for 10-12 gestational weeks; 0.43-20.28mg/ml for 26-28 gestational weeks and 0.64-28.26 mg/l for 35-37 gestational weeks (Larsson, et al. 2008).

### 4.5.3 25-hydroxyvitamin $D_3$

Levels of 25-hydroxyvitamin $D_3$ (25(OH)$D_3$) were measured from 15 MS patients and six healthy control patients at 10-12 gw, 26-28 gw, 35-37 gw, and one month, three months and six months postpartum. Measurements were performed using Agilent series 1100 high-performance liquid chromatography (HPLC) system with a binary pump as previously described (Turpeinen, et al. 2003), with some modifications. Briefly, to 0.5 ml of serum, 350 µl of methanol-2-propanol (80:20 by volume) was added. The tubes were mixed for 30 s. The analytes were extracted by mixing 3x 60 s with 3 ml of hexane. The phases were separated by centrifugation, and the upper organic phase was transferred to a conical tube and dried under nitrogen. The residue was dissolved in 100 µl of mobile phase. Calibration curves were constructed using four concentrations of 25-OH-$D_2$ and 25-OH-$D_3$ (16-129 nmol/l; cat. no. 17938, 17937; Fluka). The standards were calibrated by spectrophotometry of ethanolic solutions using a molar absorption coefficient of 18200 for both metabolites at 265 nm. Separation was performed on a Discovery HS F5 column (4 x 250 mm, 5 um bead size; Supelco) maintained at 24 °C. The mobile phase was 800 ml/l methanol in water, and the flow rate 0.8 ml/min. Detection was at 265 nm, and the injected volume was 50 µl.

Vitamin D status in mothers was defined as deficient when 25(OH)D was below 50nmol/l, insufficient when it was between 50 and 75nmol/l, and sufficient when it was above 75nmol/l, according to reference values for adult population recommended by the American Endocrine Society guideline (Holick, et al. 2011). In the high latitudes of Finland (between 60°N and 70°N), months with low sunlight exposure resulting with likelihood of lower vitamin D levels (October-May) were considered as winter months.

## 4.6 Data analysis and statistical methods

In all the studies, where applicable, the Wilcoxon test for ranks sums was used for comparing the differences in medians; an unpaired *t* test was used to compare the

differences in means; the $\chi^2$-test or the Fishers exact test was used for comparing the differences in proportions; and one way ANOVA was used to compare the differences in continuous variables between the groups. Statistical analyses were performed with JMP version 10.0, GraphPad Prism version 5 or SAS System for Windows, version 9.3 (SAS Institute Inc., Cary, NC, USA). *P* values $< 0.05$ were considered statistically significant for all analyses.

In **Study I** the annualized relapse rates per patient during each three-month period during pregnancy and postpartum were compared with the relapse rate during the year before pregnancy using the paired two-tailed t-test. Comparisons of EDSS values at different time points were also performed using the paired two-tailed t-test. All comparisons between categorical variables in the study cohort and in the Finnish Medical Birth Register were performed using the Chi square test or the Fisher's exact test. Comparisons between continuous variables in the study cohort and in the Finnish Medical Birth Register were performed using the one sample t-test procedure.

In **Study II** patients were classified to groups with no relapse, with at least one relapse, and with two or more relapses during the year preceding pregnancy to investigate whether pre-pregnancy disease activity would influence the mother's breastfeeding behavior. The respective proportions in these groups were compared with Fishers exact test. The mean breastfeeding times of respective groups were compared with unpaired two-tailed Student t-test. Because the prospective follow-up time of the study was six months and a significant proportion of patients reported breastfeeding for over six months, the over six month time was regarded as equal to six months for calculation of the mean breastfeeding times. MS patients were also classified to four groups according to the breastfeeding duration to assess differences in the annual relapse rates of these respective groups. The mean annual relapse rates were compared with Student t-test.

In **Study III** the alteration of the mean serum TPO-Ab and TG-Ab concentrations were analyzed by repeated measures ANOVA, which was fitted for log-transformed response variables. Unstructured covariance structure was used to control the between-subjects variation due to unequal variances between time points. Comparisons between different time points were statistically analyzed with Dunnet-Hsu post-test. Percentages of individuals with elevated thyroid Ab-levels among MS patients and controls were compared using Fisher's exact test.

Mean ARR, mean age and disease duration, and mean EDSS score comparisons between patients with elevated and normal thyroid Abs were performed using Wilcoxon test for rank sums.

In **Study IV** differences in longitudinally measured CRP levels between MS patients and controls were evaluated using the repeated measures ANOVA. Where necessary,

a logarithmic transformation was applied. To assess the relation of CRP concentration with mean annualized relapse rate, mean age, mean disease duration and mean EDSS score, MS patients were categorized into two groups: patients with CRP ≤5 mg/l (n=18) and >5mg/l (n=22) during pregnancy. Comparisons between these groups were performed using the Student's t-test or Wilcoxon rank-sum test. Comparisons of CRP levels between different modes of delivery were performed with Oneway ANOVA. Comparisons between relapse-free and relapsing MS patients were performed with Wilcoxon rank-sum test. Association of elevated CRP at delivery (>25 mg/l) with later CRP concentration in the postpartum period was evaluated using Wilcoxon rank-sum test. Youden indices were calculated in order to define the best cut-off value for CRP in the beginning of pregnancy to predict gestational diabetes. The predictive value of CRP on postpartum relapses was analyzed with logistic regression analysis.

In **Study V** differences in longitudinally measured 25(OH)$D_3$ levels between MS patients and controls were evaluated using the repeated measures ANOVA. Comparisons between different time points were statistically analysed with the Dunnet-Hsu post-test. The association between vitamin D levels and pre- and postpartum relapses was analysed using linear mixed model with time point as repeated effect and season as random effect.

# 5. RESULTS

## 5.1 Characteristics of study patients

All the 60 MS patients had the relapsing-remitting form of MS and their mean age was 30.5 years (range 23–42 years) in the beginning of pregnancy. The mean duration of the disease at the onset of the study was 5.7 (SD 4.3) years (range 0–17.5 years). The total number of relapses before the study onset was on average 4.1 (SD 2.8, range 1–12). Half of the patients (n=33; 54.1%) were on disease modifying therapy (DMT) before pregnancy; twelve (36.3%) of them continued medication until first trimester and twenty-one (63,7%) discontinued their treatment already before pregnancy on average 6,2 (SD 7,2; range 0.5-33) months before conception. The average relapse rate during the year before pregnancy was 0.82 (SD 0.98). Approximately half of the patients experienced no relapses during the year preceding the pregnancy (30/61; 49.2%).

## 5.2 The course of Multiple sclerosis during pregnancy and after delivery

### 5.2.1 Relapse rate during pregnancy

Mean annualized relapse rates in the year before pregnancy, each trimester of pregnancy and during 0–3 months and 3–6 months after delivery are presented in Figure 2. Seventy percent (43/61) of patients experienced no relapses during pregnancy. The mean annual relapse rate was lowest during the third trimester of pregnancy (0.40 (SD 1.21). The relapses during pregnancy did not cause severe disability and therefore no corticosteroid or intravenous immunoglobulin (IvIg) treatment was required during pregnancy.

### 5.2.2 Relapse rate after delivery

The relapse rate was highest during the first three months after delivery (1.33 (SEM 0.25). Almost half (45.9%) of the patients experienced a relapse during the six months after delivery.

Thirty-three patients (54.1%) experienced no relapses during the six months following the delivery. Twenty-four patients (39.3%) had one relapse. Three patients had two relapses and two patients had three relapses during the follow-up. Of these relapses, 22 (61.1%) occurred during the first three months after the delivery and 14 relapses (38.9%) during 3–6 months after the delivery.

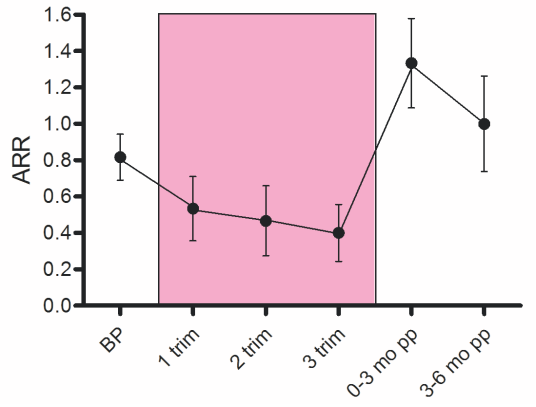


**Figure 2.** Mean (SEM) annualized relapse rates (ARR) in 61 pregnancies of women with MS during the year before pregnancy (BP) [0.82(0.13)], the three trimesters of pregnancy [0.53(0.18), 0.47(0.19) and 0.40(0.16), respectively] and 0–3 and 3–6months postpartum period (pp) [1.33(0.25) and 1.00(0.26), respectively]. Vertical bars represent the SEM. ARR BP vs. 1st trimester p=0.171; ARR BP vs. 2nd trimester p=0.1478; ARR BP vs. 3rd trimester **p=0.026**; ARR BP vs. 0–3months pp **p=0.030**; and ARR BP vs. 3–6months pp p=0.504; paired two- tailed t-test.

### 5.2.3 Expanded disability status scale

The EDSS score followed a course similar to the relapse rate; the mean EDSS was lowest at mid pregnancy 26-28 gestational weeks (gw) [1.33(SEM 0.18)] and highest at 4–5 weeks after delivery [1.63(SEM 0.21)] (Figure 3).

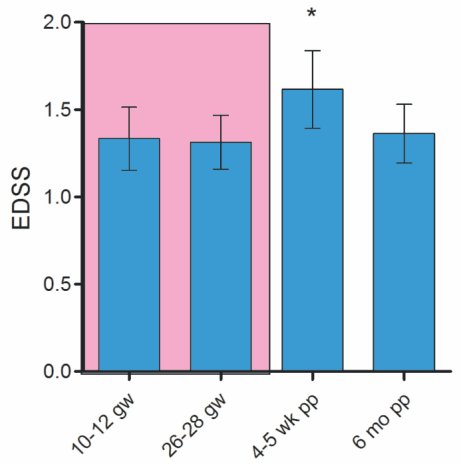


**Figure 3.** Mean (SEM) Expanded Status Scale (EDSS) score of the patients with MS during the 61 pregnancies at 10–12 gestational weeks (gw) [1.33(0.18)], 26–28 gw [1.32(0.15)] and at 4–5 weeks [1.63(0.21)] and six months postpartum (pp) [1.40(0.15)]. Vertical bars represent the SEM. The asterisk marks a statistically significant alteration in the EDSS compared with the EDSS measured in early pregnancy. EDSS 10–12gw vs. EDSS 26–28gw p=0.760; EDSS 10–12gw vs. EDSS 1 month pp **p=0.045**; EDSS 10–12gw vs. EDSS 6 months pp p=0.0675.

## 5.3 Pregnancy and delivery outcomes of Multiple sclerosis patients

### 5.3.1 Pregnancy characteristics and outcomes

In this study 60 MS mothers were followed during 61 pregnancies. One mother was followed twice during her two pregnancies. Two thirds (40/61) of study MS patients were primigravida. One study patient gave birth to twins. Three (3/61: 4.9%) pregnancies were artificially inseminated. Three MS mothers (4.9%) presented with pre-eclampsia. There was a trend towards an increased prevalence of gestational diabetes (GDM) in the MS patient group when compared with the general population, but this difference did not reach statistical significance (14.8% vs. 8.4%, p=0.0736). The rate of adverse pregnancy outcomes was similar among MS mothers and the Finnish mothers in general (Original publication I, Table 1). The mean birth weight of newborn was 3413g (SD 557) among MS mothers and it did not significantly differ from the average birth weight in Medical Birth register (3518 g, p=0.1707*).* Use of disease modifying therapy during early pregnancy did not have a major effect on pregnancy as the twelve exposed pregnancies did not differ in regards of pregnancy outcome or birth weight of the newborn compared to non-exposed pregnancies (Table 4). Nine of these mothers were exposed to IFN-β and three to glatiramer acetate (GA).

**Table 4.** Disease modifying treatment (DMT) exposure in early pregnancy.

| | **Exposed ,%** | **N** | **Unexposed,%** | **N** | **p-value** |
|---|---|---|---|---|---|
| Tot | 19.7 | 12/61 | 80.3 | 49/61 | |
| Mean maternal EDSS | 1.88 ± 1.60 | | 1.17 ± 1.03 | | 0.0618 |
| Operative delivery, all | 50.0 | 6/12 | 30.6 | 15/49 | 0.3534 |
| Caesarean section (tot) | 33.3 | 4/12 | 12.2 | 6/49 | 0.0958 |
| Planned | 25.0 | 3/12 | 8.1 | 4/49 | 0.1300 |
| Acute | 8.3 | 1/12 | 4.1 | 2/49 | 1.0000 |
| Vacuum extraction | 16.7 | 2/12 | 16.2 | 8/49 | 1.0000 |
| Mean birth weight | 3628(SD713) | | 3384(SD507) | | 0.1746 |
| Birth weight <2500g | 8.3 | 1/12 | 4.1 | 2/49 | 0.4881 |
| Pregnancy complications[1] | 33.3 | 4/12 | 28.6 | 14/49 | 0.7359 |
| Delivery complications[2] | 8.3 | 2/12 | 10.5 | 5/49 | 0.6618 |

[1] Pre-eclampsia, preterm delivery, gestational diabetes mellitus, placenta praevia

[2] Prolonged 2nd stage of delivery, manual separation of the placenta

### 5.3.2 Delivery characteristics of the Multiple sclerosis patients

Among study MS patients, the mean gestational age at birth was 39+2 gw (range 33-42). Four MS mothers (6.6%) gave preterm birth (<37 gw) and two mothers (3.3%) delivered post-term (>42 gw). Three MS mothers (4.9%) developed pre-eclampsia. Forty-one mothers (67.2%) delivered vaginally without assistance, ten mothers (16.4%) needed vacuum extraction and ten mothers (16.4%) delivered by caesarean section, of which seven were planned and three acute. Compared to Finnish mothers in general MS mothers

presented somewhat higher frequency in the need of vacuum assistance during delivery (6.5% vs 16.4%, p=0.0017) but the rate of caesarean sections was equal (16.6% vs 16.4%, p= 0.965). Forty-three percent (22/51) of vaginally delivered MS mothers got epidural analgesia during delivery and 6 % (3/51) got spinal analgesia. Twenty-three percent (12/51) got no analgesia during delivery. Patients with MS gave birth more often without any analgesia than did women in the general population (23.5% vs. 13.8%; p=0.0440).

### 5.3.3 Pregnancy complications and C-reactive protein

C-reactive protein (CRP) was measured from two MS mothers with preterm delivery and one with pre-eclampsia. CRP in the beginning of pregnancy was significantly elevated in two of these mothers (2/3) compared to none in non-complicated pregnancies (0/38), p=0.0037. The first-trimester CRP levels of these two MS mothers were 20.1 and 24.7 mg/l (reference interval 0.32-11.9mg/l (Larsson, et al. 2008). The third mother with preterm birth on the gestational week 35 presented with moderate CRP during first and second trimesters of pregnancy (4.07 and 5.49mg/ l respectively) and slightly higher, but within reference area, CRP on the third trimester (18.44 mg/l; reference interval 0.64-28.26 mg/l (Larsson, et al. 2008)).

## 5.4 Breastfeeding behaviour among Multiple sclerosis patients

The majority of the MS mothers (55/61, 90.2%) breastfed their babies. Thirty-three mothers (54.1%) continued breastfeeding for six months or longer (Figure 4 a).

### 5.4.1 Breastfeeding duration and disease activity

We found that breastfeeding was less frequent among mothers with active pre-pregnancy disease (Figure 4b). Moreover, the duration of breastfeeding was shorter among patients with active pre-pregnancy disease (Figure 4c). Five of the six mothers who did not breastfeed resumed IFN-β therapy within two weeks after delivery. One mother developed postpartum depression and could not breastfeed at all. One mother started IFN-β treatment two weeks after the delivery while also breastfeeding for five months with no observed harm to the baby.

Mothers who did not breastfeed at all (n=6) were observed with significantly higher annual relapse rate (ARR) during the year before pregnancy compared to mothers who breastfed six months or longer (n=33) [1.83(SEM 0.54) vs 0.49 (SEM 0.15), p=0.0013, Figure 4 b]. There was also a significant difference in pre-pregnancy ARR between mothers breastfeeding 2-5.9 months (n=16) compared to mothers continuing breastfeeding for six months or longer (n=33) [1.06(SEM 0.17) vs 0.49(SEM 0.15), p=0.0392, Figure 4b]. Between these respective groups, there was also a significant difference in the

mean ARR after delivery [1.63(SEM 0.42) vs 0.73(SEM 0.19), p=0.0282, Figure 4b]. Differences between other breastfeeding subgroups were not significantly different. Postpartum mean ARR was relatively low [1.00(SEM 0.45)] in the non-breastfeeding group, in which five of the six mothers continued DMT early after delivery.

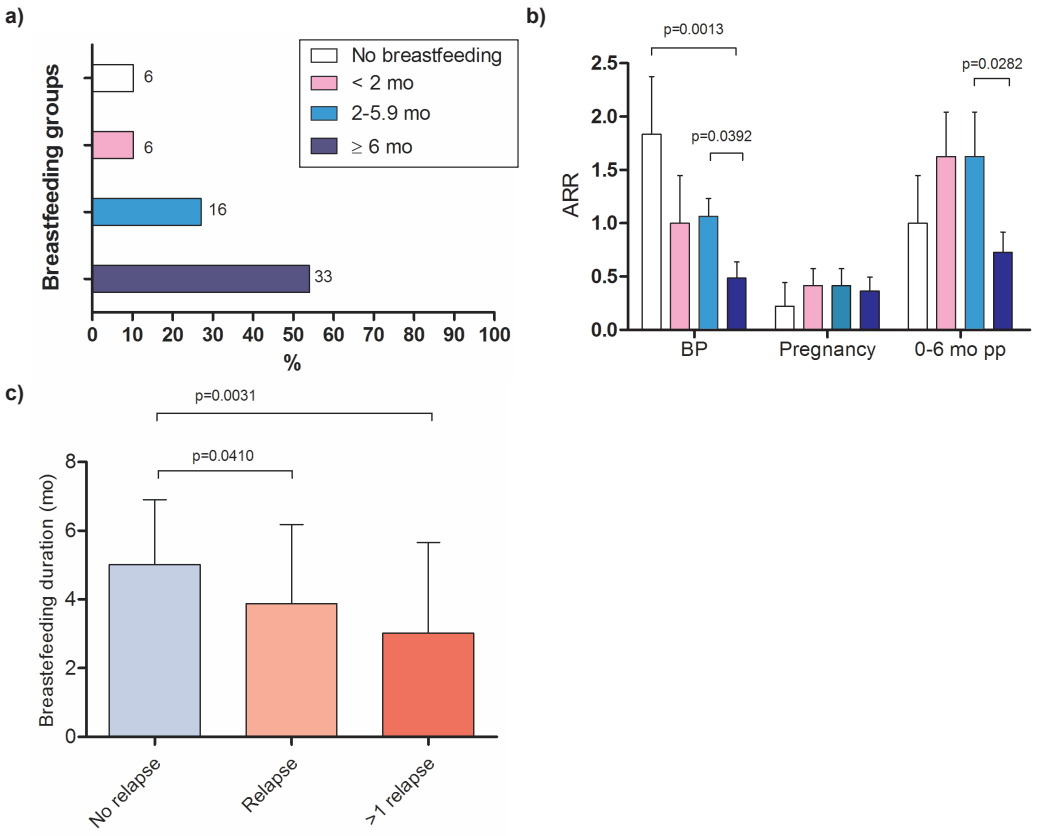


**Figure 4.** Breastfeeding duration and the disease activity of study MS patients. **a.)** Percentage and number of patients according to the breastfeeding duration. **b.)** Mean (SEM) annual relapse rate of the respective breastfeeding groups. The annual relapse rate (ARR) during the year preceding pregnancy was significantly higher among mothers not breastfeeding at all compared to mothers who breastfed ≥ 6 months. There was a significant difference in the mean ARR between mothers who breastfed 2-5.9 months compared to mothers who breastfed ≥ 6 months during the year preceding pregnancy and during the six months postpartum. **c.)** Mean (SD) duration of breastfeeding among patients with no relapse [n=30, 5.0 (1.9) months], at least one relapse [n=31, 3.9 (2.3) months] and over one relapse [n=15, 3.0 (2.6) months] during the year before pregnancy. Differences were statistically significant.

## 5.5 Pregnancy and postpartum related thyroid autoimmunity

### 5.5.1 Thyroid antibodies during and after pregnancy

TPO-Ab and TG-Abs were measured from 46 study MS patients. The mean antibody concentrations gradually decreased during pregnancy and increased again after the delivery

with maximal levels at six months postpartum (Figure 5, overall p<0.0001). Two MS patients (2/37, 5.4%) were observed with elevated TG-Abs in the beginning of pregnancy, another one of them also with elevated TPO-Abs (Figure 6). This patient with both TG- and TPO-Abs elevated developed later postpartum thyroiditis. In the control group one (1/34, 2.9%) individual had elevated level of TPO-Abs in the first trimester. There was no significant difference in the proportions of patients with elevated antibodies in early pregnancy between MS patients and controls (5.4% vs 2.9%, p= 1.000). At six months postpartum, a significantly higher proportion of MS patients 12/34 (35.3%) presented signs of thyroid autoimmunity (elevated levels of either TPO-Abs or TG-Abs), compared to the measurements during the first trimester (5.4% vs 35.3%, p= 0.0022). Two (2/35, 5.7%) control patients were observed with elevated TPO-Abs and none with elevated TG-Abs at six months postpartum. At this time-point the proportion of MS mothers with elevated thyroid Abs was significantly higher compared to control mothers (35.3% vs 5.7%, p= 0.01).

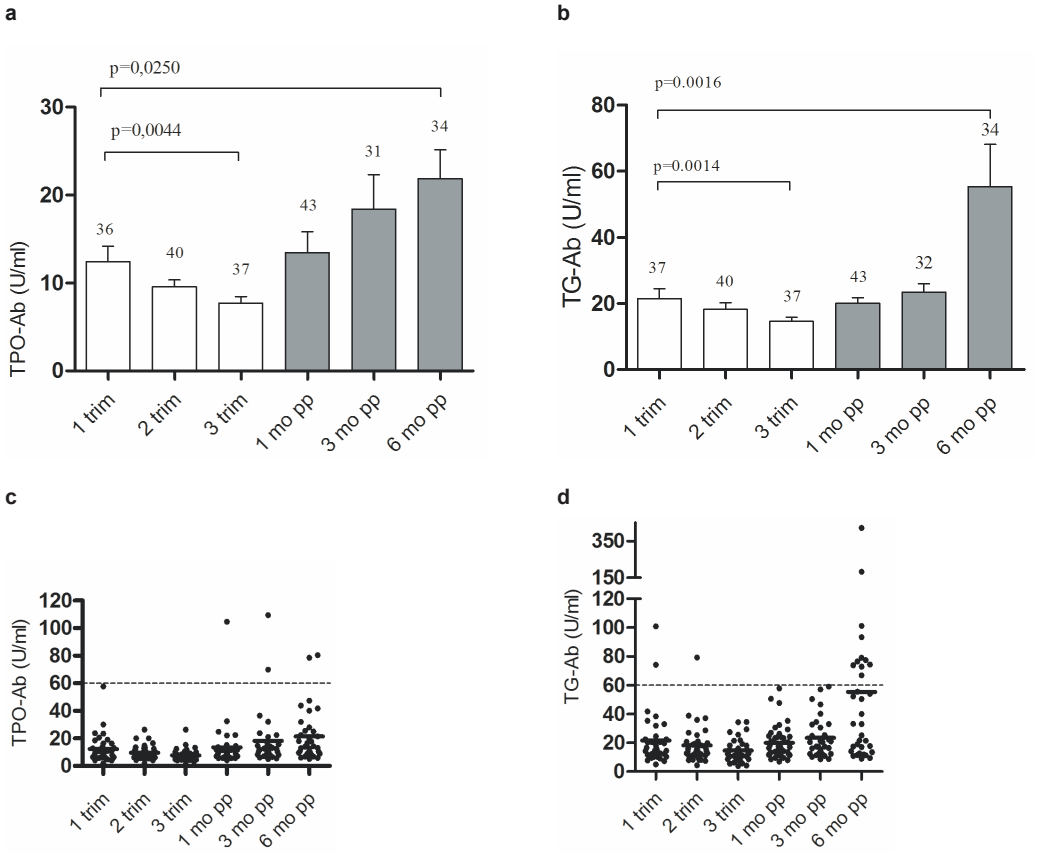


**Figure 5.** Mean thyroid antibody levels and the distribution of individual thyroid antibodies during pregnancy and the postpartum period among MS patients. Evolution of mean (SEM) a) thyroid peroxidase antibodies (TPO-Abs), b) thyroglobulin antibodies (TG-Abs) and distribution of c) TPO-Abs and d) TG-Abs during the three trimesters of pregnancy and at one month, three months and six months postpartum. The number of samples available on each time-point is indicated above each column. The dotted line indicates the limit (60U/ml) for elevated Abs.

### 5.5.2 TSH and $FT_4$ values at six months postpartum

TSH and $FT_4$ values were measured from 29 MS patients and 35 controls at six months postpartum. Only one (1/29, 3.4%) MS patient presented with mild hyperthyreosis at six months postpartum, with low TSH value (0.018mU/l; normal range 0.35-4.9mU/l) and elevated $FT_4$ value (20.78pmol/l; normal range 9-19pmol/l). Accordingly, this particular patient presented with significantly elevated TG-Abs and moderately elevated TPO-Abs in early pregnancy and was observed with significant increase in both TG- and TPO-Ab levels postpartum (Figure 6). In the control group one individual (1/35; 2.9%) was observed with subclinical hypothyreosis at six months postpartum with high TSH value (22.6mU/l). Her $FT_4$ value was in the lower reference area (11.23pmol/l). She had elevated TPO-Ab levels both in early pregnancy and postpartum.

There was no significant difference in the demographic variables, MS disease activity or level of disability between MS patients with elevated or normal thyroid Ab levels (Original publication III, Table 1).

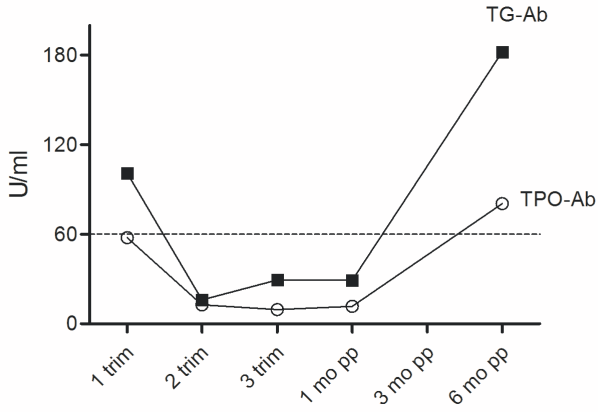


**Figure 6.** Evolution of the thyroid antibody levels of the MS patient with postpartum thyroiditis. Cut-off for elevated antibody levels was considered >60U/ml (dotted line) for both thyroid peroxidase antibodies (TPO-Ab) and thyroglobulin antibodies (TG-Abs).

### 5.5.3 Thyroid autoimmunity in relation to obstetric outcomes

Thyroid antibody positivity was not associated with any increase in the adverse pregnancy outcomes, such as pre-term delivery or pre-eclampsia (Original publication III, Table 2). In addition, the rate of caesarean section or vacuum assistance was similar between the patients with elevated (n=12) and normal (n=34) thyroid Abs (Original publication III, Table 2). The only significant difference between these respective groups was the rate of gestational diabetes, as all three MS mothers with gestational diabetes were thyroid Ab positive (p=0.024). Characteristics of the newborns were

similar in the respective groups (Original publication III, Table 2). The only MS patient with laboratory confirmed postpartum thyroid dysfunction gave birth to a baby with low birth weight (≤2500g). This patient presented with elevated thyroid antibodies (TPO-Ab 57.7 U/ml, TG-Ab 100.9U/ml)) already during first trimester (Figure 6). She had a recent diagnosis of MS and was considered to have a relatively active disease with over twenty demyelinating lesions in the diagnostic MRI scan. She had not been on disease modifying therapy before pregnancy. Despite the initiation of disease modifying therapy (IFN-β) already at one month postpartum in order to prevent postpartum relapses, she experienced a relapse at six months postpartum in parallel with the postpartum thyroiditis.

## 5.6 Fatigue during pregnancy and postpartum

The prevalence of fatigue did not significantly differ between pregnancy and postpartum period in the cohort of study MS patients. The FSS scores of present study patients are presented in Figure 7a. Fatigue was observed in 45.0% of patients during pregnancy, and in 53.8% of patients in the postpartum period (p=0.5026). Presence of elevated thyroid Abs was not a predisposing factor for fatigue as fatigue was observed in 66.7% of patients with elevated thyroid Abs compared to 57.1% of patients with normal thyroid Ab levels, (p = 0.7294).

MS patients with fatigue had a slightly higher mean EDSS than patients with no fatigue [1.83 (SD1.06) vs. 1.20 (SD 1.02), p = 0.0503] and they were observed with significantly higher CRP levels during pregnancy compared to mothers without fatigue (p=0.0353, Figure 7 b). Postpartum the difference in CRP levels between these groups disappeared.

At six-month postpartum time-point, 15 (15/37, 40.5%) patients presented with fatigue with a mean FSS score of 5.2. Among the 22 (22/37) patients with no fatigue at this time point, the mean FSS score was 2.3.

Of the patients reporting fatigue at six months postpartum, 40.0% had elevated thyroid Ab levels, whereas only 22.7% of the patients with no fatigue had elevated thyroid Ab levels (p=0.2955). Fatigue after delivery affected the duration of breastfeeding. Only 30% of mothers experiencing fatigue continued to breastfeed for longer than six months, whereas 70% of mothers with no reported fatigue breastfed for longer than six months (p=0.0238).

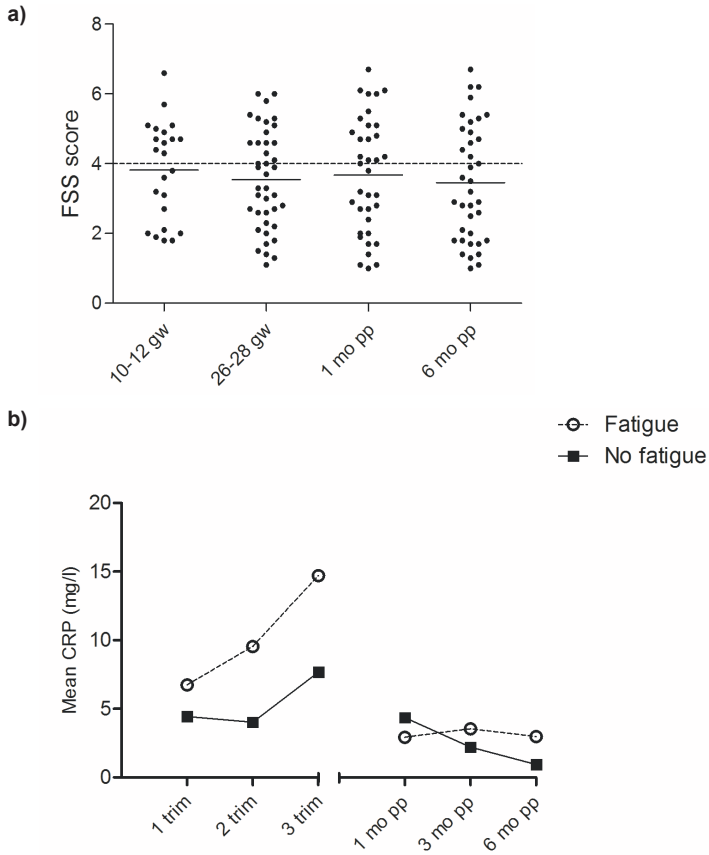


**Figure 7.** Prevalence of fatigue and its relation to C-reactive protein (CRP) levels. **a)** Fatigue Severity Scale (FSS) scores of 40 MS patients at 10–12 and 26–28 gestational weeks (gw) and four to five weeks and six months postpartum (pp). The dotted line indicates the cut-off value for clinically significant fatigue (≥ 4 points). Horizontal bars represent the mean. **b)** CRP levels of the MS patients with fatigue and no fatigue during pregnancy and postpartum. Patients with fatigue were observed with significantly higher mean CRP during pregnancy, but not postpartum.

## 5.7 Depressive symptoms during pregnancy and postpartum

Depressive symptoms of study MS patients were measured by the CES-D questionnaires. The CES-D scores of study patients are presented in Original publication III (Figure 4b). Depression was observed in 30.0% of patients during pregnancy and in 25.6% of patients postpartum ($p = 0.8027$). Thyroid antibody positivity was not associated with depressive symptoms. Depression was observed in 41.6% of patients with elevated thyroid antibodies compared to 35.7% in patients with normal antibodies ($p = 0.7357$).

## 5.8 C-reactive protein during pregnancy and postpartum

The evolution of CRP levels followed a similar course during pregnancy and postpartum period among patients with MS and healthy controls. The median CRP concentrations of these groups did not significantly differ in any of the time points (p=0.9484, Figure 8a). Nor was there any difference in median CRP concentrations between relapsing and relapse-free MS patients (Figure 8b). The median CRP was significantly higher during pregnancy than postpartum (MS patients 4.33 mg/l (QR 2.49-7.38) vs. 1.27 mg/l (QR 0.64-3.2); p=<0.0001 and controls 3.97 (QR 2.45-5.43) vs. 1.22 mg/l (QR 0.69-1.86), p=<0.0001). Mode of delivery did not have a significant influence on the CRP measured within three days of delivery, as the mean CRP concentrations were 32.6 mg/l (SD 14.5, n=21) after a normal vaginal delivery, 36.6 mg/l (SD 17.4, n=6) after vacuum assisted delivery and 37.9 mg/l (SD 4.8, n=4) after caesarean section (p=0.7197). Patients with lower CRP (≤5mg/l) during pregnancy gave birth to babies of 3562g (SD 560, n=18) mean birth weight with no significant difference compared to patients with higher CRP (>5mg/l; 3410g (SD 587, n=22), p=0.4114). No significant difference was observed in relapse rates or EDSS scores between these respective groups (Table 5).

To assess the effect of higher delivery CRP on postpartum inflammatory activity and relapses, patients were classified into lower (CRP <25mg/l) and higher (≥25mg/l) CRP groups. No association on postpartum relapses could be found in these respective groups, but patients with higher delivery CRP persisted with significantly higher CRP levels until three months postpartum compared to patient with lower delivery CRP (Figure 9a). Similar prolonged peripheral inflammatory reaction was not seen among respective groups in healthy controls. Instead, the control patients with higher delivery CRP were observed with slightly lower levels during the following six months postpartum (Figure 9b). MS patients with gestational diabetes (n=6) were observed with higher CRP concentrations thorough the study period and CRP >7 mg/l in the beginning of pregnancy predicted gestational diabetes with sensitivity and specificity of 83%.

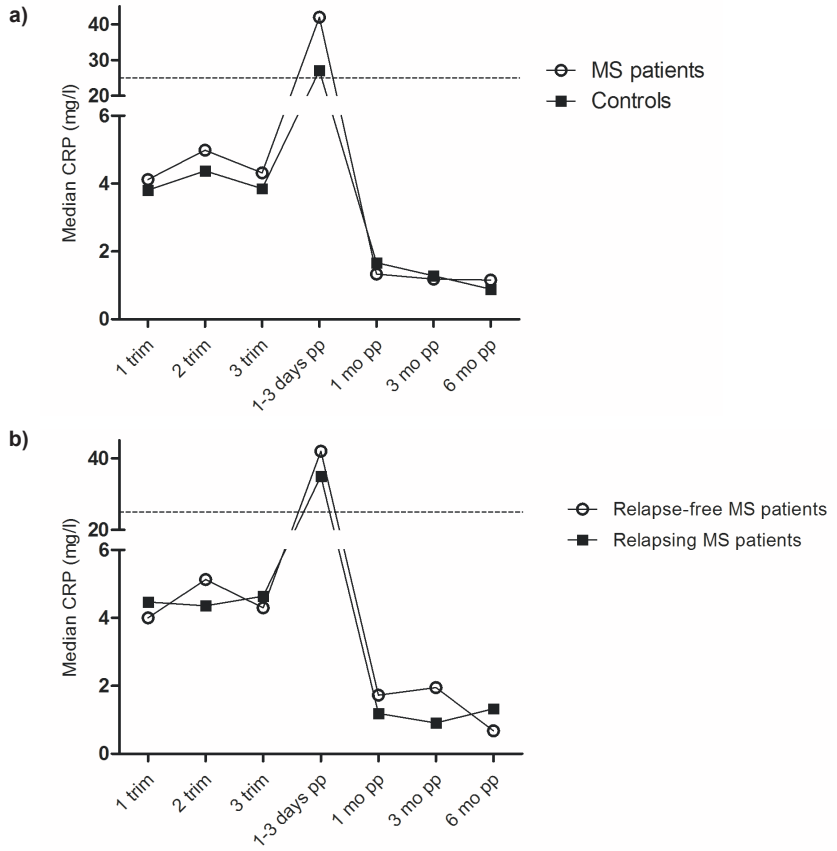


**Figure 8.** Evolution of the median CRP concentration of **a)** MS patients and healthy controls **b)** relapse-free and relapsing MS patients. Cut-off 25mg/l was used to further compare the postpartum inflammatory reactions and relapse activity between patients with higher and lower delivery CRP.

**Table 5.** The mean annualized relapse rates (top panel) and EDSS scores (bottom panel) of MS patients with CRP ≤5 mg/l and >5 mg/l during pregnancy.

| | **CRP*≤5; n=18 ARR, mean(SD)** | **CRP>5; n=22 ARR, mean(SD)** | ***p*** |
|---|---|---|---|
| 1st trim | 0.89 (1.71) | 0.55 (1.41) | 0.4898 |
| 2nd trim | 0.89 (2.19) | 0.18 (0.85) | 0.1720 |
| 3rd trim | 0.44 (1.29) | 0.36 (1.20) | 0.8374 |
| 0-3 mo pp | 1.77 (2.05) | 1.63 (2.36) | 0.8426 |
| 3-6 mo pp | 1.11 (2.30) | 1.27 (2.27) | 0.8250 |
| | **CRP≤5; n=18 EDSS, mean(SD)** | **CRP>5; n=22 EDSS, mean(SD)** | ***p*** |
| 1st trim | 1.58 (1.59) | 1.43 (1.14) | 0.7455 |
| 2nd trim | 1.39 (1.50) | 1.45 (0.99) | 0.8689 |
| 1 mo pp | 1.69 (1.38) | 1.75 (1.33) | 0.9004 |
| 6 mo pp | 1.57 (1.00) | 1.55 (1.01) | 0.9556 |

Abbreviations: ARR, annual relapse rate; EDSS, expanded disability status scale score; trim, trimester of pregnancy; mo pp, months postpartum

*CRP was measured at three time-points during pregnancy; 10-12 gestational weeks (gw), 26-28 gw and 35-36 gw

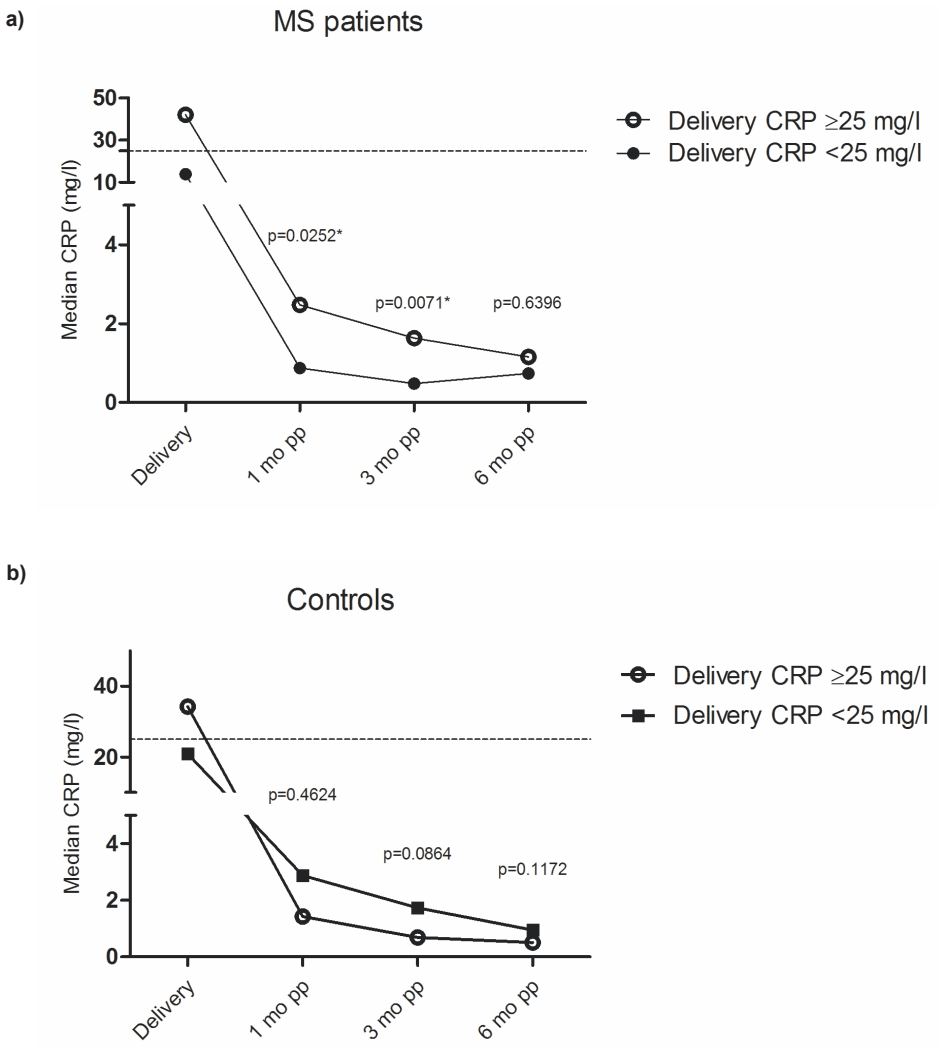


**Figure 9. a)** The median postpartum CRP levels of MS patients with higher (≥25 mg/l, n=22) and lower delivery CRP (<25 mg/l, n= 8). The median CRP concentration remained significantly higher at one month postpartum (2.47(QR 1.03-4.67) vs. 0.88(QR 0.33-2.16); p=0.0252) and three months postpartum (1.3(QR 0.93-3.97) vs. 0.48(QR 0.026-0.85); p=0.0071) in the group of MS patients with higher delivery value of CRP. The asterisks indicate statistically significant difference. The cut-off level is indicated with a dotted line. **b)** Similar prolonged inflammatory reaction after higher delivery CRP (≥25mg/l) could not be observed among the healthy controls.

## 5.9 Vitamin D during pregnancy

Patients with MS presented with lower vitamin D concentrations during pregnancy when compared to healthy controls, (p=0.0368, Original publication V, Figure 2). Vitamin D

levels diminished significantly after the delivery and reached the minimum concentration at one month postpartum in both groups (3rd trim vs. 1 mo pp: MS patients 46.9 (SD5.3) vs 36.5 (SD4.6)nmol/l, p= 0.0207 and controls 62.7(SD7.5) vs 52.8 (SD4.1)nmol/l, p=0.5359). At this point, as many as 80% of the MS patients (12/15) were deficient of vitamin D (concentration <50nmol/l) while all of them continued breastfeeding.

Overall, 80 % (12/15) of MS patients and 50 % (3/6) of controls presented with serum $25(OH)D_3$ concentrations <50nmol/l (p=0.2906, Fisher's exact test). During pregnancy, 73 % (11/15) of MS patients presented with vitamin D deficiency. After delivery 80% (12/15) of patients were vitamin D deficient. Majority of the samples were drawn during winter months (October- May) both in the MS cohort (60/90; 67%) and in the control group 22/36; 61%). The distribution of the samples drawn during summer and winter seasons were similar during late pregnancy and early postpartum period in both groups (Original publication V, Figure 1).

# 6. DISCUSSION

## 6.1 The natural course of Multiple sclerosis during pregnancy and postpartum

Previously described amelioration of MS during pregnancy and significant disease reactivation after delivery was confirmed in present study.

## 6.2 Pregnancy of Multiple sclerosis patients

According to our results, it is as safe for MS mothers to have children as it is for healthy mothers in general. However, the use of artificial insemination was more frequent among present study MS mothers compared to Finnish mothers in general. It is possible that mothers with MS are more urged to successful conception and thus lack time to wait for it to happen spontaneously. MS patients were previously counseled to discontinue disease modifying therapy before conception knowing the risk that stopping the treatment can result in disease activation. Use of IFN-β or GA during early pregnancy resulted in no negative influence on the pregnancy outcome in present study and similar findings have been reported also by others (Houtchens and Kolb. 2013). According to this it has been suggested that MS patients, particularly with an active disease, could continue IFN-β or GA until first trimester of pregnancy (Ruuskanen, et al. 2013, Ghezzi, et al. 2013, Tsui and Lee. 2011).

## 6.3 Delivery outcomes of Multiple sclerosis patients

In present study MS mothers needed instrumental assistance more often than healthy mothers in general. Similar findings have previously been reported in a Norwegian retrospective study with a cohort of 649 MS patient births (Dahl, et al. 2008). Possible factors contributing to this difference could be higher risk of fatigue with exhaustion and pelvic muscle weakness or spasticity among MS patients. On the other hand, in our study mothers with MS presented with equal rate of caesarean sections as healthy mothers. Similar results have been reported also by others (van der Kop, et al. 2011).

In our patient population MS mothers delivered more often without any analgesia than healthy mothers, but epidural analgesia was used in similar proportions. Some concerns have previously existed on the safety of epidural analgesics among MS patients. According to present conception, however, use of epidural analgesia is safe among MS patients and is not associated with disease activation (Dorotta and Schubert. 2002, Bader, et al. 1988). Thus pain alleviation during delivery should be provided for MS mothers as often and on the similar basis as for healthy mothers.

## 6.4 Breastfeeding and MS disease activity

The association of breastfeeding and lower postpartum relapse rate has been gaining a lot of attention for recent years. Exclusive breastfeeding has been suggested to protect MS patients from relapses after delivery (Langer-Gould, et al. 2009, Hellwig et al. 2009). According to our results in this relatively small Finnish cohort it seemed that association between longer breastfeeding and lower risk of postpartum relapses might simply reflect different patient behavior, biased by the disease activity before pregnancy and after delivery. Active disease before pregnancy decreased the frequency to breastfeed and shortened its duration in our cohort, as there was a significant difference between <2 months and ≥2 months breastfeeding groups in pre-pregnancy disease activity but not in postpartum disease activity (Original publication II, Figure). Five of the non-breastfeeding six mothers with highest relapse rates during the year preceding pregnancy chose DMT instead of breastfeeding, and this might have been effective in decreasing their annual relapse rate postpartum (Figure 4b). It is a shame that the starting time of supplements was not accurately recorded in this study. A significant difference in relapse rates both before pregnancy and after delivery, however, was observed between the groups breastfeeding 2-5.9 months and six months or longer (Figure 4b). One could assume the mothers with six months or longer breastfeeding were the most successfull "heavy breastfeeders" and perhaps the ones who breastfed most likely and longest exclusively and thus might also have gained some protection against relapses. A more in-depth comparison of the breastfeeding subgroups in present study was rather limited due to the small sample size (Figure 4a).

Breastfeeding is strongly promoted in Northern Europe and in Finland, and there is also a good reason to encourage breastfeeding among mothers with MS regarding its benefits for the mother-baby relations and wellbeing of the offspring (Oddy. 2004,Vukusic, et al. 2004). Breastfeeding mothers are recommended not to start DMT after birth, as the clinical data on drug transfer into milk and their effect on newborn has been limited (Coyle, et al. 2004, Houtchens and Kolb. 2013). Nevertheless, according to the growing knowledge in the field transfer of GA into breast milk seems unlikely and IFN-β has been reported to be present only in extremely small quantities (Ilett and Kristensen. 2005, Hale, et al. 2012). However, data on the subject is limited and promoting breastfeeding should be done carefully after evaluation of individual risk, especially with the mothers who have high pre-pregnancy disease activity and are possibly in the need of postpartum medication (Achiron, et al. 2004,Vukusic, et al. 2004). In the future we might attain enough information on the safety of IFN-β or GA use during breastfeeding to be able to update our recommendations regarding the issue (Ruuskanen, et al. 2013).

## 6.5 Postpartum thyroid autoimmunity in Multiple sclerosis patients

The susceptibility of MS mothers to postpartum thyroiditis has not been evaluated before. It is known that certain MS treatments predispose MS patients to autoimmune thyroid dysfunction (Coles, et al. 1999, Jones. 2009, Monzani, et al. 2004, Somers, et al. 2009). In addition, postpartum autoimmune thyroiditis is a common co-morbidity in certain other autoimmune diseases, such as type one diabetes and rheumatoid arthritis (Cotsapas, et al. 2011, Atzeni, et al. 2008, Stagnaro-Green. 2012). In present study, two patients with MS were observed with elevated thyroid antibodies in the beginning of pregnancy and another one of them developed postpartum thyroiditis. This is well in line with previous studies, according to which 50 % of mothers with elevated thyroid antibodies in the beginning of pregnancy develop postpartum thyroiditis (Stagnaro-Green. 2012). In our MS patient cohort the titers of thyroid autoantibodies followed rather the course seen in patients with autoimmune thyroid disease than that of healthy eu-thyroid women (Weetman AP. 2010). Accordingly, MS mothers were observed with elevated thyroid antibodies at six months postpartum significantly more often than healthy controls. Yet actual thyroid hormonal disturbance was observed in similar rates between MS and control mothers during our follow-up period of six months postpartum. It is possible that some mothers developed thyroid hormonal dysfunction during the following six months outside the scope of our study. However, the possibility of postpartum thyroiditis should be kept in mind when following MS patients after delivery, as the symptoms of thyroid insufficiency can easily be confused with those of fatigue.

### 6.5.1 Relation of thyroid antibodies and obstetric outcome

Previous studies have reported association between TPO-Abs and adverse pregnancy outcome (Glinoer, et al. 1991, Stagnaro-Green, et al. 1990, Casey. 2006, Stagnaro-Green, et al. 2005, Abbassi-Ghanavati, et al. 2010, Haddow, et al. 2011). In present study, prevalence of elevated thyroid antibodies in the beginning of pregnancy was not significantly different among MS patients and controls. Increased rate of elevated thyroid antibodies among MS patients, mainly TG-Abs, were observed during the postpartum period, which explains why no relation to adverse pregnancy outcomes could be found in present study. Only one MS patient presented with relatively elevated TPO-Abs in the beginning of pregnancy, and she gave birth to a baby with low birth weight and developed also postpartum thyroiditis. TPO-Abs can interfere with the complement system, which is why their pathologic role in the pregnancy complications is probably more significant than that of TG-Abs (Chiovato, et al. 1993). We do not know why present study MS patients were observed with increased rates of the very TG-Abs and not TPO-Abs. According to literature, elevated titers of exclusively TG-Abs are rarely seen (Muller, et al. 2001).

## 6.6 Gestational diabetes

The incidence of diagnosed gestational diabetes was slightly higher among our MS patient cohort when compared to Finnish mothers in general (14.8 vs. 8.4, p=0.0736). Gestational diabetes is associated with increased risk for adverse pregnancy outcome, which is why active screening and adequate treatment of this state of impaired glucose metabolism is important (Knight, et al. 2012). Gestational diabetes and hyperglycemia during pregnancy have also been associated with higher prevalence of thyroid autoimmunity (Vitacolonna, et al. 2012). The prevalence of thyroid antibodies among MS patients with gestational diabetes would be interesting to assess more carefully in future studies. Only three MS mothers with gestational diabetes were tested with thyroid antibodies in this study. Interestingly, all of them presented elevated levels. The CRP levels were also generally higher among MS patients with gestational diabetes (n=6) in present study, which is line with previous work (Ozuguz, et al. 2011). CRP level >7 mg/l in the beginning of pregnancy predicted gestational diabetes in our MS population with 83% sensitivity and specificity. As the incidence of gestational diabetes among MS patients was notable in present study, routine screening of pregnant MS patients with glucose tolerance test can be considered.

## 6.7 Fatigue and depression during pregnancy and postpartum

Fatigue was associated with significantly higher CRP concentrations during pregnancy but not postpartum in our cohort of MS patients. Reason why this association was observed only during pregnancy, could be the previously reported pregnancy imposed inflammatory response in the mother (Belo, et al. 2005). CRP levels in present study were in general significantly higher during pregnancy compared to levels after delivery. According to our results, the increased and altered inflammatory activity during pregnancy could perhaps generate conditions that promote the induction of fatigue related processes. Our results thus support the low-grade inflammation etiology hypothesis of fatigue (Cho, et al. 2013, Dimsdale and Dantzer. 2007). The prevalence of fatigue, however, slightly increased from pregnancy (45%) to postpartum period (54%) perhaps reflecting the increased disease activity or the changes in conditions and demands due to the baby after delivery. Furthermore, the pregnancy associated amelioration process of the disease might also protect MS patients from fatigue during pregnancy, although the association between inflammation and fatigue can be more clearly seen during pregnancy than postpartum.

According to the CES-d scores, 30% of MS mothers reported depressive symptoms during pregnancy and 25 % postpartum. The prevalence of postpartum depression in general population varies between 10-19 % depending on the measuring method and time after delivery (Cox, et al. 1993, O'Hara and McCabe. 2013). The symptoms of fatigue may have affected the depression reporting in our MS cohort, as the proportion of depressed individuals

was elevated and higher already during pregnancy. According to our results it seems, that the postpartum period as such does not predispose MS patients to depression. Thyroid Ab positivity did not increase the risk for postpartum depression or fatigue in present study.

## 6.8 C-reactive protein was not predictive of disease activity

CRP measured with a high sensitivity assay was not predictive of MS relapses in present study which is in line with previous observations (Giovannoni, et al. 2001b, Ristori, et al. 1998, Soilu-Hanninen, et al. 2005). This might be due to the many factors that influence the level of CRP. To begin with, there is inter-individual variation in physiological CRP levels along pregnancy, which are also influenced by the genetic and environmental exposures such as diet and other lifestyle factors (Belo, et al. 2005, Shen and Ordovas. 2009). Furthermore, CRP as an acute phase protein is involved in the initiation of the systemic inflammatory cascade. The process of MS relapse is complex and takes place on the other side of the blood brain barrier. The period of increased MS relapses in association to different infections is long, as it has been observed to start even one week before the symptoms of the infection appear and to continue up to five weeks after the infection has terminated (Panitch. 1994). Although MS disease activity has been shown to be associated with elevated CRP values (Soilu-Hanninen, et al. 2005, Giovannoni, et al. 2001b), it is perhaps the lack of specificity that limits its use in predicting postpartum relapses.

MS patients with higher delivery CRP were observed with prolonged inflammatory activity until three months postpartum. Similar prolonged inflammation could not be found among the study control patients. The intensity of immune reconstitution after delivery varies individually and some individuals have been observed to present exaggerated postpartum pro-inflammatory cytokine rebound (Elenkov, et al. 2001). However, the factors controlling this phenomenon are not well known. The proportion of individuals with this exaggerated rebound after delivery might be higher among mothers with MS or other autoimmune diseases compared to healthy mothers.

### 6.8.1 C-reactive protein and pregnancy complications

Two of the three mothers with complicated pregnancy (pre-eclampsy or preterm delivery) and CRP assessment were observed with significantly elevated CRP (>20mg/l, reference interval 0.32-11.9mg/l (Larsson, et al. 2008)) in early pregnancy. None of the MS mothers with normal pregnancy outcome presented with significantly elevated CRP at this point. Elevated CRP level in early pregnancy has been observed to associate with preterm delivery, pre-eclampsia, insulin resistance, gestational diabetes and overt diabetes mellitus in previous studies (Festa, et al. 2000, Ozuguz, et al. 2011, Ford. 1999, Pitiphat, et al. 2005, Tjoa, et al. 2003). The mothers with prematurity or pre-eclampsia in this study were so few

that determination of the predictive value of CRP in regards of these complications was not possible. However, we were able to define the predictive value among patients with gestational diabetes. In our cohort, CRP over 7 mg/l in early pregnancy predicted patients with gestational diabetes with 83% sensitivity and specificity.

## 6.9 Vitamin D

Mothers with MS were observed with significantly lower levels of $25(OH)D_3$ than healthy controls during present study. Higher prevalence of vitamin D deficiency among MS patients has previously been suggested to result from MS-related disability (EDSS>3) and limited activity outdoors (van der Mei, et al. 2007). MS related disability was likely not the major cause of this difference in our cohort, as our MS patients were observed with low mean EDSS score of 1.0. Behavioural and nutritional factors may still explain some of the difference in present study. Serum vitamin D levels have been suggested to depend more strongly on sunlight exposure than oral vitamin D intake (Perampalam, et al. 2011). However, above and below latitudes of approximately 33°, vitamin D synthesis in the skin is very low or absent during most of the winter, and people are dependent of nutritional vitamin D intake (Holick, et al. 2011). In present study, majority of the samples in the early postpartum period in both groups were drawn during the winter season, suggesting that the differences observed between MS patients and controls at least in this time period were not affected by the season (Original publication V, Figure 1). Small cohorts limit the power of present study and the results can be viewed only as preliminary. However, the mean concentration of $25(OH)D_3$ among healthy controls in present study was in line with the results in a previous comparable study performed on healthy pregnant women in Finland (Lamberg-Allardt, et al. 1984).

During the time of present study follow-up in 2003-2005, vitamin D was not considered as significant for health as it is today and only about 40% of Finnish pregnant mothers in general population were observed to use vitamin D-supplementation according to the recommendations (Arkkola, et al. 2006). Apart from vitamin D obtained from supplements, also the socio-economic factors affect our diet. Unfortunately vitamin D intake was nor recorded in our study.

Interestingly, $1,25(OH)_2D$ concentration, the biologically active form of vitamin D, and MS disease amelioration present a similar course during pregnancy (Holick, et al. 2011). The relation of high oestrogen and vitamin D function during pregnancy is also worth considering, as the protective function of vitamin D has been demonstrated to be controlled by oestrogen in EAE mice (Nashold, et al. 2009). The protective effect of vitamin D in MS might thus be restricted to females and could perhaps explain some of the increasing sex bias in MS prevalence.

# 7. FUTURE CONSIDERATIONS

The knowledge on the field of MS has been growing exponentially during the last two decades and we now have several treatment options available for different stages of severity this disease presents itself. However, a lot remains yet to be clarified and several issues need to be assessed in detail. The exact etiology and molecular pathophysiology of the disease has not yet been confirmed, although several hypotheses and associations have been established. Undoubtedly, the origin of the disease is multifactorial. The research on the interaction of the genes and the environment open countless new fascinating directions to aim our curiosity. Immune changes during child-bearing makes the period of pregnancy permanently interesting in search of the factors that protect from the disease worsening. The growing incidence of MS among women, but not among men is an argument for the gender specific risks that exist in our environment, behavior or epigenetics. As we gather more information on how and why MS is triggered in an individual, we may be able to start effective prevention of the disease on a population level. On the other hand, if the increased hygiene proves to be causative, how will it be managed? Perhaps the result could be found in vaccination or probiotics.

Our study was limited with a relatively low sample size, but the results of this study could be considered as preliminary results for further research. Our results showing increased thyroid autoimmunity among MS patients in the postpartum period should be confirmed in a larger study with a longer follow-up period. Similarly it would be interesting to measure the changes of vitamin D and its interactions with immune cell subtypes during pregnancy in a larger cohort of pregnant MS patients.

In the near future as we acquire more information on the safety of the use of first line immune-modulatory treatments during pregnancy and breastfeeding, it might be possible to continue the use of these treatments along pregnancy. This might result in better control or even prevention of the disease worsening after delivery. The problem of the present more effective immune-modulatory treatments is that they lack specificity to the central nervous system autoimmunity. In case we are able to develop more targeted treatments that influence only locally at the site of disease process without systemic side effects, it might be safe to use them during pregnancy and breastfeeding without any harm to the baby. The ultimate aim would be to find a cure for this disabling disease affecting young adults at an early stage of their lives. It remains to be seen, whether gene therapy with viral vectors provides us a resolution.

# 8. SUMMARY AND CONCLUSIONS

The purpose of this study was to evaluate (I) the course of the disease during pregnancy and the obstetric outcomes, (II) the breastfeeding manners, (III) the risk of postpartum thyroid autoimmunity, (IV) the predictive value of CRP measurement in regards of disease activity or pregnancy related co-morbidities and (V) the levels of vitamin D during pregnancy and postpartum period of patients with MS.

We confirmed the finding in previous studies that MS patients present with favorable pregnancy outcome as often as healthy mothers in general. The population of 60 Finnish pregnant MS patients were no more likely to experience pregnancy complications than are Finnish mothers in general, nor were their infants more likely to be delivered preterm or to be of low birth weight. MS patients needed instrumental assistance during delivery and delivered without analgesia more often than healthy mothers. MS patients presented postpartum thyroid autoimmunity more often than healthy controls, which possibly reflects the shared predisposition to postpartum immune reconstitution syndrome among the autoimmune diseases. Despite the increased prevalence of elevated thyroid Abs postpartum, patients with MS were not more prone to thyroid dysfunction when compared to control mothers. Thyroid autoimmunity did not increase the risk for fatigue or postpartum depression in this study. However, as the symptoms of MS fatigue and thyroid insufficiency can easily be confused, the possibility of thyroid dysfunction should be kept in mind in the postpartum follow-up of these patients. Elevated CRP in the beginning of pregnancy was predictive of gestational diabetes among MS patients, but not of relapse rate during pregnancy or postpartum period. MS mothers presenting fatigue during pregnancy were observed with elevated CRP levels, which further supports the inflammation etiology hypothesis of fatigue. Most of our MS mothers were vitamin D deficient during pregnancy and lactation, which is why adequate vitamin D supplementation with at least 35 µg daily can be recommended during this period. To conclude, screening of thyroid antibodies and impaired glucose metabolism during pregnancy of MS patients can be recommended as tools for diagnosing common pregnancy-related co-morbidities among MS patients.

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