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ABSTRACT

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Pregnancy-related immune alterations in multiple sclerosis

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Annales Universitatis Turkuensis, Turku, 2018

Background: Pregnancy induces alterations in the maternal immune system. Multiple sclerosis (MS) is an inflammatory autoimmune disease of the central nervous system (CNS), which is ameliorated during pregnancy. The exact mechanisms leading to this are poorly understood, but it is likely that the immunological changes which contribute to a successful pregnancy also help control autoimmunity. Pregnancy-related alterations may also affect the immunosurveillance of opportunistic pathogens, which can render the mother susceptible to certain infections such as listeriosis and JCV-induced PML.
Aim: To investigate immunological mechanisms contributing to amelioration of MS during pregnancy. To evaluate how pregnancy-related immunological alterations affect the susceptibility to an opportunistic infection particularly relevant in care of MS, namely JCV-induced PML.
Methods: Blood and cerebrospinal fluid (CSF) samples and clinical data were collected longitudinally during pregnancy and 6 months postpartum from MS-patients participating in a nationwide Finnish Multiple Sclerosis and Pregnancy Study. Alterations in peripheral blood lymphocyte subpopulations, MS-associated CSF determinants (IgG-index, number of oligoclonal bands and lymphocytes) and humoral responses to JCV were evaluated during and after pregnancy.
Results: MS-pregnancy is characterized by non-altered proportions of T- and B-cells, increased proportion of regulatory $CD56^{bright}$ NK-cells, decreased proportions of CD16+ and $CD3\text{-}CD56^{dim}$ NK-cells, and increased Th2/Th1 cytokine ratio. The CSF IgG-Index was significantly higher during pregnancy than postpartum. JCV-Ab-indices increased postpartum.
Conclusions: Regulatory $CD56^{bright}$ NK-cells might facilitate the control of autoimmune inflammation during pregnancy and thus contribute to the reduced MS activity during pregnancy. Hightened postpartum Ab-responses to JCV likely reflect altered immunity towards JCV during pregnancy.

Keywords: Multiple sclerosis, pregnancy, NK-cells, John Cunningham Virus, anti-JCV-index

TIIVISTELMÄ

Maija Saraste

Raskauden aikaiset immunologiset muutokset MS-taudissa

Turun yliopisto, Lääketieteellinen tiedekunta, Kliininen laitos, Neurologia, Turun kliininen tohtoriohjelma

Annales Universitatis Turkuensis, Turku, 2018

Tausta: Raskauden aikana äidin immuunijärjestelmän toiminta muuttuu. Multippeliskleroosi eli MS-tauti on keskushermoston tulehduksellinen autoimmuunisairaus, jonka oireet lievittyvät raskauden aikana. Lievittymiseen vaikuttavia mekanismeja ei tunneta tarkasti, mutta todennäköisesti immunologiset muutokset jotka edesauttavat raskauden onnistumista, hillitsevät autoimmuniteettiä. Raskauden aikaiset immunologiset muutokset voivat vaikuttaa myös opportunististen patogeenien immuunivalvontaan, mikä lisää äidin herkkyyttä sairastua tiettyihin infektioihin, kuten listerioosiin ja JC-viruksen (JCV) aiheuttamaan progressiiviseen multifokaaliseen leukoenkefalopatiaan (PML).

Tavoitteet: Selvittää niitä immunologisia mekanismeja, jotka myötävaikuttavat MS-taudin lievittymiseen raskauden aikana. Tutkia, vaikuttavatko raskauden aikaiset immunologiset muutokset immuniteettiin MS-taudin hoidon kannalta merkittävää opportunistista virusta eli JC-virusta kohtaan.

Menetelmät: Kansalliseen MS-tauti ja raskaus –seurantatutkimukseen osallistuvilta MS-potilailta kerättiin veri- ja selkäydinnestenäytteitä sekä kliinisiä tietoja raskauden aikana ja kuusi kuukautta synnytyksen jälkeen. Perifeerisen veren tulehdussolujen alaluokkien prosentuaalisissa määrissä ja selkäydinnesteen MS-tautiin liittyvissä tekijöissä (IgG-indeksi, oligoklonaalisten juovien ja tulehdussolujen määrä) tapahtuvia muutoksia sekä JC-virukseen kohdistuvaa vasta-aine välitteistä immuunivastetta mitattiin raskauden aikana ja synnytyksen jälkeen.

Tulokset: Raskauden aikana MS-potilaiden T- ja B-solujen prosentuaalisissa osuuksissa ei tapahtunut muutoksia. Regulatoristen $CD56^{bright}$ NK-solujen (natural killer, luonnollinen tappajasolu) prosentuaalinen määrä lisääntyi, mutta CD16+ ja CD3-$CD56^{dim}$ NK-solujen pieneni raskauden aikana. Th2/Th1 sytokiinien suhde on suurempi raskauden aikana. Selkäydinnesteen IgG-indeksi oli merkittävästi suurempi raskauden aikana kuin synnytyksen jälkeen. JCV-vasta-aine-indeksi kohosi synnytyksen jälkeen.

Johtopäätökset: Regulatoriset $CD56^{bright}$ NK-solut voivat osallistua autoimmuunitulehduksen säätelyyn raskauden aikana ja täten myötävaikuttaa raskauden aikaiseen MS-taudin lievittymiseen. Synnytyksen jälkeiset muutokset JCV-vasta-aine-tuotannossa viittaavat, että raskaus vaikuttaa JC-viruksen immunokontrolliin.

Avainsanat: MS-tauti, raskaus, NK-solut, JC-virus, JCV-vasta-aine-indeksi

TABLE OF CONTENTS

ABBREVIATIONS

Ab	Antibody
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
ARR	Annualized relapse rate
BBB	Blood-brain-barrier
CSF	Cerebrospinal fluid
CMV	Cytomegalovirus
CNS	Central nervous system
DMF	Dimethyl fumarate
DMT	Disease modifying treatment
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
EDSS	Expanded disability status scale
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box P3
FSC	Forward scatter
GA	Glatiramer acetate
Gd	Gadolinium
Gr	Granzyme
gw	Gestational week
GWAS	Genome wide association study
HBSS	Hanks buffered salt solution
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
IFN-β	Interferon-beta
IFN-γ	Interferon-gamma
IF-staining	Immunofluorescence-staining
IgG	Immunoglobulin G
IL	Interleukin
JCV	John Cunningham virus
LFA-1	Lymphocyte function-associated antigen 1
L. monocytogenes	Listeria monocytogenes
MRI	Magnetic resonance imaging

MS	Multiple sclerosis
NEDA	No evident disease activity
NF-κB	Nuclear factor kappa B
NK-cell	Natural killer –cell
OCB	Oligoclonal band
OD	Optical density
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PE	Phycoerytherin
PMA	Phorpol myristate acetate
PML	Progressive multifocal leukoencephalopathy
PP	Postpartum
PSGL-1	P-selectin glycoprotein ligand 1
RA	Rheumatoid arthritis
rmANOVA	Repeated measures analysis of variance
RPM	Rounds per minute
RR	Rate ratio
RRMS	Relapsing remitting multiple sclerosis
SD	Standard deviation
SSC	Side scatter
SEM	Standard error of mean
SPMS	Secondary progressive multiple sclerosis
TGF	Tumor growth factor
Th	T helper
TNF	Tumor necrosis factor
Treg	Regulatory T-cell
VCA	Viral capsid antibody
VCAM-1	Vascular cell adhesion molecule 1
VLA-4	Very late activation antigen 4
VLP	Virus-like particle

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications which are referred in the text by numbers I-V.

I Saraste MH, Kurki T, Airas LM. Postpartum activation of multiple sclerosis: MRI imaging and immunological characterization of a case. Eur J Neurol 2006; 13: 98-99

II Saraste M, Väisänen S, Alanen A, Airas L and The Finnish Multiple Sclerosis and Pregnancy Study Group. Clinical and immunological evaluation of women with multiple sclerosis during and after pregnancy. Gend Med 2007; 4: 45-55

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IV Saraste M, Ryynänen J, Alanen A, Multanen J, Färkkilä M, Kaaja R, Airas L. Cerebrospinal fluid findings in multiple sclerosis patients before, during and after pregnancy. J Neurol Neurosurg Psychiatry 2006; 77: 1195-1196

V Saraste M, Atula S, Hedman K, Hurme S, Jalkanen A, Sneck M, Surcel H-M, Maghzi AH, Airas L. Humoral response to John Cunningham virus during pregnancy in multiple sclerosis. Mult Scler Relat Disord 2018; 21: 11-18

1 INTRODUCTION

Multiple sclerosis (MS) is a chronic, inflammatory, demyelinating and neurodegenerative disease of the central nervous system (CNS) (Hernández-Pedro et al., 2013; Reich et al., 2018). MS is considered as an autoimmune disease, in which autoreactive activated lymphocytes cross the blood-brain-barrier (BBB) and form focal inflammatory lesions. This leads eventually to demyelination and axonal damage causing a large range of symptoms. It is thought that MS arises in genetically susceptible individuals with stochastic events and environmental factors influencing disease penetrance (Dendrou et al., 2015). MS is more common among women than in men, and the typical age of onset is between 20 and 40 years. Thus, MS affects usually women at childbearing age.

Pregnancy induces multiple changes in the maternal immune system. During pregnancy the immune system of the mother must accept the semi-allogeneic fetus, but also protect the mother against infections (Figueiredo and Schumacher, 2016). Adaptive immune responses, including T-cell activity, are down-regulated, whereas the innate immune system is activated (Luppi, 2003). As a "side-effect" of these changes the disease activity of autoimmune diseases may alter during pregnancy (Patas et al., 2013). The disease activity of MS is ameliorated during pregnancy. The rate of relapses decreases 70 % during the third trimester of pregnancy, but peaks soon after the delivery before returning to pre-pregnancy level (Confavreux et al., 1998). The alterations in MS relapse rate coincide with changes in levels of sex-hormones estrogen and progesterone, but the biological mechanisms leading to amelioration of MS disease activity during pregnancy are not well understood (Patas et al., 2013).

The pregnancy-induced changes in the maternal immune system may also alter the susceptibility to certain infections. Some infections, such as influenza and hepatitis E, are more severe in pregnant women and pregnancy also increases the susceptibility of some infections, such as listeriosis and malaria (Kourtis et al., 2014). John Cunningham virus (JCV) is an opportunistic polyomavirus that causes progressive multifocal leukoencephalopathy (PML) associated with certain immunosuppressive treatments of MS (Wollebo et al., 2015). Reactivation of JCV has been observed during pregnancy in healthy women (Coleman et al., 1980). Therefore, it would be important to know whether pregnancy related immune alterations affect immunosurveillance for JCV in MS-patients, who might be exposed to immunosuppressive medications before and during pregnancy.

2 REVIEW OF LITERATURE

2.1 Multiple sclerosis

2.1.1 Prevalence

Multiple sclerosis (MS) is the most common disabling neurological disease of young adults affecting more than 2.5 million individuals worldwide (Compston and Coles, 2002; Steinman, 2014). MS disease prevalence and incidence rates vary greatly with geography and ethnicity (Howard et al., 2016). The overall prevalence is around 120 per 100 000 and the yearly incidence about seven per 100 000. The lifetime risk of MS is one in 400. (Compston and Coles, 2002). MS is more common among women and the female to male ratio has increased from 2:1 to 3:1 mainly because of increased incidence in women (Compston and Coles, 2008).

2.1.2 Risk factors

A major fraction of MS risk can be explained by currently known risk factors such as genetic predisposition, female sex, high latitude, Epstein-Barr virus (EBV) infection, smoking, low vitamin D levels caused by insufficient sun exposure and dietary intake and adolescence obesity. Most non-genetic factors seem to have the greatest effect during adolescence. (Olsson et al., 2017). For example, infectious mononucleosis caused by EBV in adolescence or adulthood increases the risk of developing MS even threefold. Primary EBV-infection induces strong innate immunity activation which may promote the activation and expansion of lymphocytes which recognize both viral-antigens and myelin antigens through molecular mimicry between EBV and self-proteins. The geography of MS correlates strongly with the duration and intensity of ultraviolet radiation from sunlight, which is an important determinant of serum vitamin D level. (Ascherio et al., 2012). Among non-Hispanic white individuals, the risk of developing MS is 62% lower in those having highest 25(OH)D levels than in those having lowest levels (Munger et al., 2006). Smoking increases the risk of MS, and the risk is directly associated with the duration and intensity of smoking. The effect of smoking seems to be stronger in males than in females. (Ascherio et al., 2012). Obesity at adolescent is associated with two-fold risk of MS in women (Munger et al., 2009), and there is a strong correlation between adolescent body mass index and subsequent risk of MS (Hedström et al., 2016). Obesity remains as a risk factor

for MS after adjusting for other, both genetic and environmental, risk factors (Gianfrancesco et al., 2014).

Clustering of MS within families is probably explained by genetic factors (Ascherio et al., 2012). The familial recurrence rate of MS is about 20 %. The concordance rate of MS is five times higher in monozygotic twins than in dizygotic twins (25% vs. 5%), although this difference has not been seen in all studies. The recurrence risk of siblings is 5% and of parents and of children 2%. (Compston and Coles, 2008). The human leukocyte antigen (HLA) gene complex contains genes that influence susceptibility to MS. Major susceptibility genes encode polymorphic cell-surface HLA-class II molecules, which have an important role in immune recognition of self from non-self. (Oksenberg et al., 2008). HLA-DRB5 and HLA-DQB1 are associated with increased susceptibility to MS whereas HLA-C seems to have a protective effect (Compston and Coles, 2008; Oksenberg et al., 2008). However, the HLA-locus accounts for only 20-60% of the genetic susceptibility of MS (Oksenberg et al., 2008). Genome wide association studies (GWAS) have identified other genes associated with MS that encode for example receptors for interleukin (IL)-2 and IL-7 and CD58 (Hafler et al., 2007). Recently more than 200 new candidates for MS susceptibility genes were identified using GWAS with noise reduction including key regulators of nuclear transcription factor κB (NF-κB) signaling (Hussman et al., 2016). Large part of identified genes interacted in a pathway that regulates induction and infiltration of pro-inflammatory T helper (Th)1 and Th17 cells and maintenance of immune tolerance by regulatory T-cells (Hussman et al., 2016).

2.1.3 Clinical course

Clinical course of MS is heterogeneous with four recognized patterns. Onset of MS is typically at age of 20 to 40 (Compston and Coles, 2002). 85% of patients present with relapsing-remitting disease (RRMS), in which periods of exacerbations are followed by complete or partial recovery (Hauser and Oksenberg, 2006). A relapse is defined as a neurological deficit lasting for 24 h or more, and reflects acute inflammation in the CNS (Steinman, 2014). Relapse rate seldom exceeds 1.5 relapses per year (Compston and Coles, 2008). Between relapses patients may be clinically stable and/or experience gradual progression of disability (Hauser and Oksenberg, 2006). In the beginning of MS, patients usually recover fully from relapses, but with time recovery becomes partial and persistent disability begins to accumulate (Compston and Coles, 2002). About 65% of patients with RRMS convert to secondary progressive MS (SPMS) at around 40 years of age (Compston and Coles, 2008). SPMS is characterized by gradual

progression of disability with or without superimposed relapses (Hauser and Oksenberg, 2006). 10% of patients present with primary progressive MS, in which disability is gradually accumulated from onset without superimposed relapses. About 5% of patients have progressive relapsing MS in which gradual progression of disability is accompanied by one or more relapses. (Hauser and Oksenberg, 2006).

2.1.4 Symptoms

Patients suffering from MS may have numerous varying symptoms depending on the site of inflammatory lesions in the CNS. In most patients motor, sensory, visual and autonomous systems are involved (Compston and Coles, 2008). Common initial symptoms include monocular visual loss i.e. optic neuritis, double vision, a sensory disturbance, limb weakness, gait instability or ataxia. In later stages bladder dysfunction, fatigue, and heat sensitivity occur in most patients (Hauser and Oksenberg, 2006). Expanded disability status scale (EDSS) is used to rate disability (Kurtzke, 1983). EDSS scores range from 0 (no abnormality) to 10 (death caused by MS), and patients are scored based on neurological examination and walking ability.

2.1.5 Diagnosis

The diagnosis of MS is based on the demonstration of dissemination of lesions in space and time with the exclusion of alternative diagnoses (Milo and Miller, 2014), and relies on the integration of clinical, magnetic resonance imaging (MRI), and cerebrospinal fluid (CSF) laboratory findings (Thompson et al., 2018a). The 2017 McDonald criteria for diagnosis of MS in patients with attack at onset is shown in Table 1. If the criteria are fulfilled and there is no better explanation for the clinical presentation the diagnosis is MS (Thompson et al., 2018a). CSF oligoclonal immunoglobulin G (IgG) bands are seen in about 90% of MS-patients (Krumbholz et al., 2012). The presence of oligoclonal bands suggests intrathecal immunoglobulin synthesis and confirms that the underlying pathology is inflammatory (Compston and Coles, 2002, 2008).

Table 1. 2017 McDonald criteria for diagnosis of MS (modified from Thompson et al., 2018).

Number of clinical attacks	Number of lesions with objective clinical evidence	Additional data needed for MS diagnosis
≥ 2	≥ 2	None
≥ 2	1 with historical evidence of a previous attack involving lesion in a distinct anatomical location	None
≥ 2	1	DIS demonstrated by an additional clinical attack implicating a different CNS site or by MRI (≥1 T2-hyperintense lesions characteristic of MS in two or more of following CNS areas: periventricular, cortical or juxtacortical, infratentorial, spinal cord)
1	≥ 2	DIT demonstrated by an additional clinical attack or by MRI (simultaneous presence of Gd-enhancing and non-enhancing lesions at any time or by a new T2-hyperintense or Gd-enhancing lesion on follow-up MRI, with reference to a baseline scan, irrespective of the timing of the baseline MRI) OR demonstration of CSF-specific oligoclonal bands
1	1	DIS demonstrated by an additional clinical attack implicating a different CNS site or by MRI AND DIT demonstrated by an additional clinical attack or by MRI OR demonstration of CSF-specific oligoclonal bands

MS = multiple sclerosis; DIS = dissemination in space; CNS = central nervous system; MRI = magnetic resonance imaging; DIT = dissemination in time; Gd = gadolinium; CSF = cerebrospinal fluid

2.1.6 Treatment

2.1.6.1 Treatment of relapses

Treatment of moderate or severe relapses may shorten the duration of the relapse and decrease the associated disability. Relapses are primarily treated with high dose methylprednisolone course of 3-5 days administered either orally or intravenously. The second line treatment option of relapses is plasmapheresis. (Berkovich, 2013).

2.1.6.2 Disease-modifying treatment

Disease-modifying treatment (DMT) of MS has developed greatly within the past 20 years with more than 10 approved DMTs for RRMS. As MS cannot be cured, the aim of the disease-modifying treatment is to prevent relapses and progression of disability, although it has been discussed whether so-called no evident disease activity (NEDA), meaning absence of relapses, disability progression and MRI activity, should be achieved. It is important to start the disease-modifying treatment as early as possible after MS diagnosis as response to treatment seems to be better in the early phases of MS and decreased inflammatory activity may prevent early occurring irreversible nervous damage. (Comi et al., 2017).

DMTs can be divided to first line and second line therapies. In escalation strategy treatment is started with a moderately effective, but potentially safer, first line treatment which can be changed to a more effective second line treatment if relapses are continuous. In case of very active disease induction strategy can be used. In induction strategy treatment is started directly with a highly effective therapy. (Thompson et al., 2018b). Table 2 summarizes the mechanisms of action, efficacy on relapses and side-effects of DMTs.

2.2 Multiple Sclerosis pathogenesis

MS pathogenesis is not fully understood. Although considered as a prototypic CNS-specific autoimmune disease, the autoimmune pathogenesis of MS is complex and involves multiple cell types of both adaptive and innate immunity as well as CNS-resident innate immune cells with inflammatory capacity (Dendrou et al., 2015; Martin et al., 2016). Invading peripheral immune cells and blood-brain-barrier (BBB) disruption can be seen in MS-lesions at early stages. High numbers of macrophages and CD8+ cytotoxic T-cells and lower numbers of CD4+ helper T-cells, B-cells and plasma cells can be found. (Dendrou et al., 2015). Natural killer (NK)-cells are also found in active MS lesions (Gross et al., 2016b). As MS disease proceeds the immune cell infiltration from periphery is decreased but the chronic CNS inflammation and neurodegeneration are increased (Dendrou et al., 2015).

Table 2. Disease-modifying treatments of multiple sclerosis. Modified from Thompson et al., 2018b and supplemented by Cocco and Marrosu, 2014, Gold et al., 2013 and Gold et al., 2016.

Drug (route of administration)	Mechanisms of action	Efficacy on relapses	Serious adverse effects
First line therapies			
Interferon-β 1a/1b (i.m. or s.c.)	Reduces antigen presentation and T-cell proliferation, alters cytokine expression, restores suppressor function	33%	Liver toxicity (very rare)
Glatiramer acetate (s.c.)	Alters T-cell differentiation inducing proliferation of anti-inflammatory lymphocytes	29%	None
Dimethyl fumarate (p.o.)	Reduces the release of inflammatory cytokines and activates antioxidant pathways	51%	PML (1:50 000)
Teriflunomide (p.o.)	Reversible inhibitor of dihydroorotate-dehydrogenase, inhibits the proliferation of autoreactive B- and T-cells and induces a shift to an anti-inflammatory profile	35%	None
Second line therapies/Induction strategy			
Fingolimod (p.o.)	Functional antagonist of sphingosine 1-phospate receptors, inhibits egress of lymphocytes from lymph nodes and their recirculation	52%	PML (1:12 000), macular oedema, varizella zoster virus, herpes encephalitis
Natalizumab (i.v.)	Monoclonal anti-VLA-4 antibody, prevents lymphocytes from entering the CNS across the BBB	68%	PML (4.19 per 1000 patients), hypersensitivity reactions
Alemtuzumab (i.v.)	Monoclonal anti-CD52 antibody, depletes B- and T-cells	52% (vs. IFN)	Secondary autoimmune diseases, listeria-infection
Third line therapy/Induction strategy			
Mitoxantrone (i.v.)	Inhibits proliferation of B-cells, T-cells and macrophages		Cardiomyopathy, acute leukemia
New therapies for highly active MS			
Cladribine (oral)	Synthetic deoxyadenosine analogue, depletes B- and T-cells	58%	Pulmonary tuberculosis, malignancy
Ocrelizumab (i.v.)	Monoclonal anti-CD20 antibody, depletes B-cells	47% (vs. IFN)	Increased risk of malignancy
Daclizumab	Monoclonal anti-CD25 antibody, expands $CD56^{bright}$ NK-cells, inhibits T-cell activation	50%	Withdrawn in March 2018 because of reports of serious inflammatory brain disorders

i.m.=intramuscular; s.c.=subcutaneous; p.o.=peroral; i.v.=intravenous; PML=Progressive multifocal leukoencephalopathy; VLA-4=Very late activation antigen -4; IFN=interferon; CD=cluster of differentiation

2.2.1 Autoimmunity

T-cells and B-cells that recognize self-antigens are mostly deleted in thymus and bone marrow during their development (central tolerance), but some autoreactive cells are released into the periphery (Dendrou et al., 2015). Accordingly, myelin-specific T-cells can be observed in the peripheral blood of both MS-patients and healthy subjects (Raddassi et al., 2011; Van der Aa et al., 2003). Normally regulatory cells can control the activity of autoreactive cells (peripheral tolerance), and thus prevent autoimmune reaction. However, if the function of regulatory cells is reduced or defected or the resistance of effector cells is increased, autoreactive cells may be activated and attack their target tissue causing autoimmune disease. (Dendrou et al., 2015). There seems to be functional differences between the myelin-reactive T-cells between MS-patients and healthy controls, as it was shown in a T-cell library assay that myelin-reactive T-cells of MS-patients secrete more pro-inflammatory cytokines whereas T-cells of healthy subjects secreted more IL-10 (Cao et al., 2015).

2.2.2 Migration through blood-brain-barrier

In order to form an inflammatory MS lesion, lymphocytes must enter the CNS. Lymphocytes can enter CNS by migrating either through the BBB, blood-CSF-barrier or blood-meningeal barrier (Michel et al., 2015). The BBB is formed by endothelial cells, which are surrounded by basement membranes, pericytes and astrocytes and it prevents toxic substances and limits inflammatory cells from entering CNS (Takeshita and Ransohoff, 2012). The migration of lymphocytes through BBB involves multiple steps, which are tightly regulated by adhesion molecules and chemokines. In the first step, rolling, adhesion molecules P-selectin glycoprotein ligand 1 (PSGL-1) and very late activation antigen 4 (VLA-4) expressed on the surface of leukocytes bind to P-selectin and vascular cell adhesion molecule 1 (VCAM-1), respectively, and the velocity of the cell reduces. The rolling leukocyte is then activated by binding of chemokines on the endothelial cells to chemokine receptors on leukocytes, which leads to upregulation of VLA-4 and lymphocyte function-associated antigen 1 (LFA-1, CD11a/CD18). The avidity or affinity of the interaction between these adhesion molecules and their cellular partners is also increased, and leukocytes are attached firmly to endothelial cells. After this, arrested leukocytes crawl to preferred site for migration, and

finally migrate across endothelial cells to perivascular space and across the glia limitans to brain parenchyma. (Michel et al., 2015; Takeshita and Ransohoff, 2012).

2.2.3 Formation of inflammatory lesions

According to a major hypothesis in MS lesion development, autoreactive T-cells recognizing CNS antigens may be activated in the periphery for example by molecular mimicry and bystander activation. Low number of activated cells might enter the CNS and be re-activated with their cognate antigen. (Hemmer et al., 2015). Re-activated T-cells release pro-inflammatory cytokines, for example interferon-γ (IFN-γ), which activate microglia (Mallucci et al., 2015). IFN-γ may also contribute to the disruption of BBB and promote trans endothelial migration of CD4+ T-cells (Sonar et al., 2017). Chemokines secreted by activated microglia attract more T-cells, monocytes and plasma cells to the CNS (Hemmer et al., 2015; Mallucci et al., 2015). BBB disruption by inflammatory mediators leads to formation of inflammatory lesions. Activated peripheral innate and adaptive immune cells, microglia and astrocytes then promote demyelination and oligodendrocyte and neuroaxonal injury through direct cell-contact dependent mechanisms and through action of myelin sheath and glial cells targeting antibodies, and soluble inflammatory and neurotoxic mediators, such as IFN-γ and IL-17. (Dendrou et al., 2015; Hemmer et al., 2015). There is, however, also alternative hypothesis, in which an initiating event within the CNS causes activation of microglia, which then leads to secondary activation of peripheral immune cells in deep cervical lymph nodes (Hemmer et al., 2015).

2.3 Peripheral immune cells and multiple sclerosis

Circulating immune cells contribute to the pathogenesis of MS. Alterations in the composition and function of immune cell subsets has been associated with MS and its disease activity. (Jones et al., 2017). At clinical onset MRI-active and MRI-inactive patients display significant differences in lymphocyte subsets suggesting that brain inflammation is associated with distinct changes in peripheral blood lymphocyte subsets (Rinaldi et al., 2006). According to a recent study a decrease in peripheral blood B-cell and NK-cell populations and a marked reduction of naïve CD4+ Th-cells were the best predictors of a relapse and disability progression in patients after a first demyelinating event (Posová et al., 2017).

2.3.1 T-cells

Changes in activated peripheral blood T-cells correlate with clinically and radiologically determined MS disease activity (Khoury et al., 2000). Multiple differences in T-cell subsets in MS-patients compared to healthy subjects have been observed (Jones et al., 2017), for example decreased proportions of naïve CD4+ cells and peripheral blood CD8+ T-cells, increased proportion of CD4+ and CD8+ effector memory cells and CD8+ central memory cells, and increased CD4/CD8 ratio (Haegele et al., 2007; Liu et al., 2007; Mikulkova et al., 2011, Pender et al., 2014). The main T-cell subsets implicated in MS are CD4+ Th1 cells producing pro-inflammatory cytokine IFN-γ and Th17 cells producing Il-17, but the relative importance of these subtypes is not clear (Dendrou et al., 2015). Th17 cells might contribute to the disruption and early penetration of the BBB (Kleinewietfeld and Hafler, 2013). The proportion of peripheral blood Th17 cells including cells specific for myelin basic protein is increased in active MS-patients compared to in-active MS-patients or healthy subjects whereas the proportion of Th1 cells is not changed (Durelli et al., 2009).

Many current disease-modifying treatments of MS have effects on peripheral blood T-cells. Daclizumab therapy is associated with decreases in absolute numbers and proportion of CD4+ and CD8+ T-cells (Bielekova et al., 2006). Fingolimod decreases the absolute number and the proportion of CD4+ T-cells, which leads to decreased CD4/CD8 ratio (Claes N., 2014; Kovarik et al., 2011). Natalizumab on the other hand increases absolute numbers of circulating T-cells (Mellergård et al., 2013; Putzki et al., 2010) but does not change the relative proportion of CD4+ or CD8+ T-cells (Skarica et al., 2011). Dimethyl fumarate (DMF) reduces the proportion of Th1 and Th17 cells but increases the proportion of Th2 cells (Wu et al., 2017). Interferon-β (IFN-β) suppresses Th17 cell differentiation (Ramgolam et al., 2009), and enhances their apoptosis in vitro (Durelli et al., 2009).

2.3.1.1 Regulatory T-cells

Regulatory T-cells can suppress and regulate adaptive and innate immune responses, and they play an important role in maintaining peripheral tolerance. Deficits in the function of regulatory T-cells appear to be a common cause of human autoimmune diseases. Several subtypes of CD4+ regulatory T-cells have been described. Forkhead box P3 (FoxP3) is a transcription factor expressed on CD4+CD25+ natural regulatory T-cells that controls their phenotype and function. (Kleinewietfeld and Hafler, 2013). The suppressive functions of regulatory T-cells

have been reported to be impaired in MS-patients (Frisullo et al., 2009; Haas et al., 2005; Venken et al., 2006; Viglietta et al., 2004). In addition, the proportions of circulating CD4+CD25+FoxP3+ and CD8+FoxP3+ T-cells seem to be lower during relapses than during remission (Frisullo et al., 2009; Frisullo et al., 2010). The frequency of peripheral blood CD4+CD25+ and CD4+CD25+FoxP3 regulatory T-cells on the other hand seems to be similar in MS-patients and healthy controls (Frisullo et al., 2009; Haas et al., 2005; Venken et al., 2006), although one study has found decreased frequency of CD4+CD25+FoxP3+ T-cells in RRMS-patients compared to healthy controls (Venken et al., 2008). In addition to FoxP3+ regulatory T-cells, also IL-10 producing T regulatory type 1 cells have been linked to MS-disease (Kleinewietfeld and Hafler, 2013).

2.3.2 B-cells

The role of B-cells in the pathogenesis of MS has become more evident after the development of anti-CD20-antibodies, rituximab and ocrelizumab, which are highly efficient in RRMS. CD20 is expressed on nearly all B-cells, it is absent only from pro-B-cells and antibody producing plasma cells. (Disanto et al., 2012). B-cells have multiple functions that may contribute to MS pathogenesis (von Büdingen et al., 2015). The most obvious function is the ability to produce antibodies. Oligoclonal bands, which arise from the intrathecal synthesis of clonal IgG, are present in the CSF of more than 95% of MS-patients (Disanto et al., 2012). Oligoclonal bands indicate that B-cells are abnormally activated within the CNS of MS-patients (Disanto et al., 2012), but the target of these antibodies has not been identified (Krumbholz et al., 2012). CSF antibody levels seem to be rather stable in patients receiving anti-CD20 treatments, which contest their importance in the pathogenesis of MS (Disanto et al., 2012; von Büdingen et al., 2015). Besides producing antibodies B-cells can act as antigen presenting cells and efficiently present their cognate antigen to T-cells thus activating them. In addition, B-cells can active T-cells by producing pro-inflammatory cytokines. On the other hand, B-cells can regulate T-cell activity by producing anti-inflammatory cytokine IL-10. (Krumbholz et al., 2012). B-cells also participate in the formation of ectopic lymphoid tissues, which are tertiary lymphoid tissues established at the sites of inflammation that have been found associated with meningeal tissues in MS (von Büdingen et al., 2015).

Circulating B-cells are affected also by other than anti-CD20-treatments. Natalizumab-treatment increases both the absolute numbers and proportions of B-cells in circulation (Putzki et al., 2010; Saraste et al., 2016) and especially the number of pre-B-cells is increased (Krumbholz et al., 2008). DMF-treatment

modulates the B-cell compartment by shifting the balance between pro- and anti-inflammatory B-cell responses (Li et al., 2017) and increasing the numbers of circulating B-cells with regulatory capacity (Lundy et al., 2016).

2.3.3 Natural killer –cells

NK-cells are part of the innate immune system. They are large granular lymphocytes that do not express rearranged antigen receptors (Artis and Spits, 2015). NK-cells can secrete pro-inflammatory cytokines IFN- γ and tumor necrosis factor (TNF). They induce granule-mediated cytotoxicity through perforin and granzyme (Gr) expression, and they participate in tumor surveillance, eliminate virus-infected cells and amplify inflammatory responses. (Walker et al., 2013). NK-cells can be phenotypically defined by an absence of CD3 and expression of CD56 (Caligiuri, 2008). NK-cells, which comprise about 10% of all peripheral blood lymphocytes, can be divided to functionally distinct subpopulations based on relative expression of CD56 and CD16. Majority, about 90%, of circulating NK-cells express low numbers of CD56 and high numbers of CD16 and belong to $CD56^{dim}CD16^{bright}$ subpopulation. (Poli et al., 2009). Only about 10% of circulating NK-cells express high numbers of CD56 and belong to $CD56^{bright}CD16^{-/dim}$ subpopulation (Poli et al., 2009), but the proportion of $CD56^{bright}$ NK-cells is increased in sites of peripheral inflammation and nearly 100% of NK-cells found in secondary lymphoid tissues, ie. lymph nodes and tonsils, are $CD56^{bright}$ (Caligiuri, 2008). In the CSF of both healthy individuals and MS-patients most of NK-cells are $CD56^{bright}$ (Gross et al., 2016b), and the NK-cells of endometrium and early pregnancy decidua are mainly $CD56^{bright}$ (Poli et al., 2009). $CD56^{bright}$ NK-cells are considered to be immunoregulatory. $CD56^{bright}$ NK-cells produce significantly greater levels of IFN-γ, TNF-α and IL-10 in response to monokine stimulation than $CD56^{dim}$ NK-cells but are less effective mediators of antibody-dependent cellular cytotoxicity and natural cytotoxicity (Cooper et al., 2001). $CD56^{bright}$ can control the proliferation of CD4+ T-cells through a cytotoxic mechanism (Laroni et al., 2016). $CD56^{bright}$ cells are also able to produce high amounts of adenosine and inhibit autologous CD4+ T-cell proliferation suggesting that the regulatory function of $CD56^{bright}$ NK-cells is at least in part related to adenosine production (Morandi et al., 2015).

NK-cells may influence MS pathogenesis in multiple, either protective or exacerbating ways (Høglund and Maghazachi, 2014). The number of circulating NK-cells seems to be reduced in MS-patients (Gross et al., 2016b; Segal, 2007). Reductions in the functional activity of NK-cells have been associated with clinical relapses and development of new or enlarging active lesions in MRI (Kastrukoff

et al., 2003; Kastrukoff et al., 1998). It has been shown that NK-mediated control of T-cell activity is dysregulated in MS (Gross et al., 2016b). In untreated MS-patients the cytotoxic suppression of the proliferation of autologous CD4+ T-cells by activated $CD56^{bright}$ NK-cells is impaired, despite the similar $CD56^{bright}$ NK-cell phenotype and frequency in MS-patients and healthy controls. The impaired suppression could be related to the increased T-cell HLA-E expression in MS-patients compared to healthy controls enhancing the inhibition of $CD56^{bright}$ NK-cells. (Laroni et al., 2016).

Many MS-treatments influence peripheral NK-cells. IFN-β enhances NK-cell activity and significantly increases the percentage of peripheral blood $CD56^{bright}$ NK-cells (Chanvillard et al., 2013; Saraste et al., 2007; Vandenbark et al., 2009). In addition, an expansion of blood $CD56^{bright}$ NK-cells after IFN-β treatment have been shown to be related to a positive clinical response (Martinez-Rodriguez et al., 2011). Daclizumab-therapy leads to a selective expansion of $CD56^{bright}$ NK-cells (200%), which correlates strongly with decreased brain inflammation measured as average number of contrast enhancing lesions on brain MRI (Bielekova et al., 2006). Daclizumab-treatment also increases the cytotoxicity of NK-cells toward autologous activated T-cells. $CD56^{bright}$ NK-cells use GrK- and GrA-mediated cytotoxicity when killing these cells. (Jiang et al., 2011). An increase in the proportions and numbers of $CD56^{bright}$ NK-cells have been observed also under alemtuzumab-treatment (Gross et al., 2016a), whereas natalizumab and fingolimod seem to decrease the proportion of circulating $CD56^{bright}$ NK-cells (Johnson et al., 2011; Mellergård et al., 2013).

2.4 Multiple sclerosis and pregnancy

MS often onsets during childbearing years and affects preferentially women. Thus pregnancy-related questions are relevant for many MS-patients. Generally, pregnancy in women with MS is not considered high risk (Pozzilli et al., 2015). The effect of MS on fertility is not clear, but fertility in women with MS could be affected by reproductive decision making, decreased libido or sexual function, lower estrogen levels, thyroid autoimmunity, temporary therapy-related amenorrhea or premature ovarian failure (Bove et al., 2014). The use of artificial insemination may be more common among women with MS (Jalkanen et al., 2010). MS does not seem to increase the risk of early trimester pregnancy loss, preterm delivery, stillbirth or fetal malformations (Bove et al., 2014; Finkelsztejn et al., 2011a). However, a meta-analysis has suggested that the prevalence of caesarean sections, abortions, prematurity and low birth weight might be relatively higher among women with MS (Finkelsztejn et al., 2011a). Women with MS may

also need more often instrumental assistance during the delivery (Jalkanen et al., 2010).

2.4.1 Disease activity during and after pregnancy

RRMS disease activity is typically decreased during pregnancy. In a large European study involving 254 women with MS the annualized relapse rate (ARR) decreased by 70 % during the third trimester compared to the year before pregnancy. However, during the first three months postpartum the relapse rate increased again and was even higher than before pregnancy. After this, relapse rate returned to the pre-pregnancy level. (Confavreux et al., 1998). These results were recently confirmed in a register study including 893 pregnancies (Hughes et al., 2014) and similar results have been achieved also in smaller studies (De Las Heras et al., 2007; Fernández Liguori et al., 2009; Jalkanen et al., 2010), although in some studies the postpartum relapse rate was smaller than before pregnancy (Finkelsztejn et al., 2011b). A Lebanese study found decreased relapse rate during each trimester of pregnancy and in the first year of postpartum (Fares et al., 2016). In this study the relapse rate was returned to pre-pregnancy level during the second year of postpartum (Fares et al., 2016). Considering that the reduction induced by current MS treatments ranges from 30-60%, the reduction during pregnancy is remarkable (Voskuhl and Gold, 2012). Use of high-efficacy DMTs before pregnancy may, however, decrease the positive effect of pregnancy. This was observed in a recent cross-sectional retrospective study including 87 RRMS patients with 99 pregnancies (Alroughani et al., 2018). Most patients in this study received IFN-β, fingolimod or natalizumab before pregnancy. The total number of relapses during the year before pregnancy was four in this cohort, during pregnancy 17 and during the ten weeks postpartum period 13. The four-fold increase in the relapse occurrence during pregnancy was driven mostly by patients experiencing re-bound disease activity after cessation of natalizumab or fingolimod. A longer washout period was significantly associated with relapse occurrence. (Alroughani et al., 2018).

Although a peak in disease activity after delivery has been observed in several studies, only minority of women experience a relapse during the first three months postpartum and more than half of women seem to remain relapse free during the year after pregnancy (Aharoni, 2013; Hughes et al., 2014; Vukusic et al., 2004). Postpartum relapses are mainly predicted by the relapse rate before and during pregnancy, a higher ARR before pregnancy predicting increased risk of early postpartum relapse (Hughes et al., 2014; Portaccio et al., 2011; Vukusic et al., 2004). A more recent study, in which most of the women received DMT [IFN-β

(53%), GA (23%), natalizumab (11%), intravenous immunoglobulins (1%), and rituximab (0.5%)] before pregnancy found no association between pre-pregnancy and postpartum relapse rates (Hellwig et al., 2015). Whether DMT use before pregnancy is associated with postpartum relapses is not clear (Hellwig et al., 2015; Hughes et al., 2014; Portaccio et al., 2011), whereas breastfeeding seems to have no effect on postpartum relapse rate (Portaccio et al., 2011; Vukusic et al., 2004). The pre-pregnancy disease activity might affect the mother's decision of whether to breastfeed or not (Airas et al., 2010). Some studies have, however, suggested that relapse in the first 6 months postpartum may be diminished by exclusive breastfeeding (Hellwig et al., 2015).

2.4.2 Effect of pregnancy on the long-term disease course

Pregnancy does not seem to have harmful effect on the long-term disease course of MS (Airas, 2015; Langer-Gould and Beaber, 2013). Studies have found either no effect of pregnancy on disability progression (Karp et al., 2014; Roullet et al., 1993; Vukusic et al., 2004) or a positive effect (D'hooghe et al., 2010; Jokubaitis et al., 2016; Keyhanian et al., 2012; Runmarker and Andersen, 1995; Verdru et al., 1994). However, pregnancy might accelerate the rate of transition to SPMS (Karp et al., 2014).

2.4.3 Disease modifying treatment during pregnancy

Information about the safety of DMTs during pregnancy is limited, and thus in general patients are advised to discontinue their use prior to conception (Pozzilli et al., 2015). IFN-β and GA are recommended to be discontinued prior to conception or once pregnancy is known (Aharoni, 2013; Lu et al., 2013). Fingolimod should be discontinued 2-3 months before cessation of effective contraception (Cree, 2013; Pozzilli et al., 2015). Women using alemtuzumab are advised not to get pregnant for 4 months after a course of alemtuzumab (Alroughani et al., 2016). Teriflunomide has caused teratogenicity in animal studies and is contraindicated in pregnant women or women not using reliable contraception. The elimination process of teriflunomide is slow and can take up to two years, thus accelerated elimination procedure should be used if patient is planning pregnancy. (Cree, 2013).

Natalizumab is recommended to be discontinued 3 months prior to pregnancy and should be used during pregnancy only if the clinical condition of the woman requires it (Cree, 2013; Houtchens and Kolb, 2013). There are few reported cases

in which natalizumab has been used throughout pregnancy to avoid return of disease activity or natalizumab has been restarted during pregnancy because of severe worsening in clinical condition (Ciron et al., 2016; Fagius and Burman, 2014; Haghikia et al., 2014; Hoevenaren et al., 2011; Massey et al., 2017a).The Tysabri Pregnancy Exposure Registry included 369 MS patients who discontinued natalizumab during the 3-month period prior to conception or during pregnancy (Friend et al., 2016). The spontaneous abortion rate was consistent with that of the general population and registry outcomes showed no specific pattern of malformations that would suggest a drug effect although the overall rate of birth defects was higher than expected (Friend et al., 2016). In a German study 101 RRMS patients exposed to natalizumab during first trimester of pregnancy were compared to RRMS patients not exposed to natalizumab, and natalizumab did not appear to increase the risk of adverse pregnancy effects (Ebrahimi et al., 2015). However, majority of patients in these studies discontinued natalizumab prior to or within the first trimester (Ebrahimi et al., 2015; Friend et al., 2016). Smaller studies have implied that natalizumab used throughout pregnancy or during the third trimester of pregnancy may lead to hematological abnormalities in the newborn, including low platelet count, thrombocytopenia, anemia and leukocytosis (Ciron et al., 2016; Haghikia et al., 2014).

2.5 Immune system during pregnancy

Pregnancy affects the activity of autoimmune diseases. The diseases driven primarily by cell-mediated immunity, such as MS and RA, are ameliorated, but diseases driven primarily by antibody-mediated immunity, such as systemic lupus erythematosus (SLE), are worsened (Voskuhl and Gold, 2012). The effect of pregnancy on autoimmune diseases can be considered as a “side-effect” of pregnancy-related immune alterations (Patas et al., 2013).

During pregnancy the immune system of the mother must accept the semi-allogeneic fetus, but at the same time also protect the mother against infections (Figueiredo and Schumacher, 2016). This is achieved by modulation of the immune system rather than by general immune suppression, as some aspects of innate immunity, for example blood phagocytes and dendritic cells are increased during pregnancy (Kraus et al., 2012; Mor and Cardenas, 2010; Pazos et al., 2012). Fetal antigens may participate in the modulation by interacting directly with maternal immune system and inducing regulatory T-cells (Gold and Voskuhl, 2016). Although the maternal immune system is not generally strongly suppressed, adaptive immune responses are weakened during pregnancy and the activity of T-

cells is suppressed, which could impact the amelioration of cell-mediated autoimmune diseases during pregnancy (Luppi, 2003; Pazos et al., 2012).

Pregnancy has been previously considered as an anti-inflammatory state, during which maternal immune system favors Th2-type immune responses to protect the fetus (Mor and Cardenas, 2010; Wegmann et al., 1993). A systemic Th1 to Th2 shift during mid-gestation starting in the second trimester and lasting until pre-parturition suppresses the cytotoxic T-cell response decreasing the robustness of cell-mediated immunity (Kourtis et al., 2014; Patas et al., 2013) although animal studies have suggested that Th2 cytokines are not essential for fetal survival (Patas et al., 2013; Voskuhl and Gold, 2012). Based on Th1 and Th2 shifts pregnancy can be considered as either pro-inflammatory or anti-inflammatory state depending on the stage of pregnancy. The pro-inflammatory phase during first trimester is followed by an anti-inflammatory phase at second trimester during which anti-inflammatory cytokines such as IL-10 and TGF-β are present, but at the end of pregnancy the pro-inflammatory phase is returned to promote the delivery (Mor and Cardenas, 2010; Racicot et al., 2014). This can be reflected also to increased inflammatory activity in the CNS, as appearance of active MS-lesions has been observed in MRI performed at the end of pregnancy and in a large majority of MS-patients the signs of disease activity in MRI were observed soon after delivery (Paavilainen et al., 2007).

2.6 Factors that may impact disease activity in multiple sclerosis during and after pregnancy

The exact biological mechanisms that lead to the pregnancy-related alterations in the disease activity of MS are not thoroughly understood (Patas et al., 2013). Several studies have investigated alterations in many systemic immunological parameters as well as changes in serum hormone levels and gene expression during and after pregnancy in MS-patients to clarify this phenomenon. The sizes of study populations, time of sampling, studied parameters and key findings of these studies are summarized in Table 3.

Table 3. Alterations in genetic, hormonal and immunological parameters during and after pregnancy in MS-patients according to present literature.

Study	n(MS)	n(HC)	Samples	Parameters (methods)	Key findings
(Airas et al., 2007)	33[a]	15	10-12 w of preg 26 w of preg 4-5 w PP	up-and down regulation of immune-related genes with microarray analysis; HLA-G mRNA and expression in PBMC (PCR and FCM); soluble serum HLA-G levels (ELISA)	HLA-G is differentially regulated in MS vs. controls; % of HLA-G+CD4+ and HLA-G+CD8+ decreased PP vs. pregnancy in active patients; soluble HLA-G decreased PP vs. pregnancy in active MS
(Al-Shammri et al., 2004)	8		1. trim 2. trim 3. trim PP	IL-4, IL-10, IFN-γ, TNF-α production by PBMC (ELISA)	increased Th2/Th1 ratio during pregnancy in 6 of 8 patients
(de Andrés et al., 2017)	30 (pr) 30 (npr) 10 (men)	13 (pr) 32 (npr) 9 (men)	1. trim 2. trim 3. trim PP	% and CD69 expression of $CD56^{bright}$ and $CD56^{dim}$ NK-cells and CD3+CD56+CD8+ NKT-cells (FCM)	increased activation of NKT-cells during pregnancy and PP vs. non-pregnant MS; increased activation of $CD56^{bright}$ at 2. and 3. trim vs. non-pregnant MS
(Gilli et al., 2010)	32	16	before preg 3, 6 and 9 mo of pregnancy PP	gene expression profiles during pregnancy in MS vs. controls (micro-array, PCR, Western Blotting	complementary changes in expression of 7 inflammation-related transcripts; expression of gene expression profiles correlated with clinical outcome
(Gilmore et al., 2004)	4	3	3. trim (36.7) 7 w PP	IL-4, IL-10, IFN-γ, TNF-α production by PBMC (ELISA or bioassay)	IFN-γ/IL-10 ratio increased at PP in 3 of 4 patients; IFN-γ/IL-4 no clear pattern
(Iorio et al., 2009)	10[b]	20	before preg[c] 1. trim (12-14) 2. trim (24-26) 3. trim (36-38) 4-6 w PP	CD4+CD25+FoxP3+ Treg %; FoxP3 MFI in circ. CD4+CD25+ cells; pSTAT1, pSTAT3, T-bet expression in circ. CD4+ and CD8+ cells (FCM); IFN-γ, IL17, IL-10 production by PBMC (ELISA);	Treg% and FoxP3 MFI increased at 1. and 2. trim vs. before, 3. trim and PP; higher expression of pSTAT1, pSTAT3 and T-bet and increased production of IFN-γ at 3. trim and PP in active group

Table 3. continued

Study	n(MS)	n(HC)	Samples	Parameters (methods)	Key findings
(Langer-Gould et al., 2010)	26	24	1., 2., 3. trim, 2, 4, 6, 9, 12 mo PP	16 functional cell types (FCM)	decline in circulating CD4+IFNγ+ cells leads to PP relapses
(López et al., 2006)	7[d]		before preg 1. trim (10.7) 2. trim (23.4) 3. trim (35.7) 12 w PP	% of T, B, NK-cells, monocytes expressing CCR3, CCR4, CCR5, CXCR3, CXCR4 (FCM); IL-10 and IFN-γ mRNA expression (PCR)	% of CXCR3+CD4+ and CXCR3+CD8+ decreased at 2. trim, % of CXCR4+CD4+ and CXCR4+CD8+ increased at 2. trim; IL10/IFN-γ ratio increased at 3. trim vs. PP
(Neuteboom et al., 2009a)	36[e]		before preg[f] 1. trim (10-12) 3. trim (28-30) 4-8 w PP	serum IL-8 levels (ELISA)	decrease at 3. trim vs. 1.trim; high levels at 1. trim associated with high risk of PP relapse
(Neuteboom et al., 2009b)	36[e]	17	before preg[f] 1. trim (10-12) 3. trim (28-30) 4-8 w PP	serum leptin levels; soluble leptin receptor levels (ELISA)	leptin increased in 3. trim and dropped PP. Drop associated with occurrence of PP relapse
(Neuteboom et al., 2010)	9 (Th17) 15 (Treg)	8 (Th17) 15(Treg)	before preg 1.trim (10-12) 3. trim (28-30) 4-8 w PP	Th17 % (CD4+CD45RO+IL17A+) of CD4+; CD25+FoxP3+CD127low Treg of CD3+CD4+ (FCM)	no fluctuations in Th17%; Treg % decreased at 1. and 3. trim vs. before and PP
(Sánchez-Ramón et al., 2005)	13	21	1. trim (1-13) 2. trim (14-28) 3. trim (29-del) 1 mo PP	counts and % of CD4+CD25+ Treg, CD4+CD25^{high} Treg and CD4+CD38+HLA-DR+ activated cells (FCM)	CD4+CD25+ Treg % increased at 3. trim vs. PP; CD4+CD25^{high} Treg % decreased at 3. trim vs. PP; CD4+CD38+HLA-DR+ % decreased at 2. and 3. trim vs. 1. trim

MS = multiple sclerosis; HC = healthy control; w = week; preg = pregnancy; PP = postpartum= HLA = human leukocyte antigen; mRNA = messenger ribonucleic acid; PBMC = peripheral blood mononuclear cells; PCR=polymerase chain reaction; FCM = Flow cytometry; ELISA=enzyme linked immunosorbent assay; CD = cluster of differentiation; trim = Trimester of pregnancy; IL = Interleukin; IFN-γ = interferon-gamma; TNF-α = tumor necrosis factor -alfa; Th = T helper; pr = pregnant; npr = non-pregnant; NK = natural killer; mo = month; FoxP3 = Forkhead box P3; Treg = regulatory T-cell; MFI=mean fluorescent intensity; circ = circulating; pSTAT = phosphorylated signal transducer and activator of transcription; CCR3, CCR4, CCR5, CXCR3, CXCR4 = chemokine receptors; deliv = delivery

[a] micro-array n=3; PCR n=6; FCM n=10; ELISA n=27; [b] divided in two groups based on disease activity; [c] sample taken during remission; [d] FCM n=7; ELISA n=6; [e] all four samples n=16; [f] sample taken 6 mo before pregnancy only from MS-patients

2.6.1 Sex hormones and Th1/Th2 shift

During pregnancy the levels of circulating steroid hormones such as estrogens, progesterone, glucocorticoids and some, but not all, androgens are elevated (Makieva et al., 2014; Patas et al., 2013). The peak in the level of these hormones is reached during the third trimester of pregnancy, thus coinciding with the maximal decrease in MS relapse rate (Figure 1). After delivery the levels of these sex hormones are rapidly decreased with a simultaneous increase in relapse rate. (Patas et al., 2013). This implies that pregnancy related alterations in MS disease activity may be at least partially associated with the hormonal changes. In a small study treatment of MS-patients with oral estriol significantly decreased the numbers and volumes of Gd-enhancing lesions (Sicotte et al., 2002). A randomized, double-blinded, placebo-controlled phase 2 trial studied the effect of daily oral estriol combined with GA (Voskuhl et al., 2016). The annualized confirmed relapse rate was smaller in estriol group than in placebo group (0.25 vs 0.37, p=0.077) reaching the statistical significance level of 0.10 used in the study (Voskuhl et al., 2016). However, others have considered the results of this study to be negative (Langer-Gould, 2016).

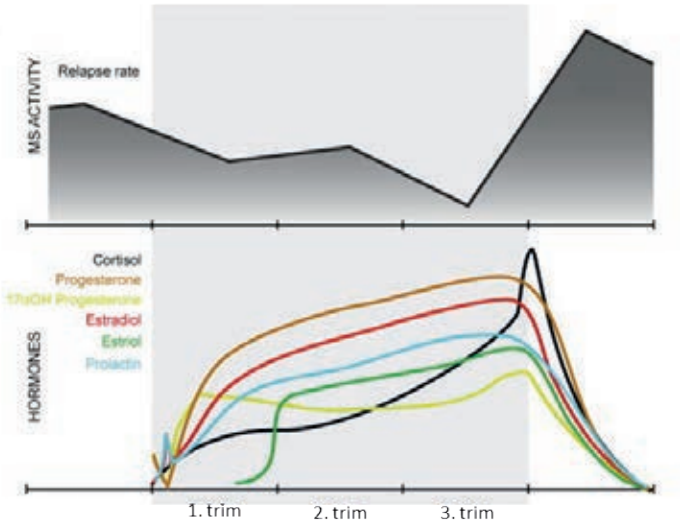

Figure 1. Alterations in multiple sclerosis relapse rate and steroid hormone levels during and after pregnancy. Modified from Patas et al., 2013.

The immunomodulatory effects of steroid hormones are largely mediated through binding to specific nuclear receptors expressed in most cells of the immune system that act as transcription factors. In vitro studies have shown that estradiol and estriol have dose dependent effects on the production of IL-10 and IFN-γ and biphasic effect on the production of TNF-α (Gilmore et al., 1997; Javadian et al., 2014). Progesterone seems to enhance the secretion of IL-4 (Correale et al., 1998; Lissauer et al., 2015). A Th1 to Th2 shift have been observed during pregnancy in MS-patients in few small studies (Al-Shammri et al., 2004; Gilmore et al., 2004; López et al., 2006). Oral estriol treatment of MS was associated with a partial Th1 to Th2 shift which consisted of increased intra-cellular and secreted levels of IL-5 and IL-10, and decreased levels of TNF-α from stimulated peripheral blood mononuclear cells. These changes correlated with reduced volumes of Gd-enhancing lesion on MRI. (Soldan et al., 2003). Progesterone induces reduction of Foxp3+ regulatory T-cells in PBMC of non-pregnant women (Mjösberg et al., 2009) and induces apoptosis of peripheral blood NK-cells (Arruvito et al., 2008). At pregnancy levels estradiol and estriol inhibit NF-κB (Straub, 2007; Zang et al., 2002). Pregnancy levels of estriol inhibit the migration of T-cells isolated from MS-patients (Zang et al., 2002) and estradiol decreases membrane expression of adhesion molecules and cell adhesion to endothelial cells (Straub, 2007). Estradiol also enhances humoral immunity and stimulates antibody secretion at high levels (Straub, 2007).

2.6.2 Regulatory T-cell and Th17 balance

The balance between regulatory T-cells and Th17 cells has an important role in mediating the maternal tolerance to the fetus, and the disruption of this balance by decreasing levels of regulatory T-cells or increasing levels of Th17 cells may lead to preterm birth, pre-eclampsia or unexplained recurrent pregnancy loss (Figueiredo and Schumacher, 2016). Early studies have observed increased proportions of peripheral blood CD4+CD25+ regulatory T-cells peaking at either first or second trimester have been observed in healthy women (Heikkinen et al., 2004; Somerset et al., 2004). However, more recent studies have observed non-altered or reduced regulatory T-cells during pregnancy (Dimova et al., 2011; Mjösberg et al., 2009; Tilburgs et al., 2006). The FoxP3+ Treg/IL-17 expressing CD4+ T-cell ratio has been observed to increase in the end of normal pregnancy (Santner-Nanan et al., 2009). The increased Treg/Th17 ratio during normal pregnancy might have an impact on the amelioration of MS-disease activity during pregnancy (Figueiredo and Schumacher, 2016). However, the frequency of Th17 cells in MS-patients seems to be unaltered during pregnancy (Neuteboom et al., 2010) and the results about regulatory T-cell frequencies in MS-patients during

pregnancy are inconclusive (Iorio et al., 2009; Neuteboom et al., 2010; Sánchez-Ramón et al., 2005).

2.6.3 Fluctuations in the peripheral blood immune cells

Fluctuations in the numbers and proportions of peripheral blood lymphocyte subsets have been observed during pregnancy in healthy woman, but whether fluctuations occur also in MS-patients during pregnancy seems to be largely unknown. Decrease in the proportion of CD4+ T-cells producing IFN-γ have been observed during pregnancy in MS (Langer-Gould et al., 2010). In healthy women the absolute numbers of peripheral blood lymphocytes have been shown to decrease during pregnancy and increase postpartum compared to non-pregnant controls (Watanabe et al., 1997). Two longitudinal studies and one cross-sectional study have shown that number of total lymphocytes is first decreased from first to second trimester, and then started to increase (Lurie et al., 2008; Mosimann et al., 2013; Valdimarsson et al., 1983). Several, but not all, studies have observed decreases in absolute numbers of peripheral blood T-cells during normal pregnancy either compared to non-pregnant controls or in samples obtained six months after delivery (Kraus et al., 2012; Mahmoud et al., 2001; Tallon et al., 1984; Valdimarsson et al., 1983; Watanabe et al., 1997). The proportion of CD3+ T-cells seems to be unchanged during pregnancy (Kraus et al., 2012; Luppi et al., 2002; Mahmoud et al., 2001; Tallon et al., 1984) although few studies have observed increased proportion of T-cells during early pregnancy (Fiddes et al., 1986; Valdimarsson et al., 1983). Some studies have found increased proportions of CD8+ T-cells at third trimester of pregnancy compared to non-pregnant controls (Fiddes et al., 1986; Luppi et al., 2002), and decreased proportions of CD4+ T-cells (Luppi et al., 2002; Mahmoud et al., 2001) whereas others have not observed any alterations in these T-cells subsets during pregnancy (Kraus et al., 2012; Kühnert et al., 1998; Tallon et al., 1984). Similarly, the proportion of B-cells has been reported to be either decreased (Valdimarsson et al., 1983), slightly increased (Mahmoud et al., 2001) or not changed during pregnancy (Kühnert et al., 1998; Luppi et al., 2002).

In healthy women the number of peripheral blood NK-cells seems to decrease during third trimester of pregnancy compared both to non-pregnant controls (Mahmoud et al., 2001; Watanabe et al., 1997) or first trimester (Mosimann et al., 2013). Some but not all studies have observed decreases also in the proportion of NK-cells (Luppi et al., 2002; Mahmoud et al., 2001; Mosimann et al., 2013). During third trimester of pregnancy the number of $CD56^{dim}$, but not $CD56^{bright}$, NK-cells is decreased compared to first trimester and to six months postpartum

(Mosimann et al., 2013; Kraus et al., 2012). The proportion of $CD56^{bright}$ on the other hand is increased in healthy pregnant women compared to non-pregnant healthy women (de Andrés et al., 2017), and the proportion of activated $CD56^{bright}$ NK-cells is increased during pregnancy both in healthy women and MS-patients (de Andrés et al., 2017; Mosimann et al., 2013).

2.7 Virus susceptibility during pregnancy

The suppressed adaptive immunity during pregnancy impacting the amelioration of cell-mediated autoimmune diseases affects also anti-viral responses (Kourtis et al., 2014; Pazos et al., 2012). A shift from Th1 to Th2 immunity that leads to suppression of cytotoxic T-cell responses during pregnancy could explain the increased severity of infections involving cell-mediated immunity, although the overall susceptibility to infections seems to be unaltered during pregnancy (Kourtis et al., 2014). Pregnant women are at a higher risk of severe illness and mortality due to viral infections such as influenza (Silasi et al., 2015). Also, infections with Hepatitis E and Herpes simplex viruses are more severe in pregnant women, and patients with immunosuppressive treatment or a disease leading to immunosuppression, and pregnant women are the largest groups of adults with disseminated Herpes simplex virus infection (Kourtis et al., 2014). Certain intracellular pathogens, such as *Listeria monocytogenes* (*L. monocytogenes*) and *Mycobacterium tuberculosis*, may also cause more severe infections during pregnancy (Mateus et al., 2013; Warner et al., 1992). In addition to the increased severity of infections during pregnancy, also the susceptibility of some infections such as listeriosis and malaria is increased during pregnancy (Kourtis et al., 2014). Listeriosis is an opportunistic infection caused by *L. monocytogenes* (Hernandez-Milian and Payeras-Cifre, 2014). All pregnant women are advised to avoid ingestion of vacuum-packed, cold-smoked and fresh-salted fish products, un-pasteurized milk and products made from un-pasteurized milk, soft cheeses even if made from pasteurized milk, unheated frozen vegetables, and uncooked or undercooked meats to avoid food-borne listeriosis (thl.fi/fi/web/infektiotaudit/taudit-ja-mikrobit/bakteeritaudit/listeria).

2.8 John Cunningham virus

John Cunningham virus (JCV) is a ubiquitous polyomavirus (Bellizzi et al., 2013), with both archetypic and neurotropic strains (Jelcic et al., 2015). Most people are infected with JCV in childhood through mouth and nose (Wollebo et al., 2015), and the initial infection is typically asymptomatic and caused by archetypic JCV

strains (Beltrami and Gordon, 2014; Jelcic et al., 2015). During initial infection JCV is spread from tonsils and gastrointestinal tract through blood circulation to the kidney and bone marrow, where it persists mainly inactively (Wollebo et al., 2015).

JCV seroprevalence varies geographically, and seroprevalence observed in different studies have varied between 30% and 90% (Schwab et al., 2017; Wollebo et al., 2015). Seroprevalence increases with age and is higher in men than in women, but there is no big difference between the seroprevalence of MS-patients and healthy controls (55% vs. 59%, respectively) (Schwab et al., 2017).

Under conditions of immunosuppression JCV may be reactivated (Wollebo et al., 2015). Reactivation of this opportunistic virus may cause PML, which is a lethal demyelinating disease of the CNS that is mainly seen in patients with HIV or acquired immune deficiency syndrome (AIDS), but also in patients with impaired cell-mediated immunity including MS-patients receiving natalizumab (Wollebo et al., 2015). The multiple expanding regions of demyelination seen in the CNS of PML patients are caused by the lytic infection of oligodendrocytes and astrocytes by the neurotropic form of JCV, which has mutations and rearrangements in the noncoding control region compared to the more common archetype (Wollebo et al., 2015). Neurotropic forms of JCV have been detected in peripheral blood leukocytes and in the brain of healthy, non-immunocompromised individuals (Wollebo et al., 2015), but it seems that immunosuppressed patients are more likely to have JCV DNA in the brain (Bayliss et al., 2011). This study did not include patients treated with natalizumab or other monoclonal antibodies (Bayliss et al., 2011).

Circulating anti-JCV-antibodies (anti-JCV-Abs) produced during the primary infection fail to protect against JCV reactivation and development or progression of PML (Antoniol and Stankoff, 2014; Beltrami and Gordon, 2014). Cell-mediated immunity is more effective in clearing JCV infection before occurrence of PML (Monaco and Major, 2015). CD8+ T cells eliminate JCV from the brain, but efficient recognition of JCV variants by CD4+ T cells is also required (Jelcic et al., 2015; Jelcic et al., 2016). Thus pregnancy-related suppression of cell-mediated immunity could increase the likelihood of PML. Reactivation of JCV has been observed also during pregnancy (Coleman et al., 1980).

Although circulating anti-JCV-Abs are inefficient in the protection against PML, they are important in the clinical risk-assessment of PML in natalizumab-treated MS-patients. PML can occur only in patients who are seropositive for JCV, and the risk is further increased with longer duration of natalizumab-treatment and previous use of immunosuppressive medication (Antoniol and Stankoff, 2014). Higher levels of serum or plasma anti-JCV-Abs measured as higher anti-JCV-

index by 2-step second-generation enzyme-linked immunosorbent assay (ELISA) are associated with higher PML risk (Plavina et al., 2014). Patients without previous use of immunosuppressive medication could be further divided into lower and higher PML risk groups based on anti-JCV-index. The risk was smallest for patients with anti-JCV-index <0.9 and highest for patients with anti-JCV-index >1.5. (Plavina et al., 2014).

3 AIMS OF THE STUDY

To investigate the immunological mechanisms which contribute to amelioration of MS disease during pregnancy, and which lead to reactivation of MS disease after delivery. To evaluate how the pregnancy-related immunological alterations affect the susceptibility to an opportunistic infection particularly relevant in care of MS, namely JCV-induced PML.

The specific aims of the sub-studies were:

I To describe MRI findings and immunological characterization of peripheral blood and CSF mononuclear cells in a patient experiencing unusually severe activation of MS-disease after delivery.

II To study alterations in the proportions of peripheral blood T-cells, B-cells and NK-cells during and after pregnancy in a cohort of MS-patients and compare these to non-pregnant MS-patients and pregnant and non-pregnant healthy controls.

III To study alterations in the proportions of peripheral blood regulatory T-cells, IFN-γ and IL-4 producing cells, and $CD56^{bright}$ and $CD56^{dim}$ NK-cells during and after pregnancy in MS-patients.

IV To investigate alterations in MS-related CSF parameters during and after pregnancy.

V To evaluate pregnancy-related alterations in humoral response to JCV in MS-patients by measuring serum JCV-Ab-indices during and after pregnancy in MS-patients and during pregnancy in healthy controls, and by comparing these results to total IgG-levels and to levels of antibodies against other viruses (anti-EBV-Ab, anti-CMV-Ab and anti-measles-Ab).

4 MATERIALS AND METHODS

4.1 Study design

This study was part of "The Finnish Multiple Sclerosis and Pregnancy Study". During the study period of three years from January 2003 to 2005 all Finnish central hospital neurology units were asked to recruit study participants fulfilling inclusion criteria, which were a confirmed pregnancy status and MS diagnosed according to Poser's or McDonald's criteria (McDonald et al., 2001; Poser et al., 1983). Seven blood and serum samples were prospectively collected from the study participants. Three samples were taken during pregnancy (at 10-12, 26-28 and 35-37 gestational weeks [gw]) and four samples after pregnancy (at 2-3 days, 4-5 weeks or 1 month, 10-12 weeks or 3 months and 6 months postpartum). In addition, two CSF samples were drawn; one at 26-28 weeks of pregnancy and another one at 4-5 weeks postpartum. The timing of samples was designed to match the alterations of clinical MS disease activity during and after pregnancy (Confavreux et al., 1998).

In addition to biological samples, clinical data was also collected. Neurological examinations were performed twice during pregnancy (at 10-12 and 26-28 gw) and twice after pregnancy (4-5 weeks and 6 months postpartum). Disability was assessed using the EDSS (Kurtzke, 1983) and information about the number of relapses and the possible use of medication was collected. A relapse was defined as an appearance of neurologic dysfunction lasting more than 24 hours and evaluated according to each unit's practice. Data on demographics and on MS disease course before study entry was also recorded.

4.2 Study participants

4.2.1 Patients with multiple sclerosis

A total of 78 female MS-patients participated in the study. 59 of MS-patients were pregnant, and 18 MS-patients with freshly diagnosed and non-treated RRMS were used as non-pregnant controls. Only one blood sample was obtained from non-pregnant MS-patients. The numbers and the characteristics of the patients participating in each study are shown in Table 4.

Table 4. Characteristics of pregnant and non-pregnant MS-patients participating in the study

	Pregnant MS-patients				Non-pregnant MS-patients
Study	**I**	**II and III**	**IV**	**V**	**II and III**
n	1	42	6	59	18
recruitment time	2004	1/2003-6/2004	1/2003-6/2004	1/2003-2005	
age (years)	25	29.5 (3.8)	26 (5)	30.5 (4.2), 23-41	35.7 (10.4)
disease duration (years)[1]	3	5.6 (4.2), 0-17	4.2 (4.2), 1-12.5	5.3 (4.1), 0-17.5	1.6 (3.0), 0.5-12
number of relapses[2]	2	4.3 (2.7), 1-12	3.2 (1.2), 2-5	4.2 (2.8)	2.6 (1.0), 1-5
DMT (n)	0	24	1	30	0
No DMT (n)	1	18	5	29	18

Mean (standard deviation) values with range are presented unless specified otherwise. DMT = disease modifying treatment (IFN-β or glatiramer acetate) before pregnancy;
[1] Duration of MS disease at study onset (from beginning of symptoms)
[2] Total number of relapses experienced before the study onset

4.2.2 Healthy controls

A total of 95 healthy controls participated in the study. 20 pregnant healthy women with mean [standard deviation (SD)] age of 30.3 (3.9) years, and 18 non-pregnant healthy women with mean (SD) age of 29.7 (5.2) years were used as controls in studies II and III. Three blood samples were obtained during pregnancy at 10-12, 26-28 and 35-37 gw and four after delivery (1-3 days, 4-5 weeks, 10-12 weeks and 6 months postpartum). From non-pregnant controls only one blood sample was obtained.

57 age- and parity-matched pregnant healthy women with mean (SD) age of 29.8 (4.0) years were used as controls in study V. Serum samples were obtained from Finnish Maternity Cohort serum bank (FMC, Oulu, Finland), supported by National Institute for Health and Welfare. Samples have been prospectively collected during pregnancy (one per trimester) and stored at -25°C.

4.3 Ethics

The study protocol was approved by the Ethical Board of the Turku University Hospital and written informed consent was obtained from all subjects.

4.4 Laboratory assessments

4.4.1 Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from 20 ml of heparinized blood within four hours of sample drawing if patients were from Turku. From samples collected outside Turku PBMC were isolated the following day, as samples were shipped to Turku. This did not significantly reduce the viability of the cells. The lymphocyte subtype distribution or the expression of studied cell surface molecules were not affected (data not shown). PBMC were isolated by density gradient centrifugation using Ficoll-Hypaque™ PLUS (Amersham Biosciences, Uppsala, Sweden), and washed twice with Ca- and Mg-free Hanks buffered salt solution (HBSS) containing 25 mM hydroxyethyl piperazineethanesulfonic acid (HEPES) and 0,014 % $NaHC0_3$. Cells were resuspended in RPMI-1640 containing 10 % fetal calf serum (FCS), 10 mM HEPES, 4 mM glutamine, 100μg/ml streptomycin and 100 U/ml penicillin. Portion of the cells were used immediately for immunofluorescence (IF) staining and the rest were stored for later use in 10 % dimethyl sulfoxide at – 135 °C. When frozen cells were used, cells were melted at room temperature and used immediately for the staining. Thawing did not significantly alter the expression level of the studied molecules (data not shown). All analysed samples from a given study subject were thawed and stained simultaneously to avoid the effect of potential day-to-day variation in the staining procedure.

4.4.2 Preparation of CSF cells

CSF cells and supernatant were separated by centrifugation for 10 min at 1500 rounds per minute (RPM) within few hours of sampling (samples collected in Turku) or following day after shipping of samples either on ice or at room temperature (samples collected outside Turku). The number of CSF cells were determined by microscopy. Supernatants were stored at -83°C. The cell pellet was resuspended in RPMI-1640 containing 10 % FCS, 10 mM HEPES, 4 mM glutamine, 100μg/ml streptomycin and 100 U/ml penicillin, and cells were used for IF-staining in study I.

4.4.2.1 CSF laboratory analyses

IgG-index of CSF can be calculated from serum and CSF albumin and IgG levels: IgG-index = CSF-IgG/serum-IgG x CSF-albumin/serum-albumin. In study IV IgG-index during and after pregnancy was determined from frozen CSF samples in TYKSlab clinical laboratory. Number of oligoclonal bands during and after pregnancy was determined from frozen CSF samples in TYKSlab clinical laboratory by using the Paragon Protein (SPE-II) Electrophoresis Kit (Beckman Coulter, Fullerton, California, USA).

4.4.3 Immunofluorescence staining

4.4.3.1 Principle of immunofluorescence staining

The principle of IF-staining is illustrated in Figure 2. In direct IF-staining only one fluorochrome labelled antibody is used. In in-direct IF-staining two antibodies are used. The primary antibody is un-labelled, and the secondary antibody is a fluorochrome labelled anti-isotype-antibody, which binds to the primary antibody. In double-colour staining two antibodies recognising different antigens and labelled with different fluorochromes are used.

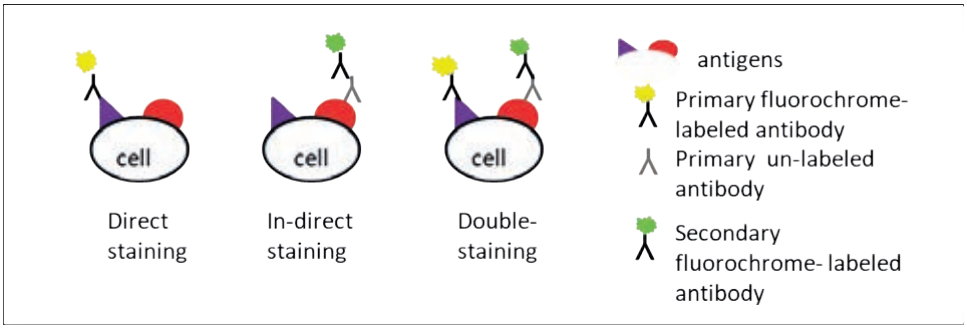

Figure 2. Principles of direct, in-direct and double immunofluorescence staining

4.4.3.2 Immunofluorescence staining of PBMC and CSF cells

The percentages of CD3+, CD4+ and CD8+ T-cells, CD19+ or CD20+ B-cells and CD16+ NK-cells and the percentages of cells expressing adhesion molecules VLA-4, PSGL-1 and CD18, and chemokine receptors CCR7 and CXCR3 were determined using single colour direct or indirect IF-staining with fresh PBMC or

CSF cells. The percentages of $CD56^{bright}$ and $CD56^{dim}$ NK-cells were determined using double-colour IF-staining with antibodies against CD56 and CD3 with viably frozen PBMC. Details of the antibodies used are presented in Table 5.

Table 5. Monoclonal primary antibodies, secondary antibodies and negative isotype control antibodies used in immunofluorescence staining of peripheral blood mononuclear cells and cerebrospinal fluid cells

Antibody	Clone	Detected cell type	IF-type	Study
Primary Antibodies				
anti-CD3-PE[1]	HIT3a	T cells	direct	I
anti-CD4-FITC[1]	RPA-T4	helper T cells	direct	I,II, III
anti-CD19-PE[1]	HIB19	B cells	direct	I, II, III
anti-CD16/Leu-11c-PE[1]	B73.1	NK cells	direct CD16-CD56 double	I, II, III
anti-CD56-PE[1]	NCAM16.2	NK cells	CD3-CD56 double	III
anti-CD56[1]	B156	NK cells	CD16-CD56 double	III
anti-CD3[2]	CRL8001	T cells	indirect CD3-CD56 double	II, III
anti-CD8[2]	CRL8014	killer T cells	indirect	II, III
anti-CD20[2]	IF5.3	B cells	indirect	II
anti-CD18[2]	HB203	β-chain of β2-integrines	indirect	I
anti-CD49D[3]	HP2/1	VLA-4+ cells	indirect	I
anti-CD162[4]	PL1	PSGL-1+ cells	indirect	I
anti-CCR7[1]	3D12	CCR7+ cells	indirect	I
anti-CD183[1]	1C6/CXCR3	CXCR3+ cells	indirect	I
Secondary Antibodies				
anti-mouse-IgG-FITC[5]			indirect	I, II, III
anti-rat-FITC			indirect (CCR7)	I
Negative controls				
anti-chicken T cell[6]	3G6	negative control	indirect	II, III
anti-mouse-CD4-PE[7]	CT-CD4	negative control	direct	II, III

[1]BD Biosciences, San Diego, CA, USA; [2] Monoclonal antibodies produced by hybridoma cell lines, American Type Culture Collection, ATCC, Manassas, VA, USA; [3]Immunotech, Marseille, France; [4]Ancell, Bayport, MN, USA; [5]Sigma-Aldrich, Saint Louis, Missouri, USA; [6]Salmi, 1992, used as hybridoma supernatant; [7]CALTAG Laboratories, Burlingame, CA, USA

IF=Immunofluorescence staining; CD=Cluster of differentiation; PE=Phycoerytherin; FITC=Fluorescein isothiocyanate; NK=Natural killer; VLA=Very late activation antigen; PSGL=P-selectin glycoprotein ligand; CCR7, CXCR3=chemokine receptors

When direct IF-staining was used, $4x10^5$ cells were washed twice and incubated with saturating concentration of labelled antibodies. After incubation cells were washed twice and fixed in phosphate buffered saline (PBS) containing 1% formaldehyde. When in-direct IF-staining was used, $4x10^5$ cells were washed twice and incubated with saturating concentrations of primary antibodies. After two washes cells were further incubated with secondary antibody at 1:100 dilution containing 5% Ab-serum, washed twice and fixed in PBS containing 1% formaldehyde. In double colour staining cells were first incubated with primary and secondary antibodies using in-direct IF-staining protocol as described above and then further incubated with labelled antibody. Cells were washed twice and fixed in PBS containing 1% formaldehyde. All washes were done using PBS containing 2% FCS and 0.01% NaN_3 and all incubations were for 15 min on ice in dark.

4.4.4 Flow cytometer analysis

With flow cytometry the physical properties, including size and granularity, and fluorescent properties of cells in a single-cell suspension can be analysed simultaneously as laser light hits cells flowing through the instrument at high speed (Pockley et al., 2015). Different cell populations can be recognised by detecting the forward and side scattered light. The forward scatter (FSC) is relative to the size of the cells and the side scatter (SSC) is relative to the granularity or complexity of the cells.

IF- and intracellularly-stained cells were analysed using a two-colour FACScan flow cytometer (Becton Dickinson). 30 000 events were collected from each tube. Lymphocytes were gated according to side- and forward-scatter properties and analysed using the CellQuest software to determine the percentages of positive cells in each studied lymphocyte subclass.

4.4.5 Detection of IFN-γ and IL-4 producing cells

The percentages of *in vitro* stimulated PBMC producing Th1- and Th2-type cytokines IFN-γ and IL-4 during and after pregnancy (at 35-37 gw and 4-5 weeks postpartum, respectively) were analysed in ten MS-patients and ten healthy controls by intracellular staining using viably frozen cells. To induce maximal cytokine production $0.5\text{-}1x10^6$ melted cells at a density of $2x10^6$ cells/ml were stimulated with 5 ng/ml phorbol myristate acetate (PMA, Sigma-Aldrich, St Louis, MO, USA) and 500ng/ml ionomycin (Calbiochem, San Diego, CA, USA) at 37°C.

Unstimulated cells were used as control population. After 2 hours 10 μg/ml brefeldin A, a protein transport inhibitor used to retain the cytokines within the cell, was added and stimulation was continued for an additional 3 hours. Stimulated cells were washed twice with PBS containing 0.5% bovine serum albumin and 0.01% atside and either fixed directly with 4% paraformaldehyde for 15 min on ice or in case of IFN-γ and CD3 double staining, surface stained with phycoerytherin (PE) conjugated anti-CD3-antibody (BD Biosciences, San Diego, CA, USA) for 15 min on ice before fixing. Fixed cells were washed with PBS containing 0.5% bovine serum albumin and 0.01% atside and permeabilized with 0.5% saponin in PBS containing 0.5% bovine serum albumin and 0.01% atside. After this, intracellular cytokine staining with fluorescein isothiocyanate (FITC) conjugated anti- IFN-γ- and PE-conjugated anti-IL-4 antibodies (Galtag Laboratories, Burlingame, CA, USA) for 20 min on ice was performed. After three washes with PBS containing 0.5% bovine serum albumin and 0.01% atside cells were analyzed using a FACScan flow cytometer (Becton Dickinson) as described in section 4.4.4.

4.4.6 Detection of CD4+CD25^{High}FoxP3+ regulatory T-cells

The proportion of peripheral blood CD4+CD25^{High}FoxP3+ regulatory T-cells during and after pregnancy (at 35-37 gw and 4-5 weeks postpartum, respectively) was detected in eight MS-patients using anti-human FoxP3 staining kit according to the manufacturer's instructions (eBioScience, San Diego, CA, USA). Viably frozen cells were first stained with anti-CD4-PerCP (Peridinin chlorophyll protein) and anti-CD25-FITC antibodies (clones SK3 and M-A251, respectively, BD Biosciences). Surface stained cell were then fixed, permeabilized and stained intracellularly with anti-FoxP3-PE antibody (clone PCH101) and isotype control antibody (both eBioscience). Cells were analysed using flow cytometer. CD4+ lymphocytes were gated according to SSC and FSC properties and CD4 staining and analysed for CD25^{high} and FoxP3 expression.

4.4.7 Measurement of serum antibodies

Serum of MS-patients was separated by centrifugation for 10 min with 2200 RPM and stored at -40°C in small aliquots for later use.

4.4.7.1 Anti-JCV-antibodies

JCV-Ab-index was determined by a two-step second–generation ELISA (STRATIFY JCV™ DxSelect™) (Lee et al., 2013) at Unilabs (Copenhagen, Denmark). In the assay diluted serum samples are incubated in microwells that are precoatet with JC virus-like particles (VLP). JCV-Ab-index is calculated from optical density (OD) values by dividing the sample OD by cutoff calibrator OD. Cutoff calibrator is prepared from pooled sera of anti-JCV-Ab positive healthy humans. Assay results are expressed as index values and as JCV-Ab positivity (index > 0.40) or negativity (index < 0.20). Index values between 0.20 and 0.40 denotes indeterminate response and the test result is acquired in a confirmation test, in which diluted sample is pre-inhibited with JC VLP. The sample is positive if the inhibition percent is >45%. (Lee et al., 2013; Plavina et al., 2014).

4.4.7.2 Total IgG

Total IgG levels of serum samples were measured by immunoturbidimetric method, which is based on an agglutination reaction induced by the antigen-antibody binding (Koivunen and Krogsrud, 2006, Labmedicine). Total IgG levels of MS patients were analyzed at Unilabs (Copenhagen, Denmark) using polyethylene glycol enhanced immunoturbidimetric method on an automated clinical biochemistry analyzer (Siemens ADVIA chemistry systems). Total IgG levels of healthy controls were determined by the immunoturbidimetric Tina quant assays using Roche Modular(P) chemistry analyzers (Roche Diagnostics) in Helsinki, Finland.

4.4.7.3 Anti-EBV, anti-CMV and anti-measles antibodies

Anti-EBV-nuclear antigen-1-Ab (anti-EBNA-1-Ab) and anti-Viral capsid antibody-Ab (anti-VCA-Ab) levels were measured with the automated Liaison® quantitative chemiluminescent assay as described previously (Farrell et al., 2009). Anti-measles-Ab and anti-CMV-Ab levels were measured with commercial ELISA kits from Virion Serion (Launch diagnostics) as described (Farrell et al., 2009). Results are expressed as ODs.

4.5 Statistical analyses

Statistical analysis of the data was performed by SAS System for Windows versions 9.1 (studies II, III and IV) and 9.4 (study V, SAS Institute Inc., Cary, NC) and GraphPad Prism. Analysis of variance (ANOVA) for repeated measurements with suitable post-test was used to evaluate variations in longitudinally measured variables. Two-sided p values < 0.05 were considered statistically significant for all analyses.

In study II repeated measures ANOVA was used to evaluate variations in longitudinally measured proportions of CD3+, CD19+ or CD20+ and CD16+ cells during and after pregnancy in MS-patients and healthy controls. Paired 2-sided t-test was used to compare the ratio of CD4+ and CD8+ cells at early vs. late pregnancy and relapse rate vs. each three-month period during and after pregnancy. Unpaired 2-sided t-test was used to compare proportions of CD3+, CD19+ or CD20+ and CD16+ cells, and ratio of CD4+ and CD8+ cells between samples of non-pregnant subjects and samples of pregnant subjects taken during the third trimester of pregnancy. Unpaired 2-sided t-test was also used to compare differences in the proportion of CD16+ cells between patient groups (DMT initiated after pregnancy vs. DMT not initiated after pregnancy and relapse during pregnancy vs. no relapse during or after pregnancy).

In study III repeated measures ANOVA with Tukey-Kramer multiple comparison post-test was used to evaluate variations in annualized relapse rate before, during and after pregnancy, and in longitudinally measured proportions of CD16+, CD3+, CD4+, CD8+ and CD19+ cells during and after pregnancy. Tukey Kramer test evaluates the significance of the difference between each pair of means that are based on different sample sizes (Salkind et al., 2007). Repeated measures ANOVA with Bonferroni post-test for multiple comparisons was used to evaluate variations in longitudinally measured proportions of CD56+CD3- cells expect paired Student's t-test was used to compare the proportions of $CD56^{bright}$ cells of all PBL between late pregnancy and early postpartum period. ANOVA with Tukey's post-test was used to compare differences in the proportions of CD16+ cells between non-pregnant subjects and samples taken during and after pregnancy.

In study IV repeated measures ANOVA with Newman-Keuls multiple comparison post-test (Salkind et al., 2007). was used to evaluate variations in relapse rate and IgG-index before, during and after pregnancy. Paired, two-sided t-test was used to compare EDSS values during late pregnancy and at 6 months postpartum.

In study V repeated measures ANOVA with heterogeneous compound symmetry covariance structure followed by Tukey-Kramer test for multiple comparisons was

used to evaluate variations in longitudinally measured JCV-Ab-indices, IgG levels and anti-EBNA-1-Ab, anti-VCA-Ab, anti-measles-Ab and anti-CMV-Ab levels during and after pregnancy, and to compare differences between sample sets from MS-patients and healthy controls. If data was not normally distributed, log-transformations were made. Mann-Whitney U-test was used to compare the change of JCV-Ab-indices and IgG levels from 1st to 3rd trimester in healthy controls vs. MS-patients. Pearson's chi-squared test was used to compare the proportions of JCV-Ab positive MS- patients and healthy controls during pregnancy. To assess the proportional changes of JCV-Ab indices and IgG levels linear mixed models followed by Bonferroni test for multiple comparisons were used after log transformation of the response variables. Time, type (IgG level and JCV-Ab-index) and their interaction were included in the models. Estimates of differences were back transformed and represent rate ratios (RR). Data is presented as mean (SD) and range of values. For non-normally distributed variables medians and 25th and 75th percentiles are used.

5 RESULTS

5.1 Disease activity during and after pregnancy

5.1.1 Case report (I)

A patient described in study I experienced first relapse at age of 23 years with motor symptoms and balance disturbance. Multiple periventricular demyelinating lesions were seen in brain MRI, and CSF findings were typical for MS-disease. Three years later shortly before pregnancy the patient experienced her second relapse with sensory symptoms. She was completely symptom-free during pregnancy, but her disease activation after delivery was unusually severe, as in a brain MRI taken 8 weeks after delivery, the patient had 200 active, Gd-enhancing lesions (Figure 3). A course of methylprednisolone diminished the number of Gd-enhancing lesions seen in MRI. No alterations were observed in the proportions of peripheral blood and CSF T-cells, B-cells or NK-cells, or in the proportions of cells expressing adhesion molecules VLA-4, PSGL-1 and CD18 before and two weeks after the treatment. However, the proportions of CXCR3+ and CCR7+ peripheral blood lymphocytes were increased by 135% and 56%, respectively (Figure 3).

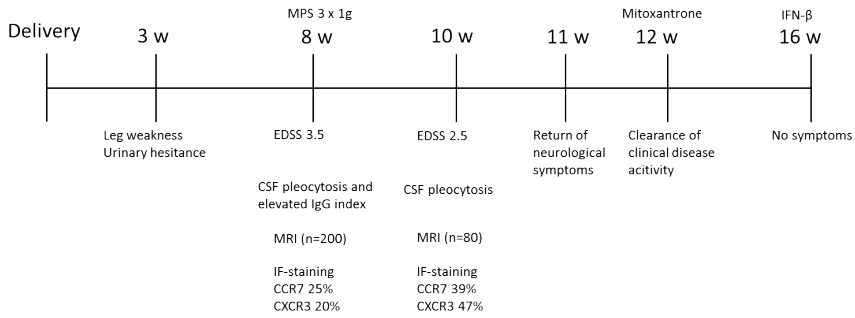

Figure 3. Description of the MS-disease activation after pregnancy of a patient. Shown are medications (above the timeline), symptoms, CSF findings, numbers of gadolinium-enhanced lesions seen in MRI and proportions of peripheral blood lymphocytes expressing chemokine receptors CCR7 and CXCR3. MPS=methylprednisolone; IFN-β=Interferon-β; EDSS= Expanded Disability

Status Scale; CSF = cerebrospinal fluid; IF-staining = immunofluorescence staining

5.1.2 EDSS during and after pregnancy

Pregnancy did not affect the clinical disability of MS-patients, as mean (SD) EDSS was not changed during the follow-up [1. trim vs. 6 mo PP, 1.5 (1.3) vs. 1.4 (0.9)].

5.1.3 Relapse rate during and after pregnancy

Details about the numbers of patients experiencing relapses during and after pregnancy are presented in Figure 4.

The mean ARR of MS-patients decreased significantly during pregnancy compared to the previous year (Figure 5). Relapse rate was decreased already during the first trimester when compared to the year before pregnancy although this decrease did not reach statistical significance [0.5(1.3) vs. 1.0(1.0), respectively]. The decrease was statistically significant during the third trimester [0.2(0.9) vs. 1.0(1.0), respectively, p=0.02]. Soon after delivery during 1-3 months postpartum the relapse rate was significantly increased compared to the third trimester of pregnancy (1.4(1.9) vs. 0.2(0.9), respectively, p=0.003]. During 3-6 months postpartum the relapse rate returned to the prepregnancy level.

In study II nine patients started immunomodulatory treatment (IFN-β n=8, GA n=1) during the first three months after delivery. At 1-3 months postpartum the annualized relapse rate was similar in treated and untreated patients. During 3-6 months postpartum the mean relapse rate of the treated patients was 64 % smaller compared to the non-treated [0.4(1.3) vs. 1.1(1.8)], although before and during pregnancy the relapse rate of patients initiating immunomodulatory treatment was higher (Figure 5), indicating that the treated patients had more active disease overall.

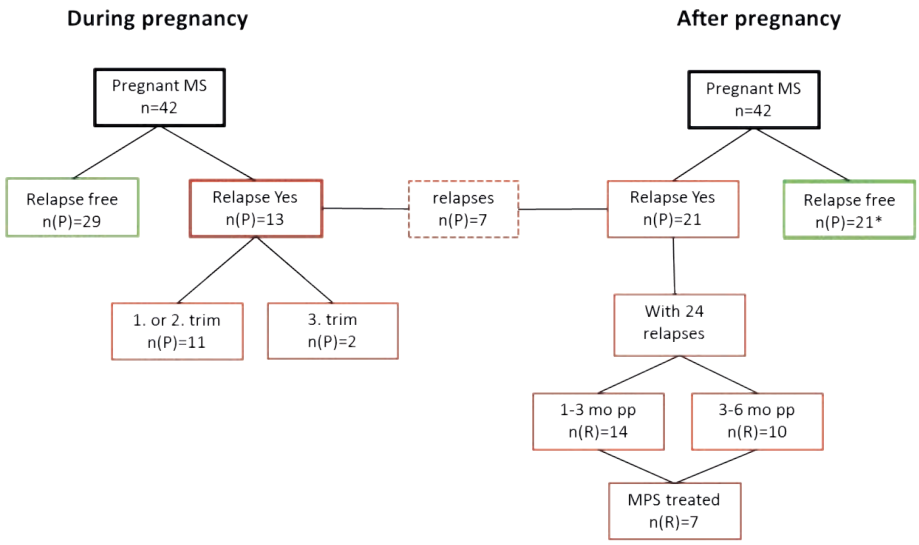

Figure 4. Numbers of MS-patients experiencing relapses during and after pregnancy. n(P) = number of patients; n(R) = number of relapses; MPS = methylprednisolone; * of which 4 patients were treated with disease modifying treatment

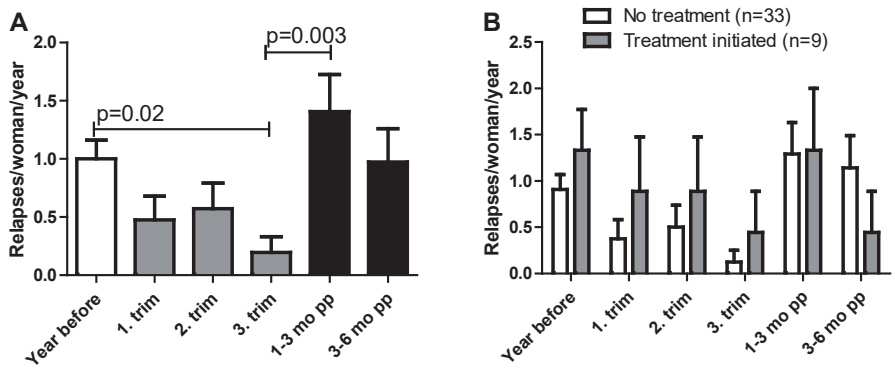

Figure 5. Annualized relapse rates of MS-patients during the year before pregnancy, during pregnancy and during the first six months after pregnancy. Mean with standard error of mean is shown. (A) The mean relapse rate of MS-patients (n=42) decreased during pregnancy and increased after delivery. (B) The mean relapse rates of MS-patients, who did not receive immunomodulatory treatment after pregnancy (n=33, white bars) and of MS-patients, who started immunomodulatory treatment 1-3 months postpartum (n=9, grey bars). trim = trimester; mo pp = months postpartum

5.2 The effect of pregnancy on peripheral blood immune cells

The proportions of CD19+, CD3+, CD4+, CD8+ and CD16+ cells were measured in 42 pregnant MS-patients and 20 pregnant healthy controls. Numbers of available samples at different timepoints varied from 21 to 35 in case of MS-patients and from 9 to 18 in case of healthy controls.

5.2.1 B-cells during and after pregnancy in patients with multiple sclerosis and healthy controls

The proportion of B-cells did not alter during or after pregnancy in MS-patients or in healthy controls (Figure 6). The mean (SEM) proportions measured during third trimester were similar to non-pregnant controls both in MS-patients and healthy controls [(6.1(0.8) vs. 8.2(0.9) and 5.2(0.8) vs. 7.5(0.6), respectively)].

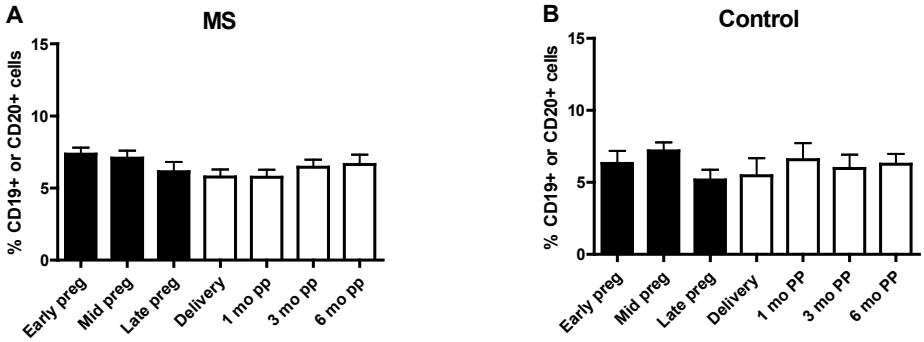

Figure 6. The mean (SEM) proportions of CD19+ or CD20+ peripheral blood B-cells of all peripheral blood lymphocytes in MS-patients and healthy controls during and after pregnancy. (A) The proportion of B-cells of MS-patients did not change during the follow-up. (B) The proportion of B-cells of healthy controls did not change during the follow-up. Early preg = 10-12 gestational weeks; Mid preg = 26-28 gestational weeks; Late preg = 35-37 gestational weeks; mo pp = months postpartum

5.2.2 T-cells during and after pregnancy in patients with multiple sclerosis and healthy controls

The proportions of CD3+ T-cells did not alter during or after pregnancy in MS-patients or in healthy controls (Figure 7). The mean (SEM) proportions measured

during third trimester were similar to non-pregnant controls both in MS-patients and healthy controls [66.8 (2.6) vs. 73.8(1.9) and 71.3(2.4) vs. 76.6 (1.8), respectively]. Although there were no statistically significant changes in the mean proportion of CD4+ or CD8+ T-cells, a slight increase in the proportion of CD4+ T-cells and a slight decrease in the proportion of CD8+ T-cells were observed leading to an increased CD4/CD8 ratio from first to third trimester(1.9(1.0) vs. 2.7(1.0)], respectively, p=0.004, Figure 7). However, there was no difference in the CD4/CD8 ratio during the third trimester compared to untreated non-pregnant MS-patients (2.4 vs. 2.7, respectively). In contrast to MS-patients, in healthy controls the CD4+/CD8+ ratio was decreased during pregnancy from the first to the third trimester [(2.4 (0.7) vs. 1.6(0.7), respectively], but the decrease was not statistically significant. The CD4+/CD8+ ratio of non-pregnant healthy controls was similar to the value of the third trimester (1.8 vs. 1.6, respectively).

5.2.2.1 Regulatory T-cells

The proportion of CD4+CD25^{high}FoxP3 natural regulatory T-cells was analysed in paired pregnancy and postpartum samples of eight MS-patients. There were no alterations between the third trimester sample and 4-5 weeks postpartum sample.

5.2.3 Natural killer –cells

5.2.3.1 CD16+ NK-cells during and after pregnancy in patients with multiple sclerosis and healthy controls

The proportion of peripheral blood CD16+ NK-cells was gradually decreased during pregnancy in MS-patients (Figure 8). The mean (SEM) proportion of CD16+ NK-cells decreased 40% from first to third trimester [9.7% (1.6) vs. 6.0 (0.95), p=0.02)]. The proportion was significantly decreased at third trimester also compared to non-pregnant MS-patients [5.6% (0.9) vs. 12.4% (1.6), p<0.001]. Although in pregnant healthy controls the decrease in the proportion of CD16+ NK-cells observed during pregnancy was not statistically significant, there was a significant decrease at third trimester compared to non-pregnant healthy controls [6.3% (0.7) vs. 11.4% (1.0), p<0.001, Figure 8].

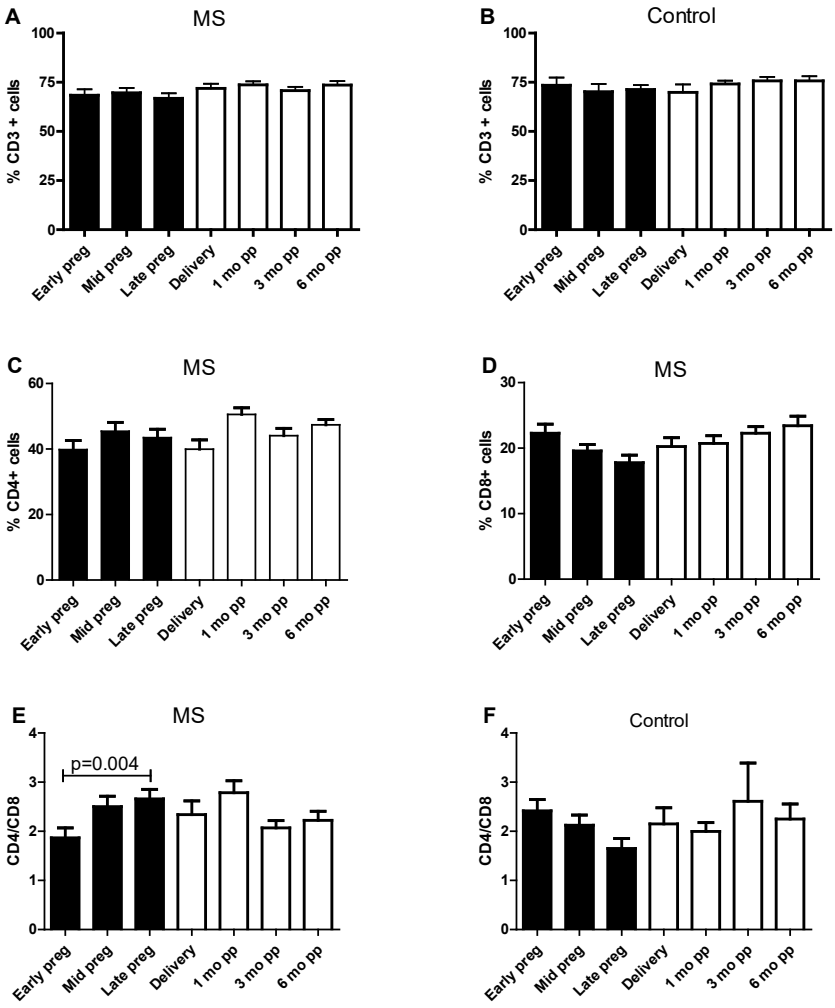

Figure 7. The mean (SEM) proportions of peripheral blood T-cells of all peripheral blood lymphocytes (A-D) and the ratio of CD4+ cells to CD8+ cells (E-F) in MS-patients and healthy controls during and after pregnancy. (A) The proportion of CD3+ T-cells of MS-patients did not change during the follow-up. (B) The proportion of CD3+ T-cells of healthy controls did not change during the follow-up. (C) The proportion of CD4+ T-cells of MS-patients did not change during the follow-up. (D) The proportion of CD8+ T-cells of MS-patients did not change during the follow-up. (E) The CD4+ to CD8+ ratio of MS-patients was significantly increased during pregnancy. (F) The CD4+ to CD8+ ratio was not changed during the follow-up. Early preg, 10-12 gestational weeks; Mid preg, 26-28 gestational weeks; Late preg 35-37 gestational weeks; mo pp, months postpartum

Compared to third trimester of pregnancy, the mean (SEM) proportion of NK-cells was increased after delivery in both MS-patients and healthy controls. In MS-patients the increase was statistically significant at 3 and 6 months postpartum [6.0 (0.95) vs. 11.7(1.3) and 10.3 (1.4), p=0.0002 and p=0.004, respectively]. In healthy controls the increase was significant at 1 and 3 mo pp (p=0.005 and p=0.03, respectively).

Nine out of 42 MS-patients in study II initiated immunomodulatory treatment (IFN-β n=8, GA n=1) 1-3 months postpartum. In contrast to the non-treated patient group, in which the mean (SEM) proportions of CD16+ cells was higher at 3 and 6 months postpartum than at 1 month postpartum [13.6% (1.2) and 11.6% (1.3) vs. 9.3 (1.2), respectively, original publication II, Figure 4A], the proportion of CD16+ cells of the treated patient group was lower at 3 and 6 months postpartum than at 1 month postpartum [6.3% (2.1) and 5.4% (0.3) vs. 8.7% (2.3), respectively, original publication II, Figure 4B]. At 3 months postpartum the proportion of CD16+ cells was significantly lower in the treated group than in the non-treated group [6.3% (2.1) vs. 11.6% (1.3), respectively, p=0.005]. However, the mean proportion of CD16+ cells of the treated group was lower also during pregnancy.

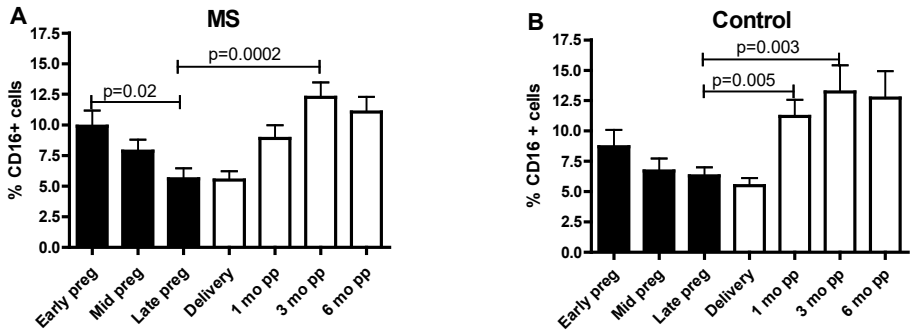

Figure 8. The mean (SEM) proportions of CD16+ peripheral blood natural killer (NK)-cells of all peripheral blood lymphocytes in MS-patients and healthy controls during and after pregnancy. (A) In MS-patients the proportion of NK-cells was decreased during pregnancy and increased after delivery. (B) In healthy controls the proportion of NK-cells did not change during pregnancy but was increased after delivery. Early preg, 10-12 gestational weeks; Mid preg, 26-28 gestational weeks; Late preg 35-37 gestational weeks; mo pp, months postpartum

5.2.3.2 CD56+ NK-cells during and after pregnancy in patients with multiple sclerosis

As our results of CD16+ NK-cells implied that NK-cells could be involved in pregnancy-related alterations of MS disease activity, the effect of pregnancy on $CD56^{bright}$ and $CD56^{dim}$ NK-cell subpopulations was analysed using viably frozen peripheral blood mononuclear cells of 12 MS-patient representative of the study cohort. The mean (SEM) proportion of CD3-$CD56^{dim}$ NK-cells was significantly changed during the follow-up (p=0.02 rmANOVA, original publication III, Figure 3). There was a statistically significant decrease from early pregnancy to sample taken 2-4 days after delivery [9.8% (1.6) vs. 6.6 (1.2), respectively, $p < 0.05$, original publication III, Figure 3b]. The proportion of $CD56^{bright}$ NK-cells of all peripheral blood lymphocytes and of all CD3-CD56+ NK-cells was higher at late pregnancy than at early postpartum [0.89% (0.14) vs. 0.70% (0.09), p=0.03, and 11.45(1.36) vs. 9.11(0.88), $p < 0.05$, original publication III, Figure 3].

5.2.4 Cells producing IFN-γ and IL-4

The effect of pregnancy on peripheral blood lymphocytes producing Th1- and Th2-type cytokines was analysed in 10 MS-patients and 10 healthy controls. In MS-patients the mean (SEM) proportion of cells producing Th1-type cytokine IFN-γ was significantly lower during third trimester of pregnancy than 4-5 weeks postpartum ($p<0.01$). Double IF-staining with anti-CD3 and anti-IFN-γ antibodies performed after PMA and ionomycin treatment showed that all cells producing IFN-γ were CD3+ T-cells (original publication III, Figure 1b). The mean (SEM) proportion of cells producing Th2-type cytokine IL-4 was not changed statistically significantly. Thus, the IL-4/IFN-γ ratio was higher during than after pregnancy (0.36 vs. 0.23, respectively).

In healthy controls the proportion of IFN-γ producing cells was not changed, but the proportion of IL-4 producing cells was significantly higher during third trimester than postpartum ($p<0.05$). Thus, also in healthy controls the IL-4/IFN-γ ratio was higher during than after pregnancy (0.32 vs. 0.13, respectively).

5.3 CSF-parameters

The IgG-index of CSF was higher during than before or after pregnancy (2.1 vs. 1.5 and 1.6, respectively, p=0.04). The number of oligoclonal bands remained stable in the samples taken during and after pregnancy, the numbers before

pregnancy were not available. The number of CSF lymphocytes was in most patients lower during than after pregnancy even if the patient did not experience relapse after pregnancy. In samples taken during relapses the number of lymphocytes was higher. During pregnancy there were 0-2 x 10^6 lymphocytes/l.

5.4 JCV-results

In study V, JCV-Ab-index and total IgG levels were measured in all available serum samples from MS-patients (first trimester n=33; second trimester n=40; third trimester n=17; 4 to 5 weeks postpartum (w pp) n=26; 10-12 w pp n=28 and 6 months postpartum n=30). From healthy controls samples taken only throughout pregnancy (first, second and third trimester) were available. From these samples JCV-Ab-indices (n=44; n=48; n=44) and IgG levels (n=57 at every time point) were measured. MS-patients and healthy controls were divided to JCV-Ab-positive (n=17 and n=33, respectively) and JCV-Ab-negative subgroups (n=32 and n=16, respectively) based on JCV-Ab-positivity or negativity in the first available measurement timepoint during pregnancy.

5.4.1 JCV-seroprevalence

At first trimester the rate of seropositivity in the total cohort of MS-patients was 27%, at second trimester 35% and at third trimester 41%. The rates of seropositivity in the total cohort of healthy controls during pregnancy were 68%, 65% and 64%, respectively. Thus, the seroprevalence was lower in MS-patients than in controls at first and second trimesters but not at third trimester (p=0.0009, p=0.011 and p=0.19, respectively). In MS-patients the rates of seropositivity at 1, 3 and 6 months after pregnancy were 46%, 48% and 43%, respectively. Interestingly, 22% (7/32) of initially (first available sample taken at first or second trimester) seronegative MS-patients converted to seropositive during follow up (1.125 years) with a mean index increase of 0.99 (range 0.05-3.1). One patient seroconverted during pregnancy and six after pregnancy. Thus 19% of seronegative patients seroconverted per year and 6.3% (5.6% per year) of seronegative patients had high index values (2.77 and 3.38) after seroconversion.

5.4.2 JCV-Ab-indices in patients with multiple sclerosis and healthy controls

In MS-patients the mean JCV-Ab-indices did not change during pregnancy (overall p=0.8, rmANOVA, first vs. third p=0.99, Table 6). The mean JCV-Ab-

indices increased after delivery and were higher at a one, three and six months postpartum compared to first and second trimesters (Table 6). Similar patterns were observed in the JCV-Ab-positive and JCV-Ab-negative subgroups (n=17 and n=32, respectively, Table 6).

In healthy controls the mean JCV-Ab-indices decreased significantly during pregnancy (first vs. third trimester p=0.0007, Table 6). The mean JCV-Ab-indices were similarly decreased from first to third trimester in the JCV-Ab-positive subgroup (p=0.004, Table 6) but remained stable in the JCV-Ab-negative subgroup (first vs. third trimester, p=0.97, Table 6). Comparison of the change from first to third trimester of JCV-Ab-indices in MS-patients vs. healthy controls revealed a significantly different change between the two cohorts [(median change (25^{th} and 75^{th} percentiles), 0.0 (-0.08, 0.09) and -0.13 (-0.42, 0.0), respectively, p=0.03)].

In this cohort the JCV-Ab-indices of all MS-patients were statistically significantly lower compared to the matched healthy controls during each trimester (p=0.003 at first trimester, p=0.006 at second trimester and p=0.01 at third trimester, Table 6).

5.4.3 Total IgG levels in patients with multiple sclerosis and healthy controls

Total serum IgG levels were gradually decreased during pregnancy both in MS-patients and healthy controls (first vs. third trimester, p<0.0001 and p<0.0001, respectively, Table 6). The decrease was similar in MS-patients and healthy controls (p=0.12). In MS-patients the IgG levels were significantly increased after pregnancy compared to each trimester of pregnancy and reached the maximal level at six months postpartum (Table 6). When the overall pregnancy-related alterations in IgG levels and JCV-Ab-indices were compared to each other, it was noted that the IgG changes were different from the JCV-Ab-index changes among MS-patients (proportional change; RR=0.77, CI 0.67-0.89, p=0.002) but not among healthy controls (RR=0.91, CI 0.83-0.99, p=0.07).

5.4.4 Antibody levels for EBV, CMV and measles viruses in patients with multiple sclerosis

Levels of anti-EBNA-1-Ab, anti-VCA-Ab and anti-measles-Ab decreased significantly during pregnancy and increased again after delivery (first vs. third trimester p=0.004, p=0.02 and p=0.03, respectively, Table 6). The anti-CMV-Ab level did not change during or after pregnancy (first vs. third trimester, p=1.0, Table 6).

Table 6. Serum total IgG levels and levels of antibodies against JCV, EBV, Measles and CMV viruses in MS-patients and healthy controls during and after pregnancy

MS-patients								
	1.trim	2.trim	3.trim	Change[d]	1mo pp	3mo pp	6mo pp	Change[e]
IgG	10.15 (1.94)	8.62 (1.77)	7.72 (2.81)	↓	9.36 (2.82)	11.06 (2.10)	11.94 (1.93)	↑
JCV (All)[a]	0.62 (0.90)	0.80 (1.1)	0.77 (0.99)	↔	1.12 (1.12)	1.20 (1.30)	0.98 (1.29)	↑
JCV (+)[b]	1.72 (1.11)	1.92 (1.16)	1.84 (1.00)	↔	2.01 (1.01)	2.49 (1.04)	2.27 (1.16)	↑
JCV (-)[c]	0.19 (0.10)	0.20 (0.11)	0.19 (0.11)	↔	0.47 (0.82)	0.48 (0.75)	0.51 (0.79)	↑
EBNA1	1.41 (0.53)	1.30 (0.53)	1.21 (0.54)	↓	1.31 (0.51)	1.36 (0.55)	1.51 (0.56)	↑
VCA	0.94 (0.48)	0.89 (0.41)	0.84 (0.45)	↓	0.90 (0.45)	0.90 (0.47)	1.01 (0.47)	↑
Measles	1.20 (0.53)	1.19 (0.53)	1.13 (0.52)	↓	1.36 (0.51)	1.2 7(0.53)	1.26 (0.49)	↑
CMV	0.63 (0.50)	0.59 (0.48)	0.5 7(0.49)	↔	0.57 (0.48)	0.61 (0.50)	0.70 (0.50)	
Healthy controls								
	1.trim	2. trim	3. trim	Change[d]				
IgG	10.96 (1.73)	9.79 (1.89)	8.76 (2.01)	↓				
JCV (All)[a]	1.78 (1.36)	1.66 (1.39)	1.66 (1.35)	↓				
JCV (+)[b]	2.51 (1.01)	2.38 (1.13)	2.28 (1.14)	↓				
JCV (-)[c]	0.22 (0.07)	0.21 (0.06)	0.20 (0.06)	↔				

IgG = total immunoglobulin G level as g/l; JCV = level of antibodies against John Cunningham Virus as JCV-Ab-index; EBV = Epstein-Barr virus; EBNA = level of antibodies against EBV nuclear antigen 1 measured as optical density; VCA = level of antibodies against EBV viral capsid antigen measured as optical density; Measles = level of antibodies against Measles virus measured as optical density; CMV = level of antibodies against Cytomegalovirus measured as optical density; MS = multiple sclerosis; trim = trimester; mo pp = months postpartum

[a] Entire study population

[b] MS-patients or healthy controls who were JCV seropositive in first available sample obtained during pregnancy

[c] MS-patients or healthy controls who were JCV seronegative in first available sample obtained during pregnancy

[d] Pregnancy vs. postpartum

[e] 1. trim vs. 3 trim

↓ Significant decrease

↑ Significant increase

↔ No significant alterations

6 DISCUSSION

This thesis was part of the prospective and longitudinal national Finnish Multiple Sclerosis and Pregnancy study. Amelioration of MS disease activity during pregnancy and its reactivation soon after delivery provides unique opportunity to study immunological mechanisms contributing to alterations of MS disease activity. A better understanding of these mechanisms could help predicting clinical course of MS in individual patients and bring new information of the pathogenesis of MS. Alterations in immunological mechanism during pregnancy has been investigated in several studies with the number of participating pregnant MS-patients ranging from 4 to 36. However, only one of these studies had more than one sample taken postpartum (Langer-Gould et al., 2010). Our largest cohort including 59 MS-patients followed longitudinally with frequent blood samples taken during pregnancy in each trimester, few days after delivery and one, three and six months postpartum is thus quite exceptional. The constitution of a cohort with as encompassing longitudinal samples also postpartum is challenging and requires large commitment from both study participants and personnel. This could be one reason why there are no other similar studies. CSF immune parameters may reflect closely the immune responses in the brain (Stangel et al., 2013). Thus, analysis of these parameters may characterize the immunological pathways involved in the pathogenesis of MS and in pregnancy-related disease activity changes. To our knowledge our study was the first to analyze CSF immune parameters of pregnant MS patients.

There are few methodological limitations in our study that need to be addressed. We did not study the statistical correlations of changes in cellular parameters to clinical disease activity or relapse rate. However, we did collect information of clinical disease activity with number of relapses occurring also before pregnancy and in our cohort the decrease of relapse rate during pregnancy and increase postpartum was very similar to a previous larger study (Confavreux et al., 1998). The disease activity alterations in our study population during and after pregnancy were thus typical to MS and describe well the natural course of MS in relation to pregnancy. Therefore, our study population is well suitable for the immunological characterization to understand the mechanisms leading to MS amelioration during pregnancy and activation after delivery. In addition, the number of samples taken at different time-points varied because of enrollment at later stages of pregnancy, no-shows, and drop-outs. We did not collect pre-pregnancy samples. Most IF-stainings and flow cytometry-analyses were done with freshly isolated PBMC so day to day variations in these procedures could have affected the results during long study period.

6.1 Disease activity of multiple sclerosis during and after pregnancy

In our study the annualized MS relapse rate was significantly decreased during the third trimester of pregnancy compared to year before pregnancy and increased postpartum. These results are in line with a previous larger longitudinal study and with a more recent register study (Confavreux et al., 1998; Hughes et al., 2014). In our study the mean EDSS was not significantly altered during pregnancy or six months after pregnancy. This result is in line with a more recent register study (Hughes et al., 2014).

In the study II nine of 42 MS-patients started disease-modifying therapy, either IFN-β or GA, immediately during the first three months after delivery. The decision to start DMT was made by the treating neurologist of each patient. The efficacy of treatment was seen 3-6 months postpartum as decreased relapse rate. Our observation suggests that disease-modifying IFN-β or GA treatment may be effective in reducing the relapse rate after delivery. However, as our study was not designed to study the effect of postpartum treatment to prevent postpartum relapses, the number of treated patients was small, and the statistical significance of our observation was not determined. In a more recent Italian study 74 out of 350 MS-patients started disease-modifying treatment, most of them IFN-β, within three months from delivery (Portaccio et al., 2014). The authors concluded that early introduction or resumption of disease-modifying treatment after delivery may be a suitable strategy to reduce risk of postpartum relapse, although the marginal reduction in the risk did not reach statistical significance (Portaccio et al., 2014). Another study, in which 17 patients started disease-modifying treatment within two weeks of delivery, observed that early treatment does not dramatically reduce the risk of having a first postpartum relapse but may reduce the risk of subsequent relapses in the postpartum year (Beaber et al., 2014). This is understandable as the treatment effect of IFN-β and GA is normally considered to be seen with quite a long delay (Johnson et al., 1995; Rice et al., 2001).

A better understanding of mechanisms and factors that might participate in the regulation of MS disease activity during and after pregnancy might help to identify patients that may be more susceptible to relapses after pregnancy. In our study only half of the patients experienced relapses after delivery, most of them only one. However, in some patients the postpartum reactivation of disease may cause significant problems. In study I we describe a patient who experienced an unusually severe disease activation soon after delivery. Our observation of increased proportions of CXCR3 and CCR7 expressing peripheral blood lymphocytes simultaneously with a significant decrease in the number of Gd-enhancing MRI lesions warrants further study of the role of CXCR3 and CCR7 expressing lymphocytes in MS. It would have been interesting to know whether

the increases were uniform among different lymphocyte populations, but unfortunately the specific lymphocyte populations expressing CXCR3 and CCR7 were not determined. CXCR3 is expressed on activated T-cells (Loetscher et al., 1996), and it is essential for the recruitment of lymphocytes towards sites of inflammation (Uzawa et al., 2010). CXCR3 is expressed on lymphocytes in active MS-lesions (Sørensen et al., 1999), and it is involved in the intrathecal accumulation of T-cells in MS (Kivisäkk et al., 2002). CCR7 on the other hand is expressed on naïve and central memory T-cells that are capable of homing to lymph-nodes and lack inflammatory and cytotoxic function (Sallusto et al., 1999). Decrease of the migration of immune cells into tissue is one of the mechanism of actions of MPS (Sloka and Stefanelli, 2005). Thus, our result of increased proportion of CXCR3+ cells after MPS treatment could reflect decreased migration of activated immune cells into the CNS due to the drug, and these activated cells thus being rendered "trapped in circulation".

6.2 Pregnancy-related immune alterations in MS-patients

6.2.1 T-cells

The studies investigating immunological alterations during pregnancy in MS-patients have mainly focused on peripheral blood regulatory T-cells and cytokine production. To our knowledge there are no other studies about pregnancy-related alterations in the proportions of all T-cells in MS-patients. Thus, our result of unaltered proportions of CD3+, CD4+ and CD8+ T-cells during pregnancy brings new knowledge and forms a good foundation for other studies investigating more specific subpopulations of T-cells.

In study II we observed in MS-patients a significant increase in the CD4/CD8 ratio during third trimester compared to first trimester. The lack of difference in CD4/CD8 ratios at third trimester and non-pregnant controls may be due to inter-individual differences. The significance of our observation is not clear. The CD4/CD8 ratio is increased in non-pregnant untreated MS-patients compared to healthy controls (Pender et al., 2014), but to our knowledge there are no other studies about the CD4/CD8 ratio in pregnant MS-patients. CD4+ T-cell population can contain Th1 and Th17 cells considered harmful in MS and Th2 and regulatory T-cells considered beneficial for MS. As the phenotypes of CD4+ and CD8+ T-cells were not analyzed more closely in our study, the changes leading to increased CD4/CD8 ratio can be only speculated. In other studies both increases and decreases in regulatory T-cells (Iorio et al., 2009; Neuteboom et al., 2010;

Sánchez-Ramón et al., 2005), unaltered proportions of Th17 cells (Neuteboom et al., 2010), and decreases in the proportion of activated CD4+HLA-DR+CD38+ T-cells and CD4+CD45RA- cells producing IFN-γ have been observed in MS-patients during pregnancy (Langer-Gould et al., 2010; Sánchez-Ramón et al., 2005). In theory, the increased CD4/CD8 ratio in MS-patients could be another phenomenon of "activated autoreactive cells trapped in circulation".

It has been hypothesized that the amelioration of MS-disease during pregnancy is associated with the anti-inflammatory Th1 to Th2 shift during pregnancy (Patas et al., 2013). We observed in study III among MS-patients a higher Th2/Th1 ratio during third trimester of pregnancy than postpartum. There are only few other small studies with MS-patients that have studied Th1 and Th2 cytokine secretion patterns during and after pregnancy. Al-Shammri et al. showed shifts from a Th2 cytokine bias during pregnancy towards a Th1 cytokine bias after delivery and Gilmore et al. observed increased secretion of IFN-γ leading to increased IFN-γ/IL-10 ratio postpartum whereas the IFN-γ/IL-4 ratio showed no clear pattern for postpartum elevation (Al-Shammri et al., 2004; Gilmore et al., 2004). An increase in the IL-10/IFN-γ ratio during third trimester has been observed in MS also at the mRNA expression level (López et al., 2006). However, differences between patients were large in these two studies. Iorio et al. observed differences in the expression of IFN-γ and IL-10 in patients with or without disease activation after delivery (Iorio et al., 2009). In patients with disease activation the expression of IFN-γ was increased at third trimester with no changes in the expression of IL-10, and in patients without disease activation the expression of IFN-γ was not changed, but the expression of IL-10 was increased during second and third trimesters (Iorio et al., 2009). A larger study of 26 pregnant women with MS observed a decrease during pregnancy in CD4+ T-cells producing IFN-γ that was strongly associated with postpartum relapses, whereas no significant fluctuations were observed in CD4+ T-cells producing IL-4 or IL-10 or CD8+ T-cells producing IFN-γ (Langer-Gould et al., 2010). Although our study brings additional evidence of increased Th2/Th1 ratio during pregnancy in MS-patients, more and larger studies with analysis of correlations between disease activity and cytokine production are needed to clarify the relevance of alterations in anti- and pro-inflammatory cytokines to pregnancy-related alterations in MS disease activity.

Regulatory T-cells play an important role in the maintenance of peripheral tolerance and loss of this tolerance may lead to autoimmune diseases and loss of fetal-maternal tolerance during pregnancy (Kleinewietfeld and Hafler, 2013). Increased proportions of CD4+CD25+FoxP3+ regulatory cells have been observed during remission in MS-patients (Frisullo et al., 2009) and it has been proposed that expansion of regulatory T-cells during pregnancy may be associated with amelioration of MS (Sánchez-Ramón et al., 2005). However, our results do not

support this hypothesis as we did not observe alterations in the proportion of CD4+CD25^{high}FoxP3+ regulatory T-cells between third trimester and postpartum time. However, the small number of studied samples may have increased the risk of Type II error.

Our results are in line with one previous study (Iorio et al., 2009), but in contrast to other studies that have found increases in the proportion of CD4+CD25+ and decreases in the proportion of CD4+CD25^{high} T-cells and CD25+FoxP3+CD127^{low} during third trimester compared to postpartum (Neuteboom et al., 2010; Sánchez-Ramón et al., 2005). Differences in our results and results of these two studies could be explained at least partly by two things. Firstly, slightly different antibody combinations were used to identify regulatory T-cells. Secondly, third trimester was determined to include either weeks 28-30 of pregnancy or longer period from week 29 to delivery, whereas third trimester samples used in our study were obtained at gestational weeks 35-37. In addition, the number of study participants in all these studies including ours is relatively small. Our results do not exclude the possibility that pregnancy enhances the functional properties of regulatory T-cells, which could promote amelioration of MS during pregnancy. Indeed, the suppressive functions of regulatory T-cells seems to be impaired in MS-patients compared to healthy controls despite of similar frequencies in the peripheral blood (Frisullo et al., 2009; Haas et al., 2005; Venken et al., 2006). Overall, the results about regulatory T-cells during MS pregnancy are inconclusive and the significance of regulatory T-cells in the amelioration of MS activity during pregnancy should be clarified in larger studies including also functional assays.

6.2.2 NK-cells

There are only few other studies that have studied NK-cells during pregnancy in MS-patients. A small previous study did not observe alterations in the NK-cells expressing chemokine receptors (López et al., 2006). A recent study found increased activation of CD3+CD56+CD8+ NKT-cells during pregnancy and postpartum compared to non-pregnant, non-treated women with MS, who had non-active disease at the time of sample collection (de Andrés et al., 2017). Thus, study II was the first study to describe pregnancy-related alterations in the proportion of CD16+ NK-cells in MS-patients with decrease during third trimester of pregnancy compared both to first trimester and non-pregnant, non-treated MS-patients, and increase after delivery. Our observations of lower proportions of NK-cells during time of lowered disease activity at third trimester of pregnancy and after initiation of immunomodulatory treatment and higher proportion postpartum simultaneously with increased disease activity imply that NK-cells might contribute to the

amelioration of MS-disease during pregnancy. Peripheral blood NK-cells might migrate to decidua during pregnancy, where they differentiate to decidual NK-cells, which differ phenotypically from peripheral blood NK-cells (Manaster and Mandelboim, 2010).

6.2.2.1 CD56^{bright} and CD56^{dim} NK-cells

NK-cells can be divided to CD56^{bright} and CD56^{dim} subpopulations with distinct functional properties based on expression level of CD56 (Poli et al., 2009). In study III we analyzed for the first time the proportions of these NK-cell subpopulations in pregnant MS-patients. We demonstrated that the proportion of CD3-CD56^{dim} NK-cells was decreased during pregnancy and increased after delivery in MS-patients. Thus, alterations of this subpopulation were like the alterations of CD16+ NK-cells. In contrast the proportion of CD3-CD56^{bright} NK-cells was increased during third trimester of pregnancy and decreased postpartum. Therefore, we can determine that the increased proportion of CD56^{bright} is one mechanism that associates with the amelioration of MS disease activity during pregnancy and a decrease in the proportion after delivery associates increased relapse rate. It remains to be seen whether these associations also have functional consequences. A recent study demonstrated increased activation of CD56^{bright} cells in MS-patients during second and third trimesters compared to non-pregnant MS-patients (de Andrés et al., 2017). They did not find difference in the proportion of CD56^{bright} or CD56^{dim} NK-cells in pregnant and non-pregnant MS-patients (de Andrés et al., 2017), but it seems that pregnant MS-patient group included samples taken at different trimesters, which could have affected their results.

Our observations are in line with the evidence that daclizumab-therapy leads to a selective expansion of CD56^{bright} NK-cells and more importantly this gradual expansion correlates strongly with decrease in brain inflammatory activity (Bielekova et al., 2006). The proportion of peripheral blood CD56^{bright} cells is increased also during IFN-β and alemtuzumab-treatments (Chanvillard et al., 2013; Gross et al., 2016a; Saraste et al., 2007; Vandenbark et al., 2009). Similar findings of increased proportion of CD56^{bright} NK-cells during pregnancy and MS-treatments implicate that studying changes during pregnancy may bring important knowledge of MS disease mechanisms and reveal potential targets for new treatments.

CD56^{bright} NK-cells can regulate both adaptive and innate immune cells (Gross et al., 2016c). Stimulated CD56^{bright} NK-cells produce more cytokines than CD56^{dim} NK-cells (Cooper et al., 2001). In addition CD56^{bright} NK-cells can kill activated autologous T-cells through a perforin-dependent degranulation pathway (Jiang et

al., 2011), inhibit proliferation of autologous T-cells through adenosine production (Morandi et al., 2015) and suppress proliferation of autologous CD4+ T-cells through direct cytotoxicity which may indicate that $CD56^{bright}$ NK-cells have a role in prevention of excessive activation of CD4+ T-cells (Laroni et al., 2016). The suppression of proliferation and NK-mediated control of T-cell activity is dysregulated in MS (Gross et al., 2016b; Laroni et al., 2016). Thus, in the future it would be interesting to study whether pregnancy affects the functional properties of $CD56^{bright}$ NK-cells and their ability to control activity of T-cells. It would also be interesting to study whether there is a statistically significant correlation between the proportions of $CD56^{bright}$ NK-cells and clinical or radiological disease activity during or after pregnancy.

6.2.3 B-cells

The pregnancy-related alterations of B-cells seem to be largely unstudied in MS-patients. There is only one small study which found no pregnancy-related alterations in the chemokine receptor expression of B-cells (López et al., 2006). In study II we observed for the first-time unaltered proportions of peripheral blood B-cells during and after pregnancy in MS-patients.

In study IV we observed higher CSF IgG-index during than after pregnancy, which indicates enhanced production of intrathecal antibodies during pregnancy. This observation may directly reflect the shift towards Th2-type immune function during pregnancy. Overall, the pathogenicity of CSF antibodies is not well understood (Disanto et al., 2012) and unchanged IgG-index six months after initial dose of anti-CD20-antibody rituximab has been demonstrated (Cross et al., 2006). Our observation of non-altered numbers of OCBs during and after pregnancy is not surprising as the pattern of OCBs is remarkably stable in the long term of MS disease course and is usually not affected by therapies (Krumbholz et al., 2012). The long-term persistence of OCBs indicates that the CNS of MS-patients continuously supports the development and maturation of B-cells (Krumbholz et al., 2012). The alterations in the number of CSF lymphocytes observed in our study are in line with the notion that the total number of leukocytes in the CSF correlates with disease activity and with treatment response (Stangel et al., 2013). Our B-cell related results might imply that B-cells do not have an impact in the pregnancy-related disease activity alterations of MS.

6.2.4 Differences between patients with multiple sclerosis and healthy controls

Based on our results the proportions of T-cells, B-cells or NK-cells are altered similarly in MS-patients and healthy controls during and after pregnancy. This is in line with the general opinion that the immunological changes which contribute to a successful pregnancy also help control autoimmunity. Previous results about alterations in peripheral blood immune cells in healthy controls are not coherent. Our results of unaltered proportions of peripheral blood T-cells or B-cells in healthy controls are in line with other longitudinal studies of healthy pregnant women during pregnancy (Fiddes et al., 1986; Kraus et al., 2012; Kühnert et al., 1998). We did not find differences in the proportion of T-cells or B-cells during third trimester compared to non-pregnant controls thus confirming results from a previous study with healthy women (Luppi et al., 2002). However, other studies have observed small increases in the proportion of B-cells but not of T-cells or vice versa during third trimester of healthy women compared to non-pregnant controls (Kühnert et al., 1998; Mahmoud et al., 2001). Our result of unaltered CD4/CD8 ratio in healthy controls during pregnancy is partially in line with previous results as both unaltered and decreased CD4/CD8 ratios of healthy women during pregnancy have been observed in previous studies (Fiddes et al., 1986; Kühnert et al., 1998; Luppi et al., 2002). Our results of alterations of NK-cells during pregnancy in healthy controls are partly in line with a previous study showing slight but not significant decreases during pregnancy in the number and proportion of CD16+ NK-cells compared to non-pregnant controls (Kühnert et al., 1998), whereas other studies have not observed differences in the proportion CD16+CD56+ or CD56+ NK-cells between third trimester and non-pregnant controls (Luppi et al., 2002; Mahmoud et al., 2001).

6.3 Effect of pregnancy induced immune alterations on protection against microbial attacks

Adaptive immune responses are weakened, and the activity of T-cells is suppressed during pregnancy (Luppi, 2003; Pazos et al., 2012). A Th1 to Th2 shift suppresses the cytotoxic T-cell response decreasing the robustness of cell-mediated immunity (Kourtis et al., 2014; Patas et al., 2013). The amelioration of cell-mediated autoimmune diseases and increased severity of some infectious pathogens, such as Influenza A, Hepatitis E and Herpes simplex viruses, and opportunistic infections, such as listeriosis, during pregnancy emphasize the immunosuppressive effects of pregnancy (Pazos et al., 2012; Mateus et al., 2013; Kourtis et al., 2014). Interestingly, there are similarities between opportunistic infection risks of

pregnancy and immunosuppressive MS-treatments as cases of listeriosis have been reported in alemtuzumab-treated MS-patients (Holmøy et al., 2017; Rau et al., 2015). Cell-mediated immunity is essential in the defense against both listeriosis and JCV-induced PML, an opportunistic infection particularly relevant in the care of MS with natalizumab (Hernandez-Milian and Payeras-Cifre, 2014; Monaco and Major, 2015). Early studies have observed reactivation of JCV in pregnant healthy woman (Coleman et al., 1983). Thus pregnancy-related immunosuppression of cell-mediated immunity might also influence the susceptibility to JCV-induced PML. So far, this question has not been addressed in MS-patients. Pregnancy might not protect from relapses in patients treated with natalizumab before pregnancy due to strong tendency for rebound disease activation after cessation of the drug before a planned pregnancy (Alroughani et al., 2018). Natalizumab has therefore been used during pregnancy to prevent disease activity and postpartum to prevent relapses (Fagius and Burman, 2014; Haghikia et al., 2014; Massey et al., 2017b; Vukusic et al., 2015). It would be important to know whether the immunosuppressive effects of pregnancy contribute to the risk of JCV reactivation as this could cumulatively increase the risk of PML in patients treated with natalizumab during pregnancy.

6.3.1 Effect of pregnancy on humoral response to JCV

We studied the effects of pregnancy on humoral immune response against JCV by measuring changes in JCV-Ab-indices, which are used in the risk assessment of PML in natalizumab treated patients. To our knowledge the JCV-Ab-indices during pregnancy have not been determined previously. Many factors may affect the levels of circulating antibodies during pregnancy. Antibody levels may be decreased due to dilution effect caused by the cumulative increase in the overall plasma volume during pregnancy that starts at the sixth gestational week (Bernstein et al., 2001). Changes in the pathogen specific immune responses and disease or condition-related events effecting general or specific antibody responses may either increase or decrease antibody levels. General enhancement of Th2 immune responses or change in pathogen presence may increase levels of antibodies. Our observation of decreased total IgG and decreased levels of antibodies against EBV and Measles viruses during pregnancy is probably due to the increased plasma volume. The non-altered JCV-Ab-indices during pregnancy with increase postpartum may imply enhanced production of anti-JCV antibodies due to an alteration in the specific immune response towards JCV. Similarly to anti-JCV-indices the level of antibodies against CMV was not altered during pregnancy. Others have observed an isolated correlation between anti-JCV and anti-CMV antibody titers (Auer et al., 2016). In contrast to MS-patients the JCV-

Ab-indices of healthy controls were decreased during pregnancy. Early studies have shown high or rising titers of JCV during pregnancy in healthy women suggesting virus reactivation (Coleman et al., 1980). A more recent study found similar frequency of JCV urinary excretion in pregnant and non-pregnant woman, and the frequency or magnitude of excretion did not vary with gestational age (McClure et al., 2012).

The unexpectedly low JCV seroprevalence among MS-patients during pregnancy compared to the mean prevalence of 55% observed in other studies (Schwab et al., 2017) can be seen as a limitation to our study. However, variation in the JCV seroprevalence among different cohorts has been large (30%-90%) (Wollebo et al., 2015). The lower seroprevalence observed in our study could be partly due to our relatively young study population consisting only women, as JCV-seroprevalence is lower in women than men and increases with age (Olsson et al., 2013). Treatment on the other hand does not seem to have an influence on JCV seropositivity (Kolasa et al., 2016). The most probable explanation for the lower seroprevalence is nevertheless interindividual differences and chance, which effects the relatively small study population could have emphasized. Unfortunately, samples before pregnancy were not available and seroprevalence before pregnancy could not be determined. Therefore, the effect of pregnancy cannot be entirely excluded. However, based on our results and other studies the seroprevalence among healthy pregnant controls seems to be similar to the mean seroprevalence among non-pregnant healthy controls (McClure et al., 2012; Schwab et al., 2017).

As PML can occur only in JCV-seropositive natalizumab-treated patients, a possibility of seroconversion must be taken account. In our study 19% of seronegative patients seroconverted per year and more importantly, 5.6% of seronegative patients per year had high index values after seroconversion, so they can be considered as biologically significant seroconverters (Schwab et al., 2017). A strong increase in the levels of antibody could be due to primary infection, re-exposure to JCV or a new site of infection due to the spread of virus (Warnke et al., 2013). In a Finnish study of 67 MS-patients 21% (4/19) initially seronegative patients seroconverted during the follow-up of 4.5 years (4.7% per year) (Kolasa et al., 2016). A recent meta-analysis showed that in studies using same anti-JCV-index measurement as we an average 10.8% of natalizumab-treated MS-patients seroconvert per year and only 3.48% per year seroconverted to high index values (Schwab et al., 2017). Thus, our results may imply that pregnancy might increase both the overall seroconversion and seroconversion to high index values. A speculative reason for this could be the suppression of adaptive immunity during pregnancy. However, this observation should be confirmed in the future in a larger study including also pre-pregnancy samples.

Our results imply that the humoral immune response towards JCV is altered during pregnancy and these pregnancy-related alterations are more pronounced in MS-patients than healthy controls. However, due to relatively small sample size and surprisingly low JCV-seroprevalence among MS-patients, these conclusions are only speculative and should be confirmed in the future in larger studies. Further studies could also investigate whether cell-mediated immune response against JCV is suppressed during pregnancy due to overall suppression in cell-mediated immunity. Despite its limitations, our study brings new knowledge about the humoral immune response to JCV during MS and control pregnancy and might also have implications for the clinical decisions making and safety of natalizumab-treatment of MS during pregnancy.

7 CONCLUSIONS

The purpose of this thesis was to identify immunological phenomena that might explain the amelioration of MS disease during pregnancy, and reactivation of MS disease after delivery. To achieve this purpose, we studied alterations in various peripheral blood lymphocyte subpopulations during and after pregnancy in MS-patients and healthy controls and alterations in CSF determinants. In addition, we evaluated how pregnancy-related immunological changes might affect the susceptibility to an opportunistic infection particularly relevant in the care of MS namely, JCV-induced PML.

In the first study, we demonstrated the potential aggressiveness of postpartum immune activation by describing a MS-patient with as many as 200 Gd-enhancing lesions in the brain MRI soon after delivery. This is an exceptional finding under any circumstances, and to understand better the cellular mechanisms related to such extreme disease activation, we performed phenotyping of the peripheral blood lymphocytes. The proportions of T-cells, B-cells or NK-cells did not change, but the proportions of lymphocytes expressing chemokine receptors CXCR3 and CCR7 were increased simultaneously with significant decrease in the number of Gd-enhancing lesions after methylprednisolone treatment. This may reflect the sealing of the BBB by steroid treatment, and enrichment in blood of the lymphocytes bearing chemokine receptors which normally would guide them into the brain. In studies II and III we expanded the immunological evaluation of MS pregnancy to Finnish national level by collecting a cohort of 42 prospectively followed MS-patients from 10 central hospitals in Finland. We evaluated the alterations in peripheral blood immune cells during and after pregnancy in this cohort, with the major finding of an expansion in the regulatory NK-cell population during pregnancy. In study IV we extended the findings of immunological alterations from peripheral blood to the CNS by showing that the Th2 shift related to pregnancy leads to enhanced intrathecal immunoglobulin production, demonstrable by an increased CSF IgG-index. Finally, in study V we showed that pregnancy-related immune suppression may lead to alterations in the humoral immune response to opportunistic JCV.

It is likely that the increased estrogen levels, particularly estriol, contribute to the alterations in the cellular adaptive immune responses during pregnancy. These changes help to better control particularly the Th1 autoimmunity of MS. In this thesis several of the cellular mechanisms likely contributing to this are described in the context of MS pregnancy. During MS pregnancy the proportions of CD16+ and CD3-CD56^{dim} NK-cells are gradually decreased. In opposite, the proportion of regulatory CD56^{bright} NK-cells is increased during the third trimester of

pregnancy in MS-patients. This suggests that regulatory $CD56^{bright}$ NK-cells might facilitate the control of autoimmune inflammation during pregnancy and thus contribute to the reduced activity of MS during pregnancy. The third trimester of MS pregnancy is also characterized by increased CD4/CD8 and Th2/Th1cytokine ratios, indicating that the role of T-cell subpopulations in the pregnancy-related amelioration of MS warrants further investigation. On the other hand, the unaltered proportions of peripheral blood B-cells and unaltered numbers of CSF oligoclonal bands together with increased CSF IgG-index during pregnancy might indicate a minor role for B-cells in the amelioration of MS during pregnancy. Our results of increased JCV-Ab-indices in the postpartum setting may reflect altered immunity towards JCV during pregnancy and might have clinical implications for the use of natalizumab during and after pregnancy. The complex mechanisms leading to successful pregnancy and to the amelioration of Th1-type autoimmune disease, that based on our results are understood a little better, also lead to partial immunosuppression, which is relevant when patients are treated with therapies that also cause immunosuppression.

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