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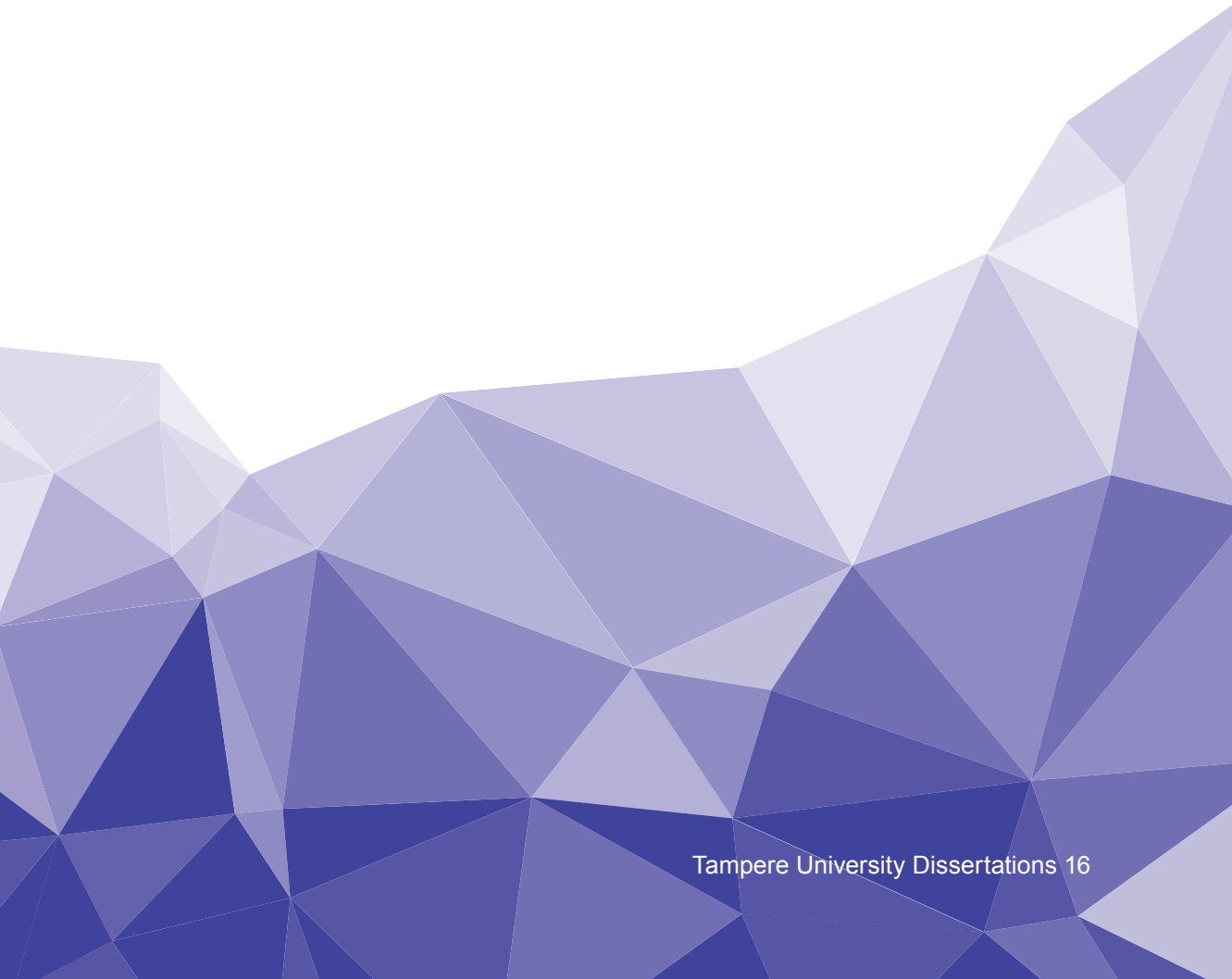
Biomarkers for Progressive Multifocal Leukoencephalopathy Risk Assessment and Disease Activity in Multiple Sclerosis



Tampere University Dissertations 16

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Biomarkers for Progressive Multifocal Leukoencephalopathy Risk Assessment and Disease Activity in Multiple Sclerosis

ACADEMIC DISSERTATION
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ACADEMIC DISSERTATION
Tampere University, Faculty of Medicine and Health Technology
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TO MY LOVING FAMILY

ABSTRACT

Currently several disease-modifying therapies (DMTs) are available for the treatment of MS, but due to clinical and pathophysiological complexities of this disease, the evaluation of its prognosis and therapeutic response is difficult. Moreover, reliable and sensitive biomarkers for use in clinical practice are still lacking. In spite of efficacy of new treatments, the long-term use of highly effective MS therapies such as natalizumab (NTZ) has been associated with the potential of developing progressive multifocal leukoencephalopathy (PML). PML is a fatal demyelinating lytic infection of the CNS caused by the reactivation of latent neurotropic virus called JC polyomavirus (JCPyV). Currently, the risk of PML has become a major challenge in the treatment of MS, because in addition to NTZ, also other effective MS therapies were reported to carry the PML risk. Since there are no clearly established biomarkers available to predict the PML risk, and currently used risk stratification parameters are not sensitive enough to rule out the complete risk of PML, one of the main aim of this thesis was to find the biomarker to identify the PML risk in individual MS patient under NTZ therapy. In this thesis, soluble (s) L-selectin and JC virus miRNAs were analysed in relapsing-remitting MS (RRMS) patients to assess their biomarker potential in predicting the risk of PML. The results from the sL-selectin study showed a positive correlation between sL-selectin and anti-JCPyV-antibody levels and most importantly sL-selectin level was found to be higher in those patients who were considered to have a high risk for PML compared to patients with low risk. Based on these data sL-selectin could be used for the assessment of PML risk in MS patients treated with NTZ. The JCPyV miRNA study detected reduced levels of 5p miRNA among NTZ-treated MS patients and an association with JCPyV seropositivity, suggesting a possible involvement of these miRNAs in support of JCPyV reactivation. Thus, these results suggested that miRNA-J1-5p can be a potential new marker for the NTZ-associated PML risk assessment in MS patients. In addition, observation of the high level of miRNA prevalence also in JCPyV seronegative patients suggested that the ELISA test currently used for the detection of anti-JCPyV antibody may be less sensitive than miRNA detection to reveal earlier acquired JCPyV infection.

The other main aim of this thesis was to identify the biomarkers of MS disease activity and to distinguish between patients with benign or aggressive disease course based on the presence of clinical activity as measured by the number of relapses, neurological disability scores, and MRI disease activity. In the study of costimulatory molecules, increased levels of sCD26 and sCD30 in MS suggested the potential of these

molecules as biomarkers consistent with relatively inactive or stable disease activity. Moreover, sCD30 molecule was considered as a marker of regulatory immune response due to its positive correlation with an anti-inflammatory cytokine IL-10, and increased levels of sCD30 in RRMS patients treated with DMTs compared with untreated patients. Gene expression study for DR3, DcR3 and TL1A in PBMC obtained from MS patients, displayed TL1A as a candidate biomarker for reflecting inflammatory activity in MS and predicting disability progression. Our findings further illustrated that TL1A may hold the ability to reflect ongoing stable disease course and as well as the marker of therapeutic response to immunomodulatory treatment in MS. However, additional studies including a larger sample size are needed to evaluate the clinical relevance of these findings.

TIIVISTELMÄ

Multippeliskleroosia (MS) voidaan hoitaa useilla eri taudin kulkua muuntavilla lääkkeillä, mutta taudin kliinisen ja patofysiologisen monimuotoisuuden vuoksi yksittäisen potilaan hoitovasteiden arviointi eri lääkeaineille on haastavaa. Kliinisesti luotettavia ja sensitiivisiä biomerkkiaineita kaivataan hoitovasteiden arviointiin. Nykytilanteessa MS-potilaita hoidetaan entistä tehokkaimmilla lääkkeillä, kuten natalizumabilla, mutta niiden pitkäaikainen käyttö lisää progressiivisen multifokaalisen leukoenkefelopatian (PML) riskiä. PML on keskushermoston fataali demyelinisoiva lyyttinen infektio, joka on seurausta neurotrooppisen JC-viruksen reaktivaatiosta. MS-potilaiden hoidon yksilöllisessä suunnittelussa on otettava huomioon PML:n riski, joka on kohonnut erityisesti natalizumab-hoidetuilla potilailla, mutta myös muiden tehokkaiden lääkeaineiden on osoitettu lisäävän riskiä tälle taudille. Tällä hetkellä kliinisessä käytössä ei ole vakiintunutta biomerkkiainetta, joka ennustaisi PML-taudin kehittymisen riskiä ja myöskään nykykäytäntöjen mukaan stratifikaatioparametrit eivät ole tarpeeksi sensitiivisiä tunnistamaan korkean riskin MS-potilaita natalizumab-hoidettujen joukosta. Väitöskirjan tavoitteena oli löytää biomerkkiaine, joka tunnistaisi PML:n riskin natalizumab-hoidetuilla potilailla.

Väitöskirjatutkimuksessa tutkittiin liukoisen L-selektiinin ja JC-virus-mikro-RNA:iden (miRNA) ilmentymistä relapsoivaa-remittoivaa MS-tautia sairastavilta selvittääksemme niiden biomerkkiainepotentiaalia ennustaa PML-taudin riskiä. Tulokset osoittivat, että liukoinen L-selektiini korreloi positiivisesti JCPyV-vasta-ainetasojen kanssa ja erityisesti L-selektiinitasot olivat korkeammat niillä potilailla, jotka voitiin luokitella korkean riskin potilaiksi. Tulokset viittaavat siihen, että liukoista L-selektiiniä voitaisiin käyttää biomerkkiaineena PML-taudin kehittymisen riskin arvioinnissa. JCPyV miRNA tutkimuksessa havaittiin miRNA-J1-5p tasojen olevan matalammalla tasolla natalizumab-hoidetuilla MS-potilailla ja tasojen olevan yhteydessä JCPyV-seropositiivisuuteen, joka mahdollisesti liittyy JC-viruksen reaktivaatioon. Tulokset osoittivat, että miRNA-J1-5p miRNA on uusi potentiaalinen biomerkkiaine JCPyV:n riskin arvioinnissa. Lisäksi JCPyV:lle seronegatiivisilta potilailta löytyi JCPyV-miRNA:ta, joka viittaa nykyisen JCPyV-vasta-aineiden ELISA- määritysmenetelmän johtavan osittain vääriin negatiivisiin tuloksiin.

Väitöskirjan toisena tavoitteena oli löytää taudin aktiivisuuden biomerkkiaineita, joiden avulla voitaisiin erotella aggressiivista ja benigniä tautimuotoa sairastavat potilaat käyttäen mittareina relapsien lukumäärää, neurologisen disabiliteetin kertymistä ja MRI:llä mitattua aktiivisuutta. Kohonneet seerumin CD26- ja CD30- tasot MS-taudissa

viittasivat molekyyleillä olevan biomerkkiainepotentiaalia ja niiden assosioituvan inaktiiviseen ja stabiiliin taudinkulkuun. Lisäksi seerumin CD30-tasot olivat koholla immunomoduloivia lääkeaineita käyttävillä potilailla ja ne korreloituivat IL-10 tasojen kanssa, joka viittaa immunoregulatorisiin tehtäviin MS- taudissa. Taudin aktiivisuusmerkkiaineista tutkittiin myös kolmen kostimulaattorimolekyylin (DR3, DcR3 ja TL1A:n) ilmentymistä geenitasolla veren mononukleaarisista soluista MS-potilailla, jossa havaittiin TL1A:n olevan yhteydessä tulehdukselliseen aktiivisuuteen ja vammautumisen kertymiseen. Tutkimuksessa havaittiin lisäksi TL1A:n tasot olivat yhteydessä stabiiliin tautimuotoon ja assosioituvan immunomoduloivilla lääkkeillä hoidettavien potilaiden hyvään hoitovasteeseen. Tässä väitöskirjatyössä tehtyjen löydösten varmentaminen kliiniseen käyttöön edellyttää lisätutkimuksia, käyttäen suurempia aineistoja.

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LIST OF ORIGINAL PUBLICATIONS

I **Basnyat, P**., Hagman, S., Kolasa, M., Koivisto, K., Verkkoniemi-Ahola, A., Airas, L., Elovaara, I. (2015). Association between soluble L-selectin and anti-JCV antibodies in natalizumab-treated relapsing-remitting MS patients. *Multiple Sclerosis and Related Disorders*, 4(4), 334-338. doi: 10.1016/j.msard.2015.06.008

II **Basnyat, P**., Virtanen, E., Elovaara, I., Hagman, S., & Auvinen, E. (2017). JCPyV microRNA in plasma inversely correlates with JCPyV seropositivity among long-term natalizumab-treated relapsing-remitting multiple sclerosis patients. *Journal of Neurovirology*, 23(5), 734-741. doi:10.1007/s13365-017-0560-x

III **Basnyat, P**., Natarajan, R., Vistbakka, J., Lehtikangas, M., Airas, L., Matinlauri, I., Elovaara, I., Hagman, S. (2015). Elevated levels of soluble CD26 and CD30 in Multiple Sclerosis. *Clinical and Experimental Neuroimmunology*, 6(4), 419-425. doi: 10.1111/cen3.12253

IV **Basnyat, P**., Sumelahti, M. L., Lehtimäki, T., Elovaara, I., Hagman, S. (2018). Gene expression profiles of Tumor Necrosis Factor-like Cytokine TL1A and its Receptors DR3 and DcR3 in Multiple Sclerosis. (Submitted)

III Publication has been previously included in the doctoral dissertation, "Biomarkers in Multiple Sclerosis: Special emphasis on Melatonin and Adipokines", by Renuka Natarajan, University of Tampere, 2016.

ABBREVIATIONS

APCs	Antigen Presenting Cells
ARR	Annualized Relapse Rate
BBB	Blood-Brain Barrier
BKPyV	BK polyomavirus
CAMs	Cell Adhesion Molecules
CD	Cluster of Differentiation
CDMS	Clinically Definite Multiple Sclerosis
CIS	Clinically Isolated Syndrome
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CXCL	C-X-C motif chemokine ligand
DC	Dendritic cells
DcR	Decoy Receptor
DMTs	Disease-Modifying Therapies
DRs	Death receptors
DTI	Diffusion Tensor Imaging
EAE	Experimental Autoimmune Encephalitis
EBV	Epstein-Barr Virus
EDSS	Expanded Disability Status Scale
ELISA	Enzyme-Linked Immunosorbant Assay
FLAIR	Fluid Attenuation Inversion Recovery
GM	Gray Matter
HHV-6	Human herpesvirus 6
HLA	Human Leucocyte Antigen
HSV	Herpes simplex virus
IFN	Interferon
Ig	Immunoglobulin
JCPyV	JC polyomavirus
IL	Interleukin
MBP	Myelin Basic Protein

MCPyV	Merkel Cell Polyomavirus
MHC	Major Histocompatibility Complex
miRNA	MicroRNA
MMPs	Matrix Metalloproteinases
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
NAWM	Normal-Appearing White Matter
NCCR	Non-coding control region
NFL	Neurofilaments Light
NFH	Neurofilaments Heavy
NF-KB	Nuclear factor kappa B
NK	Natural Killer
NMO	Neuromyelitis Optica
NO	Nitric Oxide
NTZ	Natalizumab
OCBs	Oligoclonal Bands
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase Chain Reaction
PI	Progression Index
PML	Progressive multifocal leukoencephalopathy
PPMS	Primary Progressive Multiple Sclerosis
ROS	Reactive Oxygen Species
RRMS	Relapsing Remitting Multiple Sclerosis
RT	Reverse Transcription
S	Soluble
SPMS	Secondary Progressive Multiple Sclerosis
TCR	T-cell Receptor
Th	T helper
TL1A	TNF-like ligand 1A
TNF	Tumor Necrosis Factor
TNFSF	Tumor Necrosis Factor Superfamily
Treg	Regulatory T-cell
VCAM	Vascular Cell Adhesion Molecule
VLA	Very late Activation Antigen

VZV	Varicella Zoster Virus
WM	White Matter

1 INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease of the central nervous system (CNS) that leads to significant neurological disability (Reich et al., 2018). MS mainly affects young adults, typically begins between the ages of 20 and 40 years, and is universally more prevalent in women compared to men (Krokki et al., 2011). The disease is characterized by complex pathophysiological processes, which include multifocal inflammation, demyelination, reactive gliosis, oligodendrocyte and axonal loss, and remyelination (Lassmann, 2013). Etiology of MS is unknown, but both differential genetic predisposition and environmental factors such as vitamin D deficiency, infection with Epstein-Barr virus, smoking, and obesity are considered as risk factors for MS (Olsson et al., 2017).

MS has a variable clinical course and a heterogeneous clinical presentation and the disease is classified into three main types based on clinical courses: relapsing-remitting MS (RRMS), secondary progressive MS (SPMS) and primary progressive MS (PPMS) (Lublin & Reingold, 1996; Lublin, 2014). Initially, majority of the patients, about 85%, have RRMS, which is characterized by intermittent relapses followed by partial or full recovery between initial relapses. After 10-20 years, RRMS turns into SPMS, characterized by the irreversibility of the neurological deficits due to progressive neurodegeneration (Confavreux & Vukusic, 2006; Weinshenker, 1998). About 15 % of MS patients exhibit a gradual progression of disability without relapses from onset called as PPMS (Compston, 2003; Lublin, 2014).

Immunopathogenesis of MS involves the activation of myelin-specific T cells, mainly the T helper (Th)-1 $CD4^+$ T cells and Th17 cells, that invade to CNS parenchyma from periphery through blood-brain barrier (BBB) and blood-CSF barrier (BCF) mediating neural tissue damage (Holman et al., 2011; Jadidi-Niaragh & Mirshafiey, 2011; Stromnes et al., 2008). In addition, other immune cells such as CD8+T cells, natural killer (NK) cells, B cells, and several cytokines and chemokines contribute to the pathogenesis of MS (Comabella & Khoury, 2012).

There is still no cure for MS but different disease-modifying therapies (DMTs) are available particularly for the relapsing-remitting form of the disease. These drugs reduce immune cell activity and their entry into the CNS and decrease the frequency of clinical attacks known as relapses (Torkildsen et al., 2016). Most of the MS

treatments are associated with the side effects, among which opportunistic infections are the most serious ones (Berger & Houff, 2009). Progressive multifocal leukoencephalopathy (PML) is the most serious complication and may occur in patients treated with natalizumab (NTZ) - or other immunomodulatory drugs. NTZ is an effective drug for MS, however, long-term treatment (more than 2 years) in RRMS patients is associated with the risk of developing PML (Clifford et al., 2010). PML is a JC polyomavirus (JCPyV)-mediated infection of the CNS caused by the reactivation of latent virus, followed by lytic infection of oligodendrocytes and astrocytes (Ferenczy et al., 2012; Khalili et al., 2007). Currently there is no biomarker available to predict the complete risk of PML in individual NTZ- treated MS patient and this risk has become a major challenge for clinicians, because, in addition to NTZ, also other effective biological therapies such as fingolimod and dimethyl fumarate were reported to carry the risk of PML in MS patients (Faulkner, 2015). Therefore, we analysed L-selectin and JCPyV miRNAs in our study to explore their biomarker potential for predicting NTZ-associated PML risk.

Due to the clinical and pathophysiological complexities, MS disease course including PML risk and prognosis of MS are highly unpredictable. Moreover, due to the lack of reliable and sensitive biomarkers, it is difficult to evaluate disease activity and therapeutic response (Gastaldi et al., 2017). Currently, most of the existing biomarkers in MS are not fully able to reflect the immensity of diverse MS disease activity. Therefore, our aim was to assess CD26 and CD30 molecules in sera as biomarkers of MS subtypes, and relation to inflammatory disease activity and disability in MS patients. Similarly, we analysed the relative gene expression of death receptors (DR3, DcR3) and ligand (TL1A) to detect their association with MS subtypes, inflammatory disease activity and disability in MS patients. These biomarkers will contribute to overall clinical management of MS patients with an ultimate goal to prevent the disease progression and development of long-term neurological disability.

2 REVIEW OF THE LITERATURE

2.1 Epidemiology of multiple sclerosis

Multiple sclerosis has an increasing prevalence worldwide (Cotsapas et al., 2018) and it is one of the most common causes of non-traumatic disability among young and middle-aged individuals (Leray et al., 2016; Mandia et al., 2014). MS affects more than 2 million people worldwide and it is more prevalent in women compared to men (Fox et al., 2006; Kira, 2014). A similar trend of increasing RRMS incidence and high female prevalence has been reported in Finland (Sumelahti et al., 2014). The female-to-male ratio in MS prevalence has increased over time, and it has been estimated from 1.4 in 1955 to 2.3 in 2000 (Alonso & Hernan, 2008) and even higher recently, from 2.35 to 2.73, according to a study which compared sex ratio trends of over a 60-year span (Trojano et al., 2012). However, in individuals with primary progressive disease form, there is no gender preponderance. MS prevalence varies considerably by continent and geographical latitude (Leray et al., 2016). The prevalence is highest (>30 per 100,000) in northern parts of Europe and North America; medium (5-30 per 100,000) in southern Europe and southern United States; and Central and South America (10-20 per 100,000). Low Prevalence rate has been reported (<5 per 100,000) in Asia and South America (Koch-Henriksen & Sorensen, 2010). However, it is still elusive whether this variation in the incidence rate is due to environmental or the genetic differences.

Finland belongs to a high-risk region for MS affecting around more than 9,000 people (Finnish neuro society, 2018). The incidence of MS has increased considerably from 1981 to 2010 in Finland (Fox et al., 2006; Holmberg et al., 2013; Kira, 2014). There are regional differences in MS epidemiology in Finland. In Seinäjoki and Vaasa with the highest incidence, the total incidence rate of 12.5/100,000 person and 8.3/100,000 in 2010, respectively has been reported (Sumelahti et al., 2014). The risk of MS was two-fold higher in Seinäjoki and substantially higher in Vaasa compared to the Pirkanmaa, which is considered as a region of medium-risk for MS in Finland (Sumelahti et al., 2001; Sumelahti et al., 2014).

2.2 Risk factors for multiple sclerosis

2.2.1 Genes

The etiology of MS is unknown but complex interactions between genetic background and environmental factors are responsible for disease development (Reich et al., 2018). The human leukocyte antigen (HLA) provides the highest genetic contribution to MS susceptibility; however, the exact mechanism of alternation of MS incidence in different population is not fully understood (Hemmer et al., 2015; Ramagopalan & Ebers, 2008; Ramagopalan et al., 2009). HLA class II extended haplotype HLA-DRB1*1501 is one of the most important factors that affect MS susceptibility (Hillert & Olerup, 1993; Hillert, 2010; Smestad et al., 2007). This haplotype accounts for approximately 50% of the genetic risk for MS and it is known as the strongest known MS-susceptibility marker. Although this haplotype is regarded as the strongest risk factor, it only increases the risk of MS by 2- to 4-fold and this factor is also present in approximately 20% to 30% of the healthy population (Hollenbach & Oksenberg, 2015; Nylander & Hafler, 2012). The reason why these HLA class II molecules contribute as strong risk factor for MS may be due to their role in antigen presentation to pathogenic $CD4^+$ T cells (Parnell & Booth, 2017). Several studies have shown the correlation between DRB1*1501 and disease progression or severity, and also with the presence of oligoclonal bands and increased IgG levels in the CSF of MS patients (Goris et al., 2015; Mero et al., 2013). Different other non-HLA genes (genes outside HLA region) which are found in genome-wide association studies (GWAS) are also identified as mild risk factors for MS (De Jager et al., 2009; International Multiple Sclerosis Genetics Consortium et al., 2007). These genes include IL7RA, IL2RA, CLEC16A, LFA-3, TNFRSF1A, CD6 and IRF8 (International Multiple Sclerosis Genetics Consortium (IMSGC), 2008; Zuvich et al., 2010).

2.2.2 Viral infections

Increasing evidence supports the role of several viruses such as and Epstein-Barr virus (EBV) and Human herpesvirus 6 (HHV-6) in MS disease induction and pathogenesis (Belbasis et al., 2015; Pormohammad et al., 2017). Currently these viruses are suggested as leading risk factors for MS (Pietilainen-Nicklen et al., 2014;

Virtanen & Jacobson, 2012), however their causative or pathogenic role in disease development is still unclear, because these viruses are commonly lymphotropic and they could be only passengers in the MS brains, due to the persistent presence of immune cells. Serological studies have shown higher level of EBV antibodies specific for Epstein-Barr nuclear antigen-1 (EBNA1) in the serum of MS patients in comparison to normal individuals (Ascherio & Munger, 2010). Recently EBV was shown in brain tissues in most of the MS cases further supporting the role of EBV in MS pathology (Hassani et al., 2018). Although the mechanism of interaction between HHV-6 and MS remains elusive yet, growing evidence supports the significant relationship between MS and infection with HHV-6 (Pormohammad et al., 2017). Detection of HHV-6 viral mRNA (Opsahl & Kennedy, 2005) and protein expression particularly in the oligodendrocytes in demyelinated plaques (Challoner et al., 1995) have raised the hypothesis that HHV-6 may be a driver of MS pathogenesis. In addition, the presence of HHV-6 DNA and anti-HHV-6 IgG and IgM antibodies has been shown in serum and CSF of MS patients (Challoner et al., 1995; Moore & Wolfson, 2002; Soldan et al., 2000). Recent studies have also shown correlation between HHV-6 specific oligoclonal bands (OCBs) and several clinical and magnetic resonance imaging (MRI) parameters of MS (Pietilainen-Nicklen et al., 2014). MS patients who had detectable viral DNA in CSF had significantly more contrast enhancing lesions as compared to patients who lack CSF viral DNA (Pietilainen-Nicklen et al., 2014). In addition to EBV and HHV-6, recent studies have shown an association between human endogenous retrovirus (HERV) expression with development and progression of MS (Morandi et al., 2017; Mostafa et al., 2017). An increased expression of HERV-K and HERV-H families in the blood, brain or CSF of MS patients has been reported by some studies (Christensen, 2005).

2.2.3 Vitamin D deficiency

Several studies, including genetic studies, have confirmed vitamin D deficiency as a potent risk factor for MS (Munger et al., 2004; Munger et al., 2006; Pierrot-Deseilligny & Souberbielle, 2017). Recently a large nationwide study in Finland has shown that vitamin D deficiency is linked to a higher risk of MS for women in Finland (Munger et al., 2016).Vitamin D level has been considered as an early predictor of MS disease activity and progression (Ascherio et al., 2014). Low levels of circulating 25-dihydroxyvitamin D, and an association between vitamin D status

and MS disease activity, have been reported by several studies suggesting the protective role of vitamin D in MS disease (Mowry et al., 2012; Munger et al., 2006; Runia et al., 2012; Simpson et al., 2010). Vitamin D exerts the immunomodulatory role in MS, mainly during the inflammatory stage of the disease (Pierrot-Deseilligny & Souberbielle, 2017) and decreases the risk of relapse and reduction in disease activity (Munger et al., 2006; Munger & Ascherio, 2011). Vitamin D was shown to have an effect in reducing the relapses by 50-70% (Munger & Ascherio, 2011; Pierrot-Deseilligny & Souberbielle, 2017). Due to these beneficial effects, systematic moderate supplementation of vitamin D has been recommended in MS as predicted by statistical models. A recent study has suggested that the supplementation of vitamin D with 10,400 IU daily is safe and well-tolerated in MS patients and exhibits in vivo pleiotropic immunomodulatory effects (Sotirchos et al., 2016). The immunological effects of vitamin D include the reduction of IL-17 production by $CD4^+$ T cells and decreased proportion of effector memory $CD4^+$ T cells with a concomitant increase in central memory and naïve $CD4^+$ T cells (Sotirchos et al., 2016).

2.2.4 Gender-related hormones

A growing body of evidence suggests that the gender influences MS disease susceptibility, disease course, symptoms and the severity of MS (Airas & Kaaja, 2012; Hanulikova et al., 2013). Especially, higher female predominance in MS is considered to be due to the hormonal rather than genetic factors (Leray et al., 2016). Potential elements that play role on this gender dimorphism are the effects of sex hormones on immune responses (de Andres et al., 2004; Sanchez-Ramon et al., 2005). Pregnancy plays an important role in the stabilization of MS. Several studies have found decreased clinical disease activity as decrease in the number of relapse rate by more than 70% and a modified disease course during pregnancy when concentrations of estrogen and progesterone are highest (Confavreux et al., 1998; Hanulikova et al., 2013; Salemi et al., 2004). Clinical improvement during pregnancy increases especially in the last trimester compared to a year before pregnancy (Pozzilli et al., 2015). However, the relapse rate increases postpartum and during menopause probably due to the decrease in the estrogen hormone levels and diminished immunosuppressive effects of pregnancy (Airas & Kaaja, 2012). Estrogen is considered to have potential neuroprotective effects and regulates MS pathology by increasing regulatory cytokines, decreasing demyelination, and

increasing the oxidative and energy producing activities in the cells of CNS (Christianson et al., 2015; R. Voskuhl & Momtazee, 2017). Due to the beneficial effects, estrogens and androgens have been studied for the treatment of MS (Spence & Voskuhl, 2012). Previously estriol treatment in women with MS showed significant reductions in gadolinium-enhancing lesions during the treatment compared to six months before the treatment (Sicotte et al., 2002; Soldan et al., 2003). In fact, recently the supplementation of estriol with MS drug (glatiramer acetate) has proceeded already to the clinical trial providing for reduced relapse rates in women with RRMS (R. R. Voskuhl et al., 2016). In addition, testosterone treatment has been also studied for its neuroprotective effect in men with RRMS (Gold & Voskuhl, 2006; Kurth et al., 2014; Sicotte et al., 2007).

2.3 Clinical subtypes, disease course and diagnosis

The clinical course of MS is variable and the disease is classified into relapsing-remitting MS (RRMS), secondary progressive MS (SPMS), primary progressive MS (PPMS), and progressive-relapsing MS types (Lublin, 2014). Eighty percent of the MS patients are initially diagnosed as clinically isolated syndrome (CIS) patients (Miller et al., 2012). CIS patients were defined as patients who had their first acute demyelinating event suggestive of MS (Confavreux & Vukusic, 2006; Miller et al., 2012; Polman et al., 2005; Scalfari et al., 2010). The clinical manifestation of CIS may include unilateral optic neuritis with visual disturbances, and/or spinal cord, brain stem, cerebellar, or hemispheral symptoms and signs (Miller et al., 2012).

Initially, in the RRMS disease course, the majority of the patients (85%) experience relapses, neurologic symptoms and findings characterized by a subacute onset over several days and usually followed by remissions with complete or partial recovery after several weeks or months (Yamout et al., 2013). These clinical relapses are characterized by the presence of inflammatory infiltrates and demyelination in the brain and spinal cord (Mahad et al., 2015). Later the disease enters into a progressive neurodegenerative phase characterized by the accumulation of a more severe neurological disability (Compston & Coles, 2008). After a median of 10 to 15 years, more than half of the RRMS patients undergo transition to a progressive form called SPMS that may be with or without clinical relapses but always with a gradual increase in the neurological dysfunction (Raine, 2008). Clinical relapses may occur in SPMS patients especially during the early transition period from RRMS to SPMS (Fox et al., 2006). Around 10-20 % of patients have a progressive onset of the disease

from the beginning of the disease without superimposed relapses. This form of the disease is characterized by the steady progress of an irreversible disability called PPMS and it is regarded as a non-inflammatory or less inflammatory pathologic form of MS (Compston & Coles, 2008). Different clinical stages of MS based on the disease course are shown in Figure 1.

The diagnosis of MS needs clinical and radiographic evidence. In 2001, the diagnostic criteria were developed called "McDonald Criteria" for the diagnosis of MS (Confavreux et al., 2001; McDonald et al., 2001). These criteria were revised in 2005, 2010 and 2017 to enable earlier, more sensitive and specific diagnosis of MS (McDonald et al., 2001; Polman et al., 2005; Polman et al., 2011; Thompson et al., 2018). According to the 2017 revision, the early diagnosis of MS can be made primarily in CIS patients, establishment of dissemination of space (DIS) of CNS lesions on MRI, and the presence of CSF-specific oligoclonal bands, without the requirement for demonstration of dissemination of time (DIT) of CNS lesions on MRI (Thompson et al., 2018).

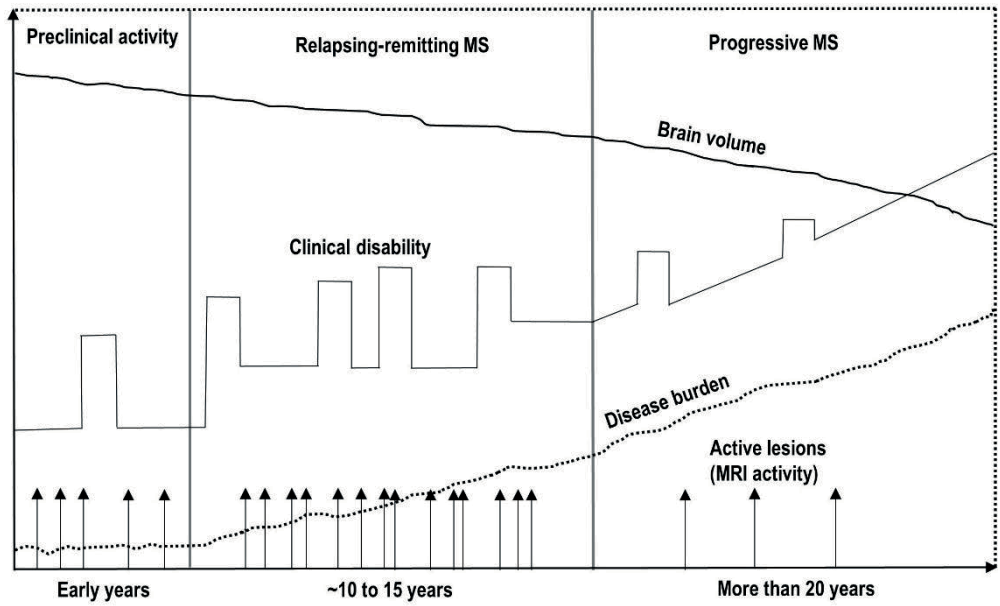

Figure 1. **Schematic diagram of Multiple Sclerosis disease course.** The disease usually starts with a preclinical phase also called clinically isolated syndrome (CIS) where clinical symptoms are suggestive of MS. MRI activity, as shown by vertical arrows, measures the number of gadolinium-enhancing lesions or new T2 hyperintense brain lesions that represents the ongoing inflammatory process where breakdown of blood-brain barrier allows migration of cells to the CNS. Subsequent decrease in brain volume as measured by atrophy and increase in disease burden as shown by number of active lesions indicates the permanent CNS tissue damage. Redrawn with permission from publisher (Olsson et al., 2017)

2.4 Neuropathology of multiple sclerosis

The central hallmark of MS pathology is the development of demyelinated areas called plaques or lesions which occur either focally or diffusely in white matter (WM) and grey matter (GM) of the CNS (Lassmann et al., 2007; Lassmann, 2013). Mostly, MS lesions comprise disruption of BBB, multifocal inflammation, demyelination, loss of oligodendrocytes, reactive gliosis, and axonal degeneration (Dutta & Trapp, 2006; Trapp & Nave, 2008; Trapp & Stys, 2009). Acute active MS lesions are hypercellular demyelinated plaques, which are hugely infiltrated by macrophages, and contain patchy infiltrates of autoreactive T cells and antigen-nonspecific monocytes and macrophages inside the area of myelin loss (Frischer et al., 2009). These inflammatory infiltrates mainly contain a higher number of clonally expanded $CD8^+$ T cells, and the lesser number of $CD4^+$ T cells, B cells, and plasma cells, which accumulate mainly in the perivascular spaces and meninges (Nylander A., 2012; Popescu et al., 2013). Chronic lesions are more frequently seen in progressive MS, which are characterized by a rim of microglia and/or macrophages without myelin debris, a well-demarcated hypocellular gliotic area characterized by the myelin loss, relative preservation of axons, and the development of astrocytic scars (Mahad et al., 2015; Stadelmann et al., 2011; Stadelmann, 2011). In addition, other immune cells such as B cells and plasma cells, macrophages containing myelin debris, and complement factors and immunoglobulin depositions are also present in the active lesions (Lassmann, 2013; Trapp & Stys, 2009). Demyelinating activity within a plaque can be assessed based on the presence or absence of specific myelin degradation products such as myelin basic protein (MBP) and myelin oligodendrocyte protein (MOG) (Popescu et al., 2013; Stadelmann, 2011).

The pathology of MS varies between relapsing and progressive disease forms (Lassmann, 2013). Active CNS tissue injury occurs in all the stages of MS but active MS lesions, mostly in cortical demyelinated lesions, are most common in RRMS form whereas become less frequent during later progressive stages of the disease (Dutta & Trapp, 2014). Four major cortical lesions have been detected in MS brains. Type I or leukocortical lesions extend through both the WM and the GM, Type II or intracortical lesions that are fully localized in cerebral cortex, Type III lesions are characterized by subpial areas of demyelination and Type IV lesions cover the entire width of the cortex (Popescu & Lucchinetti, 2012; Popescu et al., 2013). Chronic lesions do not show active inflammation or the inflammation decreases as plaques progress, and macrophages and microglia gradually disappear (Lassmann, 2014). Consequently, axonal damage and loss in normal appearing white matter (NAWM),

and prominent involvement of grey matter and subpial demyelination, and brain atrophy are the apparent pathological features of chronic MS (Dutta & Trapp, 2006; Dutta & Trapp, 2014).

2.5 Immunopathogenesis of multiple sclerosis

Immunopathogenesis of MS is a complex process in which inflammation is considered as a key mediator of events that leads to tissue damage in the CNS (Baecher-Allan et al., 2018). Both innate and adaptive immune responses play important roles in the clinical course of MS (Hemmer et al., 2015). Reactivation of myelin-specific $CD4^+$T cells in the brain initiate release of abundant proinflammatory mediators causing axonal damage and demyelination (Nylander A., 2012). Then, $CD8^+$ T cells are also regarded as potent effector cells for CNS damage as these cells are involved in the axonal damage by directly attacking neurons and oligodendrocytes through their cytotoxic and proinflammatory properties (Salou et al., 2015).

Previously MS pathogenesis was thought to be mainly driven by $CD4^+$ effector T cells; however, several immunological studies found other immune entities contributing to the disease pathogenesis, such as interleukin (IL)-17-producing T helper (Th) 17 cells, B cells, plasma cells, $CD8^+$ T cells, and both $CD4^+$ and $CD8^+$ T-regulatory (Treg) cells (Selter & Hemmer, 2013). Therefore, currently MS is defined as Th1, Th17 mediated autoimmune disease, and rather not just the Th1 mediated process (Hernandez-Pedro et al., 2013; Jadidi-Niaragh & Mirshafiey, 2011). Increasing evidence suggests that programmed cell death (apoptosis) also contribute to the pathology and tissue damage in MS, which occur either in the brain or in the peripheral level (Macchi et al., 2015; Mc Guire et al., 2011). MS immunopathogenesis consists of mainly three events: activation of immune cells in the periphery, transmigration of such cells into the CNS, and neural tissue damage (Comabella & Khoury, 2012).

2.5.1 T cell activation and proliferation

The essential component in the activation of $CD4^+$ T cells is the interaction between antigen presenting cells (APCs) with T lymphocytes (Selter & Hemmer, 2013). Dendritic cells (DCs) are the primary APCs that are activated via toll-like receptors

(TLRs) and recognize specific microbial or viral antigens (Hartung et al., 2014). After activation, APCs interact with $CD4^+$ T cells through T-cell receptors (TCRs) that recognize major histocompatibility complex (MHC) class II molecules on the APCs (Grakoui et al., 1999). Thus, this first interaction between TCR and APCs in the form of peptides bound histocompatibility molecules provides the first signal. The interaction between MHC II and TCR activates CD40 ligand on the surface of T-cells and binds to its CD40 receptor present on the surface of APCs resulting the upregulation of CD80 and CD86 molecules. These molecules then interact with CD28 and CTLA4 molecules on the surface of T cell to generate a second signal (Kasper & Shoemaker, 2010). This second signal, also called costimulatory signal, is required for the optimal activation of T cells (Kasper & Shoemaker, 2010; Loma & Heyman, 2011; Selter & Hemmer, 2013; Sharpe & Abbas, 2006). Additional third signal for the optimal activation of T cells can be provided through cytokine signaling (Kambayashi & Laufer, 2014). Schematic diagram of T cell activation is presented in Figure 2A. Naïve $CD4^+$T cells after activation differentiate into distinct T helper subsets such as Th1, Th2, Th17, and Tregs cells depending mainly upon the cytokine milieu of the microenvironment, and produce lineage-specific cytokines (Figure 2B)(Han et al., 2015; Zhu, 2017). Unlike $CD4^+$ T cells, $CD8^+$ T cells can directly interact with MHC class I/APCs and mediate damage of neurons and oligodendrocytes (Salou et al., 2015).

2.5.2 Costimulatory molecules

The CD80/CD86–CD28/CTLA4 are the most important and well known costimulatory molecules (Slavik et al., 1999), but several other costimulatory molecules, such as CD26 and CD30 are responsible for the optimal activation of T cells (Del Prete et al., 1995; Tanaka et al., 1993). These molecules are regarded as markers of Th1 and Th2 lymphocyte activation, respectively (Del Prete et al., 1995; Jafari-Shakib et al., 2009; Romagnani et al., 1995). These multifunctional proteins are expressed on different cell types and play important role in MS and in several other autoimmune diseases (Aliyari Serej et al., 2017; Kim et al., 2015; Morimoto & Schlossman, 1998; Ohnuma et al., 2011; Shinoda et al., 2015; Steinbrecher et al., 2001; Tejera-Alhambra et al., 2014). Several other ligands and receptors interactions also provide costimulatory signals to T cells, for example, TNF-like ligand 1A (TL1A), and its two receptors, i.e. death domain receptor 3 (DR3, TNFRSF25) and decoy receptor 3 (DcR3, TNFRSF6B). These ligand-receptors interactions mediate

various signaling pathways to maintain immune homeostasis and regulate the pathology of various autoimmune diseases (Meylan et al., 2008; Meylan et al., 2011; Richard et al., 2015; Sonar & Lal, 2015). The widely studied TNF superfamily molecules that provide costimulatory signals to activated T cells include tumor necrosis factor receptor 2 (TNFR2, TNFRSF1B), OX40 (CD134, TNFRSF4) and 4-1BB (CD137, TNFRSF9) (Ward-Kavanagh et al., 2016). Further, costimulatory or coinhibitory signals based on the receptor-ligand interactions are essential for innate and adaptive immune responses and are shown to be involved in several chronic inflammatory diseases including MS (Sonar & Lal, 2015).

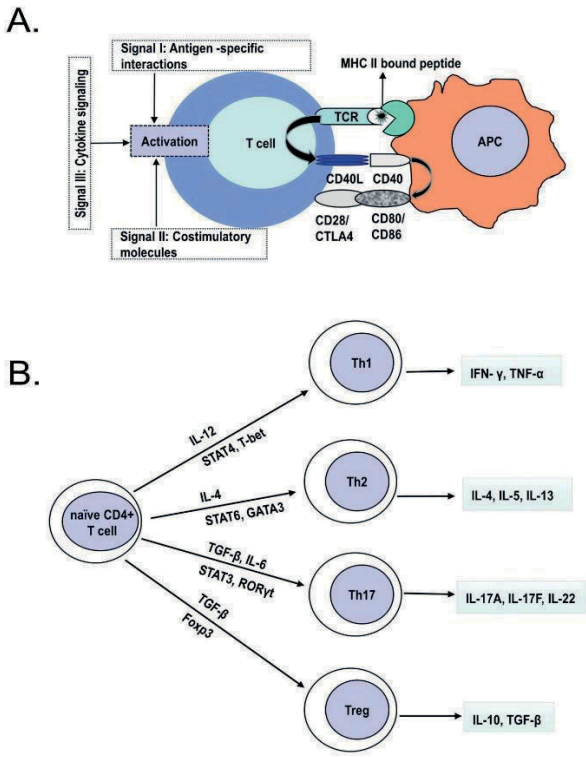

Figure 2. **T cell activation and proliferation. A.** Schematic representation of T cell activation. **B.** T cell differentiation. Th1 cells release proinflammatory cytokines such as interferon-gamma (IFN- γ), interleukin (IL)-2, and tumor necrosis factor-a (TNF-α). Th2 cells secrete regulatory cytokines such as IL-4, IL-5, and IL-10. Th17 cells secrete proinflammatory cytokines such as IL-17A and IL-17F. Underneath each arrow are the master transcription factors, which are expressed on each cell subsets and are required for the lineage commitment. Abbreviations: APC, Antigen presenting cell; TCR, T cell receptor; Foxp3, forkhead box protein 3; GATA-3, GATA-binding protein 3; RORγT, retinoic acid receptor-related orphan receptor; STAT, signal transducer and activator of transcription. Redrawn with permission from publisher (Kambayashi & Laufer, 2014; Comabella & Khoury, 2012).

2.5.3 Transmigration of immune cells to the CNS

The tight junctions between the endothelial cells of the BBB and the epithelial cells of the blood-CSF barrier limit the access of immune cells into the CNS (Ransohoff et al., 2003). Transmigration of autoreactive T cells across the BBB into the CNS is mediated by cell adhesion molecules (CAMs), chemokines, and matrix metalloproteinases (MMPs) expressed on lymphocytes (Engelhardt et al., 2001; Engelhardt, 2008; Engelhardt, 2010). MMPs are the proteolytic enzymes that disrupt the BBB by degrading the extracellular matrix and basement membranes (Comabella & Khoury, 2012). It is considered that in MS, initially the primary adhesion molecule α4β1-integrins or very late activation antigen-4 (VLA-4) expressed on the surface of activated lymphocytes interact with vascular cell adhesion molecule-1 (VCAM-1) expressed on the capillary endothelial cells (Engelhardt, 2008). This interaction is facilitated by the MMPs, and chemokines and its receptors along with other inflammatory mediators regulate the extravasation of immune cells from the periphery to CNS (Engelhardt, 2008). Classical leukocyte adhesion cascade starts from activation to transmigration and consist of four steps. i) capturing and rolling ii) activation iii) arrest and iv) diapedesis or transmigration (Luster et al., 2005). However additional steps have been integrated into this sequence such as capture or tethering, slow rolling, adhesion strengthening and spreading, intravascular crawling, and paracellular and transcellular transmigration (Engelhardt, 2010; Ley et al., 2007).

2.5.4 Mechanisms of CNS tissue damage

In CNS, activation of macrophage and microglia produce several cytotoxic molecules that promote CNS tissue injury and are abundantly present in MS lesions (Hendriks et al., 2005). Activated microglia promotes CNS inflammation by releasing proinflammatory IL-1β and TNF-α, and reactive oxygen species (ROS) and nitric oxide (NO) radicals (Bogie et al., 2014; Hendriks et al., 2005; Lassmann & van Horssen, 2011). These radicals cause the oxidative injury of oligodendrocytes and neurons (Miller et al., 2013). Oxidative stress, one of the most important mechanisms of tissue injury, leads to mitochondrial injury/dysfunction, which causes energy deficiency or virtual hypoxia initiating a cascade of deleterious events contributing to axonal degeneration in MS (Witte et al., 2014). Thus, the major cause of degeneration of chronically demyelinated axons includes an imbalance between energy demand and energy supply (Dutta & Trapp, 2014). Other components such as glutamate excitotoxicity, complement activation, proteolytic and lipolytic

enzymes, and T cell-mediated injury via T cell products contribute to oligodendrocyte, myelin, and axonal damage (Popescu et al., 2013). B-cells, plasma cells, and abundant immunoglobulins are involved in the pathology of tissue damage in MS (Cross & Wu, 2010; Cross & Waubant, 2011; Wekerle, 2017). B cells contribute to demyelination and neurodegeneration due to its role in antigen presentation, autoantibody production, cytokine regulation, and the formation of ectopic lymphoid follicles in the meninges (Howell et al., 2011; Li et al., 2015; Serafini et al., 2004). B cells travel out from the CNS and undergo affinity maturation in the lymph nodes, and re-enter to CNS mediating further damage (Dendrou et al., 2015). Moreover, apoptotic processes are also involved in the extensive cell death of oligodendrocytes, which leads to demyelination (Macchi et al., 2015; Moreno et al., 2014). Other mechanisms driving tissue damage in MS include alternation in intra - axonal ion homeostasis, imbalance of microbial community, and age-dependent iron accumulation within the brain tissue (J. Chen et al., 2016; Lassmann, 2013; Levy et al., 2017; Su et al., 2013; Witte et al., 2014). Different immunological mechanisms play important roles in the dysregulation of the immune system inside the CNS during the early and late phase of MS, which is presented in Figure 3.

2.6 MRI in multiple sclerosis

MRI is the most sensitive noninvasive tool for characterizing MS lesion profiles, detecting asymptomatic dissemination of lesions in space (DIS) and time (DIT), and it is helpful in discriminating the inflammatory and neurodegenerative processes in the brain and spinal cord (Thompson et al., 2018). MRI is useful in the assessment of disease diagnosis, evaluating disease activity and disease progression, and therapeutic monitoring (Baecher-Allan et al., 2018; Reich et al., 2018). The inflammatory element of MS is seen as gadolinium-enhancing lesions reflecting the breakdown of BBB and the movement of cells into the CNS and accumulation of disease burden (Lublin, 2014). Conventional MRI provides information on the number and distribution of focal T2 lesions and contrast-enhancing WM lesions, but it is unable to detect the actual burden of GM lesions (Kaunzner & Gauthier, 2017).

Different types of MRI images provide different information regarding disease pathology. T1-weighted and gadolinium-enhanced images reveal the presence of active lesions defining active inflammation, T2-weighted images provide information on disease burden or lesion load detecting hyperintense WM lesions, FLAIR (fluid attenuated inversion recovery) images quantify lesion and help to visualize T2

hyperintense WM lesions (Bakshi et al., 2008; Fox et al., 2011). Other advanced quantitative MR based techniques such as magnetization transfer ratio imaging (MTR), diffusion tensor imaging (DTI), functional MRI (fMRI), have improved disease diagnosis and monitoring, as well as increased deeper understanding of MS pathophysiology (Fox et al., 2011). In recent times, MRI protocols have been updated and improved and recent guidelines have been developed to facilitate the early diagnosis of MS (Dutta & Trapp, 2014; Kaunzner & Gauthier, 2017; Thompson et al., 2018).

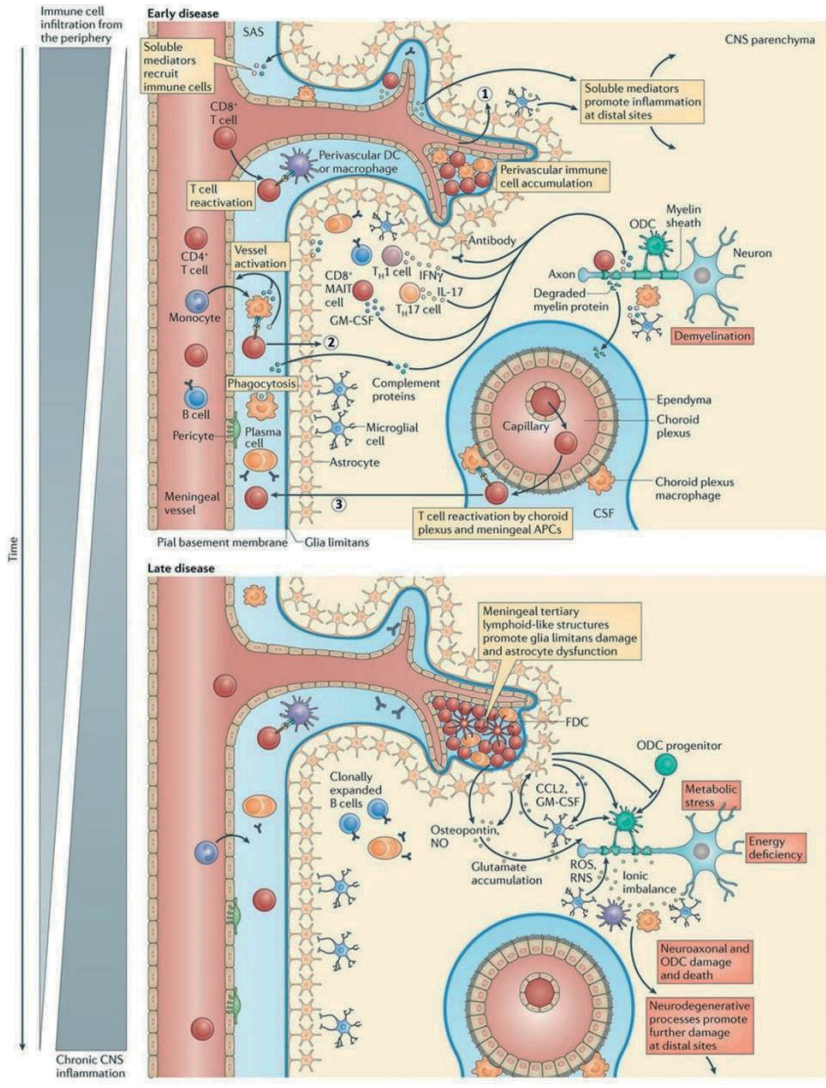

Figure 3. **Immune system dysregulation inside the CNS in early and late MS.** Early-stage MS is characterized by the immune cell infiltration from the periphery because of BBB breakdown. Peripheral immune cells together with activated CNS-resident cells mediate demyelination and ODC damage through several effects (Top panel). In the late stage, immune cell infiltration diminishes but chronic CNS-intrinsic inflammation and neurodegeneration persist. Meningeal follicles like structures may promote to late-stage inflammation in SPMS. Stimulated astrocytes secrete CCL2 and GM-CSF leading to microglial recruitment and activation. The astrocytes inhibit maturation of ODC and therefore prevents remyelination (Bottom panel). Abbreviations: APC, antigen-presenting cell; CD8+ MAIT cell, CD8+ mucosa-associated invariant T cell; CCL2, CC-chemokine ligand 2; FDC, follicular dendritic cell; GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN-γ, interferon-γ; IL-17, interleukin-17; NO, nitric oxide; ODC, oligodendrocyte; RNS, reactive nitrogen species; ROS, reactive oxygen species; Th1 cell, T helper 1 cell. Reproduced with the permission from the publisher (Dendrou et al., 2015).

2.7 Disease-modifying therapies in multiple sclerosis

The DMTs are available for MS disease; those reduce the number of relapses, manage disease symptoms, and partially control disability progression (Pardo & Jones, 2017). Currently there are eleven drugs approved for the treatment of MS (Table 1, excluding the off-label drugs) and all these drugs have immunomodulatory functions. In Finland, according to current care guidelines, interferon-β, dimethyl fumarate, glatiramer acetate, and teriflunomide are used as first-line DMTs or for active RRMS, whereas NTZ, fingolimod and alemtuzumab are used as second-line or very active therapies for MS. These drugs provide mainly anti-inflammatory effects and are more effective in the early phase of disease, but they have no significant benefit on progressive MS (Loma & Heyman, 2011; Torkildsen et al., 2016). In addition to these therapies, recently a drug called ocrelizumab has been approved for the treatment of RRMS and early PPMS patients (Sorensen & Blinkenberg, 2016). Initially MS patients are treated with drugs indicated by disease activity and careful risk-benefit stratification (Torkildsen et al., 2016) and if the patient fails to respond adequately to this first-line therapy, the use of second-line therapies should be considered (Hartung et al., 2011; Sorensen, 2011). In addition, it is suggested that oral agents dimethyl fumarate or teriflunomide should be evaluated as chosen among the other drugs for *de novo* RRMS based on the risk-benefit ratio of the approved therapies (Freedman et al., 2016; Ochi, 2015). The drugs generally differ as to efficacy, tolerance, and safety issues. Therapies for very aggressive disease are associated with an increased risk of opportunistic infections and other major adverse effects including JCPyV induced progressive PML (Clifford et al., 2010). Development of new drugs is aimed for achieving a disease-free state in patients, with no relapses, no increase in EDSS and no new or active lesions on the MRI scans. The term referred for freedom from disease activity is called no evidence of disease activity (NEDA) (Giovannoni et al., 2015; Rotstein et al., 2015; Ziemssen et al., 2016). Different disease-modifying therapies in MS are listed in Table 1

Table 1. Disease-modifying therapies in multiple sclerosis

Approved Drugs (market name)	Drug class (year of approval)	Route	Effect on immune cells
Interferon beta-1b (Betaseron)	Type I interferons (1995)	SC	Decrease migration of inflammatory cell across the BBB, decrease the production of proinflammatory cytokines, and induce anti-inflammatory cytokines. Stabilization of BBB
Interferon beta-1a (Avonex)		IM	Same as above
Interferon beta-1a (Rebif)		SC	Same as above
Pegylated interferon beta-1a (Plegridy)		SC	Same as above
Glatiramer acetate (Copaxone)	Random amino acid copolymer (1996)	SC	Preferential differentiation of $CD4^+$ T cells into Th2 cells, an increase in the number of regulatory T cells, modulation of $CD8^+$ T cells, and also the development of regulatory type II APCs
Teriflunomide (Aubagio)	Pyrimidine synthesis Inhibitor (2012)	Oral	Blocks proliferation of activated T cells
Dimethyl fumarate (Tecfidera)	Possible Nrf2 pathway activator and NFκB inhibitor (2013)	Oral	Protects against oxidative stress
Fingolimod (Gilenya)	Sphingosine-1-phosphate receptor modulator (2010)	Oral	Blocks lymphocyte egress from lymph nodes
Natalizumab (Tysabri)	Monoclonal antibody (2003)	IV	Selectively inhibits α4β1 integrins and prevents leukocyte migration across the BBB
Alemtuzumab (Lemtrada)	Monoclonal antibody (2014)	IV	Targets CD52 on lymphocytes and monocytes, and depletes B cells, T cells, monocytes, macrophages
Mitoxantrone	Immunosuppressive drug (2003)	IV	Inhibits DNA synthesis and repair by blocking type II topoisomerase. Decreases lymphocyte count
Azathioprine* (Imuran)	Immunosuppressive drug (2007)	Oral	Purine analog which is metabolized to 6-mercaptopurine and thioinosine acid, that compete with DNA nucleotides
Cyclophosphamide*	Immunosuppressive drug (2014)	IV	Suppresses IL-12 and Th1-type responses and enhancesTh2/Th3 responses
Ocrelizumab (Ocrevus)	Targets CD20 on the surface of B cells (2017)	IV	Prolonged depletion of $CD20^+$ B cells
Cladribine (Mavenclad)	Anti-metabolite (2017)	Oral	Inhibits DNA synthesis and blocks proliferation of activated T and B cells
Rituximab (Rituxan)*	Chimeric monoclonal antibody (1997)	IV	Depletes $CD20^+$ B cells through a combination of cell-mediated and complement-dependent cytotoxic effects

*Drugs for off-label use in most countries. Abbreviations: CIS, clinically isolated syndrome; IL, interleukin; IM, intramuscular; IV, intravenous; SC, subcutaneous; MHC, major histocompatibility complex; MS, multiple sclerosis; PRMS, progressive- relapsing MS; RRMS, relapsing-remitting MS; SPMS, secondary-progressive MS; Th, T helper. The table is based on the following reviews (Bielekova & Martin, 2004; Fox & Rhoades, 2012; Gajofatto & Benedetti, 2015; Pardo & Jones, 2017).

2.7.1 Safety aspects of MS treatments

Number of DMTs are available for the treatment of MS, however many are associated with the adverse effects ranging from mild to serious complications. Adverse events associated with interferon-beta treatment includes hepatic injury, anaphylaxis, depression, and some cases of acute liver failure and pancreatitis (Torkildsen et al., 2016). The treatment may induce the formation of specific neutralizing antibodies reducing the efficacy of treatment. Glatiramer acetate is a well-tolerated drug but most patients (65%) experience injection site reactions and other side effects include cutaneous necrosis, lymphadenopathy, dyspnoea and lipoatrophy, among which lipoatrophy is the most severe one (Torkildsen et al., 2016). Teriflunomide treatment is associated with hepatic injury, teratogenicity, potential immunosuppression, infection, and peripheral neuropathy. Dimethyl fumarate treatment may cause anaphylaxis and angioedema, PML, and lymphopenia. Rare side effects associated with fingolimod include myocardial infarction, PML, and opportunistic herpes simplex virus (HSV) or cryptococcus infections (Gajofatto & Benedetti, 2015). Treatment with mitoxantrone is associated with congestive heart failure, amenorrhoea and acute promyelocytic leukemia (Pardo & Jones, 2017). Recently developed ocrelizumab is associated with infusion-related reactions, infections, and neoplasms (Gajofatto & Benedetti, 2015). Most importantly, potentially life-threatening side effects associated with MS drugs include PML, which is associated with the treatment with natalizumab and other new drugs. Other risks associated with NTZ- treatment include infusion reactions, hepatotoxicity and rarely other serious opportunistic infections. NTZ-associated PML risk has become a major challenge in the treatment of MS because, in addition to NTZ, also other effective biological therapies such as fingolimod and dimethyl fumarate were reported to carry the risk of PML in MS patients (Faulkner, 2015). Potential MS drugs, which are associated with high, low, no or very low risk of PML, are listed in Table 2. Recently the new practice guideline has been developed on the efficacy and safety of DMTs in MS (Rae-Grant et al., 2018a), and thirty recommendations were made concerning starting, switching, and stopping DMTs pertinent to people with RRMS, SPMS, PPMS, and CIS of demyelination (Rae-Grant et al., 2018b).

Table 2. A PML risk stratification table for disease-modifying therapies for MS

Therapeutic Agent	Treated condition predisposes to PML?	Latency from the time of drug initiation to PML	Frequency/Incidence of PML
Class I – high potential PML risk	**No**	**Yes**	**High**
Natalizumab	MS and Crohn's disease	None<8 months; >85% of cases >24 months	1/100–1/1000
Class II – low potential PML risk	**No**	**Yes**	**Low/infrequent**
Dimethyl fumarate	MS and psoriasis	18–54 months	~1/50,000
Fingolimod	MS	18–54 months	~1/18,000
Class III – no or very low potential PML risk	**Yes**	**No**	**Very low or evident only with related drug**
Alemtuzumab Rituximab Mitoxantrone Teriflunomide Daclizumab		Unknown; no cases with MS 1/30,000	

Abbreviations: PML-progressive multifocal leukoencephalopathy. Modified with permission from (Berger, 2017).

2.7.2 Natalizumab

Natalizumab (NTZ) is the first humanized monoclonal antibody for the treatment of RRMS and received the Food and Drug Administration (FDA) approval in 2004 (Polman et al., 2006). It is generally recommended as a second-line therapy for those patients who have not responded to first-line DMTs or who have very active disease (Kappos et al., 2011). NTZ reduces the relapse rate, decreases sustained disability, and reduces the number of new lesions on MRI (Miller et al., 2003; Polman et al., 2006). NTZ has also shown its efficacy in the reduction of rate of brain volume loss and enhancement of tissue integrity (Sellebjerg et al., 2016). In a two-year phase 3 clinical trial of NTZ in RRMS patients (AFFIRM study), it reduced the risk of sustained progression of disability by 42% over two years, and reduced the rate of clinical relapse at one year by 68%, and 83 % reduction in the accumulation of new or enlarging hyperintense lesions (Polman et al., 2006). According to the current treatment guidelines, the recommended dose of NTZ for MS patients is standard 300 mg and administered intravenously over one hour every four weeks (Engelhardt & Kappos, 2008; Sheremata et al., 1999; Vollmer et al., 2004).

2.7.2.1 Mode of action

NTZ blocks the entry of inflammatory cells into the CNS across the BBB. It inhibits the interaction of α4-integrin subunit of α4β1 with VCAM-1 and of α4β7 with Mucosal addressin cell adhesion molecule-1 (MAdCAM-1), resulting in the blockage of adhesion processes of leukocytes to endothelial cells and consequently the migration of autoreactive T cells to the CNS (Hutchinson, 2007; Selewski et al., 2010). Transmigration of leukocytes into the CNS includes several steps, which was described in chapter 2.6. Since VCAM-1 is expressed on inflamed cerebrovascular endothelial cells, α4β1 is thought to be the important target of NTZ in preventing leukocyte migration into the CNS tissue in MS (Engelhardt & Kappos, 2008). NTZ is also known to alleviate ongoing CNS inflammation, by interrupting the interactions between α4-integrin-expressing leukocytes and extracellular matrix proteins such as osteopontin and fibronectin (Hutchinson, 2007). In 2008, NTZ was approved also for the treatment of Crohn's disease. The possible mechanism of action is the blockage of interaction of α4β7 with MAdCAM-1, resulting in the inhibition of the transendothelial migration of lymphocytes into the intestinal mucosa (Guagnozzi & Caprilli, 2008).

2.7.3 JC virus and PML

JC polyomavirus (JCPyV, previously named JCV) belongs to human polyomaviruses, which are nonenveloped, icosahedral viruses with a closed circular, supercoiled, double-stranded DNA genome of approximately 5130 nucleotides (Wollebo et al., 2015). The viral genome is divided into an early and a late region encoded on opposite strands. In between these two regions contains a non-coding control region (NCCR) which encodes the origin of replication (Ferenczy et al., 2012). The early region encodes two proteins, large T (tumor) antigen and small t antigen, which play regulatory functions during viral replication cycle. The late region is expressed later in the viral life cycle and it encodes three capsid proteins VP1, VP2 and VP3 forming the viral capsid, and agnoprotein responsible for DNA repair and cell cycle regulation (Ferenczy et al., 2012). Agnoprotein is an auxiliary protein in the viral late region, which have multiple functions ranging from viral transcriptional regulation to the inhibition of host DNA repair acting as a viroporin (Suzuki et al., 2010). The regulatory region NCCR of JCPyV is variable and two different types can be identified: one is archetypal form (non-pathogenic) which is shed in the urine from normal individuals, and another is neurotropic form (pathogenic) (Jelcic et al., 2015).

PML pathogenesis is almost exclusively associated with viral strains harboring neurotropic NCCR. Most probably, the originally acquired archetype virus mutates in the brain to gain the ability to replicate efficiently in glial cells. These mutant strains are found in blood and CSF, and very rarely in the urine of PML patients (Reid et al., 2011).

Respiratory inhalation was thought to be the possible route of JCPyV and tonsillar lymphocytes are considered as the primary target of initial infection. The primary infection is encountered mostly in childhood and occasionally in adult age in 60–80% of the human population and asymptomatic lifelong persistent infection is established (Ferenczy et al., 2012). However, under immunosuppressive conditions, the latent virus may reactivate, access the brain and gain neurotropic mutations, or first gain neurotropic mutations and then enter the brain, where it can replicate efficiently due to these mutations. In glial cells, JCPyV may cause a fatal demyelinating lytic infection in CNS called progressive multifocal leukoencephalopathy (PML) (Khalili et al., 2007). Reactivation and replication of JCPyV are characterized by a lytic infection of glial cells, particularly oligodendrocytes, astrocytes and neuronal cells in the CNS (Ferenczy et al., 2012; Wollebo et al., 2015).

2.7.4 Clinical manifestations and diagnosis of PML

Clinical manifestations that are consistent with PML include cognitive deficits, language problems, personality changes, motor dysfunction, visual changes, headaches, and seizures (Available at: https://pmlconsortium.org/healthcare-professionals/diagnosis/). Pathologically PML is associated with unifocal or multifocal brain lesions and its diagnostic process includes clinical examinations, MRI findings, and detection of JCPyV DNA in CSF or brain biopsy (Wollebo et al., 2015). Detection of JCPyV DNA in CSF is the widely employed approach; however, there are reports of false-negative results, although the disease course is progressive and radiological findings are indicative of PML (Babi et al., 2015). Other diagnostic approaches include detection of viral protein by immunohistochemistry and western blotting, characterization of brain lesions by histopathology on biopsy or autopsy materials (White et al., 2016). The PML diagnostic criteria has been developed for the definite diagnosis of PML, which includes the characterization of neuropathologic feature of typical histopathologic triad (demyelination, bizarre astrocytes, and enlarged oligodendroglial nuclei) along with the tools to display the

presence of virus (Berger et al., 2013; Wollebo et al., 2015). There is no cure for PML but currently employed risk stratification method has highly improved clinical decision-making process (Singer, 2017).

Currently onset of PML is defined as the time at which virus enters the brain and infects oligodendrocytes, that eventually leads to a clinically severe brain injury which is initially not detectable on MRI (Major et al., 2018). Primarily, PML was an AIDS-defining illness and it is the major cause of death in 3 to 5 patients with AIDS. More recently, however, the risk of PML has been also associated with current immunomodulatory treatments such as NTZ- treatment for MS (Major & Douek, 2013). Until August 31, 2017, the global overall incidence of NTZ-associated PML was reported as 4.22 per 1000 patients (Biogen 2017).

Due to the two cases of NTZ- associated brain infection, PML was identified in patients during two-year, phase 3 clinical trial (SENTINEL) (Rudick et al., 2006), it was removed after 3 months of its approval from the market in 2005, but was re-introduced in 2006 with a mandatory surveillance program called Tysabri Outreach Unified Commitment to Health (TOUCH) (Foley, 2010). As of September 1, 2017, 749 PML cases were confirmed, among which 746 PML cases were related with MS, and 3 with Chron's disease (Biogen 2017, https://medinfo.biogen.com). Recently a phase 3 clinical trial (ASCEND) was conducted to assess the effect of NTZ in disease progression in patients with SPMS (Kapoor et al., 2018).

2.7.5 Mechanism of natalizumab-associated PML

The exact mechanism of NTZ-associated PML is still unclear, but it is suggested that PML develops due to impaired CNS immunosurveillance (Mancuso et al., 2012). It has been proposed that blocking of T and B cells migration across the BBB into the CNS interferes with cell-mediated immunity and immunosurveillance, allowing JCPyV reactivation from latency (Berger & Houff, 2009). Thus, in the absence of functional $CD4^+$ cells and JCPyV-specific $CD8^+$ effector cells in the CNS, it is not possible to control JCPyV replication and dissemination, and this phenomenon increases the risk of developing PML (Antoniol & Stankoff, 2015). In addition, NTZ was shown to have an effect in the mobilization of mononuclear cells that harbor JCPyV DNA from the bone marrow and convey the virus into the CNS (Mancuso et al., 2012). A study had found JC virus in $CD34^+$ and $CD19^+$ cells from MS patients, suggesting that these mononuclear cells in circulation harbor JCPyV DNA in NTZ-treated MS patients, contributing to the pathogenesis of PML (Frohman et

al., 2014). NTZ-treatment also increases B cell differentiating factor Spi-B in the peripheral blood, and this Spi-B could upregulate JCPyV gene expression (and increase viral transcription) and viral replication, leading to lytic infection and tissue damage that ultimately increases the risk of developing PML (Marshall et al., 2010).

2.8 Biomarkers of natalizumab-associated PML

2.8.1 Anti-JCPyV antibodies

Currently, quantification of anti-JCPyV antibodies, measured as JCPyV index, in serum or plasma is the only available biomarker used for the PML risk stratification in patients treated with NTZ (Campagnolo et al., 2016; Cutter & Stuve, 2014; Werner & Huang, 2016). Development of PML in long-term NTZ treated patients was shown to be associated with the presence of these antibodies in the blood (Bloomgren et al., 2012; Calabresi et al., 2007). The rationale behind the determination of anti-JCPyV antibody levels is to classify the individuals with an indication of past viral exposure and those individuals who have not encountered the virus. Higher anti-JCPyV antibody level, or index, indicates higher PML risk, and earlier studies have demonstrated higher antibody levels in NTZ-associated PML compared to non-PML patients (Lee et al., 2013; Outteryck et al., 2013; Trampe et al., 2012). Anti-JCPyV antibody positivity is a risk factor for the developing risk of PML and quantification of these antibodies enables the stratification of PML risk in those patients who are JCPyV-seropositive (Faulkner, 2015). These antibodies are measured as anti-JCPyV antibodies indices in serum or plasma, with a confirmatory second-generation ELISA know as STRATIFY JCPyV™ DxSelect™, and Focus Diagnostics provides STRATIFY JCPyV testing service, exclusively licensed from Biogen, for PML risk stratification in NTZ-treated MS patients (Lee et al., 2013). In the assay, the anti-JCPyV antibody levels in a patient sample are compared with a standard sample representing robust antibody levels, whose index value has been set to 1. JCPyV index allows stratification of PML risk as high and low in NTZ-treated MS patients without prior use of immunosuppressants: patients with JCPyV index more than 1.5 are considered as higher risk for developing PML and with JCPyV index less than 1.5 as lower risk (Lee et al., 2013). Although patients have JCPyV index >1.5, PML risk is low during 1–24 months of NTZ-treatment with an estimated risk of 1.17/1000 but increases noticeably to 8.83/1000 in months 25–48

of treatment and increases to 10.12/1000 (or 1 in 99) from 49–72 months of treatment (McGuigan et al., 2016). Stratification of PML risk estimation based on the duration of NTZ-treatment and prior use of immunosuppressants are given in Table 3.

Table 3. Stratification of PML risk estimation in Natalizumab-treated MS patients who are anti-JCPyV-antibody positive.

Duration of Natalizumab treatment	Prior use of immunosuppressive drugs?	
	Yes	No
1-24 months	0.1%	<0.1%
25-48 months	1.2%	0.3%
49-72 months	1.3%	0.6%

PML risk estimates obtained from medinfo.biogen.com, 2018

2.8.2 L-Selectin-expressing $CD4^+$ T cells in peripheral blood

L-Selectin (CD62L) is a cell adhesion molecule, which plays an important role during leucocyte transmigration by mediating initial capturing and tethering of leucocytes from the blood vessels to the sites of inflammation (Rainer, 2002; Telen, 2014). Several ligands expressed on endothelial cells are known to bind L-selectin (Rainer, 2002; Tu et al., 2002) and among such ligands, P-selectin glycoprotein ligand-1 (PSGL-1) is regarded as a major ligand (Rainer, 2002). During inflammation, L-selectin mediates leukocyte–leukocyte interactions using PSGL-1 (Sperandio et al., 2003). Naive T cells express high levels of surface L-selectin and upon activation by antigens, these T cells rapidly divide and differentiate into L-selectin ^{low} effector cells resulting in the homing of T cells into the sites of inflammation (Raffler et al., 2005; Wedepohl et al., 2012). Surface L-selectin upon activation is rapidly shed from the cell surface by endoproteolytic cleavage (Wedepohl et al., 2012; Wang et al., 2010) and remains as a functionally active soluble form in the blood (Schleiffenbaum et al., 1992). sL-selectin significantly increases during acute or chronic inflammation (Smalley & Ley, 2005). Previous studies have reported the role of L-selectin in MS pathogenesis, particularly in the process of myelin damage in the CNS (Grewal et al.,

2001; Huang, Kikuta, & Rosen, 1994). Some clinical studies have detected increased levels of sL-selectin in serum and CSF of MS patients (Baraczka et al., 2000; Duran et al., 1999), and correlated with MRI findings (Hartung et al., 1995; Mossner et al., 1996).

Previously, a retrospective study proposed L-selectin-expressing $CD4^+$ T cells in peripheral blood as a biomarker of developing NTZ-associated PML risk (Schwab et al., 2013). This study found reduced levels of L-selectin-expressing $CD4^+$ T cells in long-term NTZ -treated MS patients compared to those patients who were not treated with NTZ, and healthy controls (Schwab et al., 2013). In addition, a study found the link between soluble L-selectin and JCPyV seropositivity, suggesting that soluble L-selectin could be a biomarker of PML risk (Basnyat et al., 2015a). Recently a multicentric study in international cohorts of NTZ-treated MS patients showed that utilization of both anti-JCPyV-antibody index and L-selectin could strongly reduce PML incidence up to 10-fold (Schwab et al., 2016). However, a recent study on a well-controlled cohort of NTZ-treated patients reported that the percentage of surface L-selectin is not a reliable biomarker NTZ-associated PML risk (Lieberman et al., 2016).

2.8.3 JCPyV microRNAs

MicroRNAs (miRNAs) are small, 18-25 nucleotide (nt) long, single stranded noncoding regulatory molecules, which downregulate gene expression by binding to target gene mRNA (Lagatie et al., 2013). The miRNAs exhibit high diagnostic, prognostic and therapeutic potential and can serve as potential biomarkers because of their small size, higher stability, and relative ease of detection in a variety of biological tissues and body fluids (Auvinen, 2016). JCPyV encodes a pre-miRNA, which is processed into two mature JCPyV-specific miRNAs (JCPyV-miR-J1-5p and JCPyV-miR-J1-3p) that appear late in the infection to autoregulate the early gene expression (Seo et al., 2008). Out of 13 human polyomaviruses known till date, only BK polyomavirus (BKPyV), JCPyV, and Merkel cell polyomavirus (MCPyV) encode 5p and 3p miRNAs, which are cleaved from one common precursor transcript (Auvinen, 2016). In the case of JCPyV, the sequence of JCPyV-miR-J1-5p is unique for JCPyV, but JCPyV-miR-J1-3p shares identical sequence with BKPyV-encoded bkv-miR-B1-3p, and thus its origin cannot be differentiated (Seo et al., 2008). These microRNAs are known to play a key role in controlling viral replication through downregulation of Large T-Antigen expression (Seo et al., 2008), and also control

the killer receptor NKG2D-mediated killing of virus-infected cells by NK cells through downregulation of the stress-induced ligand ULBP3 (Bauman et al., 2011). Recent studies have suggested potential of JCPyV-miR-J1-5p as a biomarker of past JCPyV infection as it can be frequently detected in plasma, urine, and CSF of both JCPyV seropositive and seronegative healthy individuals and immunosuppressed patients (Lagatie et al., 2014a; Pietila et al., 2015). Moreover, exosomal JCPyV miRNAs extracted from biological fluids have been studied for their biomarker potential of viral infection (Martelli & Giannecchini, 2017), particularly in NTZ-treated MS patients to identify the possible mechanisms of viral reactivation leading to development of PML (Giovannelli et al., 2015; Skarica et al., 2011).

2.9 Potential biomarkers in multiple sclerosis

High degree of heterogeneity in pathophysiological processes has made clinical management of MS challenging (Bielekova & Martin, 2004; Comabella & Montalban, 2014). Therefore sensitive and reliable biomarkers are needed for early diagnosis, establishing long-term prognosis, measurement of disease activity, predicting therapeutic response to treatments and potential adverse events associated with the specific treatment (Buck & Hemmer, 2014; Polman et al., 2011; Teunissen et al., 2015). A biomarker is described as a measurable indicator, which can reflect the normal biologic and pathogenic processes, and the pharmacological responses to a therapeutic intervention (Biomarkers Definitions Working Group., 2001), and an ideal biomarker is the one which is present in all MS patients but absent in other individuals (Comabella & Montalban, 2014; Tomioka & Matsui, 2014). Biomarker discovery area is very active in MS and aims to find a reliable and precise biomarker that can stratify different MS subtypes, evaluate inflammatory activity, degree of demyelination and neurodegeneration, remyelination, and depict the accurate picture of clinical status (Bielekova & Martin, 2004; Buck & Hemmer, 2014; Comabella & Montalban, 2014). There are many exploratory biomarkers available in MS, however, only a few are validated and are used in clinical practice. This fact suggests that the goal of personalized medicine in MS is still in its infancy stage. Biomarker development is a long process, which takes approximately more than 20 years from the time of biomarker discovery until its clinical implementation (Teunissen et al., 2015).

2.9.1 Biomarker samples

Different specimens such as blood, CSF, urine, tears, and saliva can be used as sources of body fluid biomarkers among which blood and CSF are the most used body fluids (Dobson, Topping et al., 2013; Gebregiworgis et al., 2013; Giovannoni & Thompson, 1998; Oehninger-Gatti et al., 2000). CSF samples are more promising for biomarker discovery due to its proximity to the CNS and hence show better pathological processes, however, multiple samples from the same patient are restricted due to the invasive sample collection procedure (Comabella & Montalban, 2014). Thus, blood-based biomarkers exhibit greater clinical value due to the easy and minimally noninvasive method of collection (D'Ambrosio et al., 2015). Blood biomarkers can provide crucial information regarding the immune trigger of MS and help to evaluate the therapeutic efficacy of MS treatments. Different biochemical compounds, mostly DNA, RNA, or proteins are measured as biomarkers in biological fluids. Most of the molecular biomarkers that are used today are protein-based biomarkers, particularly antibodies (Teunissen et al., 2015). Biomarkers in MS are classified into several groups: diagnostic and disease activity, disease progression, treatment response, and adverse event biomarkers, the latter associated with the MS therapies (Comabella & Montalban, 2014).

2.9.2 Biomarkers for diagnosis and disease activity

Diagnostic biomarkers, also called conversion markers, are used to stratify patients who have MS from patients with other neurological or autoimmune diseases, or from the healthy population (D'Ambrosio et al., 2015). These biomarkers are measured in those patients who have neurological symptoms suggestive of demyelinating disorder, patients with CIS, and patients with the radiologically isolated syndrome (RIS), or other conditions such as neuromyelitis optica (NMO). Disease activity biomarkers are measured in patients with RRMS or progressive forms and are mostly associated with pathophysiological processes of the disease. Therefore, these biomarkers can be classified as biomarkers of inflammation, demyelination, oxidative stress, glial dysfunction, remyelination, and axonal damage. Several biomarkers of disease activity are explored in MS, which belong to numerous immune profiles such as cytokines, chemokines, immune cell subsets, costimulatory molecules, antibodies, and cell adhesion molecules (Graber & Dhib-Jalbut, 2011).

2.9.3 Oligoclonal bands and Anti-aquaporin 4 IgG

Presence of **oligoclonal IgG bands (IgG-OCBs)** in CSF is regarded as the only biomarker that is validated for the definitive diagnosis of MS, although it is not unique to MS (Dobson, Ramagopalan et al., 2013; Teunissen et al., 2015; Tintore et al., 2008). IgG-OCBs are CSF restricted, thus these IgGs are not present in the serum. A strong correlation was shown between OCB positivity and disease conversion from CIS to MS (Dobson et al., 2013). CSF OCBs are the independent prognostic marker for disease conversion (Kuhle et al., 2015). Thus, the combined brain MRI lesions and the CSF oligoclonal bands reflect the inflammatory and demyelinating features of the disease (El Ayoubi & Khoury, 2017; Gastaldi et al., 2017). The diagnostic sensitivity of OCBs was reported as high (88%)(Dobson et al., 2013) and (94%)(Petzold, 2013), however, it lacks the specificity (~35%) in terms of other inflammatory CNS disorders (Owens et al., 2009). Including OCBs, other parameters such as measurement of IgG index (Link & Huang, 2006) and evaluation of daily de novo CNS IgG synthesis are used for the diagnosis of MS (Tomioka & Matsui, 2014). The principal method for the determination of CSF OCBs is isoelectric focusing followed by immunofixation (Espino et al., 2015). In addition, measurement of intrathecal IgM OCBs is suggested as a prognostic biomarker in MS and the patients with these OCBs were shown to convert earlier to SPMS (Teunissen et al., 2015). In addition, IgM OCB was also suggested as a useful prognostic biomarker in MS and recent studies have reported intrathecal IgM OCBs as a biomarker of progressive disease (Harris et al., 2017).

Anti-aquaporin 4 IgG (AQP4-IgG) allows differential diagnosis by differentiating NMO from MS (Flanagan et al., 2016; Wingerchuk & Weinshenker, 2003). AQP4-IgG are highly specific autoantibodies that target the astrocytic AQP4-IgG water channel and are present in the serum of patients with NMO (Jarius et al., 2010). Serum AQP4-IgG is a promising biomarker for NMO diagnosis and this marker specifically assists clinicians in differentiating the distinct pathophysiological features from MS (Flanagan et al., 2016). Based on several methods, the sensitivity of AQP4-IgG was found in the range from 12.5% to 100%, with a median value of 62.3% (Jarius & Wildemann, 2013). However, recently introduced techniques such as recombinant antigen-based assays and cell sorting assays have augmented the sensitivity of AQP4-IgG detection (Jiao et al., 2013).

2.9.4 Cytokines and chemokines as biomarkers of disease activity

Several **cytokines and chemokines**, and their receptors, have potential role as biomarkers in MS as they were found in MS lesions and CSF, and associate with MS disease activity and disease progression (Comabella & Montalban, 2014; Graber et al., 2007; Tomioka & Matsui, 2014). Elevated levels of proinflammatory cytokines such as IL-17, TNF-α, IFN- γ, IL-12 are frequently observed in MS compared to healthy individuals, particularly in RRMS patients with active inflammatory disease process (Kallaur et al., 2013; Obradovic et al., 2012). Occurrence of increased pro-inflammatory and decreased anti-inflammatory cytokines and chemokines are frequent during MS relapses (Dendrou et al., 2015). Regulatory or anti-inflammatory cytokines such as IL-10 and IL-4 are increased during the remission phase of the disease (Imitola et al., 2005).

C–X–C motif chemokine 13 (CXCL13) is a B cell chemoattractant which is studied as a biomarker of B-cell involvement in CNS and thus as an indicator of intrathecal B-cell responses in MS (Harris & Sadiq, 2014). This chemokine serves as a prognostic biomarker for CIS conversion and its correlation was found with relapse rate, EDSS score, and number of lesions (Brettschneider et al., 2010; Khademi et al., 2011). However, it lacks its diagnostic potential due to the specificity issue, as higher CSF levels were observed not only in MS but also in other inflammatory or infectious CNS diseases (Alvarez et al., 2013; Khademi et al., 2011). Several studies have reported increased CSF levels of CXCL13 in CIS, RRMS, SPMS, and PPMS patients compared to patients with other non-inflammatory CNS diseases (Sellebjerg et al., 2009; Stilund et al., 2015), and also higher levels were reported in serum of patients with active MS (Festa et al., 2009).

Chitinase 3-like protein 1, also called YKL-40, is a glial cells activation marker, which is expressed on activated astrocytes and microglia in the brain (Canto et al., 2015). These proteins are candidate biomarkers to identify CIS converters and thus exhibit diagnostic and prognostic potential as a biomarker in MS (Canto et al., 2015; Comabella et al., 2010; Hinsinger et al., 2015). In CSF of RRMS patients, YKL-40 was found to be associated with earlier disease progression to high EDSS scores (Martinez et al., 2015). Significantly higher CSF levels were found in converted CIS patients compared to those patients who remained as CIS (Comabella et al., 2010). YKL-40, along with neurofilaments light chain, is regarded as a significant predictor of long-term physical and cognitive disability after optic neuritis as a first

demyelinating event (Modvig et al., 2015). Recently YKL-40 in CSF was reported as a biomarker for monitoring disease activity in SPMS patients (Burman et al., 2016).

Neurofilaments light (NFL) and heavy chains (NFH) are emerging as promising new biomarkers for monitoring ongoing axonal injury and neurodegeneration in MS due to their abundant presence in CNS tissues and relative stability (Harris & Sadiq, 2014; Modvig et al., 2015). Presence of these neurofilaments in CSF reflects the degree of inflammation-mediated axonal damage as these filaments are released into the extracellular spaces during an acute and ongoing axonal injury (Martinez et al., 2015; Stilund et al., 2015; Teunissen et al., 2015). In CSF of MS patients, the levels of both NFL and NFH were shown to be elevated and they were highest especially during the relapses (Kuhle et al., 2011; Teunissen et al., 2009). A recent study has found serum NFL as a sensitive and clinically useful blood biomarker to monitor the disease activity and disability in MS patients and as well as the marker of therapeutic responses of MS therapies (Disanto et al., 2017; Novakova et al., 2017). In addition, increased levels of serum NFL in CIS patients was found to be associated with T2 hyperintense and gadolinium-enhancing lesions and with disability status (Disanto et al., 2016; Disanto et al., 2017). CSF NFL in CIS patients was also shown to be associated with gadolinium-enhancing lesions in MRI and displayed the predictive and prognostic potential for conversion and measurement of disease outcomes (Salzer et al., 2010; Teunissen et al., 2009). A recent study has highlighted the potential of serum NFL as a biomarker for subclinical MRI activity and treatment response in RRMS (Varhaug et al., 2017). Apart from MS, increased neurofilaments levels are also found in other conditions such as in Alzheimer disease (Bacioglu et al., 2016), fronto-temporal dementia (Meeter et al., 2016), and motor neuron diseases (Steinacker et al., 2016).

Anti-microbial antibodies are regarded as clinically useful biomarkers in MS. Among them are anti-EBNA-1 antibodies against EBV nuclear antigen-1 (Farrell et al., 2009; Lunemann & Ascherio, 2009), and anti-HHV antibodies against human herpesvirus type 6 (Alenda et al., 2014; Leibovitch & Jacobson, 2014; Pormohammad et al., 2017). Elevated levels of these antibodies have been reported in MS (Leibovitch & Jacobson, 2014; Lunemann & Ascherio, 2009; Lunemann et al., 2010; Skorstad et al., 2009). CSF-restricted IgG directed against neurotropic viruses such as measles, rubella, and varicella zoster (VZV), together called as MRZ have predictive values for CIS-CDMS conversion (Brettschneider et al., 2009). Approximately 80% of MS patients have antibodies against MRZ in CSF

(Felgenhauer & Reiber, 1992), and measles virus antibody index was shown to be associated with increased EDSS score and presence of Gd-enhancing lesions (Rosche et al., 2012). A recent study has reported the prevalence of positive MRZR (MRZ reaction that is composed of the three antibody indices against measles, rubella, and VZV) in PPMS and RRMS confirming its diagnostic potential in separating both MS subtypes from other inflammatory neurological diseases (Hottenrott et al., 2017).

8-iso-PGF2α, a well-recognized isoprostane in CSF, is regarded as a biomarker of disease activity and oxidative stress in MS (Mir et al., 2014). Some studies have also reported relative production of 8-iso-PGF2α in serum and urine samples of MS patients (Greco et al., 1999; Mattsson et al., 2007; Miller et al., 2011; Sbardella et al., 2013). ROS and NO lead to oxidative stress in MS and are responsible for the mitochondrial damage and tissue hypoxia in MS (Van der Goes et al., 2001). Elevated CSF levels of NO was reported in CSF of MS patients and correlated with disease activity and disability progression (Rejdak et al., 2004), and also increased urinary NO metabolites was shown to be associated with early and relapsing MS (Giovannoni et al., 1998; Giovannoni et al., 1999).

Neutralizing antibodies are used as biomarkers to identify therapeutic response to IFN- β treatment. Based on clinical experience, 40% of patients treated with IFN- β are non-responders to therapy (Rudick et al., 2004). Development of these neutralizing antibodies leads to therapeutically poor outcome and alleles such as HLA-DRB1*0401, 0408, and 1601 were shown to be associated with the higher risk of developing these antibodies (Buck et al., 2011). Quantification of myxovirus-resistance protein A (MxA) is an established tool in clinical practice for predicting the treatment non-response for IFN- β therapy (Polman et al., 2010). MxA usually upregulates upon IFN- β injection, however, the presence of anti-IFN- β neutralizing antibodies attenuates the treatment response (Polman et al., 2010). Likewise, neutralizing antibodies against NTZ-treatment are used to identify non-responders to this drug, and these antibodies have been found in 4.5-14.1% of NTZ-treated MS patients, 12 weeks after initiation of NTZ therapy (Calabresi et al., 2007; Sorensen et al., 2011). Apart from neutralizing antibodies, CSF fetuin-A (Harris et al., 2013) and circulating CD49d expression (Defer et al., 2012) have been considered as emerging candidate biomarkers to determine the therapeutic efficacy of NTZ treatment.

3 AIMS OF THE STUDY

The specific aims of the study were:

1. To identify the biomarker potential of sL-selectin for predicting the developing risk of PML in NTZ-treated MS patients (Study I)

2. To evaluate the presence and prevalence of JC virus encoded miRNAs in plasma of NTZ-treated MS patients and to investigate their biomarker potential for developing risk of PML (Study II)

3. To assess circulating levels of CD26 and CD30 in sera as biomarkers of MS subtypes, and relation to inflammatory disease activity and disability in MS patients (Study III)

4. To assess the relative gene expression of death receptors (DR3, DcR3) and ligand (TL1A) as biomarkers in MS, and to detect their association with MS subtypes, inflammatory disease activity and disability in MS patients (Study IV)

4 PATIENTS AND METHODS

4.1 Clinical characteristics of patients and ethical considerations

The Studies I-IV included in total 315 subjects, of whom 137 were MS patients, 19 CIS patients and 159 were healthy controls. RRMS patients who were included in Studies I-II were enrolled consecutively from four Finnish MS centers (Tampere, 140 patients; Helsinki, 114 patients; Seinäjoki, 98 patients; and Turku, 54 patients) between January 2012 and February 2013. These same patients participated in our Studies I-II and were selected based on the current treatment, NTZ or IFN-beta. These patients originally participated in the study of anti-JC virus seroprevalence in a Finnish MS cohort (Kolasa et al., 2015). Studies III-IV included the same patients and were enrolled from Tampere University Hospital with different subtypes including also the CIS patients. CIS patients (n=19) included in Study III, after 4 years of clinical follow-up, 10 patients converted to RRMS.

Patients were diagnosed as clinically definite MS and the diagnosis was based on the revised Mc Donald's criteria and the (Polman et al., 2005; Polman et al., 2011). All patients underwent neurological examination and blood samples were collected on the same day. Neurological disability was quantified by the expanded disability status scale (EDSS) score, which was developed by John F. Kurtzke (Kurtzke, 1983). The Ethics Committee of Tampere University Hospital approved the study, and all subjects gave informed consent. The healthy subjects had no history of any neurological disorders or immune-mediated illness. Clinical disease activity was evaluated by counting the number of relapses preceding two years of study (Studies I-IV). MRI disease activity was assessed by the detection of gadolinium-enhancing T1 lesions and volumes of fluid attenuation inversion recovery (FLAIR) lesions (Study IV). Disease progression index (PI) was calculated as EDSS/disease duration, and annualized relapse rate (ARR) was calculated as the number of total relapses/disease duration. Clinical characteristics of patients in each study are shown in Table 4.

Table 4. Clinical characteristics of MS patients enrolled for study I – IV

Study I : Soluble L-selectin measurement for PML risk assessment			
Patients cohorts (RRMS)	**NTZ-treated**	**IFN-β-treated**	**HCs**
Number of Patients	44	30	25
Sex (F/M)[a]	34/10	21/9	19/6
Age (years)[b]	38.2±7.8 (23-52)	35.5±9.9 (20-53)	33.2±11.0 (22–60)
Disease duration from diagnosis (years)[b]	9.1±5.3 (1.8–22.4)	4.6±5.4 (0.2-18.1)	-
EDSS[b]	2.7±1.9 (0-6.5)	1.4±1.6 (0-6.0)	-
Number of relapses[b,c]	1.9±1.0 (1-4)	-	-
Duration of treatment (years)[b]	2.8±1.5 (0.4-5.8)	2.5±2.8 (0-13.1)	-
Anti-JCPyV Ab index [d]	0.3 (0.1-3.1)	0.3 (0.1-2.9)	-
Anti-JCPyV Ab seropositivity[a]	21 (48%)	13 (43%)	-
JCPyV-positive Ab index [d,e]	1.1 (0.3-3.1)	1.9 (0.7-2.9)	-
Study II : Analyses of JC virus microRNAs for PML risk assessment			
Patients cohorts (RRMS)	**NTZ-treated**	**IFN-β-treated**	**HCs**
Number of Patients	49	28	25
Sex (F/M)[a]	38/11	20/8	18/7
Age (years)[b]	38.3±7.6 (23–52)	35.6±10 (20–53)	33.3±11.3 (22–60)
Disease duration (years)[b]	9.2±5.3 (1.8–22.4)	4.3±5.1 (0.2–18.1)	–
EDSS[b]	2.6±1.8 (0–6.5)	1.3±1.3 (0–5.5)	–
Number of relapses[b,c]	2.0±1.0 (0–4)	–	–
Duration of treatment (years)[b]	2.8±1.4 (0.4–5.8)	2.5±3.0 (0.1–13.1)	–
Anti-JCPyV Ab seropositivity[a]	24 (49%)	12 (43%)	–
JCPyV-positive Ab index [d,e]	1.0 (0.3–3.1)	1.9 (0.7–2.9)	–

Study III : Soluble CD26 and CD30 molecules for disease activity assessment in MS					
MS subtypes	**CIS**	**RRMS**	**SPMS**	**PPMS**	**HCs**
Number of Patients[a]	19	39	19	-	60 (sCD30) 58 (sCD26)
Gender (F/M) [a]	17/2	27/12	12/7	-	39/21; 33/25
Age (years)[b]	35.0 ± 9.0	37.4 ± 8.7	49.1 ± 8.6	-	
Disease duration (years)[b]	NA	3.9 ± 3.8	11.7 ± 9.7	-	
EDSS[b]	0.1 ± 0.3	1.4 ± 1.5	4.7 ± 1.8	-	
Number of relapses[a,c]				-	
0	4	12	15	-	
1	13	12	2	-	
2 to 5	2	15	2	-	
Therapy (NT/ IFN /GA)[a]	NT	19/18/2	19/0/0	-	
Study IV : DR3, DcR3, and TL1A gene expressions in MS for disease activity assessment in MS					
MS subtypes	**CIS**	**RRMS**	**SPMS**	**PPMS**	**HCs**
Number of Patients[a]	11	30	8	9	16
Gender F/M[a]	9/2	23/7	5/3	6/3	11/5
Age (years)[b,d]	35.4±10.2 (23-53)	36.6±10.3 (19-54)	51.3±8.3 (39-62)	60.7±8.0 (46-73)	-
Disease duration (years)[b,d]	NA	4.0±3.9 (0.0±13.7)	16.3±11.0 (1.4±32.4)	18.7±8.2 (3.3-27.2)	-
EDSS[b,d]	0.2±0.4 (0-1)	1.7±1.9 (0-7)	5.6±2.0 (2.0-7.5)	4.8±1.8 (1.5-6.5)	-
Total relapses[b,d]	1.6±0.9 (0-3)	5.7±3.9 (0-17)	7.8±8.0(0-22)		-
Relapses preceding two years[b,d]	0.8±0.6 (0-2)	1.7±1.7(0-7)	0.0±0.0		-
Annualized relapse rate[b,d]	0.0±0.0	2.0±1.6 (0-6.4)	0.4±0.4 (0-1)		-
Treatment (NT/IFN/GA/MX)[a]	NT	0/17/1/1	0/0/0/0	0/0/0/0	-

Abbreviations: Ab: antibody, CIS: clinically isolated syndrome, EDSS: expanded disability status scale, GA: glatiramer acetate, HCs: healthy controls, IFN-β: interferon-β, JCPyV: John Cunningham virus, NA: not applicable, NT: no treatment, NTZ: Natalizumab, PPMS: primary progressive MS, RRMS: relapsing-remitting MS, SPMS: secondary progressive MS

[a]Number of patients

[b]Mean ± SD (range)

[c]Two years before starting Natalizumab

[d]Median (range)

[e]Anti-JCPyV antibody index of seropositive patients only

4.2 Molecular methods

4.2.1 Blood sample collection (Studies I-IV)

Venous blood was collected into three separate tubes: i) covered test tube (BD Vacutainer® SST II Plus plastic serum tube, 8.5 ml) for serum preparation, ii) EDTA-treated tubes (BD Vacutainer® Plus plastic whole blood tube, 10 ml) for plasma preparation and iii) CPT tubes (BD Vacutainer® CPT™ Cell Preparation Tube with Sodium Citrate) for peripheral blood mononuclear cells (PBMC) separation. Serum and plasma were isolated by centrifugation at 2000 x g for 10 min and stored at -80^{o}C until use. PBMCs were separated using CPT tubes according to the manufacturer's protocol. Thereafter cells were lysed with lysing buffer for RNA isolation and stored -80^{o}C until use. Sera samples were used for Studies I and III, plasma was used for Study II, and PBMCs were used for Study IV.

4.2.2 Enzyme-linked immunosorbent assay (Articles I-III)

4.2.2.1 ELISA for soluble L-selectin, CD26 and CD30 determination (Studies I-II)

sL-selectin in sera was determined by commercially available quantitative ELISA kits according to the manufacturer's protocol (#BBE4B; Quantikine, R&D Systems Europe Ltd, Abingdon, United Kingdom). Briefly, serum samples with 1:100 dilutions were added on the 96-wells microtiter plate, which were pre-coated with a monoclonal antibody specific for human serum L-selectin and incubated for an hour RT. Thereafter, horseradish peroxidase (HRP) conjugated polyclonal antibody specific for human L-selectin, was then added in the wells. TMB (Tetramethylbenzidine) substrate was added in the plate and the blue color was allowed to develop for 30 mins. Later, the color development was stopped by adding hydrochloric acid (HCl). The absorbances were measured at wavelength of 450 nm on a Multiskan MS version 4.0 spectrophotometer (Labsystems, Helsinki, Finland). Samples from all MS subtypes and healthy controls were included in each 96 well plate in order to minimize the inter-assay variation between the plates, and also the similar batch of reagents was used. The intra- and inter-assay coefficients of variation for the sL-selectin assay was 4.1% and 7.1%, respectively. The minimum detection limit for sL-selectin ELISA according to assay protocol was 0.3 ng/mL.

The levels of sCD26 and sCD30 in sera were measured using ELISA according to the manufacturer's protocol (Human sCD26 Platinum ELISA BMS235CE and Human sCD30 instant ELISA BMS240INSTCE; eBioscience, Bender Med Systems GmbH, Vienna, Austria). The assay was performed in the same way as L-selectin (Study I). The concentration of molecules was measured by the absorbance reader Labsystems Multiskan® MCC/340 by setting 450 nm as a primary wavelength and 620 nm as a reference wavelength. A standard curve was made for each run using four parameters logistic for curve fitting (Ascent™ Software 2.0, Thermo Scientific). Sensitivity of sCD26 and sCD30 assays was 7.3 ng/ml and 0.33 ng/ml, respectively. Intra- and inter-assay reproducibility of sCD26 assay was measured using pooled plasma samples (coefficient of variation 9.3%, n=9 and 34.7%, n=10). Inter-assay reproducibility of sCD30 assay was evaluated using pooled plasma sample (3.6%, n=2).

4.2.2.2 Second generation ELISA (STRATIFY JCPyV™ DxSelect) for anti-JCPyV antibody measurement (Studies I, III)

The validated second generation ELISA, also known as the confirmatory second generation ELISA (STRATIFY JCPyV™ DxSelect™) was used to determine the anti-JCPyV antibody levels in serum or plasma, in RRMS patients treated with NTZ. The test was performed at Unilabs, Copenhagen, Denmark. The technique is licensed exclusively from Biogen and the test is not intended for donor screening. A screen index value of less than 0.2 was considered anti-JCPyV antibody negative, and of greater than 0.4 as anti-JCPyV antibody positive. The samples with a screen index between 0.2 and 0.4 were evaluated with a supplementary confirmatory inhibition test, and samples showing greater than 45% inhibition in blocking with specific antigen were classified as anti-JCPyV antibody positive (Lee et al., 2013). The detailed laboratory procedure is available at the manufacturer's website https://www.focusdx.com/pdfs/pi/OUS/EL1950.pdf.

4.2.3 Luminex assay for determination of cytokine levels (Study III)

The levels of IL-10, IFN-γ and TNF-α were measured with High Sensitivity Human cytokine LINCOplex kit (Linco Research). The data were collected and analyzed using Bio-Plex suspension array system and Bio-Plex Manager software 4.1 (Bio-Rad Laboratories, California, USA). A four-parameter regression formula was used to

calculate the sample concentration from Human cytokine LINCOplex kit. Samples from all MS subtypes and healthy controls were included in each 96 well plate in order to minimize the inter-assay variation between the plates, and also the similar batch of reagents was used. According to the manufacturer, the inter- and intra-assay values were <15%. The percent recovery of standards that was used as a detection limit for each protein ranged from 90% to 110%. The lower detection limits for IL-10, TNF-α, and IFN-γ was 0.13 pg/ml.

4.2.4 RNA extractions (Studies II and IV)

For Study III, total RNA was extracted from 620 µl of plasma using the mirVana™ PARIS™ RNA and native Protein Purification Kit (Ambion, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Total RNA was eluted into 95 µl of elution solution and spiked with 5 µl of cel-39-3p miRNA (5 fmol/µl, Integrated DNA Technologies, Coralville, IA, USA). The use of synthetic Caenorhabditis elegans miRNA was to control the success of reverse transcription (RT) and miRNA amplification, as well as for normalization of results. For Study IV, total RNA was isolated from the PBMCs with a Qiagen RNeasy plus mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol. The total RNA was eluted with nuclease-free water, and samples were stored at − 80 °C until use.

4.2.5 Reverse transcription (Studies II and IV)

For Study III, TaqMan miRNA assay was used for reverse transcription (RT) and JC virus miRNA detection (Thermo Fisher Scientific). The specific targets were JCPyV-miR-J1-5p, bkv-miR-B1-3p/JCPyV-miR-J1-3p (identical sequences), and cel-miR-39-3p. Each 15 µl RT reaction mixture contained 1× RT buffer, 0.25 mM of each dNTP, 1× RT primer, 3.33 U/µl MultiScribe RT enzyme, 0.25 U/µl RNase inhibitor, and 10 ng of total RNA. RT reactions were incubated 30 min at 16 °C, 30 min at 42 °C, and 5 min at 85 °C. If real-time PCR was performed directly after RT, the tubes were cooled to 4 °C, but for longer storage, the reactions were placed in −20°C manufacturer's protocol. For Study IV, total RNA (1 µg) was reverse transcribed to cDNA in a 20 µl reaction volume using a High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) with the standard protocol. RT reaction mixture contained 2 µl of 10x RT buffer, 0.8 µl of

25x dNTP Mix, 2 µl of 10x random hexamer primers, 1 µl of 50 U/µl MultiScribe RT enzyme, 4.2 µL of RNase-free water, and 10 µl of extracted RNA solution in RNase-free water. cDNAs were stored in -20°C until use.

4.2.6 Quantitative real-time PCR (RT-qPCR) (Studies II and IV)

For PCR amplification of JC virus-encoded miRNAs in Study II, the Applied Biosystems® 7500 Real-Time PCR System (Thermo Fisher Scientific) was used. Each 10 µl RT reaction contained 1.3 µl of diluted (1:2) RT reaction, 1× TaqMan® assay mixture, and 1× TaqMan® Universal Master Mix II, no UNG (Thermo Fisher Scientific). All miRNA assays were performed in three replicate reactions in the following conditions: enzyme activation in 95 °C for 10 min, after which 40 cycles of 15 s denaturation in 95 °C and 1 min annealing and extension in 60 °C was performed. In each 96-microwell plate, three replicate no template controls (NTC) were run for each master mix. The functionality of the miRNA assays was confirmed using synthetic oligonucleotides (Integrated DNA Technologies) representing the target sequence of each specific miRNA assay as templates.The relative miRNA expression was calculated by using standard delta delta Ct ($2^{-\Delta\Delta Ct}$) method. Further information on miRNA assays are provided in Table 5.

Table 5. MiRNA assay information (Study II)

miRNA	Assay name	Assay ID	Mature miRNA Sequence	Details are available at
jcv-miR-J1-5p	jcv-miR-J1-5p	007464_mat	UUCUGAGACCUGGGAAAAGCAU	https://www.thermofisher.com/order/genome-database/details/microrna/007464_mat?CID=&ICID=&subtype=
jcv-miR-J1-3p/bkv-miR-B1-3p	bkv-miR-B1-3p	006801_mat	UGCUUGAUCCAUGUCCAGAGUC	https://www.thermofisher.com/order/genome-database/details/microrna/006801_mat?CID=&ICID=&subtype=
cel-miR-39	cel-miR-39	000200	UCACCGGGUGUAAAUCAGCUUG	https://www.thermofisher.com/order/genome-database/details/microrna/000200?CID=&ICID=&subtype=

Likewise, in Study IV, gene expression of DR3, DcR3, TL1A and GAPDH were analyzed with TaqMan assays using the Applied Biosystems® 7900 Real-Time PCR System (Thermo Fisher Scientific). Other related information on these assays are provided in Table 6. Each PCR reaction was performed in 10 µl reaction volume in the 384 well plate, which contained 0.5 µl of 20x TaqMan® Gene Expression Assay, 5 µl of 2x TaqMan® Gene Expression Master Mix (Thermo Fisher Scientific), 2.5

μl of RNase/DNase free water and 2 μl of cDNA. Samples were run in three replicates and NTCs were run in each run. Quantitative-PCR reactions were run under standard conditions: initial denaturation at 95°C for 10min, after which 40 amplification cycles of 15sec denaturation in 95°C and 1min annealing and extension in 60°C. The gene expression data were analysed with RQ manager software (Applied Biosystems) using the comparative Ct method (ΔΔCt). The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize the results, and a HC sample, in each plate, was used as a calibrator in the data analysis.

Table 6. Information related to gene expression assays (Study IV)

Gene (Gene symbol)	Assay ID	Cat#	Details are available at
DR3 (TNFRSF25)	Hs00980365_g1	4331182	https://www.thermofisher.com/taqman-gene expression/product/Hs00980365_g1?CID=&ICID=&subtype=
DcR3(TNFRSF6B)	Hs00187070_m1	4448892	https://www.thermofisher.com/taqman-gene-expression/product/Hs00187070_m1?CID=&ICID=&subtype=
TL1A (TNFSF15)	Hs00270802_s1	4331182	https://www.thermofisher.com/taqman-gene-expression/product/Hs00270802_s1?CID=&ICID=&subtype=
GAPDH	Hs99999905_m1	4331182	https://www.thermofisher.com/taqman-gene-expression/product/Hs99999905_m1?CID=&ICID=&subtype=

4.2.7 Magnetic resonance imaging (Study IV)

All MRI examinations were performed on a 1.5 Tesla MRI Unit (Siemens Avanto, Erlangen, Germany). The MRI protocol included a T1-weighted header followed by an axial T1-weighted magnetisation prepared rapid gradient echo (MP-RAGE), and a T2-weighted turbo spin echo (TSE), fluid attenuation inversion recovery (FLAIR), magnetisation transfer contrasts (MTC), diffusion weighted imaging (DWI), and gadolinium-enhanced T1-weighted MP-RAGE sequences. T1-weighted MP-RAGE, FLAIR and T2-weighted TSE images were used for volumetric analysis. For MP-RAGE, the imaging parameters were as follows: repetition time (TR) = 1160 ms; echo time (TE) = 4.24 ms; inversion time (TI) = 600 ms; slice thickness = 0.9 mm; and in-plane resolution = 0.45 ∗ 0.45 mm. In FLAIR, the following parameters were used: TR = 8500 ms; TE = 100 ms; TI = 2500 ms; slice thickness = 5.0 mm; and in-plane resolution = 0.45 ∗ 0.45 mm. In TSE, the following imaging scheme was used: TR = 750 ms; TE = 115 ms; slice thickness = 3.0 mm; and in-plane resolution =

0.90 ∗ 0.90 mm. Volumetric segmentation of plaques in the brain was performed using semiautomatic Anatomatic™ software operating in a Windows environment, and the images were blindly analysed.

4.2.8 Statistical analyses (Studies I-IV)

Statistical analyses were performed using SPSS version 16.0 for Windows in Study I, SPSS version 18.0 in Study III, and version 22.0 in Studies II and IV (SPSS Inc., Chicago, IL, USA). GraphPad Prism 7.03 was used to prepare figures in Studies II-IV. A non-parametric, two-tailed Mann–Whitney U test was used to compare the differences between the clinical parameters of patients and levels of sL-selectin, relative expression of JC virus miRNAs, sCD26 and sCD30, and relative expression of DR3, DcR3, and TL1A genes, in different study groups (Studies I-IV). Spearman's correlation coefficient was used to analyze the association of levels of sL-selectin and JCPyV miRNA expression with anti-JCPyV antibody indices. Further, correlation was explored between the disease profiles of patients and different immune molecules (Studies III-IV). Differences in the detection rate of JCPyV miRNAs between different groups of patients treated with NTZ and IFN-β, and HCs were assessed using Fisher's exact test in Study II. Moreover, a linear regression model was used to observe the correlation between relative 5p JCPyV miRNA expression levels and anti-JCPyV antibody indices in Study II. In Studies I-IV, a p-value less than 0.05 was considered statistically significant. In Study IV, the p-value from each analysis was corrected for multiple group comparisons using the Benjamini and Hochberg method to control the false discovery rate (FDR) at a level of 0.05 (Benjamini & and Hochberg, 1995). For correlation analyses, Spearman's correlation coefficient was used to explore the association between relative gene expression levels and clinical or MRI parameters. A p-value less than 0.05 was considered statistically significant.

5 RESULTS

5.1 Biomarkers for detecting PML risk in natalizumab-treated RRMS patients (Study I-II)

5.1.1 Soluble L-selectin in sera as a biomarker for developing PML risk (I)

In Study I, the levels of sL-selectin were determined in patients treated with NTZ, IFN-β, and HCs. The results showed no difference in sL-selectin levels between these study groups (Figure 4A). In addition, there was no difference between the levels of JCPyV seropositive and JCPyV seronegative patients in NTZ-treated (1330.5±476.2 vs. 1076.0±500.9 ng/ml (mean±SD); p=0.10) or IFN-β-treated groups (1225.5±611.4 vs. 1403.6±575.9 ng/ml (mean±SD); p=0.28). When NTZ-treated patients were classified based on their anti-JCPyV antibody index into groups with high risk for developing PML (anti-JCPyV antibody index higher than 1.5, n=8) and low risk group for developing PML (anti-JCPyV antibody index≤1.5, n=36), the levels of sL-selectin was found to be significantly higher in the high-risk group compared to that with low risk group for developing PML (1621.3±506.9 vs. 1103.3±453.2 ng/mL (mean±SD); p=0.01, Figure 4B).

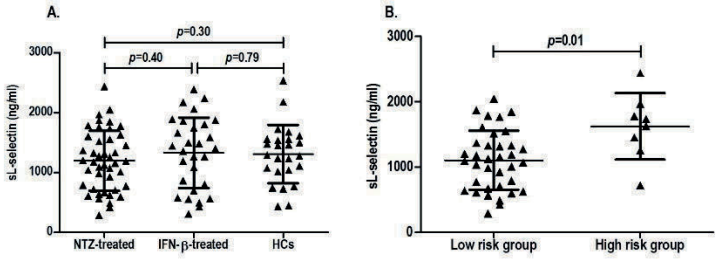

Figure 4. The levels of sL-selectin did not differ between the NTZ-, IFN-β-treated or HC, B. The levels of sL-selectin were significantly higher in the high-risk group compared to that with low risk group for developing PML in NTZ-treated patients. (Modified with permission from (Basnyat et al., 2015a).

Correlation analyses showed a positive correlation between the levels of sL-selectin and the anti-JCPyV antibody indices in all NTZ-treated patients (r=0.402; p= 0.007; n=44, Figure 5A), but not in the IFN-β-treated patients (r=−0.262; p=0.161; n=30; Figure 5B). In approximately 80% of NTZ-treated (34/44) patients who were treated for more than 18 months, sL-selectin levels correlated with anti-JCPyV antibody indices (r=0.385; p=0.025; n= 34; Figure 5C), and the strength of this correlation increased specially among JCPyV seropositive patients treated for more than 18 months (r=0.529; p=0.043; n=15; Figure 5D). No significant correlations between sL-selectin and anti-JCPyV index were found when JCPyV seropositive (r=0.356 p=0.113, n=21) and JCPyV seronegative (r=0.360; p=0.092; n=23) NTZ-treated patients were analysed as separate groups. There was no correlation between anti-JCPyV antibody index and treatment duration in patients treated with either NTZ-or IFN-β.

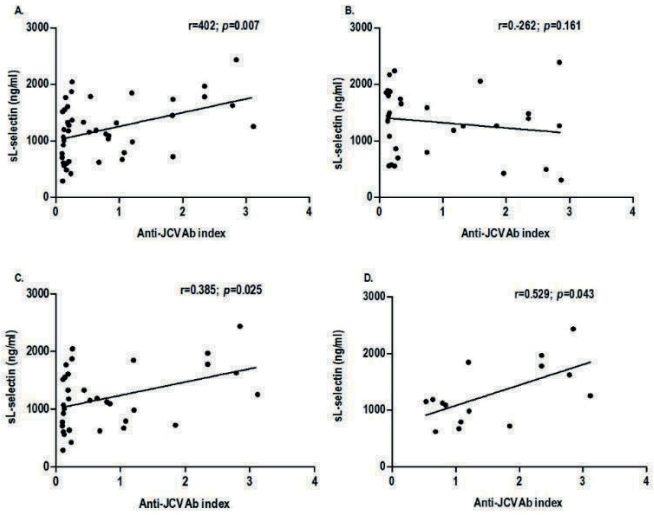

Figure 5. Correlation analyses between sL-selectin and the anti-JCPyV antibody index in **(A)** NTZ-treated patients, **(B)** IFN-β-treated patients, **(C)** Long-term NTZ-treated (>18 months) patients and **(D)** JCPyV seropositive long-term (>18 months) NTZ-treated patients. (Reproduced with permission from (Basnyat et al., 2015a).

5.1.2 JC virus-encoded microRNAs in plasma as the biomarker for developing PML risk (II)

The presence of JCPyV-encoded 5p and 3p miRNAs was studied in altogether 102 plasma samples from 77 RRMS patients and 25 HCs. The overall detection rate for 5p miRNA among NTZ-treated patients, IFN-β-treated patients, and HCs was found to be similar (Table 7). Furthermore, the detection rate of 5p miRNA among JCPyV seropositive and seronegative patients treated with either NTZ or IFN-β was also found to be similar (Table 7). Surprisingly, the rate of 5p miRNA detection among JCPyV seronegative patients treated with NTZ was higher than among IFN-β-treated patients (Table 7), although the difference was not statistically significant ($p > 0.5$). True positive 3p miRNA signals could not be indisputably detected in any of the samples.

Table 7. JCPyV-miR-J1-5p detection rates in the different patient groups. The overall detection rate among all groups was 85/102 (83%)

	All MS patients	NTZ-treated	IFN-β-treated	HCs	*p-value*
All	62/77 (80.5%)	41/49 (84%)	21/28 (75%)	23/25 (92%)	>0.5
JCPyV Ab$^+$	30/36 (83%)	20/24 (83%)	10/12 (83%)	NA	>0.5
JCPyV Ab$^-$	32/41 (78%)	21/25 (84%)	11/16 (69%)	NA	>0.5

JCPyV Ab+ anti-JCPyV antibody positive

JCPyV Ab- anti-JCPyV antibody negative

NA Not available. (Reproduced with permission from (Basnyat et al., 2017).

Relative 5p miRNA expression levels were studied in different patient cohorts: The 5p miRNA expression levels were found to be similar in the whole MS cohort compared to HCs ($p=0.06$, Figure 6A). In groups based on treatment, the expression levels were found to be lower in NTZ-treated patients as compared to patients treated with IFN-β ($p=0.027$) but not as compared to HCs ($p=0.454$, Figure 6A). IFN-β-treated patients had relatively higher levels of 5p miRNA than NTZ or HCs as shown by fold change ($p=0.001$, Figure 6A). 5p miRNA expression levels between JCPyV seropositive and seronegative patients in the different MS cohorts, treated either with NTZ- or IFN-β, were similar ($p>0.5$). When NTZ-treated patients were further classified as high risk or low risk for developing PML as in Study I, the levels

of 5p miRNA expression did not differ between the high-risk group and low-risk group of developing PML (p=0.38).

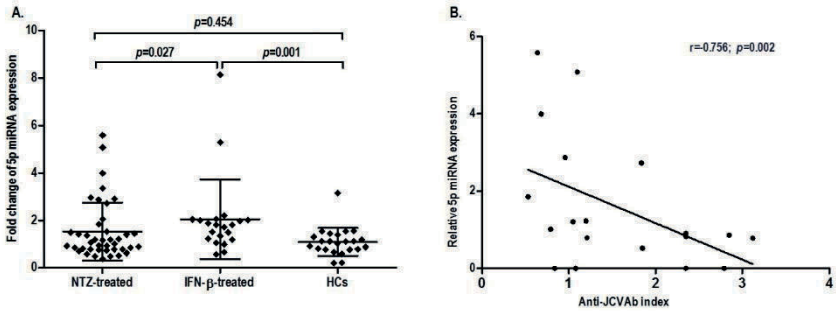

Figure 6. **(A)** Scatterplot of relative JCPyV-miR-J1-5p expression levels. Bars indicate mean ± standard deviation. The values on the y-axis reflect fold change of 5p miRNA expression among individual NTZ-treated patients and IFN-beta-treated patients as compared to HCs. **(B)** Relationship between JCPyV miRNA expression and anti-JCPyV antibody index. (Modified with permission from Basnyat et al., 2017).

Association between plasma 5p miRNA levels and anti-JCPyV antibody index was further studied by correlation analyses along with other clinical characteristics of MS patients (age, disease duration, drug duration, and EDSS). There were no significant correlations found between 5p miRNA expression and clinical characteristics of patients. Among all NTZ-treated patients (n = 49), and patients who had been treated more than 18 months (n = 40), 5p miRNA expression did not correlate with anti-JCPyV antibody index. Interestingly, a significant inverse correlation between 5p miRNA expression and anti-JCPyV antibody index was found in those patients (n = 19) who had been treated long-term with NTZ and were JCPyV seropositive (r = −0.756, p=0.002, Figure 6B). The mean duration of NTZ treatment among JCPyV seropositive MS patients was 3.0 years (SD ±1.3 years, range 1.6–5.3 years). Moreover, further analyses by linear regression model showed that the observed correlation between relative 5p miRNA expression levels and anti-JCPyV antibody indices was not affected when adjusted for age, sex, or for both.

5.2 Immune cells as biomarkers for MS subtypes, inflammatory disease activity and disability in MS patients (Study III-IV)

5.2.1 Soluble CD26 and CD30 levels and association with MS clinical spectrum

In all the study groups (CIS, RRMS and SPMS), the levels of sCD26 and sCD30 were found to be higher than in the controls (Figures 7A-B), although no differences were detected between the disease subtypes. Among the 19 CIS patients, 10 converted patients showed increased levels of sCD30 compared to controls (23.0 ± 6.4 vs 16.3 ± 7.4 ng/mL, p= 0.009), whereas no difference was observed between unconverted patients and controls (Figure 7C). The levels of sCD26 did not differ in converted CIS patients when compared with controls and unconverted CIS patients (891.1 ± 145.2 vs 778.4 ± 237.7 ng/mL, p=0.086; 891.1 ± 145.2 vs 911.4 ±135.9ng/mL, p=0.905). Moreover, the levels of cytokines IL-10, TNF-α and IFN-γ in sera did not differ between the MS subtypes and controls (p > 0.05).

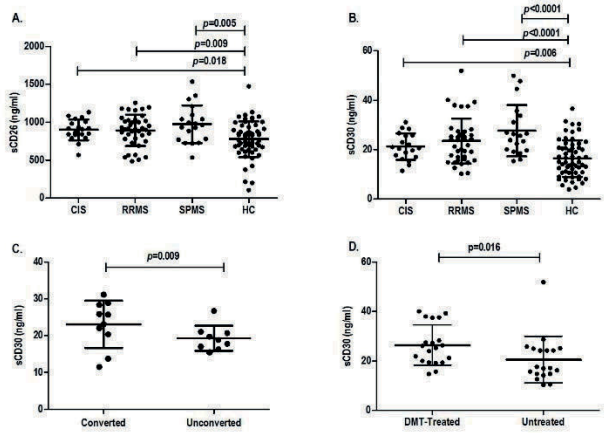

Figure 7. Different levels (mean ± SD) of **(A)** sCD26 and **(B)** sCD30 in patients with CIS, RRMS, SPMS and HCs. **(C)** sCD30 levels in converted and unconverted CIS patients. **(D)** sCD30 levels in DMT-treated and untreated RRMS patients. (Reproduced with permission from (Basnyat et al., 2015b).

To evaluate whether the sCD26 and sCD30 reflect the inflammatory disease activity in MS, their levels were further correlated with cytokines IL-10, TNF-α and IFN-γ. The levels of IL-10 correlated positively with the level of sCD30 in the MS

group (RRMS and SPMS) ($r = 0.583$, $p < 0.0001$, $n = 43$; Figure 8A), but the levels of sCD26 and sCD30 did not show any correlation with other cytokines analyzed. Correlation analyses were performed between sCD30 and sCD26, and the result showed a weak, but statistically significant positive correlation between these molecules, including both in RRMS and SPMS patients ($r = 0.262$, $p = 0.046$, $n = 58$; Figure 8B). Furthermore, investigation of association of sCD26 and sCD30 with pre-study disease activity (number of relapses) and EDSS scores, showed no significant correlation between these molecules and clinical measures in patients. Because half of the RRMS patients (51%) were treated with DMTs (Table 4), the effect of treatment was explored on these molecules. The results showed significantly higher levels of sCD30 in treated RRMS patients compared with untreated patients (26.4 8.0 vs 20.5 ± 9.4 ng/mL, $p = 0.016$; Figure 7D).

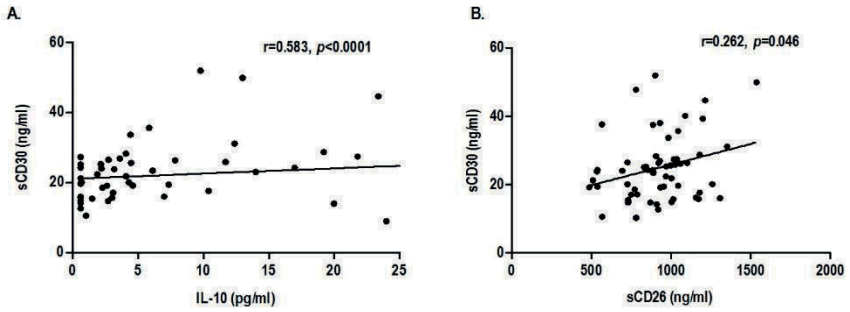

Figure 8. Correlation analysis between the levels of **(A)** sCD30 and interleukin IL-10 and between the levels of **(B)** sCD30 and sCD26 in MS patients. (Modified with permission from Basnyat et al., 2015b).

5.2.2 Gene expression profiles of Tumor Necrosis Factor-like Cytokine TL1A and its Receptors DR3 and DcR3 in MS

Relative gene expression levels of DR3, DcR3, and TL1A were compared among study groups, which revealed significantly lower expression level of TL1A in relapsing onset MS group as compared to patients with CIS, PPMS, and HCs ($p<0.05$, Figure 9C). Relapsing onset group includes RRMS patients and SPMS patients. Expression levels of DR3 and DcR3 did not differ between MS groups, CIS, and HCs ($p>0.05$, Figures 9A-B). Although relapsing onset MS showed lower levels of DR3 expression and higher levels of DcR3 expression compared to HCs

(p=0.034, p=0.032, respectively), these differences were not significant after p-value correction (Figures 9A-B).

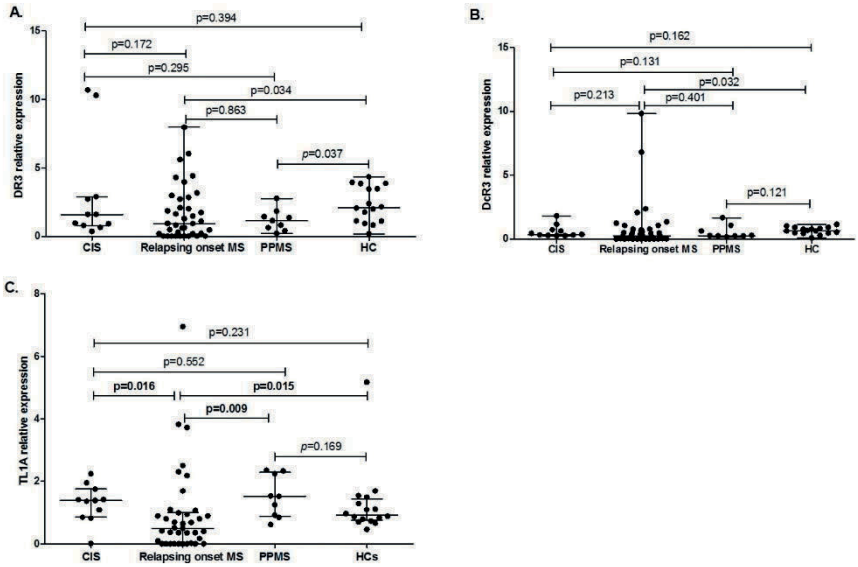

Figure 9. Scatter plot showing the relative gene expression levels of DR3, DcR3, and TL1A in different MS patients, CIS and HCs. The bars indicate the median and interquartile range.

Although the relapsing onset patients showed a lower expression of TL1A as compared to PPMS/CIS/HC groups, the subgroup analysis showed a significantly higher TL1A expression in patients with severe disability (EDSS from 3.5 to 7.5) compared to patients without disability (EDSS = 0) (p=0.016). Such difference was not observed between patients with mild disability and no disability (p=0.578), or between patients with mild disability and severe disability (p=0.127, Figure 10).

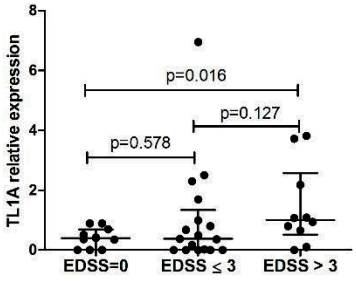

Figure 10. Scatter plot showing the relative gene expression levels of TL1A in relapsing onset MS patients based on their EDSS. The bars indicate the median and interquartile range.

The effect of DMTs on gene expressions of DR3, DcR3, and TL1A, was compared among the relapsing onset MS patients who were treated and those who were treatment naïve. Those patients who were treated showed significantly decreased expression of DcR3 and TL1A compared to treatment naïve patients ($p<0.01$, n=19, Figures 11B-C) whereas DR3 expression did not differ between these groups ($p=0.430$, Figure 11A).

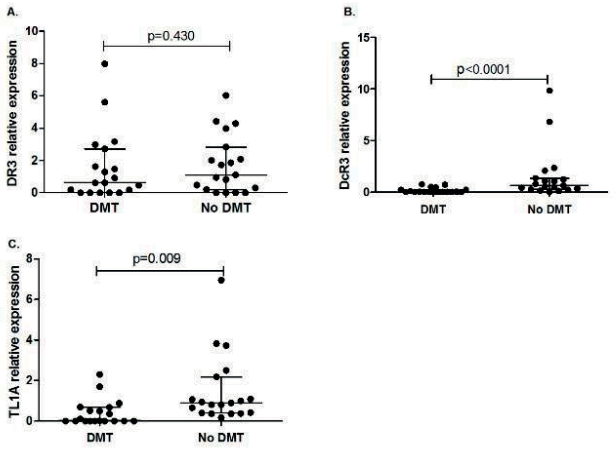

Figure 11. Relative DR3, DcR3, and TL1A gene expression levels among relapsing onset MS patients treated with immunomodulatory drugs versus those who were treatment naïve. The bars indicate the median and interquartile range.

Moreover, the association of relative DR3, DcR3, and TL1A gene expression with clinical and MRI parameters was explored in different patient groups. Among the three genes analysed, only in relapsing onset MS, TL1A significantly correlated with EDSS score ($r=0.387$, $p=0.016$, n=37, Figure 12A), the volume of T1-weighted lesions ($r=0.376$, $p=0.022$, Figure 12B), FLAIR lesions ($r=0.366$, $p=0.026$, Figure 12C), change in the volumes of FLAIR lesions (delta FLAIR) ($r=0.422$, $p=0.009$, Figure 12D), and the time from first symptoms ($r=0.341$, $p=0.036$, Figure 12E). Among CIS and PPMS patients, none of the genes reveal any correlations with clinical or MRI parameters.

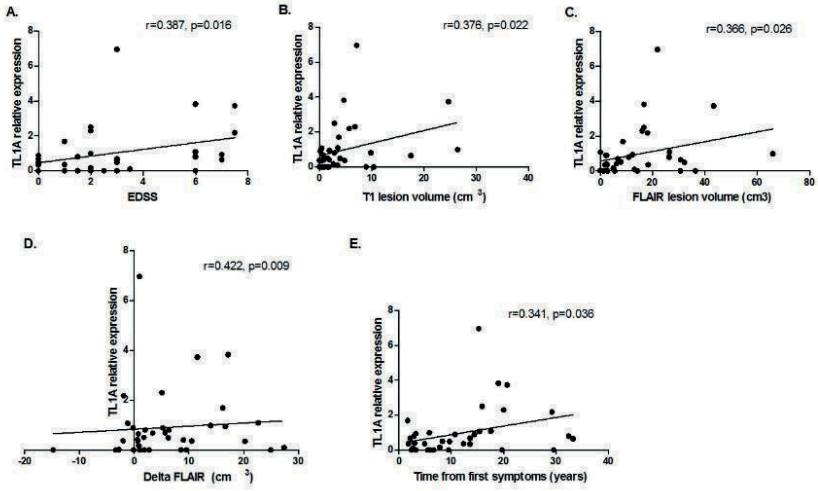

Figure 12. Association of TL1A gene expression to clinical (**A.** EDSS, **E.** Time from the first symptoms) and MRI (**B.** volumes of T1 lesions **C.** volumes of FLAIR lesions and **D.** Change in the volumes of FLAIR lesions) parameters.

6 DISCUSSIONS

6.1 Biomarkers for the PML risk assessment in natalizumab-treated MS patients

The current PML risk stratification method mainly based on the measurement of anti-JCPyV antibody has proven not effective enough in the assessment of PML (Cutter & Stuve, 2014). Anti-JCPyV antibodies are present in approximately 60-80 % of the healthy population, and 60–70% of the MS patients (Ferenczy et al., 2012; Kolasa et al., 2016; Olsson et al., 2013). However, reactivation of the JCPyV and the development of PML are rare events in healthy individuals (Ferenczy et al., 2012). Previous attempts mainly based on leukocyte cell membrane markers such as CD11a, CD49d, and CD62L still lack their clinical utility in the PML risk assessment (Basnyat et al., 2015a; Jilek et al., 2010; Schwab et al., 2013; Schwab et al., 2014). Moreover, even the detection of JCPyV DNA in the urine and blood samples from MS patients was much less sensitive than antibody measurement in ruling out the precise individual risk of PML (Rudick et al., 2010). A recent report has detected the NTZ-related PML cases in those MS patients who were negative for JCPyV antibody (Gagne Brosseau et al., 2016). Therefore, due to these reasons, we evaluated soluble L-selectin and JCPyV miRNA as new biomarkers that would narrow down the in-risk population and identify the individual patient with a higher risk of developing PML in NTZ-treated MS patients.

6.1.1 L-selectin and prediction of the risk of PML

To investigate the biomarker potential of sL-selectin for predicting the risk of PML, we analysed the levels of sL-selectin in sera obtained from NTZ-treated RRMS patients. The results showed a positive correlation between the levels of sL-selectin anti-JCPyV antibody indices in all MS patients treated with NTZ. Interestingly, this correlation was significantly stronger among those patients who were considered anti-JCPyV-antibody positive and treated with NTZ for more than 18 months. Long-term NTZ treatment duration and JCPyV antibody positive status are

considered as potent risk factors for developing the risk of PML in MS patients (Bloomgren et al., 2012). Schwab et al. 2013, reported that lack of cellular L-selectin on the surface of $CD4^+$ T cells was indicative of increased PML risk in MS patients treated with NTZ (Schwab et al., 2013). These authors further reported the positive correlation between cellular L-selectin expression and anti-JCPyV antibody index values in NTZ-treated patients (Schwab et al., 2014). Interestingly, low cell-surface L-selectin levels were shown to be associated with increased serum levels of sL-selectin and this increased soluble level was due to the shedding of L-selectin from the cell surface. This phenomenon possibly explains the reduced leukocyte migration into CNS from the periphery (Jackson et al., 2005). This mechanism, therefore, explains our result of increased level of sL-selectin. Our observation of the positive correlation between increased sL-selectin levels and increased anti-JCPyV antibody indices only in NTZ-treated patients but not in IFN-beta treated MS patients suggests that sL-selectin measurement could be useful for the assessment of PML risk among those MS patients treated with NTZ. This observation is sustained further by our finding of increased sL-selectin level in those patients who have high anti-JCPyV antibody index (>1.5) and are considered at highest risk for developing PML (Lee et al., 2013). In fact, it would be therefore rational to measure the level of sL-selectin in the serum of those MS cohort included by Schwab et al., who lacked L-selectin on $CD4^+$ T cells, and who later developed PML (Schwab et al., 2013). Our assumption is that they also would have increased levels of sL-selectin. Thus, our observations suggest a biological connection between shedding of cellular L-selectin from the cell surface and rising anti-JCPyV antibody levels in the blood of RRMS patients. This association indicates that NTZ-treatment influences two biological factors in serum: loss of L-selectin via shedding and rising JCPyV index values.

Importantly, measurement of the soluble form of L-selectin in serum using an ELISA assay is comparatively easy, reliable and can be applied to any laboratory, then cellular L-selectin measurement on cryopreserved PBMCs using flow cytometry method which was used by Schwab et al. 2013. This method is technically demanding and prone to errors related such as during cell handling, which might lead to shedding (mechanical) of L-selectin from the cell surface, which may influence the purity of results. A study has reported that surface L-selectin is not a reliable biomarker for predicting PML risk because of the issues related to sample collection, processing procedure, and assay methodologies (Lieberman et al., 2016).

6.1.2 JCPyV encoded miRNAs: a potential new marker of PML

This study evaluated the presence and prevalence of JC virus-encoded miRNAs in plasma of NTZ-treated MS patients and investigated their biomarker potential for developing risk of PML. Detection of viral miRNAs offers the new tool for the improved diagnosis, prognosis and risk assessment for chronic and persistent viral diseases (Auvinen, 2017). Earlier studies have proposed JCPyV miRNAs as potential biomarker of viral infection in gastrointestinal tract and these miRNAs were detected also in the brain tissues obtained from patients with PML (Link et al., 2014; Seo et al., 2008). Therefore, we evaluated the JCPyV encoded miRNAs in an attempt to assess its predictive potential as a new biomarker for the risk of developing PML in NTZ-treated MS patients. Human blood miRNAs have been studied for their biomarker potential in predicting NTZ-associated PML (Munoz-Culla et al., 2014) but studies utilizing the JC virus miRNAs as biomarkers are lacking. Recent studies have reported the frequent detection of JCPyV miRNAs, both JCPyV-miR-J1-5p and JCPyV-miR-J1-3p, in plasma, urine, and CSF of both anti-JCPyV antibody positive and anti-JCPyV antibody negative healthy subjects and patients with immunosuppression (Lagatie et al., 2014b; Pietila et al., 2015). Thus, these studies highlighted the possibility that the JCPyV miRNAs exhibit biomarker potential and could serve as PML risk assessment tool better than the method based on JCPyV serology.

Our results showed that the 5p miRNA was detected in altogether 83% of plasma samples. Similar to the previous study, detection rate of 5p miRNA in our samples was similar between JCPyV seropositive and seronegative patients, which further sustain the previously reported finding that negative anti-JCPyV-antibody status does not exclude the possibility of absence of viral infection (Lagatie et al., 2014b). Higher levels of 5p miRNA was also reported in tissue samples of patients with colonic neoplasia compared to healthy individuals (Link et al., 2014), in PBMC and in exosomes from plasma and urine of NTZ-treated MS patients as compared to untreated and healthy individuals (Giovannelli et al., 2015). Although the exact mechanisms of JCPyV reactivation leading to PML lack experimental evidence, two main conceptions prevail: rearrangements may occur in the genome of persistent archetype form of virus either in B cells where immunoglobulin gene rearrangement machinery can be exploited or by the homologous recombination phenomenon taking place during virus replication.

Asymptomatic reactivation of JCPyV may occur in NTZ-treated MS patients (Y. Chen et al., 2009), which may lead to the increased viral replication, rearrangements

of archetype form of virus, including putative alterations within the regulatory region NCCR of virus genome. JCPyV with archetype NCCR is usually present in asymptomatic individuals whereas viral strains with rearranged NCCR forms are the characteristics in PML (Martelli & Giannecchini, 2017). Several processes including increased transcription, DNA replication, and expression of viral gene products would enable the better detection of virus infection by both innate and adaptive immune systems of the host. On the other hand, substantial downregulation of archetype BK polyomavirus DNA replication was shown due to viral miRNA expression, aiding to maintain persistent virus in a healthy host despite a functional immune system (Broekema & Imperiale, 2013). In analogy, downregulation of miRNA expression could release viral early gene transcription and DNA replication from the negative regulation by miRNA and in the context of JCPyV, allow viral replication resulting in the formation/emergence of rearranged neurotropic strains. This could also explain our observation of lower expression of 5p miRNA of JCPyV in NTZ-treated patients as compared to IFN-β-treated patients, although the expression levels were similar in comparison with healthy controls.

Moreover, we observed an association of 5p miRNA level with JCPyV seropositivity in NTZ-treated MS patients. In a previous study, no such association between JCPyV DNA positivity in blood or urine, and a risk of developing PML among NTZ-treated MS patients was observed (Rudick et al., 2010). Although no reports are available to compare our result of correlation, two recent studies have detected similar inverse correlations of 5p miRNA expression with JCPyV T-Ag expression in colorectal cancer tissues (Link et al., 2014), and with JCPyV DNA load in blood and CSF of those HIV patients who were at risk of developing PML (Rocca et al., 2015). These observations confirm that miRNA expression may actually restrict replication of virus in order to suppress immune responses towards the virus by the host.

Both studies on sL-selectin and JCPyV miRNAs have similar limitations, the absence of pre-PML or PML samples associated with the NTZ-treatment. Actually, only two NTZ- associated PML cases among MS patients, both deceased, were reported in Finland so far. It would be also interesting to measure levels of sL-selectin and JCPyV miRNA expression in the prospective follow-up study on the same group of MS patients who were evaluated as patients with high risk for PML to see whether any of the patients developed eventual PML. Therefore, future studies, including PML samples, will have to determine the clinical relevance of sL-selection and JCPyV miRNA as biomarkers for predicting PML risk associated with

NTZ-treatment in MS. It would be also interesting to measure some human miRNAs as well in NTZ-treated JCPyV seropositive MS patients.

6.2 Novel immune molecules as biomarkers of MS disease spectrum

6.2.1 Soluble CD26 and CD30 as markers of MS disease activity

This study assessed whether the circulating levels of CD26 and CD30 in sera are associated with MS subtypes, inflammatory disease activity and disability in MS patients. The identification of candidate immunological biomarkers that correlate with disease profiles in MS is highly needed for the design of personalized therapeutic strategies. Moreover, the current treatment algorithms are not effective enough to depict the underlying complex pathogenic heterogeneity of MS. Therefore, the levels of sCD26 and sCD30 were analysed to explore their biomarker potential in MS patients, including also the CIS patients. CD26 and CD30 molecules provide costimulatory signals for the optimal activation of T cells and have been implicated in autoimmune pathophysiology (Del Prete et al., 1995; Tanaka et al., 1993). Levels of sCD26 and sCD30 were found to be increased in RRMS, SPMS and CIS patients compared to the controls. Previous studies have reported increased expression of CD26 on T cells in the blood of patients with relapsing MS (Khoury et al., 2000) and progressive MS (Hafler et al., 1985). On the contrary, the concentration of soluble form of CD26 in plasma, and its enzymatic activity, was found to be decreased in MS (Tejera-Alhambra et al., 2014). Interestingly, this study observed higher levels of sCD26 in MS patients who were in remission compared to patients who had relapses (Tejera-Alhambra et al., 2014), supporting our observation of increased levels of sCD26 in MS patients who had relatively stable disease. Another study had reported elevated sCD26 in CSF of MS patients and also in relapsing NMO patients, but they found unchanged serum levels in MS patients compared to controls (Narikawa et al., 2006). In line with these observations, increased CD26 levels suggest the predominant presence of regulatory immune response characteristics of the stable phase of MS disease course. In addition, we also presented the possible effect of IFN-β treatment resulting increased sCD26 in sera. It was shown that IFN-β treatment decreases the percentage of CD26 cells expressing on the surface of $CD8^+$ T cells in MS patients that might stimulate the

shedding of cell surface CD26 to the circulation as a soluble form (Jensen et al., 2006).

Similar to sCD26, increased levels of sCD30 were observed in MS and CIS, but without group difference among MS subtypes. In line with this observation, increased sCD30 were previously reported in blood and CSF of MS patients and, in particular, among RRMS patients who were at clinical remission compared to patients at relapse (McMillan et al., 1998). Therefore, our data suggest that the increased levels of sCD30 might reflect a relatively inactive disease course in MS. CD30 was considered as a marker of Th2-type immune responses and was shown to be involved in immunoregulatory activities to maintain the physiological balance between Th1 and Th2-type immune responses (Pellegrini et al., 2003; Pellegrini et al., 2005). The regulatory role of sCD30 was further sustained by our observation of increased levels of sCD30 in RRMS patients treated with DMTs compared to treatment naïve patients. IFN-β treatment induces the immune shift from Th1-type to Th2-type responses by enhancing the production of regulatory cytokines such as IL-10 and IL-4, and decreasing the production of pro-inflammatory cytokines, such as IFN-γ (Jensen et al., 2006; Šega et al., 2004). This supports our finding of a positive correlation between IL-10 and sCD30 and further supports sCD30 as a marker of regulatory immune responses in stable MS patients. In addition, increased levels of sCD30 in converted CIS patients before they were converted to RRMS when compared with the controls might indicate the presence of regulatory immune responses already at the CIS stage, which might indicate the goal of the immune system to inhibit or balance the pro-inflammatory events responsible for the development of tissue damage. Some of the limitations of this study were the lack of MRI data and small sample size. Therefore, further studies, including longitudinal follow-up analyses with concurrent MRI analyses, are needed to confirm this preliminary result.

6.2.2 Association of DR3, DcR3, and TL1A gene expressions with MS

This is the first study to the best of our knowledge which evaluated the relative gene expression of death receptors (DR3, DcR3) and ligand (TL1A) of tumor necrosis factor superfamily in different MS subtypes and investigated their association with clinical and MRI characteristics. Due to the evidence indicating the association of DR3, DcR3, and TL1A with neuroinflammation and autoimmunity (Sonar & Lal, 2015), PBMC gene expression of these molecules obtained from MS patients were

analyzed and evaluated whether these molecules may contribute as biomarkers in MS. The results showed that only TL1A expression was decreased in relapsing onset MS compared to PPMS, CIS and HCs. However, no group difference was observed for DR3 and DcR3. TL1A is considered as a marker of active inflammation since its binding to DR3 receptor induces activation of NF-kappaB, and therefore promoting inflammation, but also regulating apoptotic cell death by activating caspase cascade (Oh & Ghosh, 2013; Schreiber et al., 2010; Schreiber & Podack, 2013). Expression of DR3 is primarily upregulated on the activated immune cells such as T cells, monocytes, B cells and dendritic cells (Siakavellas et al., 2015).

All relapsing onset MS patients recruited in this study had relatively stable clinical disease activity; therefore, the observation of lower TL1A expression most likely reflects the state of reduced inflammatory activity or the state of immune system homeostasis that is mediated by the tight regulation of apoptosis (Macchi et al., 2015). In MS, inflammatory and apoptotic responses may occur either at the peripheral level or at the CNS, and in particular, stable phase of the disease that is characterized by the inhibition of peripheral inflammatory response, as in the case of healthy individuals (Gurevich & Achiron, 2012; Macchi et al., 2015). Moreover, as shown in other diseases, decreased expression of TL1A in relapsing onset group might partly be explained by the effect of treatment with DMTs, resulting the significant reduction of these molecules as a response to anti-inflammatory therapy (Bamias et al., 2012). IFN- β has immunoregulatory properties and the treatment induces the expression of large number of genes that encode proteins, which have immunomodulatory and apoptosis promoting functions (Dhib-Jalbut & Marks, 2010; Sellebjerg et al., 2008). In relapsing onset MS group, half of the patients were treated with DMTs and those patients showed to have even lower expression of TL1A as compared to treatment naïve patients. Moreover, in line with our previous study, DMT treated relapsing onset patients were classified as partial responders and responders (Rinta et al., 2008). Interestingly, the levels of TL1A tended to be upregulated in patients with partial DMT responders in comparison to responders indicating the presence of inflammatory activity in this subgroup. These findings suggest that TL1A expression may hold the ability to reflect ongoing stable disease course and as well as the marker of therapeutic response to immunomodulatory treatment in MS.

To date, there are no available reports of TL1A association to clinical or radiological parameters related to disease activity and progression in MS. However, some experimental studies have found an association to EAE disease pathogenesis mediated through Th1- and Th17 T-cells (Meylan et al., 2008; Pappu et al., 2008). In

addition, TL1A association with disease activity profiles was shown in several other diseases such as Crohn's disease, RA and SLE (Bamias et al., 2008; Xu et al., 2017). In the present study, TL1A correlated positively with EDSS score, volumes of hypointense T1 and FLAIR lesions in relapsing onset group indicating the connection of low TL1A expression to both disability freedom and lower hypointense T1 and FLAIR lesion load pointing at the role of TL1A in inflammatory activity. In particular, the expression of TL1A was associated to increase of EDSS score over a year and increase of FLAIR lesion volumes over a year suggesting its role to reflect the ongoing inflammatory activity in MS and predicting disease progression, however, further investigation is required in a larger sample size.

7 CONCLUSIONS

PML risk assessment is a challenging field in which the identification of sensitive biomarkers could significantly contribute to the improved identification of patients at high risk. This thesis has highlighted the biomarker potential of sL-selectin and JC virus miRNAs for predicting the risk of developing PML in long-term NTZ-treated MS patients. The Study I concluded that the level of sL-selectin is biologically connected to the anti-JCPyV antibody levels and possibly also the cell surface L-selectin in NTZ-treated MS patient. This observation is noteworthy in order to further evaluate the biomarker potential of soluble L-selectin to predict the PML risk. The Study II showed that JCPyV 5p miRNA could be a new marker for the PML risk assessment in MS. We hypothesized that a low level of miRNA in plasma may suggest a possible involvement in the release of viral reactivation and indicative of high replication of JCPyV, which may consequently cause PML. In addition, the observation of high level of miRNA prevalence also in JCPyV seronegative patients reconfirmed that the ELISA test that is currently used for the detection of JCPyV antibody has insufficient sensitivity.

Studies III and IV were aimed for finding biomarkers to evaluate the disease activity, disease progression, and neurological disability in MS patients. In Study III, the observation of increased levels of sCD26 and sCD30 in MS suggested the potential of these molecules as biomarkers of relatively inactive disease course in MS or the markers of stable disease activity. Immunologically, increased levels of these molecules indicated the state of homeostasis between pro-inflammatory Th1 and anti-inflammatory Th2-type immune responses as a reflection of stable disease activity in MS. Moreover, sCD30 was evaluated as a regulatory molecule or the marker of regulatory immune response due to its positive correlation with IL-10, and increased levels in RRMS patients treated with DMTs compared with untreated patients. To the best of our knowledge, Study IV is the first work reporting the gene expression of DR3, DcR3 and TL1A in PBMC obtained from MS patient, and evaluated their biomarker potential in MS. This study concluded TL1A as a candidate biomarker for reflecting inflammatory activity in MS and predicting disability progression. The findings of this study further illustrated that TL1A may hold the ability to reflect ongoing stable disease course and as well as the marker of

therapeutic response to immunomodulatory treatment in MS. However, additional studies including a larger sample size are needed to evaluate the clinical relevance of these findings.

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Pabitra Basnyat

Pabitra Basnyat
Tampere, Finland, January 11, 2019

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10 PUBLICATIONS

PUBLICATION I

Association between soluble L-selectin and anti-JCV antibodies in natalizumab-treated relapsing-remitting MS patients

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Association between soluble L-selectin and anti-JCV antibodies in natalizumab-treated relapsing-remitting MS patients

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ABSTRACT

Objective: In relapsing-remitting MS (RRMS) patients treated with natalizumab, the low level of L-selectin-expressing CD4+ T cells has been associated with the risk of progressive multifocal leukoencephalopathy (PML). In this study, our aim was to correlate the levels of soluble L-selectin and the anti-JCV antibody index in the sera of RRMS patients treated with natalizumab.
Methods: This study included 99 subjects, including 44 RRMS patients treated with natalizumab, 30 with interferon beta (IFN-β) and 25 healthy controls. The levels of soluble L-selectin (sL-selectin) in sera were measured by ELISA, and the anti-JC Virus (JCV) antibody index was determined by the second–generation ELISA (STRATIFY JCV™ DxSelect™) assay.
Results: A significant correlation was found between the levels of sL-selectin and anti-JCV antibody indices in sera in the natalizumab-treated patients ($r=0.402$; $p=0.007$; $n=44$), but not in those treated with IFN-β. This correlation became even stronger in JCV seropositive patients treated with natalizumab for longer than 18 months ($r=0.529$; $p=0.043$; $n=15$).
Conclusion: The results support the hypothesis of sL-selectin being connected to the anti-JCV antibody index values and possibly cellular L-selectin. Measurement of serum sL-selectin should be evaluated further as a potential biomarker for predicting the risk of developing PML.

1. Introduction

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) that is characterized by complex pathophysiological processes, including inflammation, demyelination, axonal loss and remyelination (Goldenberg, 2012). Natalizumab is a humanized monoclonal antibody and an α4 integrin (CD49d) antagonist that prevents the migration of peripheral leukocytes across the blood–brain barrier (BBB) (Hutchinson, 2007). It has been shown to reduce the relapse rate, decrease sustained disability, and reduce the number of new lesions on magnetic resonance imaging (MRI) (Miller et al., 2003; Polman et al., 2006). Despite its efficacy, long-term treatment (mostly more than 18 months) of natalizumab is associated with the substantial complication of developing progressive multifocal leukoencephalopathy (PML), a demyelinating lytic infection of the CNS caused by John Cunningham Virus (JCV) (Clifford et al., 2010). The precise mechanism of natalizumab-associated PML is still unclear, but it is suggested that PML occurs when immunosurveillance in the CNS is impaired (Mancuso et al., 2012). It has been proposed that blocking lymphocyte trafficking through the BBB during natalizumab therapy would decrease cell-mediated immunity, allowing the reactivation of the JC virus from latency (Berger and Houff, 2009). According to recent data, the relative incidence of natalizumab-associated PML is higher than 2/1000 patients (Buck and Hemmer, 2014). In addition to long-term natalizumab treatment, the prior use of immunosuppressants and the presence of anti-JCV antibodies have been established as contributing risk factors for developing PML (Bloomgren et al., 2012). Although anti-JCV antibodies are widely used for predicting the risk of developing PML, such antibodies are also measured in approximately 60–80% of healthy individuals. However, JCV reactivation and the development of PML are only rarely seen in healthy subjects (Ferenczy et al., 2012). Therefore, there is a high need for more

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sensitive biomarkers that would identify patients with a higher risk of developing natalizumab-associated PML.

Recently, a retrospective study showed that reduced levels of L-selectin-expressing CD4+ cells in blood were associated with a risk of developing natalizumab-associated PML (Schwab et al., 2013). Due to this observation, the authors proposed L-selectin as a possible biomarker for individual PML risk in MS patients. L-selectin (CD62L) is a cell adhesion molecule expressed on the surface of most circulating leukocytes, including T cells. It is also present as a functionally active soluble form in the blood (Raffler et al., 2005), which significantly increases during acute or chronic inflammation (Smalley and Ley, 2005). In this study, our aim was to evaluate whether the soluble form of L-selectin is associated with anti-JCV antibody indices in natalizumab-treated RRMS patients, which would suggest the potential of sL-selectin in the assessment of PML risk.

2. Patients and methods

2.1. Patients

This cross-sectional study included a total of 99 subjects of whom 44 RRMS patients were treated with natalizumab, 30 patients with IFN-β (21 patients with Rebif 22 μg and 9 with Rebif 44 μg) and 25 subjects were healthy controls (HC). MS patients were enrolled consecutively from four Finnish MS centers (Tampere, Helsinki, Seinäjoki, and Turku) between January 2012 and February 2013 based on their ongoing immunomodulatory therapy. The clinical characteristics of these patients are shown in Table 1. All patients underwent clinical and neurological examinations before blood sampling. The diagnosis of MS was based on the revised McDonald Criteria (Polman et al., 2005), and the diagnosis was definite. Neurological disability was evaluated by the expanded disability status scale (EDSS) score (Kurtzke, 1983). The study was approved by the Ethics Committee of Tampere University Hospital, and all subjects gave informed consent. The healthy individuals had no previous history of any neurological disorders or immune-mediated diseases.

2.2. Determination of sL-selectin /CD62L concentrations in serum

The collected blood was allowed to clot and was centrifuged for 15 min at 1500xg. Sera were separated from blood, aliquoted and stored at −80 °C until use. sL-selectin levels were determined by commercially available quantitative enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol (#BBE4B; Quantikine, R&D Systems Europe Ltd, Abingdon, United Kingdom). Briefly, serum samples with 1:100 dilutions were mixed with a monoclonal antibody that is specific for human serum L-selectin, which was pre-coated on a 96-well microtiter plate. Horseradish peroxidase (HRP) conjugate, an enzyme-linked polyclonal antibody specific for human L-selectin, was then added. Color developed after TMB (Tetramethylbenzidine) substrate addition was stopped by adding hydrochloric acid (HCL) as a stop solution. The absorbances were measured at wavelength of 450 nm on a Multiskan MS version 4.0 spectrophotometer (Labsystems, Helsinki, Finland). The intra- and inter-assay coefficients of variation for the sL-selectin assay was 4.1% and 7.1%, respectively. The minimum detection limit for sL-selectin assay was 0.3 ng/mL.

2.3. Determination of the anti-JCV antibody index

A confirmatory second–generation ELISA (STRATIFY JCV™ DxSelect) was used to test sera for anti-JCV antibodies at the Unilabs, Denmark (Lee et al., 2013). A screen index value of less than 0.2 was considered anti-JCV antibody negative and of greater than 0.4 as anti-JCV antibody positive. The samples with a screen index between 0.2 and 0.4 were evaluated with a supplementary confirmatory test, and results greater than 45% were classified as anti-JCV antibody positive (Lee et al., 2013).

2.4. Statistical analysis

Statistical analyses were performed using SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL, USA). A non-parametric, two-tailed Mann–Whitney U test was used to compare the differences between the clinical parameters and levels of sL-selectin in different groups. Spearman's correlation coefficient was used to analyze the correlation between the sL-selectin levels and anti-JCV antibody index. A *p*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Clinical data

Natalizumab-treated patients had a longer disease duration

Table 1
Clinical characteristics of MS patients and healthy controls.

Characteristics	Natalizumab	IFN-β	HC	*p*-value
	***n*=44**	***n*=30**	***n*=25**	
Sex (F/M)[a]	34/10	21/9	19/6	
Age (years)[b]	38.2 ± 7.8 (23-52)	35.5 ± 9.9 (20-53)	33.2 ± 11.0 (22–60)	NS
Disease duration from diagnosis (years)[b]	9.1 ± 5.3 (1.8–22.4)	4.6 ± 5.4 (0.2-18.1)	–	$p < 0.001$
EDSS[b]	2.7 ± 1.9 (0–6.5)	1.4 ± 1.6 (0-6.0)	–	$p=0.002$
Number of relapses[b,c]	1.9 ± 1.0 (1-4)	–	–	–
Duration of treatment (years)[b]	2.8 ± 1.5 (0.4–5.8)	2.5 ± 2.8 (0–13.1)	–	NS
Anti-JCV Ab index [d]	0.3 (0.1–3.1)	0.3 (0.1–2.9)	–	NS
Anti-JCV Ab seropositivity[a]	21 (48%)	13 (43%)	–	NS
JCV-positive Ab index[d,e]	1.1 (0.3–3.1)	1.9 (0.7–2.9)	–	NS

IFN-β – interferon-β, **HC –** healthy controls, **EDSS** – expanded disability status scale, **JCV –** John Cunningham virus, **NS –** not significant

[a] Number of patients.
[b] Mean ± SD (range).
[c] Two years before starting Natalizumab.
[d] Median (range).
[e] Anti-JCV antibody index of seropositive patients only.

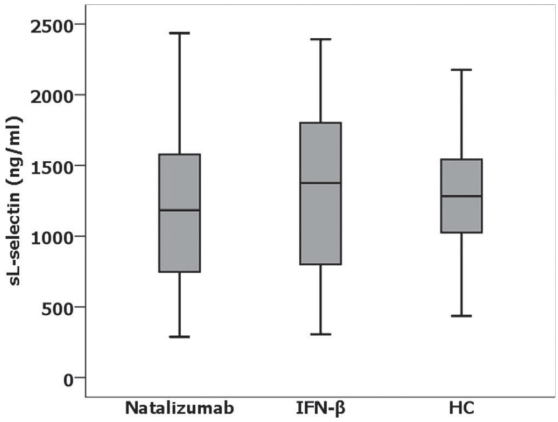

Fig. 1. The levels of sL-selectin did not differ between the natalizumab-, IFN-β-treated or healthy controls (HC) ($p > 0.05$, Mann–Whitney U test). The length of the box represents the interquartile range within which 50% of the values were located. The middle line in each box represents the median value. The lower and upper bars show the standard deviation.

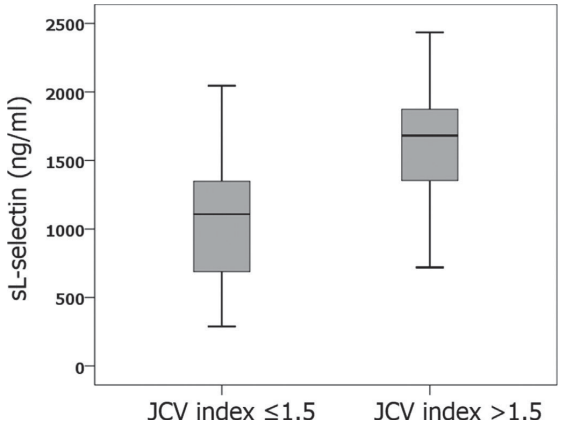

Fig. 2. The levels of sL-selectin were significantly higher in the high-risk group (anti-JCV antibody index > 1.5, $n=8$) compared to that with low risk group (anti-JCV antibody index ≤ 1.5, $n=36$) for developing PML in natalizumab-treated patients ($p=0.01$, Mann–Whitney U test). The length of the box represents the interquartile range within which 50% of the values were located. The middle line in each box represents the median value. The lower and upper bars show the standard deviation.

and higher EDSS scores than IFN-β-treated patients, while the age and duration of therapy did not differ between the groups (Table 1). Two years before initiating natalizumab therapy, all 44 patients had relapses, and 41 of them were previously treated with other immunomodulatory or immunosuppressive drugs (12 patients with only IFN-β, 28 patients with IFN-β and other immunomodulatory or immunosuppressive (IS) drugs and 1 with only the IS drug), and the remaining 3 patients did not have any pretreatment. The mean duration of natalizumab therapy was 2.8 (± 1.5 (SD), 0.4–5.8 (range)) years. During natalizumab therapy, relapses were observed in 8 (18%) patients in whom the treatment duration ranged from 1.7 to 5.8 years. The seroprevalence of anti-JCV antibodies was 48% in natalizumab-treated and 43% in IFN-β-treated patients. In JCV-seropositive natalizumab-treated patients ($n=21$), the mean duration of this therapy was 2.5 (± 1.4 (SD), 0.4–5.3 (range)) years.

3.2. Association between sL-selectin and the anti-JCV antibody index

The levels of sL-selectin did not differ between the patients treated with natalizumab, IFN-β or healthy controls (Fig. 1), or between the JCV seropositive and JCV seronegative patients in natalizumab-(1330.5 ± 476.2 vs. 1076.0 ± 500.9 ng/ml (mean ± SD); $p=0.10$) or IFN-β-treated groups (1225.5 ± 611.4 vs. 1403.6 ± 575.9 ng/ml (mean ± SD); $p=0.28$). The natalizumab-treated patients were further stratified according to their anti-JCV antibody index into groups with high risk for developing PML (anti-JCV antibody index higher than 1.5, $n=8$) and low risk group for developing PML (anti-JCV antibody index ≤ 1.5, $n=36$). The levels of sL-selectin were significantly higher in the high-risk group compared to that with low risk group for developing PML (1621.3 ± 506.9 vs. 1103.3 ± 453.2 ng/mL (mean ± SD); $p=0.01$) Fig. 2.

Association between sL-selectin and anti-JCV antibody index was also studied by correlation analyses. A positive correlation was found between the levels of sL-selectin in sera and the anti-JCV antibody indices in all natalizumab-treated patients ($r=0.402$; $p=0.007$; $n=44$, Fig. 3A), but not in the IFN-β-treated patients ($r=-0.262$; $p=0.161$; $n=30$; Fig. 3B). Approximately 80% of natalizumab-treated subjects (34/44) patients were treated with natalizumab for more than 18 months. In this group, sL-selectin levels correlated with anti-JCV antibody indices ($r=0.385$; $p=0.025$; $n=34$; Fig. 3C), and this correlation became even more significant in JCV seropositive patients treated with natalizumab for more than 18 months ($r=0.529$; $p=0.043$; $n=15$; Fig. 3D). No significant correlations between sL-selectin and anti-JCV index were found when JCV seropositive ($r=0.356$ $p=0.113$, $n=21$) and JCV seronegative ($r=0.360$; $p=0.092$; $n=23$) natalizumab-treated patients were evaluated as separate groups. There was no correlation between anti-JCV antibody index and treatment duration in patients treated with either natalizumab or IFN-β.

4. Discussion

In this study, we have shown that the levels of sL-selectin in sera of natalizumab-treated RRMS patients correlate positively with their anti-JCV antibody indices. Earlier studies have shown higher anti-JCV antibody index values in natalizumab-associated PML compared to non-PML patients (Outteryck et al., 2013; Plavina et al., 2014; Trampe et al., 2012), indicating the potential of JCV index for predicting PML risk. The demonstration of association between increased sL-selectin levels and increased anti-JCV antibody indices suggests that sL-selectin measurement could be useful for the evaluation of PML risk among patients treated with natalizumab. This observation is further supported by our result of increased sL-selectin level in natalizumab-treated patients with high anti-JCV antibody index (> 1.5) that are considered at highest risk for developing PML (Lee et al., 2013).

Recently, Schwab et al. studied the expression of L-selectin on the surface of CD4+ T cells and proposed that lack of L-selectin on CD4+ T cells was indicative of increased PML risk in natalizumab-treated MS patients (Schwab et al., 2013). Moreover, in the recent study of these authors, the positive correlation between cellular L-selectin expression on T cells and anti-JCV antibody index values in natalizumab-treated patients was detected (Schwab et al., 2014). Interestingly, low cell-surface L-selectin levels have been shown to be associated with increased serum levels of sL-selectin that is due to shedding of L-selectin from the cell surface, a mechanism possibly explaining reduced leukocyte extravasation into tissues (Jackson et al., 2005). These observations are in line with our result of increased level of sL-selectin indicative of increased PML risk in

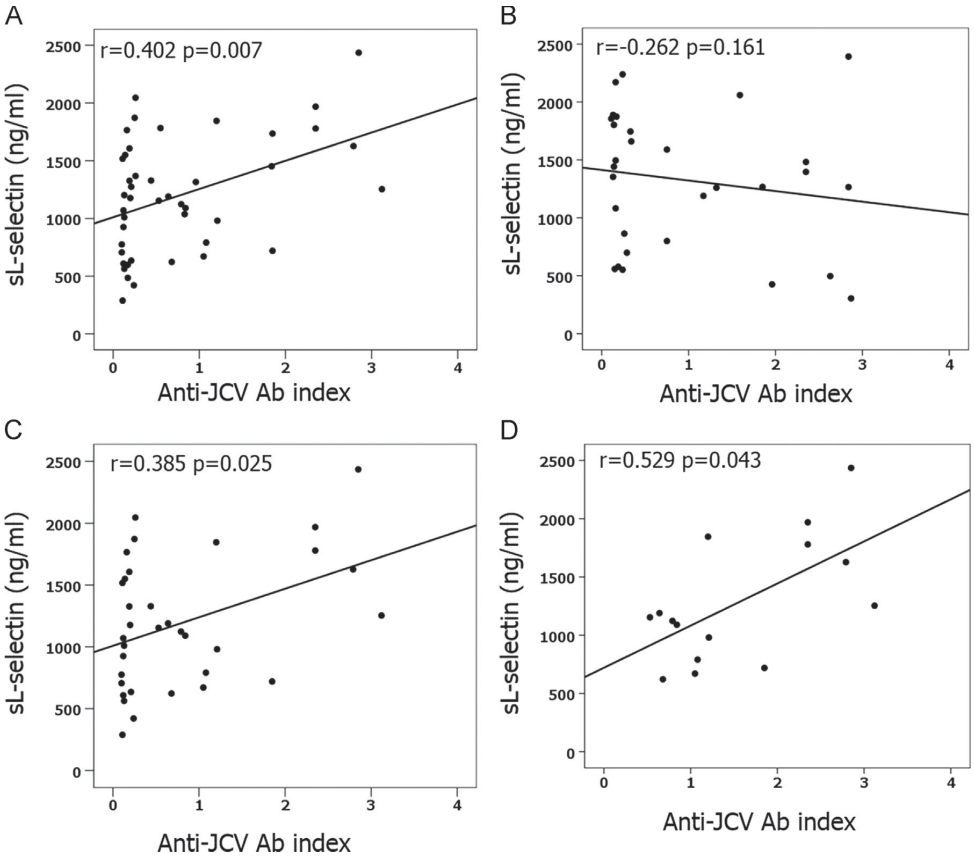

Fig. 3. Correlation analyses between sL-selectin and the anti-JCV antibody index in (A) Natalizumab-treated patients, (B) IFN-β-treated patients, (C) Long-term natalizumab-treated (> 18 months) patients and (D) JCV seropositive long-term(> 18 months) natalizumab-treated patients.

natalizumab-treated patients. Since we could not measure the sL-selectin levels in the pre-PML or PML samples, use of sL-selectin as a biomarker of PML risk need to be evaluated in the future studies. Indeed, it would be interesting to measure the level of sL-selectin in the serum of those patients described by Schwab et al., who lacked L-selectin on CD4+ T cells, and who later developed PML (Schwab et al., 2013). Our hypothesis is that they also would have increased levels of sL-selectin, and our observations might hence be indicative of a biological association between shedding of cellular L-selectin and rising anti-JCV antibody levels in natalizumab treated RRMS patients.

Role of L-selectin in MS pathogenesis has been studied earlier. Different studies have shown that L-selectin is involved in myelin damage processes in the CNS (Grewal et al., 2001; Huang et al., 1994). L-selectin is constitutively expressed on lymphocytes, and it mediates the initial capturing and tethering of leukocytes to the endothelium. Following leukocyte activation by cytokines and chemotactic factors, L-selectin is rapidly shed from the cell surface by endoproteolytic cleavage (Wedepohl et al., 2012; Wang et al., 2010). Activation-independent shedding of L-selectin may also occur during crosslinking with specific monoclonal antibodies (Palecanda et al., 1992). It is suggested that shedding L-selectin from activated lymphocytes prevents cell re-entry to the peripheral lymph nodes and sites of inflammation, likely by preventing the L-selectin-dependent mechanism of lymphocyte adhesion and transmigration (Wedepohl et al., 2012; Ivetic, 2013; Schleiffenbaum et al., 1992). Earlier studies showed increased levels of sL-selectin in serum and CSF in RRMS patients (Duran et al., 1999; Baraczka et al., 2000). Association of serum sL-selectin with active, gadolinium enhancing lesions in MRI has also been reported in different studies (Mossner et al., 1996; Hartung et al., 1995), suggesting its role in the inflammatory processes of active MS.

Natalizumab has proven to be therapeutically highly efficient for treatment of active MS. However, the risk of PML hampers its use and hence biomarkers for prediction of PML risk of individual patients are sorely needed. Importantly, measurement of soluble serum L-selectin using an ELISA assay is easy, reliable and applicable to any laboratory, unlike measurement of L-selectin on the surface of frozen T cells using flow cytometry, which is technically demanding and prone to errors related e.g. to cell handling, which might lead to shedding of L-selectin from the cell surface and thus influence the results (Schwab et al., 2013). In future studies, it will be highly interesting to measure the sL-selectin levels in a natalizumab-treated MS patient cohort with eventual PML to see whether they will have high sL-selectin levels.

In conclusion, our preliminary data demonstrate the positive correlation between sL-selectin and the anti-JCV antibody levels in the sera of natalizumab-treated MS patients, and suggest that measurement of serum sL-selectin should be evaluated further as a potential biomarker for predicting the risk of developing PML in this patient cohort.

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PUBLICATION II

JCPyV microRNA in plasma inversely correlates with JCPyV seropositivity among long-term natalizumab-treated relapsing-remitting multiple sclerosis patients

Basnyat, P., Virtanen, E., Elovaara, I., Hagman, S., & Auvinen, E

Journal of Neurovirology, 23(5), 734-741
Doi: 10.1007/s13365-017-0560-x

PUBLICATION III

Elevated levels of soluble CD26 and CD30 in Multiple Sclerosis

Basnyat, P., Natarajan, R., Vistbakka, J., Lehtikangas, M., Airas, L., Matinlauri, I., Elovaara, I., Hagman, S

Clinical and Experimental Neuroimmunology, 6(4), 419-425
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ORIGINAL ARTICLE

Elevated levels of soluble CD26 and CD30 in multiple sclerosis

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Keywords
sCD26; sCD30; interleukin-10; multiple sclerosis

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Abstract

Objective The activation of autoreactive T cells is a major event in the initiation of autoimmune responses in multiple sclerosis (MS). In addition to the T cell receptor stimulation, optimal activation of T cells requires various costimulatory molecules, such as CD26 and CD30, which has not been extensively studied in MS. Our aim was to explore whether the circulating levels of CD26 and CD30 in sera are associated with MS subtypes, inflammatory disease activity and disability in MS patients.

Methods The study included 195 participants: 39 relapsing–remitting MS patients, 19 secondary-progressive MS patients, 19 clinically isolated syndrome patients, 58 controls for sCD26 analysis and 60 for sCD30 analysis. The levels of sCD26 and sCD30 in sera were analyzed using enzyme-linked immunosorbent assay, and the levels of interleukin-10, tumor necrosis factor-α and interferon-γ were analyzed with the Luminex assay.

Results We observed increased levels of sCD26 and sCD30 in relapsing–remitting MS, secondary-progressive MS, and clinically isolated syndrome patients compared with the controls ($P < 0.05$). Furthermore, elevated levels of sCD30 were noticed in treated relapsing–remitting MS patients than in untreated patients ($P = 0.016$), and also in converted CIS patients than in unconverted patients ($P = 0.009$). Although sCD26 and sCD30 could not associate with clinical measures, such as the disability score or disease activity, the levels of sCD30 correlated positively with interleukin-10 levels ($r = 0.583$, $P < 0.0001$) and sCD26 levels ($r = 0.262$, $P = 0.046$) in MS patients.

Conclusion The present results suggest that the elevated levels of sCD30 are associated with the regulatory immune responses predisposing to clinically stable phase of MS.

Introduction

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system, characterized by inflammation, myelin damage and axonal degeneration.[1] Current studies have shown the critical role of myelin-specific CD4+ Th1 and Th17 cells in the initiation of autoimmune responses in MS.[2] During this process, autoreactive CD4+ T cells are activated in the periphery and migrate to the central nervous system through the blood–brain barrier, initiating neuroinflammation that eventually leads to neuronal damage. The activation of these T cells is a tightly regulated process that is dependent on two signals: the first signal is provided by the interaction between the T cell receptor and major histocompatibility complex (MHC) class II molecules, whereas the second signal is provided by costimulatory molecules. CD80/CD86–CD28/CTLA4 are the most important and best known costimulatory signals,[3] but several other costimulatory molecules, such as CD26 and CD30,

are known to mediate the optimal activation of T cells.[4,5]

CD26 (also known as dipeptidyl peptidase IV) is a 110-kD transmembrane glycoprotein that plays an important role in the inactivation of incretin hormones, and is also involved in the inactivation of chemokines CXCL9–11 and CXCL12, which are known to be secreted after T cell activation.[6–9] In addition to the enzymatic function of CD26, membranous CD26 also binds to its ligands, adenosine deaminase and caveolin-1, to provide a costimulatory signal that enhances the activation of T cells.[10,11] CD30 is a 120-kD transmembrane glycoprotein that belongs to the tumor necrosis factor receptor superfamily, and is predominantly expressed on the surface of activated T cells, B cells and NK cells.[12,13] Previously, it was shown that CD30 activation promotes the development of Th2 cells, and it was therefore considered to be an activation marker of Th2 cell populations. CD30 binds with its ligand, CD30L, which induces cell proliferation by the activation of nuclear factor-kappa B in T cells, but it might also induce growth arrest and apoptosis.[14,15] Both CD26 and CD30 exist in soluble forms that are released into the blood stream on T cell activation; however, their biological functions are not yet fully understood.

Until now, the role of CD26 and CD30 in MS has not been extensively studied. In previous studies, a higher number of CD26+CD4 T cells and CD8 T cells in the blood was shown to be associated with the clinical and magnetic resonance imaging (MRI) measurements of disease activity in MS.[16–19] Additionally, lower levels of soluble CD26 (sCD26) in plasma and higher levels in CSF have been reported in MS patients.[20,21] Similarly, few studies have reported elevated levels of soluble CD30 (sCD30) in MS patients, but no association has been shown with clinical or MRI measures.[22,23]

In the present study, our aim was to explore whether the levels of sCD26 and sCD30 are associated with clinical phenotypes, disease activity and disability progression in MS. Because CD26 and CD30 expression might reflect the activation state of T cells, we also associated their levels to pro- and anti-inflammatory molecules to further understand the roles of these molecules in MS.

Methods

The study included 195 participants: 39 relapsing–remitting MS (RRMS) patients, 19 secondary-progressive MS (SPMS) patients, 19 clinically isolated syndrome (CIS) patients, 58 controls for sCD26 analysis: age 30.1 ± 9.4 years, 33 women, 25 men; and 60 for sCD30 analysis: age 34.0 ± 11.8 years, 39 women, 21 men (mean ± SD).

The diagnosis of MS was based on the revised McDonald Criteria, and the diagnosis was definite.[24] CIS patients were defined as patients who had their first demyelinating event that was suggestive of MS.[24] All patients underwent neurological examination, which included the determination of prestudy disease activity (number of relapses preceding 2 years of the study), expanded disability status scale (EDSS) score and the disease duration, which are summarized in Table 1.[25] The study was approved

Table 1 Clinical characteristics of patients

	All CIS *n* = 19	CIS-CIS[a] *n* = 9	CIS-CDMS[b] *n* = 10	RRMS *n* = 39	SPMS *n* = 19
Gender (M/F)[c]	2/17	1/8	1/9	12/27	7/12
Age[d]	35.0 ± 9.0	37.0 ± 8.9	33.3 ± 8.5	37.4 ± 8.7	49.1 ± 8.6
Disease duration (years)[d]	NA	NA	NA	3.9 ± 3.8	11.7 ± 9.7
EDSS[d]	0.1 ± 0.3	0.1 ± 0.3	0.1 ± 0.3	1.4 ± 1.5	4.7 ± 1.8
Number of relapses[c,e]					
0	4	1	3	12	15
1	13	6	7	12	2
2–5	2	2	0	15	2
Therapy (NT/IFN/GA)[c]	0/0/0	0/0/0	0/0/0	19/18/2	19/0/0

CDMS, clinically definite MS; CIS, clinically isolated syndrome; EDSS, expanded disability status scale; GA, glatiramer acetate; IFN, interferon-β; NA, not applicable; NT, no treatment; RRMS, relapsing remitting MS; SPMS, secondary progressive MS.

[a]Patients who remained CIS.

[b]Patients with CIS who converted to clinically definite MS.

[c]Number of patients.

[d]Mean ± SD.

[e]All relapses preceding two years before study entry.

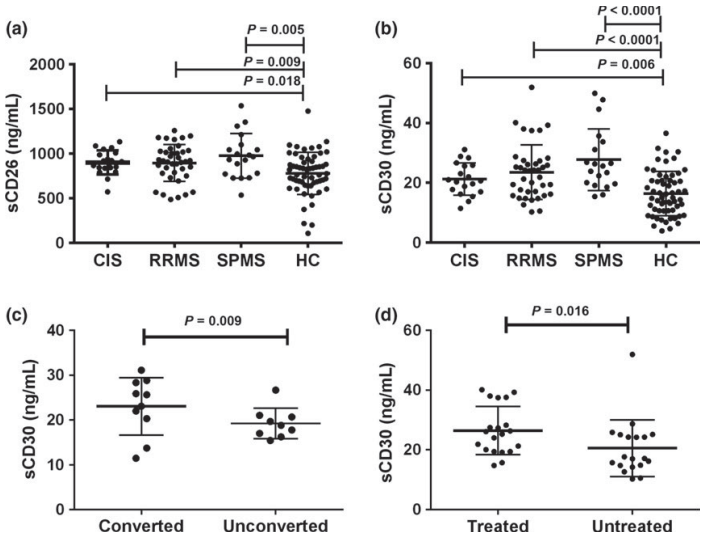

Figure 1 Different levels (mean ± SD) of (a) sCD26 and (b) sCD30 in patients with clinically isolated syndrome (CIS), relapsing–remitting multiple sclerosis (RRMS), secondary progressive MS (SPMS) and healthy controls (HC). (c) sCD30 levels in converted and unconverted CIS patients. (d) sCD30 levels in treated and untreated RRMS patients.

by the ethics committee of Tampere University Hospital, and all participants gave informed consent. Serum samples from 39 RRMS patients were obtained at the time of remission, and none of the patients had any relapse 8 weeks before study entry.

The levels of sCD26 and sCD30 in sera were analyzed using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Human sCD26 Platinum ELISA BMS235CE and Human sCD30 instant ELISA BMS240INSTCE; eBioscience, Bender MedSystems GmbH, Vienna, Austria). The levels of interleukin (IL)-10, tumor necrosis factor-α and interferon (IFN)-γ were analyzed with the Luminex assay using a high-sensitivity human cytokine LINCOplex kit (Linco Research, St. Charles, MO, USA).

Statistical analyses were carried out with SPSS version 18.0 (SPSS, Chicago, IL, USA). The Mann–Whitney *U*-test was used to analyze the differences in sCD26 and sCD30 levels and clinical parameters between the subtypes. Spearman's correlation analysis was used to explore the association of sCD26 and sCD30 with cytokines and the clinical characteristics of the patients. A *P*-value <0.05 was considered statistically significant.

Results

Clinical characteristics

The clinical characteristics of the study participants are summarized in Table 1. As expected, the patients in the SPMS group had longer disease duration and were older than the RRMS and CIS patients ($P < 0.05$). The EDSS scores were lowest in CIS when compared with RRMS and SPMS subtypes. The SPMS group had higher EDSS scores than patients with RRMS ($P < 0.05$). Two years before enrolment, 12 out of 39 (31%) RRMS patients were relapse-free, another 12 patients had one relapse and 15 out of 39 (38%) patients had two to five relapses. Out of 39 RRMS patients, 18 patients were treated with interferon-beta (IFN-β) and two other patients with glatiramer acetate. CIS patients were followed up clinically for 4 years. During that time, 10 out of 19 patients converted to RRMS (Table 1).

Serum sCD26 and sCD30 levels, and correlation with clinical parameters

In all patient groups (CIS, RRMS and SPMS), the levels of sCD26 and sCD30 appeared to be higher than in the controls (Fig. 1a,b), although no differences were detected between patient subgroups. Among the 19 CIS patients, 10 converted patients showed higher levels of sCD30 compared with the controls (23.0 ± 6.4 *vs* 16.3 ± 7.4 ng/mL, $P = 0.009$), whereas no difference was found between unconverted patients and controls (Fig. 1c). The levels of sCD26 did not differ in converted CIS patients when compared with controls and unconverted CIS patients (891.1 ± 145.2 *vs* 778.4 ± 237.7 ng/mL, $P = 0.086$; 891.1 ± 145.2 *vs* 911.4 ± 135.9

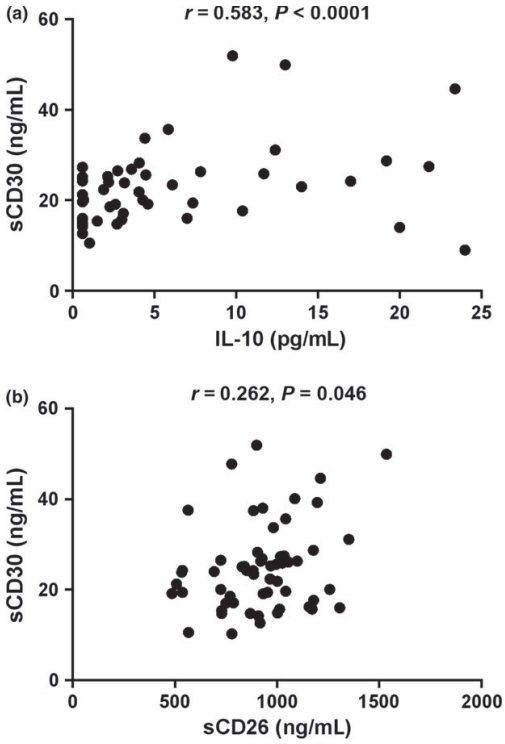

Figure 2 Spearman's correlation analysis between the levels of (a) sCD30 and interleukin (IL)-10 and between the levels of (b) sCD30 and sCD26 in multiple sclerosis (MS) patients.

ng/mL, $P = 0.905$). Furthermore, the levels of cytokines IL-10, tumor necrosis factor-α and IFN-γ in sera did not differ between the MS subtypes and controls ($P > 0.05$).

To understand whether the levels of sCD26 and sCD30 reflect the inflammatory activity in MS, we also correlated their levels with cytokines IL-10, tumor necrosis factor-α and IFN-γ, which were analyzed in 43 out of 58 MS patients. The levels of IL-10 correlated positively with the level of sCD30 in the MS group, including RRMS and SPMS patients ($r = 0.583$, $P < 0.0001$, $n = 43$; Fig. 2a), but the levels of sCD26 and sCD30 did not show any correlation with other cytokines that were analyzed in the present study. We also carried out the correlation analyses between sCD30 and sCD26, and the result showed a weak, but statistically significant, positive correlation between the sCD30 and sCD26 levels in MS patients, including both RRMS and SPMS patients ($r = 0.262$, $P = 0.046$, $n = 58$; Fig. 2b).

We investigated the levels of sCD26 and sCD30, and their association to prestudy disease activity and EDSS scores. The result showed no significant correlation between these molecules and clinical measures in MS patients. Because half of the RRMS patients (51%) were treated with immunomodulatory therapy (Table 1), the effect of treatment on these molecules was explored. The results showed significantly higher levels of sCD30 in treated RRMS patients compared with untreated patients (26.4 ± 8.0 *vs* 20.5 ± 9.4 ng/mL, $P = 0.016$; Fig. 1d).

Discussion

The objective of the present study was to explore the potential of sCD26 and sCD30 as a biomarker to depict the inflammatory disease activity or disability in MS patients that might be helpful to further understand the underlying mechanism of disease pathogenesis.

CD26 has been regarded as a marker of Th1-type immune responses,[26] and recently, its role in Th17-type immune responses was reported.[27] Several studies have shown a higher expression of CD26 on T cells in blood obtained from RRMS patients[17] and progressive MS,[28] whereas contradictory results are reported for its soluble form.[20,21] Observations from the present study showing increased levels of serum sCD26 in MS and CIS patients are consistent with the study by Narikawa et al., who reported increased levels of sCD26 in the CSF of MS patients, but unchanged serum levels compared with the controls.[21] Another study by Tejera et al.[20] showed the lower plasma levels of sCD26 in MS patients compared with controls, but the same study also reported higher levels of sCD26 in patients with remission compared with patients at relapse. In line with the same study, our observation of increased levels of sCD26 in MS most likely reflects the stable phase of the disease, as all of our RRMS patients had relatively inactive disease based on their number of relapses in the 2 years preceding the study (1.1 ± 1.2), and none of the patients had any relapse 8 weeks before the study entry. However, we cannot entirely exclude the possibility of active disease on our MS patients, as the patients did not undergo MRI examination. It is well established that the disease activity, as measured by gadolinium-enhanced MRI scans, is detected 5–10 times more frequently on MRI compared with clinical assessment of relapses.[29] In addition to inactive disease, the use of immunomodulatory treatment in our RRMS patients could also be a factor for increased

levels of sCD26. Recent study has shown that IFN-β treatment decreases the percentage of CD26 cells expressing on the surface of CD8+ T cells in MS patients that might also stimulate the shedding of CD26 from the cell surface to circulation.[19] Therefore, immunomodulatory treatment could possibly be responsible for the increased levels of sCD26 in sera. The exact role of sCD26 is not entirely known, but its shedding from the cell surface might represent the regulatory process involved in immune cell activation.[30] Therefore, the increased levels of sCD26 over the entire disease course might indicate the state of homeostasis between pro-inflammatory Th1 and anti-inflammatory Th2-type immune responses in MS.

Previously, CD30 was considered to be a marker of Th2-type immune responses, and it was shown to be involved in immunoregulatory activities to maintain the physiological balance between Th1 and Th2-type immune responses.[31,32] Increased levels of sCD30 and correlation with disease activity have been detected in different autoimmune disorders associated with Th2-type immune responses, such as systemic lupus erythematosus and systemic sclerosis.[33,34] We observed that the levels of sCD30 were increased in MS and CIS, but no differences were detected amongst the subtypes. Similar observations of increased levels of sCD30 in blood and CSF in MS patients have been previously reported by McMillan et al.[22] They reported increased levels of sCD30 in RRMS patients with clinical remission compared with the patients at relapse.[22] Therefore, our data and the data of others suggest that the increased levels of sCD30 might reflect a relatively inactive disease course in MS.

The regulatory role of sCD30 was further supported by our observation of increased levels of sCD30 in RRMS patients treated with immunomodulatory therapy compared with untreated patients, because IFN-β treatment is shown to induce a shift from Th1-type to Th2-type responses by enhancing the production of regulatory cytokines IL-10 and IL-4, and decreasing the production of pro-inflammatory cytokines, such as IFN-γ.[19,35] Furthermore, the detected positive correlation between the levels of anti-inflammatory cytokine IL-10 and sCD30 in the MS group is in line with this concept. Earlier studies have shown that CD30-positive T cells produce high levels of IL-10,[36,37] and exerts the immunoregulatory activity through the synergic action of IL-4 and IL-10.[38,39]

Consistent with these observations, our findings might suggest the predominant presence of regulatory immune response characteristics of stable phase of the MS disease course.

We also analyzed the levels of sCD26 and sCD30 in converted and unconverted CIS patients. Converted CIS patients showed increased levels of sCD30 before they were converted to RRMS when compared with the controls, while no differences were observed between the unconverted and the controls. This observation might indicate the presence of regulatory immune responses already at the CIS stage that might suggest the goal of the immune system to inhibit or balance the pro-inflammatory events responsible for the development of tissue damage. Therefore, sCD30 might be the marker of regulatory immune response also in the CIS stage of MS patients. However, the potential of sCD30 as a conversion marker should be evaluated in further follow-up studies.

Taken together, the increased levels of sCD26 and sCD30 in MS are consistent with the coexistence of both Th1- and Th2-type immune responses in the entire course of MS. Our observation of a positive correlation between IL-10 and sCD30 might suggest the role of sCD30 as a marker of regulatory immune responses in MS. Further studies, including longitudinal follow-up analyses with concurrent MRI analyses, should be carried out to confirm this preliminary result.

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Conflict of interest

None declared.

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PUBLICATION IV

Gene expression profiles of Tumor Necrosis Factor-like Cytokine TL1A and its Receptors DR3 and DcR3 in Multiple Sclerosis

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