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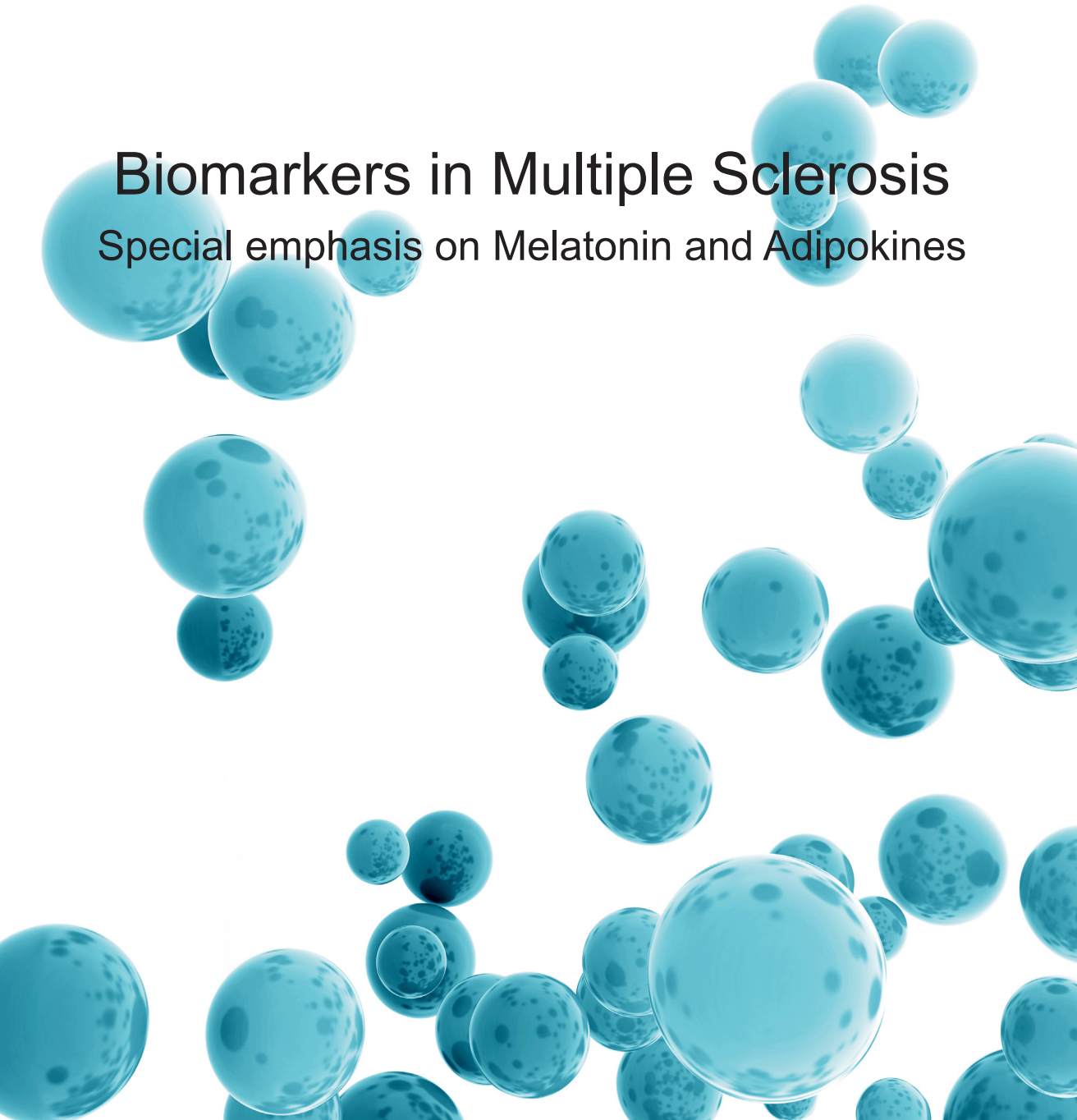
RENUKA NATARAJAN

Biomarkers in Multiple Sclerosis

Special emphasis on Melatonin and Adipokines



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Biomarkers in Multiple Sclerosis

Special emphasis on Melatonin and Adipokines

ACADEMIC DISSERTATION
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UNIVERSITY OF TAMPERE

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Finland

Supervised by
Professor Irina Elovaara
University of Tampere
Finland

Reviewed by
Professor Veijo Hukkanen
University of Turku
Finland
Professor Anne Remes
University of Eastern Finland
Finland

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To my lovable family

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I ***Natarajan R***, Einarsdottir E, Riutta A, Hagman S, Raunio M, Mononen N, Lehtimäki T, Elovaara I. Melatonin pathway genes are associated with progressive subtypes and disability status in multiple sclerosis among Finnish patients. J Neuroimmunol (2012) 15; 250(1-2):106-10.

II ***Natarajan R***, Hagman S, Hämälainen M, Leppänen T, Dastidar P, Moilanen E, Elovaara I. Adipsin is associated with multiple sclerosis: a follow-up study of adipokines. Multiple Sclerosis International (2015) 2015; 371734. doi: 10.1155/2015/371734.

III Basnyat P, ***Natarajan R***, Vistbakka J, Lehtikangas M, Airas L, Matinlauri I, Elovaara I, Hagman S. Elevated levels of soluble CD26 and CD30 in multiple sclerosis. Clinical and Experimental Neuroimmunology (2015); doi: 10.1111/cen3.12253.

IV ***Natarajan R***, Hagman S, Wu X, Hakulinen U, Raunio M, Helminen M, Maiha R, Dastidar P, Elovaara I. Diffusion Tensor Imaging in NAWM and NADGM in MS and CIS: Association with Candidate Biomarkers in Sera. Multiple Sclerosis International (2013); 265259. doi: 10.1155/2013/265259. Epub 2013 Dec 17.

ABBREVIATIONS

AANAT	Arylalkylamine-N-acetyl Transferase
ADC	Apparent Diffusion Coefficient
APCs	Antigen Presenting Cells
BBB	Blood-Brain Barrier
BMI	Body Mass Index
CCR	Chemokine Receptor
CD	Cluster of Differentiation
CDMS	Clinically Definete Multiple Sclerosis
CIS	Clinically Isolated Syndrome
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
DIS	Dissemination in Space
DIT	Dissemination in Time
DMA	Disease Modifying Agents
DTI	Diffusion Tensor Imaging
EAE	Experimental autoimmune encephalitis
EDSS	Expanded Disability Status Scale score
EIA	Enzyme-Immuno-Assay
ELISA	Enzyme-Linked Immunosorbant Assay
FA	Fractional Anisotrophy
FLAIR	Fluid Attenuation Inversion Recovery
GA	Glatiramer Acetate
Gd	Gadolinium
GM	Gray Matter
GWAS	Genome-wide Association Studies
HIOMT	Hydroxyindole-O-Methyltransferase
HLA	Human Leucocyte Antigen
HSV	Herpes simplex virus
IDO	Indoleamine 2, 3-dioxygenase
IFN	Interferon

Ig	Immunoglobulin
IL	Interleukin
LD	Linkage Disequilibrium
MBP	Myelin Basic Protein
MHC	Major Histocompatibility Complex
MIF	Macrophage-migration Inhibitory Factor
miR	microRNA
MMPs	Matrix Metalloproteinases
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
MTNR1B	Melatonin Receptor 1B
NADGM	Normally-appearing Deep Gray Matter
NAGM	Normally appearing Gray Matter
NAWM	Normally-appearing White Matter
OCBs	Oligoclonal Bands
OR	Odds Ratio
PCR	Polymerase Chain Reaction
PI	Progression Index
PPMS	Primary Progressive Multiple Sclerosis
PRMS	Progressive Relapsing Multiple Sclerosis
ROI	Region-Of-Interest
RRMS	Relapsing Remitting Multiple Sclerosis
RZR/ROR	Retinoid Z Receptors/Retinoid Orphan Receptors
SNP	Single Neucleotide Polymorphism
SPMS	Secondary Progressive Multiple Sclerosis
T1-WI	T1-weighted imaging
T2-WI	T2-weighted imaging
TCR	T-cell Receptors
TNF	Tumor Necrosis Factor
TPH2	Tryptophan Hydroxylase-2
TRAIL	TNF-related inducing ligand
Treg	Regulatory T-cell
UV	Ultra Violet
WM	White Matter

ABSTRACT

Over the last decade, several immune molecules have been studied extensively in multiple sclerosis (MS) to identify the biomarkers that could potentially predict the neurological disability, disease activity, prognosis and therapeutic responses. However, there is very little or sparse knowledge on the role of melatonin and adipokines in MS disease. These molecules are closely related with etiological factors like sunlight and obesity that may influence the risk of the disease. The studies in this thesis were conducted to investigate whether melatonin pathway genes, major adipocytokines and some exploratory immune molecules are useful as biological markers of multiple sclerosis (MS). For this purpose the single nucleotide polymorphisms (SNPs) of melatonin pathway genes, the blood levels of major adipocytokines such as adiponectin, adipsin, leptin and resistin and soluble costimulatory molecules CD26 and CD30 molecules were analyzed in different MS subtypes and their association assessed with clinical measures and the volumes of focal MS-related changes on magnetic resonance imaging (MRI). It was also evaluated whether diffusion tensor imaging (DTI) indices in the normal-appearing white matter (NAWM) and deep gray matter (NADGM) along with clinical parameters are useful in elucidating the pathogenesis of MS. The study included patients with different subtypes of MS, clinically isolated syndrome (CIS) and healthy controls that underwent neurological, MRI, and immunological examinations.

The analyses of melatonin pathway genes in MS subtypes showed an overrepresentation of T allele carriers of a functional polymorphism (G-703T, rs4570625) in the promoter region of tryptophan hydroxylase-2 (TPH2) gene in the progressive MS subtypes. The haplotype rs4570625–rs10506645TT of TPH2 gene was associated with the risk of severe disability in primary progressive MS (PPMS), while haplotype rs4570625–rs10506645TC appeared to be protective against disability in secondary progressive MS (SPMS). In the melatonin receptor 1B (MTNR1B) gene, the haplotype rs10830963–rs4753426GC was associated with the risk of SPMS, whereas another haplotype rs10830963–rs4753426GT showed an association with the risk of PPMS. Studies on adipocytokines showed that in patients with relapsing-remitting MS (RRMS), the levels of adipsin correlated with Expanded Disability Status Scale score (EDSS) and the volumes of T1-weighted and fluid attenuation inversion recovery (FLAIR) lesions as well as the changes of T1-weighted lesion volumes over the follow-up period. In the study on co-stimulatory molecules, the increased levels of costimulatory molecules sCD26 and sCD30 were found in MS and clinically isolated syndrome (CIS). In DTI study, the level of neurological disability in relapsing

remitting MS (RRMS) correlated with thalamic fractional anisotrophy (FA) values, but no predictive potential was found. In conclusion, the association of polymorphisms in the TPH2 and MTNR1B genes with the progressive subtypes of MS suggests dysregulation in melatonin pathway that may be involved in the accumulation of neurological disability and underlying neurodegeneration. Adipsin seems to exert predictive potential as a biomarker of neurological disability in RRMS. Elevated levels of sCD26 and sCD30 in MS are consistent with the dysregulation of Th1- and Th2-type immune responses in MS. The information obtained provides new evidence especially for the roles of melatonin and adipsin in MS, but further studies with larger patient series and longer follow-up periods are needed to validate these promising data.

TIIVISTELMÄ

MS-taudissa on viime vuosikymmenten aikana tutkittu laajalti useita eri immuunimolekyylejä sellaisten biomerkkiaineiden löytymiseksi, jotka kuvaisivat taudin ennustetta, aktiivisuutta, neurologista toimintakykyä ja hoitovasteita eri lääkeaineille. Melatoniinin ja adiposytokiinien yhteyttä MS-tautiin ei juurikaan tunneta. Nämä molekyylit ovat läheisesti yhteydessä etiologisiin tekijöihin, kuten auringonvaloon ja lihavuuteen, joiden katsotaan liittyvän MS-taudin kehittymiseen. Tämän väitöskirjatutkimuksen tavoitteena oli selvittää melatoniinin signalointireitin geenien, tunnetuimpien adiposytokiinien ja joidenkin uudempien immuunimolekyylien yhteyttä MS-tautiin ja niiden soveltuvuutta biomerkkiaineiksi. Näiden tavoitteiden saavuttamiseksi yhden nukleotidin polymorfismia tutkittiin melatoniinin signalointireitin geeneissä sekä verinäytteistä tutkittiin adipokiineistä adiponektiinin, adipsiinin, leptiinin ja resistiinin tasoja eri MS-taudin tautityypeissä sekä niiden yhteyttä kliinisiin tekijöihin ja magneettikuvantamisella (MK) mitattuihin fokaalisiin MS-muutoksiin. Lisäksi tutkittiin CD26 ja CD30 immuunimolekyylien tasojen yhteyttä eri MS-taudin muotoihin. Tutkimuksessa selvitettiin myös diffuusiotensorikuvantamisen (DTI) avulla normaalilta vaikuttavan valkean aineen (NAWM) ja syvän harmaan aineen (NADGM) löydösten yhteyttä MS-taudin patogeneesiin ja MS-taudin kliinisiin muuttujiin. Tutkimukseen osallistui kliinisesti eriytyneestä oireyhtymästä (KEO), aaltomaisesta ja progressiivisesta MS-taudista kärsiviä potilaita sekä terveitä verrokkihenkilöitä, jotka tutkittiin neurologisesti, aivojen kuvantamisella sekä immunologisesti.

Melatoniinin signalointireitin geenien yhteyttä MS-tautityyppeihin selvittävä tutkimus osoitti tryptofaanihydroksylaasi 2 (TPH2) geenin T-allleelin (G-703T, rs4570625) kantajien olevan yliedustettuina progressiivisessa MS-taudissa. Lisäksi TPH2 geenin haplotyyppi rs4570625–rs10506645TT oli yhteydessä vaikeaan invaliditeettiin progressiivista MS-tautia sairastavilla, kun taas TPH2:den haplotyyppi rs4570625–rs10506645TC näytti olevan suojaava invaliditeetin kehittymiselle sekundaarisprogressiivista MS-tautia (SPMS) sairastavilla. Melatoniinireseptori 1B (MTNR1B)- geenin haplotyypin rs10830963–rs4753426GC todettiin olevan yhteydessä SPMS tautityyppiin, kun taas MTNR1B:n haplotyyppi rs10830963–rs4753426GT oli yhteydessä primaaris-progressiiviseen (PPMS) MS-tautiin. Adiposytokiinien merkitystä selvittävässä tutkimuksessa osoitettiin, että aaltomaista MS-tautia (RRMS) sairastavilla adipsiinitasot korreloivat EDSS-(expanded disability status scale) asteikolla arvioituun invaliditeettiin sekä MK:lla saatujen T1- ja FLAIR leesioiden tilavuuksiin ja seurannassa ilmenneiden T1 leesioiden tilavuusmuutoksiin. Tutkimus osoitti myös CD26 ja CD30 molekyylitasojen suurentu-

mista MS ja KEO-potilailla. DTI-tutkimuksessa havaittiin toimintakyvyn huononemisen olevan yhteydessä fraktionaalisen anisotropian (FA) arvoihin talamuksen alueella, mutta prognostista potentiaalia ei todettu.

Johtopäätöksenä tutkimuskokonaisuudesta voidaan todeta, että melatoniinin signalointireitillä TPH2 ja MTNR1B geenien polymorfismit olivat yhteydessä progressiiviseen tautityyppiin, joka saattaa liittyä neurologisen toimintakyvyn huononemiseen ja neurodegeneratiivisten muutosten kertymiseen. Adiposytokiineistä adipsiinillä todettiin olevan potentiaalia ennustaa neurologisen toimintakyvyn huononemista aaltomaista MS-tautia sairastavilla. CD26 ja CD30 molekyylien suurentuneet tasot MS-taudin eri alatyypeissä liittyvät Th1 ja Th2-tyypin immunologiseen dysregulaatioon. Tiivistäen on todettavissa, että tutkimuksen keskeisimmät ja uusimmat havainnot liittyvät melatoniinin ja adipsiinin merkitykseen MS-taudissa, mutta saatujen tulosten varmentaminen edellyttää lisätutkimuksia suuremmilla potilaskohorteilla sekä pidempää seuranta-aikaa.

1 INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system. According to the present understanding MS is mediated by autoreactive $CD4^+$ T and B cells that transmigrate through the blood-brain barrier (BBB) and, together with proinflammatory cytokines, macrophages, antibodies, and complements attack the myelin sheath causing neuronal damage and axonal death (Kasper & Shoemaker, 2010; Sospedra & Martin, 2005). Pathologic and imaging studies have established that both white and gray matter and also the so-called normally appearing areas are affected by the disease (M. Calabrese et al., 2010; Filippi et al., 2011; Geurts, Calabrese, Fisher, & Rudick, 2012). MS prevalence is higher in areas distant from the Equator and the influence of sunlight exposure via Vitamin D and ultra violet (UV) radiation on MS disease has been shown in several studies (Mehta, 2010; Smolders, Damoiseaux, Menheere, & Hupperts, 2008). Another potential contributor that has recently attracted research attention is melatonin, a hormone secreted during the hours of darkness and having numerous immunomodulatory properties (Anderson & Rodriguez, 2015; Simonneaux & Ribelayga, 2003). In MS, melatonin secretion has been found to be low, phase-shifted and is found to contribute to high relapse rate in relapsing disease and neurological disability in progressive disease course (Akpinar et al., 2008; Damasceno et al., 2015; Farez et al., 2015; Gholipour et al., 2015; Sandyk & Awerbuch, 1993b; Sandyk, 1998; Sandyk, 1999). Notably, a recently increasing prevalence of MS has been connected with obesity (Strasser, 2013) but the association between adipose tissue and inflammation has not been explained. Thus, MS is multifactorial disease with different heterogeneous pathological features and variable clinical course. The heterogeneity of MS and the transition towards personalized therapy necessitate the identification of patient-specific biomarker signatures that could be used in monitoring of MS.

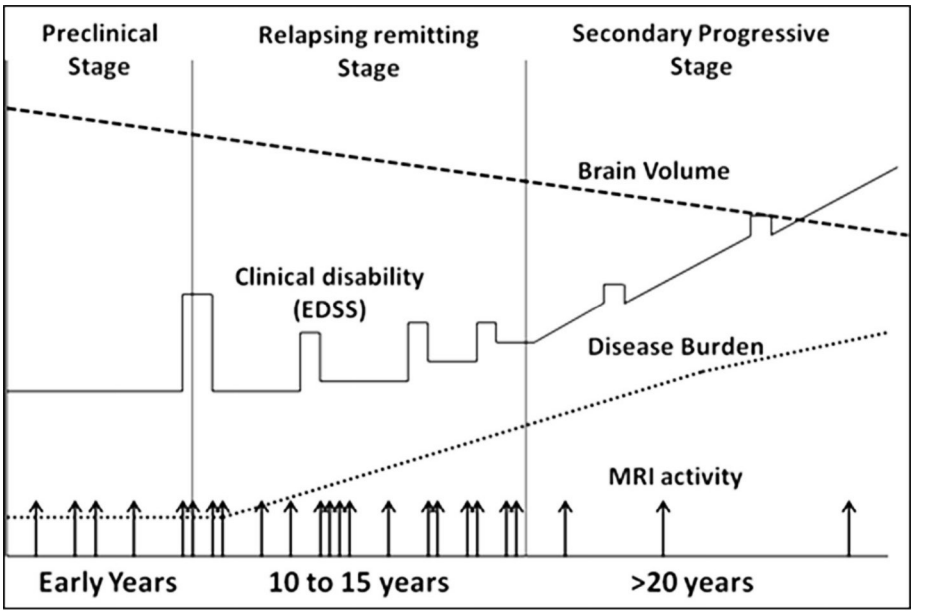
2 REVIEW OF THE LITERATURE

2.1 Multiple Sclerosis Overview

2.1.1 Clinical course and diagnosis

Multiple Sclerosis (MS) is an autoimmune demyelinating disease of CNS. It has a heterogeneous disease course with complex pathology (Compston & Coles, 2008). The pathological hallmarks of the disease are inflammation, neurodegeneration, and axonal loss (Bruck & Stadelmann, 2003; Kasper & Shoemaker, 2010; Sospedra & Martin, 2005; Compston & Coles, 2008; Noseworthy, Lucchinetti, Rodriguez, & Weinshenker, 2000; Nylander & Hafler, 2012). The first clinical manifestation is termed clinically isolated syndrome (CIS), that is, an acute demyelinating event with an acute onset usually affecting the younger population and targeting the optic nerves, brainstem, and spinal cord (Katz Sand & Lublin, 2013; D. H. Miller, Chard, & Ciccarelli, 2012; Noseworthy et al., 2000). The disease typically starts as relapsing-remitting disease (RRMS) with relapse in a sub-acute manner over days or weeks. The symptoms then gradually decline over weeks to months and resolve completely, while during disease evolution they cause persistent disability, accumulating over time with each subsequent relapse (Fisniku et al., 2008). After a period of time (10 to 20 years), in the majority of patients, the disease develops into a progressive phase with gradually increasing disability (secondary progressive MS [SPMS]). In some individuals (around 10–20%), the disease is progressive from clinical onset with occasional relapses (progressive relapsing MS [PRMS]) or without superimposed relapses (primary progressive MS [PPMS]) (Confavreux & Vukusic, 2006a; Confavreux & Vukusic, 2006b; Pandey et al., 2014; Tur & Thompson, 2015). PPMS occurs more frequently in older male subjects and is characterized by a gradual deterioration of gait and severe ambulatory dysfunction.

The diagnosis of MS at present is based on the revised McDonald criteria that have integrated clinical and MRI data in the establishment of MS (Files, Jausurawong, Katrajian, & Danoff, 2015). According to these criteria, dissemination in time (DIT) can be demonstrated by any new T2-weighted or gadolinium (Gd)-enhanced lesion in follow-up MRI satisfying Swanton's criteria, while dissemination in space (DIS) can be demonstrated by one or more T2 lesions in at least two out of four areas of the CNS (periventricular, juxtacortical, infratentorial or spinal cord).



Schematic representation of MS disease course with disability progression expressed by expanded disability status scale (EDSS); frequency of inflammatory events when studied by magnetic resonance imaging: detection of Gadolinium (Gd)-contrast enhancing T1 lesions showing the blood-brain barrier (BBB) opening (arrows); T2 lesion load reflecting disease burden (……); brain volume indicative of brain atrophy (-----). Redrawn from (Bruck & Stadelmann, 2005).

Figure 1. MS disease course.

2.2 Epidemiology and Etiology

2.2.1 Epidemiology

The geographical distribution of MS supports an association between latitude (distance from the Equator) and regional disease prevalence. The global prevalence of MS shows a latitude gradient, being lower near the Equator (<5/100,000), and higher in northern countries (>100/100,000 in northern Europe, North America, and Canada) (Koch-Henriksen & Sorensen, 2010) with few exceptions among the Norwegian Sami population and in Sardinia (Kingwell et al., 2013; Smestad, Sandvik, Holmoy, Harbo, & Celius, 2008). Finland is a high-risk area for MS situated between the latitudes 60° and 70°in northern Europe. The prevalence estimates in Finland (Seinäjoki-South) have been reported to be highest among the Nordic region (over 200/100,000) (Holmberg, Murtonen, Elovaara, & Sumelahti, 2013; Sumelahti, Holmberg, Murtonen, Huhtala, & Elovaara, 2014). Migrational studies have shown that moving from a high- to a low-risk area in childhood (during the first two decades) reduces the risk of MS to a level close to that of the final place of residence, while moving during young adulthood only reduces the risk to an

intermediate between the two areas (Ascherio & Munger, 2007; Gale & Martyn, 1995; Kingwell et al., 2013). However, there is some evidence suggesting that the latitudinal gradient may be disappearing, (Hernan, Olek, & Ascherio, 1999; Trojano et al., 2012; Wallin, Page, & Kurtzke, 2004). Recent data, including maternal parent-of-origin effects and month-of-birth risk, suggest that there are gestational or very-early-life risks conferred by environmental factors (Wingerchuk, 2011).

2.2.2 Etiology

The etiology of MS is so far unknown. Genetic susceptibility together with environmental trigger is proposed to induce the disease (Oksenberg, Baranzini, Barcellos, & Hauser, 2001). Many co-determinant factors such as viruses, bacteria, geographical latitude, and hormones have been considered (Cermelli & Jacobson, 2000; Gomez, Steelman, Young, & Welsh, 2013; Kurtzke, 1993; Loken-Amsrud, Lossius, Torkildsen, & Holmoy, 2015; Pugliatti et al., 2008; Virtanen & Jacobson, 2012). Several viruses such as Epstein–Barr-Virus (EBV), JC virus (John Cunningham virus), Herpes simplex virus (HSV) types 1 and 2, human herpesvirus 6, measles, mumps and rubella have been implicated as factors that may influence MS risk (Basnyat et al., 2015; Koch, Metz, Agrawal, & Yong, 2013; Kolasa et al., 2015). The only infectious agent that has been repeatedly shown to be associated with MS is the EBV (Aloisi, Serafini, Magliozzi, Howell, & Reynolds, 2010; Gabibov et al., 2011; Jilek et al., 2012; Pender, 2012; Serafini et al., 2010; Tzartos et al., 2012).

A. Genes

Epidemiological analysis suggests that genetic influence and variation are significant in susceptibility to MS. This was further supported by twin studies showing higher concordance rates in monozygotic twins (24–30%) than dizygotic twins (3–5%) (Fagnani et al., 2015). However, there is no increased risk for adoptive relatives and an intermediate risk for half-siblings and the offspring of conjugal pairs (O'Gorman, Lin, Stankovich, & Broadley, 2013). Linkage analysis showed that MS was associated with the major histocompatibility complex (MHC) region on chromosome 6 encoding the human leucocyte antigen (HLA) gene which exerts the strongest genetic effect on MS. Genome-wide association studies (GWAS) have detected about 100 loci associated with MS outside the HLA region, and have also confirmed the major role of the HLA-DRB1*15:01 (HLA-DR15) gene (Barcellos et al., 2006; International Multiple Sclerosis Genetics Consortium, 2013; Yeo et al., 2007). All the non-HLA associations are common variants with modest risk (odds ratio (OR) in the region of 1.1–1.3), and many are near genes with key roles in the immune system. However, all the identified loci explained only 18–24% of the heritability of MS of which HLA-DR15 explained 11% and the remaining non-HLA genes explain only a small fraction (De Jager et al., 2009; International Multiple Sclerosis Genetics

Consortium et al., 2007; International Multiple Sclerosis Genetics Consortium, 2013; Zuvich et al., 2010). Thus, genetic studies in MS are now focusing on various aspects of the gene such as rare variants, common variants, epigenetics, gene-gene interactions, pathway involvement, gene-environment interactions and structural variants to explore the missing heritability disease.

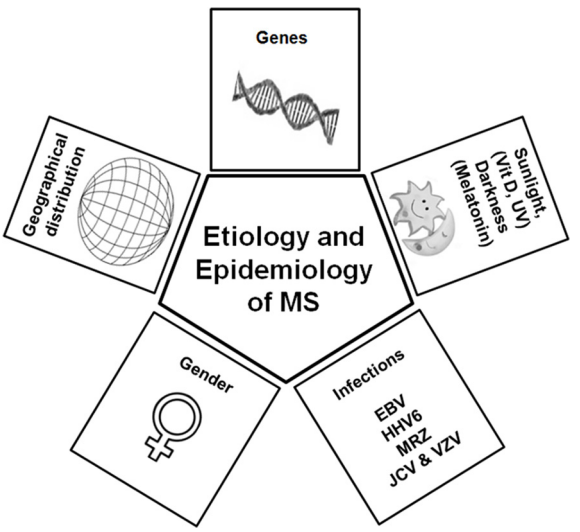
B. Gender

It is well known that gender influences the susceptibility, clinical course, symptoms as well as the severity of the disease (Nussinovitch & Shoenfeld, 2012; Tomassini & Pozzilli, 2006). Increasing incidence and prevalence of MS concerns the rising female to male (2–3F/M) ratio (Krokki, Bloigu, Reunanen, & Remes, 2011; Alonso & Hernan, 2008; Cordeau & Courtois, 2014; Debouverie, Pittion-Vouyovitch, Louis, Roederer, & Guillemin, 2007). RRMS patients have a younger age at onset and high female preponderance (F/M 2.2) while PPMS patients are older and constitute a lower female preponderance (about 1.3) (Miller & Leary, 2007; Sumelahti et al., 2014). It has been reported that motor symptoms and cognitive impairment are more commonly associated with male gender with late disease onset (Kingwell et al., 2013). The evidence reported so far suggests that sex hormones exhibit their role through their immunomodulatory actions (Airas, 2015; Christianson, Mensah, & Shen, 2015; Elloso, Phiel, Henderson, Harris, & Adelman, 2005; Laffont, Garnier, Lelu, & Guery, 2015; Pozzilli & Pugliatti, 2015).

C. Environmental factors

The exposure status of vitamin D and UV light have been postulated to be associated with MS risk (Wingerchuk, 2011). Most patients with MS have relatively low serum vitamin D levels, and the level appears to decline over time (Munger et al., 2004; Nieves, Cosman, Herbert, Shen, & Lindsay, 1994; van der Mei et al., 2007). In some studies, seasonal variation in relapse rate, with higher rates during the months of lowest vitamin D status have been observed (Smolders, Menheere, Kessels, Damoiseaux, & Hupperts, 2008; Tremlett et al., 2008). Lower vitamin D status itself has been linked to higher relapse rates and greater clinical disability in both adults and pediatric-onset MS (Simpson et al., 2010; Smolders, Menheere et al., 2008). However, other factors such as genetics, body mass, and skin color have been shown to influence vitamin D levels (Wingerchuk, 2011). Obesity in childhood or adolescence in people with the common genetic HLA risk allele is associated with an increased risk of MS (Gianfrancesco et al., 2014; Hedstrom et al., 2014), (Langer-Gould, Brara, Beaber, & Koebnick, 2013; Munger et al., 2013). Higher body mass index (BMI) is associated with lower blood levels of 25-hydroxyvitamin D leading to vitamin D insufficiency in childhood and adolescence (Munger et al., 2013). This was found to be associated with an increased risk of MS (Munger, 2013; Vierucci et al., 2013), but the exact

mechanisms underlying this association are still unclear. Obesity is associated with low-grade chronic inflammatory states which release a set of proinflammatory cytokines that may affect immune responses leading to the risk for MS (Loken-Amsrud et al., 2015). It has recently been shown that levels of melatonin contribute towards the seasonality of MS relapse rates, which further supports the concept of environmental factors modulating the disease course of MS (Farez et al., 2015).



Schematic representation of various etiological and epidemiological factors that contribute to the cause of MS disease. UV ultra violet, EBV Epstein bar virus, HHV human herpes virus, JCV John Cunningham virus, VZV variella zoster virus. Modified from (Versini, Jeandel, Rosenthal, & Shoenfeld, 2014).

Figure 2. Epidemiology and Etiology of MS disease.

2.3 Pathology in Multiple Sclerosis

The pathologic hallmark of MS is the presence of large, multifocal demyelinating plaques in multiple areas of the brain and spinal cord (Lassmann, 2014). The lesions are disseminated throughout the CNS are typically characterized by a demyelinated core separated by a very sharp border from normally myelinated surrounding tissue (Frohman, Racke, & Raine, 2006b). White matter (WM) lesions are characterized broadly as acute active lesions, chronic active lesions and chronic inactive lesions. *Acute active lesions* are more commonly seen as four different patterns in acute and RRMS stages, representing the pathologic substrate for clinical attacks (Lucchinetti et al., 1999; Lucchinetti et al., 2000; Popescu, Pirko, & Lucchinetti, 2013). *Chronic active lesions* mostly contribute towards disease progression while, *chronic inactive plaques* represent the total demyelination. As the plaque progresses from acute active to chronic inactive, its edema resolves, inflammation decreases, and macrophages and microglia gradually disappear, astrogliosis is a more prominent feature (Popescu & Lucchinetti, 2012b; Prineas et al., 2001). *Gray matter lesions* are small, focal, demyelinated lesions with the presence of activated microglia and little inflammatory

infiltration in some areas (Bo, Geurts, Mork, & van der Valk, 2006; Geurts & Barkhof, 2008; Peterson, Bo, Mork, Chang, & Trapp, 2001; Popescu & Lucchinetti, 2012a). There are three types of cortical lesions, namely cortical-juxtacortical lesions, vascular cortical lesions and sub-pial lesions present among MS patients that lead to irreversible disability, progression, and cognitive deficits (Bo, Vedeler, Nyland, Trapp, & Mork, 2003; Kidd et al., 1999; Miki et al., 1999; Peterson et al., 2001).

It has become increasingly evident that the disease processes do indeed occur in white and gray matter distant from the site of the lesions. In *normally appearing white matter* (*NAWM*) the pathological changes constitutes a diffuse inflammatory process represented by inflammatory infiltrates such as microglial cells, CD8+ T lymphocytes, markers of phagocytosis throughout the tissue thus contributing to axonal loss and injury (D. H. Miller, Thompson, & Filippi, 2003; Trapp et al., 1998); (Lassmann, Bruck, & Lucchinetti, 2007). There is therefore extensive glial pathology, with astrocyte proliferation, microglial activation and gliosis (Bergers et al., 2002; Evangelou, Esiri, Smith, Palace, & Matthews, 2000; Ganter, Prince, & Esiri, 1999; Trapp et al., 1998). These NAWM abnormalities are widespread and are present since the early stages of the disease, even before focal lesion development, thereby suggesting a pre-existing pathological process occurring in the NAWM that may predispose to focal lesion formation. In contrast, however during progressive stages NAWM abnormalities may follow lesion development that probably reflects either Wallerian degeneration or distant dysfunction of axons that have been subject to focal demyelination (Miller et al., 2003). Also, the extent of axonal loss has been observed to vary from 10 to 60% and is more severe in SPMS than in RR form of disease course (Filippi et al., 2000; Miller et al., 2003; Tortorella et al., 2000). Assessment of *normally appearing gray matter* (*NAGM*) damage has potential to define the structural substrates of disease manifestations, such as disability, cognitive impairment, and fatigue (Rovaris, Agosta, Pagani, & Filippi, 2009). NAGM abnormalities have been shown to worsen over time in relation to the clinical evolution of the disease, being more severe in patients with SPMS than in those with RRMS and CIS (Tillema & Pirko, 2013). The pathological basis of NAGM damage in MS has not been fully elucidated. Nevertheless, the abnormalities in NAGM may be due to retrograde degeneration of GM neurons secondary to white matter fiber damage (Bermel, Innus, Tjoa, & Bakshi, 2003; Cifelli et al., 2002). The retrograde degeneration is supported by the correlation found between white matter T2 lesion volume and NAGM abnormality and gray matter atrophy (Honce, 2013). However, cortical atrophy appears early in the disease process independent of WM pathology and relapses (Pirko, Lucchinetti, Sriram, & Bakshi, 2007; De Stefano et al., 2003).

2.4 Immunopathogenesis of MS

2.4.1 Immune cell activation and blood brain barrier (BBB) migration

The immunopathogenesis of MS is thought to involve a breach of self-tolerance toward myelin and other CNS antigens resulting in persistent peripheral activation of autoreactive T cells (Garg & Smith, 2015; Hafler et al., 2005). It is presumed that an environmental antigen, possibly an infectious agent such as a virus, may trigger this loss of self-tolerance. The infection could cause bystander activation of T cells or result in release of autoantigens due to cellular damage, which can then lead to activation of T cells by molecular mimicry (Aichele, Bachmann, Hengartner, & Zinkernagel, 1996; Fujinami & Oldstone, 1985; Gran, Hemmer, Vergelli, McFarland, & Martin, 1999; O'Connor et al., 2005; Wucherpfennig & Strominger, 1995). Molecules such as T-cell intrinsic [e.g., costimulatory molecules, cytokine receptors, and microRNA (miR)] and extrinsic factors (mainly regulatory T-cell (Treg) dysfunction), can trigger dysregulated $CD4^+$ T-cell responses, which ultimately leads to the pathogenicity of these cells (Gonsette, 2012). For instance, the T-cell intrinsic factors can skew the immune response toward proinflammatory Th1/Th17 cell development and also negatively impact Treg function and homeostasis.

During immune activation, *signal 1* is initiated by activated antigen presenting cells (APCs) with class II MHC interact with $CD4^+$ T cells through T-cell receptors (TCRs). This then upregulates the CD154 (CD40 ligand) on the surface of the T cell which binds to its receptor, CD40 that is constitutively expressed on APCs. The combination of this interaction further drives the upregulation of both CD80 and CD86 on the surface of APCs. CD80 and CD86 identify 2 additional receptors, CD28 and CTLA4, on the surface of the T cells to provide a *Signal 2* to APCs (Kasper & Shoemaker, 2010; Sospedra & Martin, 2005). Several other costimulatory molecules, such as CD26 and CD30 are known to mediate the optimal activation of T cells. These autoreactive T cells induce peripheral tolerance through immunosuppressive functions that leads to a decrease in naïve Treg subtypes (Haas et al., 2007; Schwarz et al., 2013). Impaired Treg function can also decrease peripheral B-cell tolerance, which can lead to the accumulation of autoreactive B-cell clones in the blood (Kinnunen et al., 2013).

Once activated on the periphery, myelin-reactive T cells with very late antigen-4 (VLA-4) are able to interact with the vascular cell adhesion molecule-1 (VCAM-1) expressed on capillary endothelial cells. This process is facilitated by the expression and upregulation of various adhesion molecules, chemokines, and matrix metalloproteinases (MMPs) (Agrawal & Yong, 2007; Gold & Wolinsky, 2011). This is followed by lymphocyte secretion of matrix metalloproteinases (MMPs) that break down the Blood-Brain Barrier (BBB) matrix, thus allowing extravasation of inflammatory cells into the CNS. Various inflammatory cells chooses specific adhesion molecule as its receptor for migration across the BBB (Glatigny, Duhen, Oukka, & Bettelli, 2011; Flanagan et al., 2012; Larochelle et al., 2012; Rothhammer et al., 2011; Lee-Chang et al., 2011; Ifergan, Kebir, Alvarez et al.,

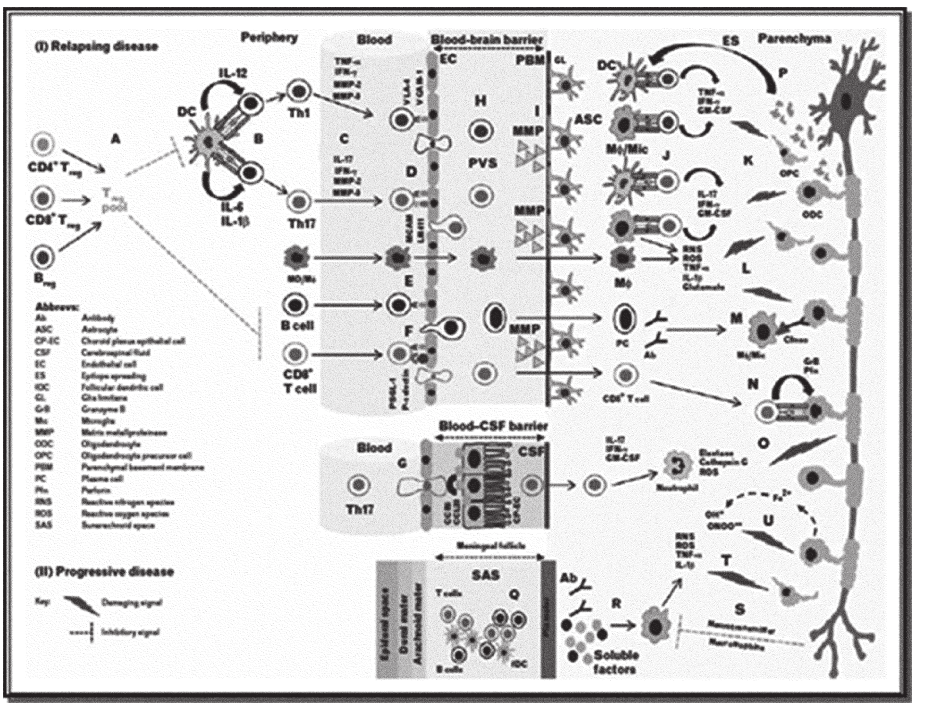
2011; Battistini et al., 2003). Immune cell infiltration leads to the formation of cuffs in the perivascular space (PVS), which are populated by $CD4^+$ T cells, $CD8^+$ T cells, B cells, and monocytes/macrophages (Wojkowska, Szpakowski, Ksiazek-Winiarek, Leszczynski, & Glabinski, 2014).

After entering the CNS, the autoreactive T cells can be reactivated upon encountering the autoantigenic peptides within the brain parenchyma expressed by local antigen presenting cells (dendritic cells, macrophages, and B cells). This trigger an inflammatory cascade leading to release of cytokines and chemokines, recruitment of additional inflammatory cells including T cells, monocytes, and B cells, and persistent activation of microglia and macrophages resulting in myelin damage (Frohman, Racke, & Raine, 2006a; Hemmer, Archelos, & Hartung, 2002). Once inside the CNS, B-cell clones specific to myelin can cause demyelination via immunoglobulin G deposition and complement-mediated mechanisms (Disanto, Morahan, Barnett, Giovannoni, & Ramagopalan, 2012). Additional pathogenicity could be mediated by their cytotoxic secretory products on oligodendroglia (Lisak et al., 2012). Apart from the cytotoxic effect of $CD8^+$ T cells, recently an interleukin-17A-producing $CD8^+$ subset (Tc17) which is noncytotoxic and proinflammatory, has been identified at high levels in the CSF during early stage of MS (Annibali et al., 2011). $CD8^+$ T cells can maintain or exacerbate ongoing multiple sclerosis inflammation by producing interferon-gamma (IFN-γ) or interleukin-17 (Lolli et al., 2013). It has been demonstrated that monocyte-derived macrophages (MDMs) mediate myelin destruction, whereas microglia-derived macrophages clear up debris in experimental autoimmune encephalitis (EAE). Also, clusters of activated microglia have been reported in NAWM and are believed to be the earliest stage (preactive lesions) of MSlesion development (van Horssen et al., 2012; Yamasaki et al., 2014; Melief et al., 2013). Activation of resident CNS glial cells (such as microglia) results in persistent inflammation even in the absence of further infiltration of exogenous inflammatory cells (O'Connor, Bar-Or, & Hafler, 2001). Activated microglia and macrophages produce a plethora of substances that are pro-inflammatory mediators that can damage neighboring glia and axons (van Horssen, Witte, Schreibelt, & de Vries, 2011). Once damaged, cells may undergo several cell death pathways leading to the activation of innate immune cells (e.g. via necrosis). The local inflammation and demyelination results in exposure of sequestered myelin autoantigens providing an additional target for self-reactive T cells, a phenomenon called "epitope spreading" (Miller et al., 1997).

2.4.2 Neural tissue damage

Several mechanisms have been proposed as contributors to the resulting damage of the myelin sheets, axons and oligodendrocytes. (i) Complement dependent antibody mediated damage, as complement deposit has been demonstrated in MS lesions (Lucchinetti et al., 2000), (ii) Digestion of surface myelin by macrophages after targeting myelin antibodies

(Trapp et al., 1998), (iii) Injury mediated by release of toxic mediator such as cytokines and proteases (Schaecher, Rocchini, Dinkins, Matzelle, & Banik, 2002), (iv) Reactive oxygen and nitrogen species (Bitsch, Schuchardt, Bunkowski, Kuhlmann, & Bruck, 2000; Rejdak et al., 2007), (v) Direct injury by $CD8^+$ cytotoxic T cells by the release of granzymes among other molecules (Sauer, Schmalstieg, & Howe, 2013). The result is tissue damage ranging in a spectrum from axons lacking myelin-sheets and loss of saltatory conduction properties, where remyelination and reconstitution of axonal function can still occur, to irreversible axonal transection and disability progression (Trapp et al., 1998). Figure 3 summarizes the various immune mechanisms involved in immunopathogenesis process.



A: Activation of autoreactive T and B cells on the periphery. B: Molecular mimicry. C: Both Th1 and Th17 disrupts the BBB by producing proinflammatory cytokines and MMPs. D, E, F and G: Passing of various T cell subsets and B cells using different proteins. H and I: Immune cell infiltration leads to the formation of perivascular cuffs in perivascular space (PVS) are filled with $CD4^+$ T cells, $CD8^+$ T cells, B cells, leucocytes, macrophages and microglia (MO/Mφ) induce the secretion of MMPs. J: Myelin-reactive T cells are reactivated by parenchymal microglia (Mic), dendritic cells or B cells. K: Reactivated T cells carry out encephalitogenic activities within the CNS by secreting a host of proinflammatory cytokines and chemokines. L: Proinflammatory cytokines from Th1/Th17 cells in the CNS activate macrophages/microglia (Mf/Mic) to mediate demyelination and axonal injury by secreting soluble products such as proinflammatory cytokines, reactive oxygen species (ROS), reactive nitrogen species (RNS), and glutamate. (M) B cells accessing the CNS differentiate into plasma cells (PC) and produce antibodies (Abs), which cause demyelination through phagocytosis or complement activation. (N) Most CNS resident cells, such as oligodendrocytes (ODCs), neurons, and astrocytes (ASC), express MHC class I under inflammatory conditions, making them potential targets for cytotoxic CD8þ T cells. (O) Neutrophils can be primed by proinflammatory molecules to produce elastase, cathepsin G, and ROS, causing CNS damage. (P) 'Epitope spreading' (ES) leads to the recognition of epitopes distinct from those that prompted the initial immune activation, leading to disease relapse and progression (Adapted from Yadav, Mindur, Ito, & Dhib-Jalbut, 2015).

Figure 3. Various immune mechanisms in immunopathogenesis of MS.

2.5 Immunomodulatory therapy for personalized medicine in MS

Currently there are ten DMTs in clinical use for RRMS and SPMS (Table 1) with varying beneficial effects in reducing relapse rate, MRI outcomes, and preserving neurological function, but their long-term outcome is still unclear (Derwenskus, 2011; Tullman, 2013). Until now, the disease-modifying ag ents (DMAs) are targeting mainly to the inflammation in MS includes interferon- β (IFN-β), glatiramer acetate (GA), natalizumab, alemtuzumab, as well as newer oral medications, including fingolimod, teriflunomide, and dimethyl fumarate. However, recent research has focused on the development of agents that can stop the progression and increase the regeneration of myelin fibers that would favor the PPMS patients. The treatment decision is often based on the risk-to-benefit ratios of each DMA and is further complicated by the inherent disease heterogeneity (Lim & Constantinescu, 2010; Piehl, 2014). Until now, sequential DMT monotherapy has been is the strategy followed to treat MS patients. The identification and validation of predictive biomarkers of therapeutic response along with genetic and imaging data help to guide optimal treatment management strategies like combination and personalized therapy in MS patients (Cutter & Kappos, 2014; Hauser, Josephson, & Johnston, 2013) (Table 1).

Table 1. Immunomodulatory therapies for MS

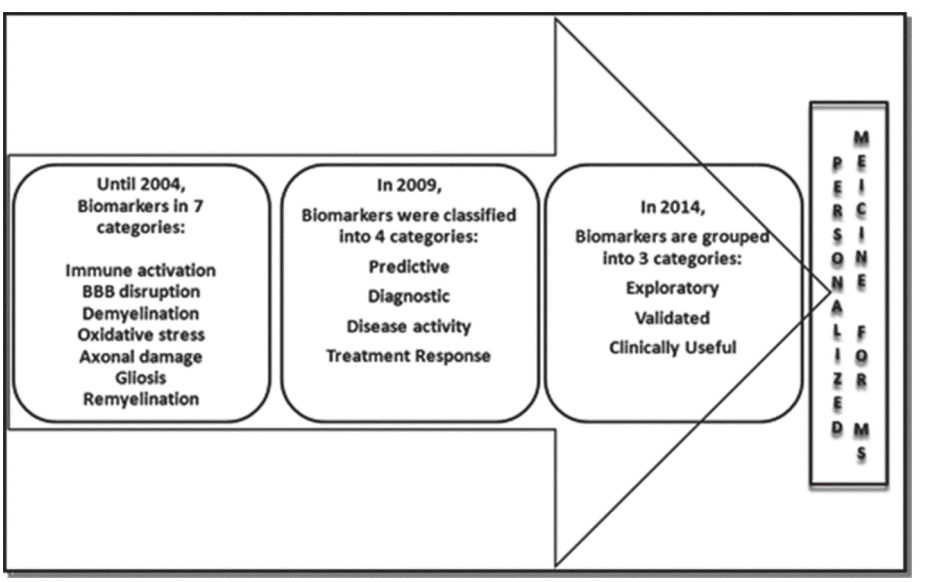
Agent	Mechanism	Route	Place in therapy
Interferon beta-1a	Decreases MHC-II expression, upregulates IL-10, and decreases Th1 and Th17 production	IM	First-line therapy for RRMS, PRMS, and CIS; slow/ delay disability
Interferon beta-1a	Decreases MHC-II expression, upregulates IL-10, and decreases Th1 and Th17 production	SC	First-line therapy for RRMS
Interferon beta-1b	Decreases MHC-II expression, upregulates IL-10, and decreases Th1 and Th17 production	SC	First-line therapy for RRMS, and CIS
Glatiramer acetate	Consists of antigen-presenting cells, which present to lymphocytes, and limits ability of lymphocytes to respond to myelin basic protein, probably stimulates regulatory T cells, shifts pro-inflammatory responses towards anti-inflammatory response	SC	First-line therapy for RRMS and CIS
Fingolimod	Sphingosine 1-phosphate receptor modulator, retains lymphocytes in the lymph nodes, preventing them from crossing the blood-brain barrier into the central nervous system and causing consequent damage	Oral	First or second-line therapy for RRMS
Teriflunomide	Pyrimidine synthesis inhibitor inhibits a key enzyme in lymphocytes—which reduces the proliferation of T and B cells, and T cell messaging.	Oral	First therapy for RRMS
Dimethyl fumarate (BG-12)	Anti-inflammatory and potential neuro- protective properties mediated through the Nrf2 pathway	Oral	First or second line therapy for RRMS
Alemtuzumab	Humanized monoclonal antibody that binds to CD52 on lymphocytes and monocytes and results in prolonged T-cell depletion	IV	First or second line therapy for active RRMS or
Natalizumab	Blocks a4 integrins, which inhibits adhesion and migration of leukocytes into the brain	IV	Second-line therapy for RRMS
Mitoxantrone	Inhibits topoisomerase II preventing lymphocyte proliferation	IV	Second-line or third-line therapy for SPMS, PRMS.

	Under Investigation		
Daclizumab	Humanized monoclonal antibody that binds to CD25 and increases CD56 bright natural killer cells, which target activated immune cells	SC	Phase 3 studies
Laquinimod	Reduces inflammation, demyelination, and axonal loss	Oral	Phase 3 studies
Ocrelizumab	Humanized anti-CD20 monoclonal antibody that results in B-cell depletion	IV	Phase 3 studies

Adapted and modified from (Tullman, 2013). 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.

2.6 Biomarkers in MS

The heterogeneity of MS and transition to personalized medicine calls for a more differentiated defining of MS patients that can be established by the development of biological markers. *A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention* (Bielekova & Martin, 2004; Harris & Sadiq, 2009; Lesko & Atkinson, 2001). Optimally, a biomarker would therefore, 1) reflect the targeted immunopathological process, 2) define patients according to predominant the pathogenic process, 3) predict responses to therapeutic interventions, and 4) provide a simple and economic monitoring tool in clinical trials and finally routine patient management (Bielekova & Martin, 2004; Harris & Sadiq, 2009; Lutterotti, Berger, & Reindl, 2007). Currently, due to the insufficient understanding of the cause of MS, its prognosis, response to therapy and as well as disease heterogeneity, it is unlikely that one single biomarker will satisfy the needs, particularly for choosing and monitoring the appropriate therapy. Moreover, it seems that the successful therapeutic strategy in MS may ultimately involve combining different therapeutics targeting several dominant pathophysiological processes. The advent of omics, genes, and transcription studies has enabled the identification and validation of many exploratory biomarkers that will identify specific biomarker panels or signatures to provide personalized care for patients (Comabella & Montalban, 2014). Figure 4 summarizes the criteria for the classification of biomarkers based on biomarker studies presented during the past decade.



Pictorial representation of biomarker research towards personalized medicine.

Figure 4. Biomarkers research in MS.

2.6.1 Sources of biomarkers

Blood and cerebrospinal fluid (CSF) are the most widely used biological fluids but others such as urine (Giovannoni & Thompson, 1998), tears (Devos et al., 2001) and saliva (Calais et al., 2010) have also been tried. CSF seems to provide more accurate information than the results obtained by examining peripheral blood since pathological processes leading to demyelination occur in the CNS (Harris & Sadiq, 2009). On the other hand, biomarkers from the *blood* are of significant clinical value because of the non-invasive collection method. Under physiological conditions blood-derived proteins enter into the CSF compartment via passive diffusion across the BBB, thus forming the majority of protein content in the lumbar CSF (Katsavos & Anagnostouli, 2013a). *Urine* is the easiest material to collect, but such tests are not yet in clinical use. Chronic urinary tract infections, asymptomatic bacterial colonization of the bladder and urinary hypohydration are more common in progressive MS patients and this may negatively affect the results. *Tears and saliva* have been successfully used for measuring Oligoclonal Bands (OCBs) and specifying soluble Human Leucocyte Antigens (HLAs) type II that are comparable to those of CSF and peripheral blood (Bielekova & Martin, 2004; Calais et al., 2010; Katsavos & Anagnostouli, 2013a; Lesko & Atkinson, 2001; Minagar et al., 2007).

2.6.2 Blood and CSF biomarkers

A. Predictive biomarkers

Predictive biomarkers could be used to identify individuals at risk of developing MS. They would be measured in neurologically asymptomatic individuals, mainly first-degree relatives of patients with MS. At present, the validated predictive biomarkers are the GWAS genes, EBNA, and Vitamin D (Casiraghi, Dorovini-Zis, & Horwitz, 2011; Comabella & Montalban, 2014; International Multiple Sclerosis Genetics Consortium, 2011).

Genes: Polymorphisms in HLA class II antigens seem to be decisive in attributing genetic burden for MS. Multiple recent MS studies have demonstrated that HLA-DRB1*1501 is the allele mainly responsible for attributing genetic risk in MS population (Bozikas et al., 2003; Oksenberg et al., 2004; Schmidt, Williamson, & Ashley-Koch, 2007). HLA-DRB1*15 was found to correlate positively with early onset MS (Van der Walt et al., 2011). DRB1*1501 positive MS patients had the worst brain atrophy scores, bigger T1 lesion burden in MRI, lower *N*-acetyl-aspartate (NAA) levels within NAWM and impaired cognitive function (Zivadinov et al., 2007). Moreover, HLA-DRB1*1501 expression is partially regulated by vitamin D through interaction at a genomic level, thus explaining the long known connection between latitude and risk for MS (Lincoln et al., 2009). Additionally, DQB1*0301 and DQB1*0602 positive had the worst brain atrophy scores, which suggests that the coexistence of certain alleles probably leads to augmentation

of the overall risk via epistatic mechanisms (Lincoln et al., 2009). Various genome-wide studies have now revealed many non-MHC single nucleotide polymorphisms as candidates for genetic burden augmentation in MS (Katsavos & Anagnostouli, 2013a). Most of them, however, had only a modest effect on susceptibility. Polymorphisms of the IL2RA and IL7RA regions seem the most promising at the moment (International Multiple Sclerosis Genetics Consortium et al., 2007). Mounting evidence has identified 57 non-MHC genes like, EVI5, TOB-1, ApoE, CD58, KIAA0350, and RPL5 genes associated with MS (International Multiple Sclerosis Genetics Consortium et al., 2011).

Anti Epstein–Barr virus nuclear antigens (***Anti EBNA***): These antigens are considered to be prognostic markers of conversion from CIS to CDMS (Comabella & Montalban, 2014), early disease onset and indicator of high inflammatory activity (Buljevac et al., 2005). High percentage of IgG antibodies and T cells against epitopes of EBNA-1 in the serum and CSF of MS patients has been reported (Lunemann et al., 2006). Infection of BBB endothelial cells has been reported, initiating disruption mechanisms, as well as proinflammatory cytokines production (Casiraghi et al., 2011).

Vitamin D: 25-Hydroxyvitamin D levels in untreated MS patients correlate inversely with radiologic disease activity (Löken-Amsrud KI et al., 2012) which may be due to the inhibitory role of vitamin D response element (VDRE) close to the HLA-DRB1*1501 coding area (Ramagopalan et al., 2009). Stewart et al. recently concluded that part of the IFN-β therapeutic effects during MS relapses may be attributed to greater production of vitamin D (Aivo, Lindsrom, & Soilu-Hanninen, 2012; Stewart et al., 2012) .

B. Diagnostic and disease activity biomarkers

Diagnostic biomarkers can be used to distinguish patients who have MS from patients with other neurological or autoimmune disorders, or from healthy individuals (Comabella & Montalban, 2014). These biomarkers can be helpful in combination with clinical and radiological disease diagnostic criteria in improving the sensitivity and specificity to identify individuals in whom the first neurological event is due to MS (Granberg, Martola, Kristoffersen-Wiberg, Aspelin, & Fredrikson, 2013; Miller et al., 2012). *Disease activity biomarkers* can also help to distinguish between patients with benign MS and those with aggressive disease courses (Comabella & Montalban, 2014).

I Biomarkers of Inflammation

Oligoclonal bands (***OCB***): The presence of OCBs in CSF is the diagnostic biomarker for MS. An increased rate of IgG synthesis and IgG index is often used as a relevant factor for conversion to CDMS (Nilsson, Larsson, Maly-Sundgren, Perfekt, & Sandberg-Wollheim, 2005; Tintore et al., 2008). Their diagnostic sensitivity is high (>90%), but their specificity is lower (<35%) among inflammatory disorders of the CNS (Katsavos & Anagnostouli, 2013a; Owens et al., 2009).

Cytokines: Active demyelinating lesions are highly inflammatory in nature leading to the release of many different cytokines within the BBB compartment. Numerous cytokines in CSF and serum have been explored as biomarkers in MS due to their extensive role in MS pathogenesis (Cannella & Raine, 1995; Hagman, Raunio, Rossi, Dastidar, & Elovaara, 2011; Harris & Sadiq, 2009; Imitola, Chitnis, & Khoury, 2005; Jadidi-Niaragh & Mirshafiey, 2011; Katsavos & Anagnostouli, 2013a; Katsavos & Anagnostouli, 2013b; Rajasekharan, Bar-Or, & CIHR/MSSC NET in Clinical Autoimmunity, 2012; Sospedra & Martin, 2005; Tumani et al., 2009; Ziemann, Wahl, Hattingen, & Tumani, 2011). Proinflammatory cytokines in the blood primarily originate from T and B cells (Romme Christensen et al., 2012). IFN-γ and TNF-α are the main products of Th1 immune response. IL-6 serves as link between B-cell and T-cell immune response as well as a Th-17 response triggering factor (Chen et al., 2012). Levels of PBMC IL-17 production are higher in patients recently diagnosed with MS than in those with longstanding disease, which may relate to recent or severe disease activity (Durelli et al., 2009; Frisullo et al., 2008; Tzartos et al., 2008). Systemic IL-10 decreases prior to clinical and MRI relapses, and increases when disease activity resolves (Imitola et al., 2005; Korsen, Bragado Alonso, Peix, Broker, & Dressel, 2015; Salmaggi et al., 1996).

Chemokines: Serum levels of CXCL8, CXCR3, CCL5 and IP-10 increase during relapse with MRI activity, while levels of CCL2 (MCP1) decline (Bartosik-Psujek & Stelmasiak, 2005; Semple, Kossmann, & Morganti-Kossmann, 2009). Chemokine CXCL13 mobilizes B cells and T-helper cells towards active demyelinating lesions by interacting with CXCR5 receptor (Kowarik et al., 2012; Szczucinski & Losy, 2007). High CSF levels of CXCL13 have been found in patients with CIS and CDMS which seems to consistently correlate with CSF B cells, plasmablasts, and intrathecal Ig synthesis (Bielekova, Komori, Xu, Reich, & Wu, 2012; Ferraro et al., 2015; Khademi et al., 2011; Sellebjerg et al., 2009).

Other molecules: The other molecules reported as diagnostic markers in conversion from CIS to CDMS in CSF are Kappa Free (KFLC) (Presslauer, Milosavljevic, Brucke, Bayer, & Hubl, 2008; Villar et al., 2012), Lambda Free Light Chains (LFLC) (Arneth & Birklein, 2009), and Measles-Rubella-Zoster Endothecal Reaction (MRZ Reaction) (Brettschneider et al., 2010).

II Biomarkers of BBB disruption

Adhesion molecules: Adhesion molecules and their receptors help activated inflammatory cells to enter the CNS. The CSF expression of sICAMs is increased during high disease activity when proinflammatory cytokines are at high levels (Acar et al., 2005; Witkowska et al., 2016) while its serum levels have recently been found to be lower in remitting patients and to be associated with clinical disability (Witkowska et al., 2016). Serum and CSF MMPs levels (like MMP9) are constantly elevated during MS relapse (Avolio et al., 2003). Soluble adhesion molecules such as soluble Platelet/Endothelial Cell Adhesion Molecule (sPECAM-1), sP-Selectin and sE-Selectin were recently found to be significantly increased

in RRMS patients during relapse compared to chronic progressive MS patients, suggesting that these molecules may be useful paraclinical markers of disease activity in MS (Basnyat et al., 2015).

Other molecules: *Ninjurin-1* was found to be up-regulated in active demyelinating lesions and plays an important role in the transmigration and localization of the latter inside the CNS (Ifergan, Kebir, Terouz et al., 2011). The *endothelin system* has recently been found to play an important role in the transmigration of monocytes through the BBB (Reijerkerk et al., 2012).

III Biomarkers of demyelination/axonal loss

Demyelination biomarkers: *Myelin Basic Protein* (*MBP*) *and αβ-Crystalline* are the two proteins reported as demyelination markers (Comabella & Montalban, 2014; Katsavos & Anagnostouli, 2013a). MBP and its fragments are found in large quantities in the CSF of most MS patients during relapse (Sellebjerg, Jaliashvili, Christiansen, & Garred, 1998). The correlation between the decrease in CSF-MBP, contrast-enhancement in MRI, and clinical disability in response to treatment with methylprednisolone suggests an association between inflammation and myelin breakdown in MS (Barkhof et al., 1992). Increased expression of the heat-shock protein αB-Crystalline is found in demyelinating lesions (van Noort et al., 2010).

Oxidative stress biomarkers: *NO and its metabolites* can cause mitochondrial damage and tissue hypoxia leading to further damage in MS lesions (Haider et al., 2011; Su, Bourdette, & Forte, 2013; Witte, Mahad, Lassmann, & van Horssen, 2013). Elevated levels of NO have been detected in acute demyelinating lesions and CSF, which further correlated with higher disability progression rates in MS (Rejdak et al., 2004). Elevated levels of NO metabolites, NO synthase nitrite and nitrate are associated with MS and its disease activity (Calabrese et al., 2002; Rejdak et al., 2004; Rejdak, Petzold, Stelmasiak, & Giovannoni, 2008).

Axonal damage biomarkers: Biomarkers of axonal damage typically consist of neuron-specific proteins, *Neurofilaments* (light chain/NF-L, intermediate chain/NF-M, and heavy chain/NF-H) which are released following axonal degeneration (Trentini et al., 2014). Several studies have reported considerably higher levels of NF-L in CSF of MS patients than in healthy controls and in patients with other neurological disorders (Modvig et al., 2013). In progressive disease forms NF-H chains are reported to be increased and seem to correlate better with disease progression (Huizinga, Gerritsen, Heijmans, & Amor, 2008; Petzold et al., 2005). The CSF levels of NF-L have been reported to return to normal in Natalizumab responders, thereby suggesting it as a biomarker of therapeutic response (Amor et al., 2014; Gunnarsson et al., 2011).

Tau protein of axons is increased in the CSF of MS patients (Martinez et al., 2015). A simultaneous elevation in Tau and NF-H values in CSF in patients with CIS has a 70% predictive value of conversion to CDMS, which is superior to the predictive value

of MRI (Brettschneider, Petzold, Junker, & Tumani, 2006). *N-AcetyloAspartate* (*NAA*) is an aminoacid, highly expressed in intact neurons. Studies have shown decreased NAA values in MS lesions and NAWM that correlates adequately with disability progression (Rahimian et al., 2013; Trentini et al., 2014; Tumani et al., 2009). Also, higher serum and CSF NAA levels in RRMS patients could be helpful in differential diagnosis between MS and neuromyelitis optica (Tortorella et al., 2011). *Glial fibrillary acidic protein* (*GFAP*) in astrocytes is a marker for astrogliosis. Most studies have reported elevated levels of GFAP and its correlation with disability in MS has been reported (Petzold et al., 2002). However, it is a non-specific marker of CNS tissue injury since it has been found to be increased in CSF of patients with other neurological diseases (Misu et al., 2009).

IV Biomarker of regeneration

Low *brain derived neurotropic factor* (*BDNF*) levels are considered to contribute in the progression of demyelination and axonal damage. In line with this notion, SPMS patients have lower CSF-BNDF levels than RRMS patients (Ziemssen, Kumpfel, Schneider, Klinkert, Neuhaus, & Hohlfeld, 2005a). *Nogo-A* is a CNS myelin protein that inhibits axonal repair. It is adequately specific for MS (Jurewicz, Matysiak, Raine, & Selmaj, 2007) and its presence in CSF of MS patients represent an adverse prognostic marker of axonal repair (Selmaj, Jurewicz, Matysiak, & Raine, 2009).

C. Treatment-response biomarkers

Treatment-response biomarkers are needed to identify individuals at risk for treatment failure or serious adverse drug reactions and hence eligible for a treatment change (Comabella & Montalban, 2014). Such treatment-specific biomarkers have been reported in MS. All protein based DMAs are potentially immunogenic – in some cases leading to development of antibodies that neutralize drug activity. Detection of neutralizing antibodies (NAbs) to DMAs in MS continues to be one of the more clinically utilized biomarkers in therapeutic decision-making. HLA-DRB1 polymorphisms influence NAbs production. TNF-related inducing ligand (TRAIL), Vit D, Myxovirus Resistance Protein-A (MxA), and sVCAM levels may also reflect response to IFN-β treatment (Gilli et al., 2006; Rieckmann, 2005). On the other hand, MS patients with prominent Th17 response are probably more harmed than benefited by treatment with IFN-β, because of IL-17 exacerbation by the drug (Axtell, Raman, & Steinman, 2011). Furthermore, the frequency of $CD4^+CD25^+$ Treg cells expressing Foxp3 has recently been successfully used to assess the effects of IFN-β therapy (Bushnell et al., 2012). A predictive role of BNDF was observed in GA responders that correlated well with clinical improvement (Ziemssen, Kumpfel, Schneider, Klinkert, Neuhaus, & Hohlfeld, 2005b). The efficacy of Natalizumab is predicted by many molecules such as, α4 integrin (on PBMCs), feutin-A, lipocalin 2, NF-L and CD49d antigens (on T cells) (Wipfler et al., 2011; Wipfler, Oppermann et al., 2011). CSF levels reduction of

sICAM-1 and sESelectin may potentially serve as biomarkers of therapeutical efficacy after cladribine treatment (Mitosek-Szewczyk, Stelmasiak, Bartosik-Psujek, & Belniak, 2010).

Table 2. Biomarkers and their usefulness in MS

Biomarker	Type	Sample	P	D	D A	TR	Validated
Genes							
HLA-DRB1*1501	gene	DNA	+	-	-	-	*
HLA-DRB1*0401, 0408, 1601	gene	DNA	-	-	-	+	*
HLA-DQB1*0301, *0602	gene	DNA	-	-	-	-	*
IL2RA and IL7RA	gene	DNA	+	-	-	-	*
EVI5, CD58, KIAA0350, and RPL5	gene	DNA	+	-	-	-	*
ApoE ε4	gene	DNA	-	-	-	-	*
TOB1	gene	DNA	+	-	+	-	*
Antibody proteins							
IgG OCB	Ptn	CSF	+	+	-	-	*
IgM OCB	Ptn	CSF	+	+	+	+	*
IgG index	Ptn	S/CSF	+	+	-	-	*
KFLC	Ptn	S/CSF	-	+	-	-	*
Anti EBNA 1	Ptn	S/CSF	+	+	+	-	*
Anti MBP	Ptn	S/CSF	+	+	-	-	*
Anti MOG	Ptn	S	+	+	-	-	*
Nabs	Ptn	S/PBMC	-	-	-	+(IFNb-R)	*
EBV Abs	Ptn	S/CSF	-	+	-	-	n
MRZ reaction	Ptn	CSF	-	+	+	-	*
Anti JC virus	Ptn	S	-	+	-	+(NZ-R)	*
anti VZV	Ptn	S/P	-	+	-	+(F-R)	*
Anti aquaporin 4	Ptn	S/CSF	-	+	-	-	*
		Biomarkers of Inflammation					
Cytokines							
IL1	Ptn	B/CSF	-	+	+	+(IFNb-R,GA-R)	E
IL6	Ptn	B/CSF	-	+	+	+(IFNb-R,GA-R)	E
IL12	Ptn/mRNA	B/CSF	-	+	+	-	*
IL15	Ptn	B/CSF	-	+	+	+(IFNb-R,GA-R)	E
IL17	Ptn/mRNA	PBMC/CSF	-	+	+	-	*
IL17F	Ptn	B/CSF	-	+	+	+(IFNb-R)	E
IL21	Ptn	B/CSF	-	+	+	+(AL-R)	E
IL23	Ptn/mRNA	B/CSF	-	+	+	-	*
TNF-a	Ptn/mRNA	B/CSF	-	+	+	-	*
Type I IFNs	mRNA	PBMC	-	-	+	+(IFNb-R)	*
Chemokines							
CCL2	Ptn	S/CSF	-	+	+	-	n
CCL5	Ptn	S/CSF	-	+	+	-	n
CXCL8	Ptn	S/CSF	-	+	+	-	n
CXCL12	Ptn	CSF	-	+	+	-	n
CXCL13	Ptn	CSF	-	+	+	-	*
CX3CR1	Ptn	S/CSF	-	+	+	-	n
CXCR3	Ptn	S/CSF	-	+	+	-	n
IP-10	Ptn	S/CSF	-	+	+	-	n

Apoptotic Molecules							
TRAIL	Ptn/mRNA	B/PBMC	-	-	+	+(IFNb-R)	E
BAFF	Ptn/mRNA	PBMC/CSF	-	-	+	-	*
sFas	Ptn	S/P	-	-	+	-	E
FasL	Ptn	S/P	-	-	+	-	E
MIF	Ptn	S/P	-	-	+	-	E
Adhesion Molecules							
sICAM-1	Ptn	CSF	-	-	+	+(CL-R)	*
sVCAM-1	Ptn	S/CSF	-	-	+	+ (IFNb-R)	*
Laminin 411	Ptn	CSF	-	-	+	-	n
α4 Integrin	Ptn	CSF	-	-	+	+(NZ-R)	n
NCAM1	Ptn	CSF	-	+	+	-	*
sPECAM	Ptn	P	-	-	+	-	*
sP-,sE-Selectin	Ptn	P/CSF	-	-	+	+(CL-R)	n
Other Molecules							
Vitamin D	Steroid	B	+	+	+	+(IFNb-R)	*
Ninjurin-1	Ptn	CSF	-	-	+	-	E
Osteopontin	Ptn	S/P/ CSF	-	-	+	-	*
Feutin-A	Ptn/mRNA	B/CSF	-	+	+	+(NZ-R)	E
Demyelination Biomarkers							
MBP	Ptn	CSF	-	+	+	-	*
αβ Crystalline	Ptn	CSF	-	-	+	-	*
Oxidative Stress Biomarkers							
NO and metabolites	Met	B/CSF/Ur	-	-	+	-	≠
7-Ketocholesterol	Met	CSF	-	-	+	-	E
Axonal loss Biomarkers							
NF-L	Ptn	S	-	+	+	+(NZ-R)	*
NF-H	Ptn	S	-	+	-	-	*
NAA	Met	CSF	-	+	+	-	*
GFAP	Ptn	CSF	-	+	+	-	*
Tau	Ptn	CSF	-	+	+	-	E
Tubulin	Ptn	CSF	-	-	+	-	E
Actin	Ptn	CSF	-	-	+	-	E
Nogo A	Ptn	CSF	-	-	+	-	n
Remyelination Biomarkers							
BDNF	Ptn	S/P/ PBMCs	-	-	+	-	*
Nogo A	Ptn	CSF	-	-	+	-	n
NT3,NT4	Ptn	CSF	-	-	+	+	E

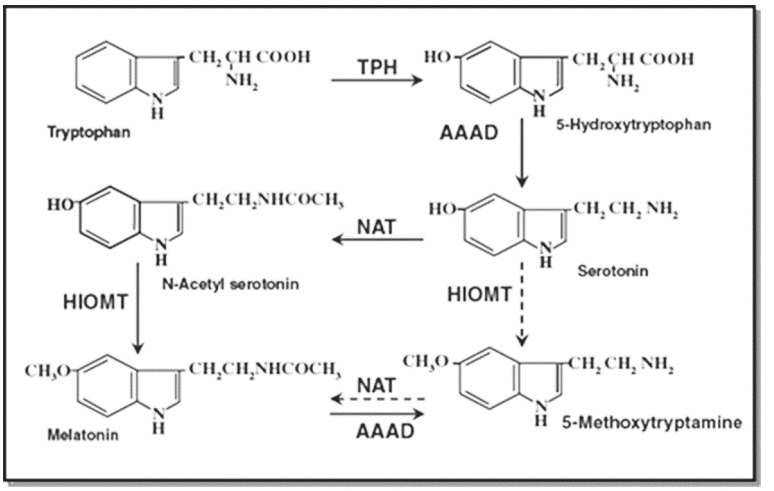
Modified and redrawn from (Comabella & Montalban, 2014; Harris & Sadiq, 2009; Katsavos & Anagnostouli, 2013a; Lutterotti et al., 2007). +: the biomarker has been analyzed for respective outcome; -: the biomarker has not been analyzed for respective outcome; *: the respective outcome has been re-analyzed in at least one independent population, ≠: the biomarker has been analyzed in at least one independent population with contradictory results; n: the biomarker has not been validated in an independent population; E: the respective outcome has been explored but not been validated. S: serum, B: blood, Ur: Urine, P: plasma, Ptn: protein, Met: metabolite, IFNb-R: interferon β response, NZ-R: natalizumab response, F-R: Fingolimod response, AL-R: Alemtuzumab response; CL-R: cladribine response.

2.6.3 Melatonin and Adipokines as Potential Biomarkers in MS disease

A. Melatonin and its immune function

Melatonin (N-acetyl-5-methoxytryptamine) is the major neurohormone that is secreted during the hours of darkness at night by the pineal gland and is involved in the regulation of circadian rhythm. It has been shown that a large number of extra-pineal sites such as thymus, mast cells, platelets, and lymphocytes are involved in the secretion (Kvetnoy, 1999). Tryptophan serves as the precursor for melatonin biosynthesis, and is taken up from the circulation and then converted into serotonin. Serotonin is then converted into N-acetylserotonin by the enzyme arylalkylamine-N-acetyl transferase (AANAT) while N-acetylserotonin is metabolized into melatonin by the enzyme hydroxyindole-O-methyltransferase (HIOMT) (Figure 5). Once formed, melatonin is released into the capillaries and in higher concentrations into the CSF (Tricoire, Moller, Chemineau, & Malpaux, 2003) and is then rapidly distributed to most body tissues (Cardinali & Pevet, 1998). Circulating melatonin is metabolized mainly in the liver to form 6-sulfatoxymelatonin (Skene et al., 2001). Melatonin is also metabolized by oxidative pyrrole-ring cleavage into kynuramine derivatives. Melatonin can activate or inhibit signal transduction cascades through receptors or independent of receptors. Two of the membrane receptors subtypes, such as MTNR1A/MT1 and MTNR1B/MT2 are GPCRs (G-Protein Coupled Receptors, are expressed mainly in the CNS, while the third, MT3, is a nuclear receptor belonging to the (Retinoid Z Receptors)/ROR (Retinoid Orphan Receptors) orphan receptor subfamily of quinone reductases and is prominently expressed both on the periphery and in the brain. The hormone binds with high affinity in the picomolar range to the membrane receptors, and/or in the nanomolar range to the nuclear receptors (RZR/ROR) as well as to Calmodulin (Dubocovich, Rivera-Bermudez, Gerdin, & Masana, 2003).

Melatonin binds to receptors expressed on the membrane of CD4 T cells, CD8 T cells, and B cells (Carrillo-Vico et al., 2005; Garcia-Maurino et al., 1997; Pozo, Reiter, Calvo, & Guerrero, 1997). Several studies have demonstrated that melatonin suppresses the production of various proinflammatory cytokines such as TNF-α, IL-1β, IL-12 and IL-6 (Carrillo-Vico et al., 2004; Carrillo-Vico et al., 2005; Majewska, Zajac, Zemelka, & Szczepanik, 2007; Raghavendra, Singh, Kulkarni, & Agrewala, 2001)). Furthermore, it also plays a protective role in mitochondrial dysfunction inhibiting the expression and levels of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and proinflammatory cytokines (Escames et al., 2007; Jung, Hong, Zheng, Lee, & Hong, 2009). All these anti-inflammatory effects have been suggested to act via the inhibition of nuclear factor-κB (NF-κB) transcriptional activity and its translocation and binding to DNA in the nucleus (Chuang, Mohan, Meltz, & Reiter, 1996; Li et al., 2009; Ozbek et al., 2009; Yip et al., 2013).



TPH, tryptophan hydroxylase; AAAD, aromatic amino acid decarboxylase; AAA, aryl acylamidase; NAT, N-acetyltransferase; HIOMT, hydroxyindole O-methyltransferase. (Adapted from Tan, Manchester, Terron, Flores, & Reiter, 2007).

Figure 5. Melatonin biosynthesis pathway.

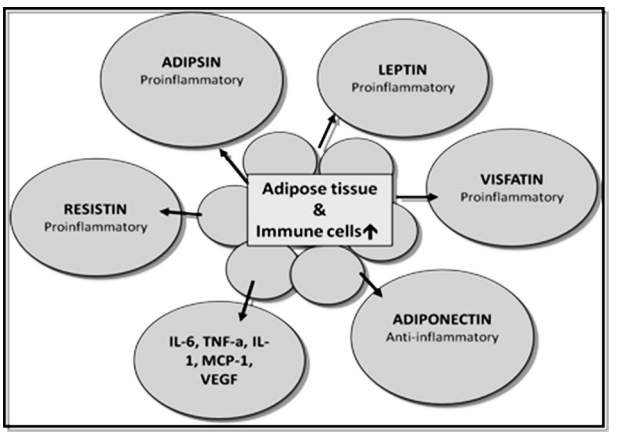
B. Melatonin in MS

It has been shown that the production of melatonin is lowered and phase-shifted in MS patients, which has been suggested to be due pineal gland dysfunction (Sandyk & Awerbuch, 1993a; Akpinar et al., 2008; Crasson et al., 2004). Furthermore, the specific inverse association detected between nocturnal melatonin secretion and the duration of clinical symptoms suggests that the function of the pineal gland declines with the progression of MS disease (Sandyk & Awerbuch, 1994). Additionally, it has also been observed that MS patients excrete a subnormal proportion of 6-sulpahtoxymelatonin in the urine at night, indicating a dysregulation of melatonin production or altered enzymatic metabolism of melatonin in MS patients (Melamud, Golan, Luboshitzky, Lavi, & Miller, 2012). Melatonin is also a factor that is affected by environmental disturbances. Aside from UVradiation, and vitamin D, sunlight has been reported to have both direct and indirect effects on melatonin biosynthesis (Hauser & Oksenberg, 2006; Schwarz, 2005). A study by Golan D et al. reported an inverse association between vitamin D levels and 6-sulpahtoxymelatonin in MS patients, which suggests that melatonin, is a potential mediator of vitamin D neuromodulatory function (Golan et al., 2013). Therefore melatonin biosynthesis, seems to be dysregulated MS, which may be associated with clinical disease manifestations and the MS disease process. Furthermore, Ghorbani A et al., recently speculated that during the initial phase of MS disease, melatonin levels may rise and trigger the immune system, but that as the disease progresses, due to pineal gland dysfunction, melatonin secretion decreases (Ghorbani, Salari, Shaygannejad, & Norouzi, 2013). It is also assumed that a shift towards the kyneurinine pathway may reduce the availability of tryptophan to synthesize

the serotonin and melatonin is possible during the course of the disease (Heyes et al., 1992; Opitz, Wick, Steinman, & Platten, 2007).

C. Adipokines in MS

Adipose tissue contains adipocytes, which are the most abundant cell type in white adipose tissue, pre-adipocytes (which are adipocytes that have not yet been loaded with lipids), endothelial cells, fibroblasts, ,leukocytes , and, most importantly, macrophages (Fantuzzi, 2005; Cousin et al., 1999). Adipocytokines are cytokines that are mainly produced by adipose tissue. Leptin, adipocnectin, resistin, adipsin, and visfatin are the most common adipocytokines that are thought to provide link between adipose tissue and related inflammatory disorders by promoting inflammation (Tilg & Moschen, 2006) (Figure 6).



Schematic representation of all adipokines secreted by adipose tissue during a state of inflammation. Various other products of adipose tissue that have important roles in immune system include: certain cytokines, such as tumor-necrosis factor (TNF), interleukin-6 (IL-6), IL-1 and CC-chemokine ligand 2 (CCL2; also known as MCP1); Vascular endothelial growth factor (VEGF). (Modifed and redrawn from Calle & Kaaks, 2004; Wellen & Hotamisligil, 2005).

Figure 6. Adipokines mediating inflammation.

Leptin: It is a proinflammatory cytokine produced mainly by adipocytes which upregulates T-cell and macrophage activation thereby facilitating the release of cytokines like IL-1, IL-12, TNF-a, and IL-6 and promoting Th1 and Th17 responses (Fantuzzi, 2005). Typically, leptin signals through its receptor leptin receptor b (OBRb) to induce activation of the mitogen-activated protein kinases (MAPKs) p38 and extracellular-signal-regulated kinase (ERK) and of signal transducer and activator of transcription 3 (STAT3) that results in the production of pro-inflammatory cytokines (Tilg & Moschen, 2006). Studies in MS have reported an increased level of leptin in both serum and CSF during relapses (Matarese, Procaccini, & De Rosa, 2008) and also in remission (Frisullo et al., 2007). In EAE models with active lesions, *in situ* secretion of leptin in foci adjacent to inflammatory lesions with T cells and macrophages has been observed (Sanna et al., 2003).

Adiponectin: This is synthesized mainly by adipocytes, but is also expressed by skeletal muscle cells, cardiac myocytes, and endothelial cells (Delaigle, Jonas, Bauche, Cornu, & Brichard, 2004; Pineiro et al., 2005). Adiponectin interacts with its cellular receptors (ADIPOR1 and ADIPOR2) leading to the stimulation and activation of peroxisome proliferator-activated receptor-α (PPARα), AMP-activated protein kinase (AMPK) and p38 mitogen-activated protein kinase33 that leads to the regulation of the anti-inflammatory function. This anti-inflammatory adipocytokine decreases the T-cell activation and proliferation and inhibits the nuclear factor kappa B (NF-kB) dependent cytokine release (TNFa and IL6) and adhesion molecule expression, but increases the levels of IL10 (Tilg & Moschen, 2006). In MS, lower levels of adiponectin have been found in sera of RRMS patients, although higher levels of adiponectin in CSF MS patients have also been reported thereby indicating the possibility of secondary intrathecal synthesis of this cytokine (Hietaharju et al., 2009; Kraszula et al., 2012).

Resistin: This is also known as FIZZ3. Resistin mRNA can be found in various tissues, including adipose tissue, the hypothalamus, adrenal gland, spleen, skeletal muscle, pancreas and gastrointestinal tract (Kusminski, McTernan, & Kumar, 2005). The receptor for resistin is unknown, but it induces the activation of p38, ERK and phosphatidylinositol 3-kinase (PI3K) to increase the production of TNF, IL-1β, IL-6 and IL-12 thereby regulating inflammatory processes (Tilg & Moschen, 2006). The mRNA expression of resistin is in turn activated by proinflammatory cytokines like IL1, IL6 and TNFa (Kaser et al., 2003). Furthermore, resistin also upregulates the expression of vascular cell adhesion molecule 1 (VCAM1), intercellular adhesion molecule 1 (ICAM1) and CCL2 by human endothelial cells and induces these cells to release endothelin-1 (Verma et al., 2003). Several factors such as pituitary, steroid, and thyroid hormones, adrenaline, β3-adrenoreceptor activation, endothelin-1 and insulin also seem to modulate resistin expression (Kusminski et al., 2005; Lehrke et al., 2004). Markedly elevated levels of resistin have recently been reported in the sera of RRMS patients (Kraszula et al., 2012).

Adipsin (***complement factor D***): This is an atypical member of the adipocytokines family, a serine protease and a rate-limiting enzyme in the alternative pathway of complement activation (White et al., 1992). The adipsin gene was isolated in 1986 and identified as an adipocytokine in 1987 (Min & Spiegelman, 1986). In human subjects it is primarily expressed in adipocytes and monocyte/macrophages (Cook et al., 1987). Adipsin, together with several other components of both the classical and alternative complement cascade, is expressed by both adipocytes and monocytes-macrophages in human subjects (Cook et al., 1987). Activation of alternative pathway results in the formation of C5 convertases and membrane attack complex, leading to the formation of anaphylatoxins, which cause inflammation and chemotaxis (Ingram, Hakobyan, Robertson, & Morgan, 2009). It has been shown that in obese subjects reduced adipsin mRNA expression and synthesis is associated with enhanced adipocyte expression of TNF-α which indicates that adipsin promotes inflammation and cellular stress by activating of proinflammatory pathways

such as, NF-kB. In MS, only one study has reported higher levels of this adipocytokine in the CSF of twins with MS in comparison to the asymptomatic co-twin (Hietaharju et al., 2009).

2.6.4 Techniques in biomarker analyses

The most common and established methods employed for analyzing the biomarkers from biological samples are Enzyme-Linked Immunosorbant Assay (ELISA), immunoflorescence, flow cytometry, polymerase chain reaction (PCR), nephelometry, western blotting and isoelectric focusing (Katsavos & Anagnostouli, 2013a). Over the past few decades new and massive technologies called, 'Omics' have come into existence and provided high-throughput and unbiased identification of biomarkers such as DNA, RNA, proteins, lipids, metabolites, and epigenetic modifications. They are categorized as genomics (Lill CM, 2014), transcriptomics (Comabella & Martin, 2007; Sanchez-Pla, Reverter, Ruiz de Villa, & Comabella, 2012), proteomics (Kroksveen et al., 2015), lipidomics (Quintana, Yeste, Weiner, & Covacu, 2012), metabolomics (Botas, Campbell, Han, & Maletic-Savatic, 2015) and epigenomics (Baranzini et al., 2010). Also, apart from MRI many new non-invasive imaging techniques such as, positron emission tomography (PET) (Oh et al., 2011), Evoked potentials (EP) (Fernandez et al., 2013; Gazioglu & Boz, 2012; Laron et al., 2010; Margaritella, Mendozzi, Garegnani, Colicino et al., 2012; Margaritella, Mendozzi, Garegnani, Nemni et al., 2012; Schlaeger et al., 2012), optical coherence tomography (OCT) (Grazioli et al., 2008; Herrero et al., 2012) and sympathetic skin response (Aghamollaii et al., 2011) have recently proved to be beneficial for detecting axonal loss, neurodegenration and disability.

2.6.5 Obstacles in biomarker validation

The main obstacles encountered during biomarker validation are; sample collection condition, timing of collection, storage and timing of sample processing and analysis. These mainly contribute to preanalytical variability of results. Many other factors, such as sex, age, diet, exercise, smoking, alcohol intake, obesity, or over-the-counter drugs, should be considered in biomarker studies because they may increase within-patient and between-patient variation. Drawbacks in study design may mask or magnify the effects of the biomarker analyzed. Some of the main aspects of study design that must be taken care of are; inclusion criteria for MS patients and controls, stratification of MS patient subtypes, insufficient sample size, prospective follow-up of cohorts because most biomarker studies are retrospective cross-sectional studies that do not allow testing the predictive nature of the biomarker and finally validating the methodology with quantitative techniques (Comabella & Montalban, 2014).

2.6.6 Imaging Biomarkers in MS

A. Conventional MRI

Magnetic resonance imaging (MRI) has been established as an important paraclinical tool in MS for the assessment of clinical diagnosis, natural history, disease progression and treatment effects (Filippi et al., 2011). It is a sensitive and reliable *in vivo* method for investigating the specific pathological changes in brain tissue and its integrity during MS disease progression (Paty et al., 1994). Conventional MR pulse sequences that are routinely used for evaluation include T2-weighted (T2-WI), proton density (PD)-weighted, and fluid-attenuated inversion recovery (FLAIR)-weighted sequences, as well as T1-weighted imaging (T1-WI) with and without gadolinium (Gd) contrast. These sequences provide a sensitive method for detecting and quantifying focal white matter disease as well as more acute inflammatory activity in MS. *T2-WI* is highly sensitive in detecting hyperintense lesions in the WM. The most typical sites in the WM are the periventricular region, corpus callosum and posterior fossa (Zivadinov & Bakshi, 2004). FLAIR, conventional spin echo and fast spin echo are the most recommended techniques for T2 hyperintense lesions. In CIS stage, T2 Lesion load is considered as prognostic biomarker of conversion to clinically definite MS (CDMS) (Barkhof et al., 1997; Filippi et al., 1995; Gauthier et al., 2007; Kappos et al., 1999; Mesaros et al., 2008). *T1-WI* is sensitive for detectingisointense lesions and also hypointense lesions known as 'black holes' that represent neurodegenration (van Walderveen et al., 1999). Most of the newly formed black holes revert to isointensity within a few months due to remyelination and resolution of the edema, while the rest remain as such representing more advanced pathologic substrates like axonal loss, wallerian degeneration or gliotic changes (Bakshi, 2005; Losseff, Miller, Kidd, & Thompson, 2001; Stevenson, Ingle, Miller, & Thompson, 2004). Thus, T1-WI scans can predict disability better than other lesion-based inflammatory MRI measures (Zivadinov & Leist, 2005) and are considered to be biomarkers of axonal damage (Sahraian, Radue, Haller, & Kappos, 2010). *Gd-enhanced T1-WI* allows the visualization of lesions due to BBB breakdown and active inflammation, which indicates a higher risk for relapse in the short term and may contribute to long-term clinical dysfunction (Losseff et al., 2001; Rovaris et al., 2005). Although the conventional MR findings are sensitive enough in revealingchanges in focal white matter, as well as the relative pathologic specificity of some imaging features, they correlate weakly with disease burden and clinical disease measures. Furthermore, conventional MRI also fails to detect the diffuse structural, metabolic, and functional abnormalities now known to be present in the normal-appearing gray and white matter.

B. Diffusion tensor imaging

Diffusion tensor imaging (DTI) is one of new advanced MRI techniques with the ability to improve the sensitivity in detecting microstructural architecture changes in normal-

appearing brain tissues (Rovaris et al., 2005). Apparent diffusion coefficient (ADC), fractional anisotropy (FA) and mean diffusivity (MD) are the three DTI parameters that are commonly used in analyses. In MS, decreased FA reflects the axonal structural integrity and increased ADC reflects the tissue inflammation in NAWM (Bammer et al., 2000; Werring, Clark, Barker, Thompson, & Miller, 1999). So far in MS DTI measures have been compared using either summary measures of the whole-brain (Nusbaum, Tang, Wei, Buchsbaum, & Atlas, 2000), the NAWM (Filippi, Cercignani, Inglese, Horsfield, & Comi, 2001) or region-of-interest (ROI) approaches (Ciccarelli et al., 2001). DTI abnormalities seem to be quite widespread in NAWM. The regional analysis of NAWM regions has revealed diffusion abnormalities in areas such as mesencephalon, capsula interna, corona radiata anterior and posterior, centrum semiovale, corpus callosum, and optic radiation. This suggests that subtle changes occur in the NAWM regions of MS patients where they are likely to represent secondary degeneration of axons damaged by remote lesions and could be causally related to disturbances which are common in MS (Ciccarelli et al., 2005; Dineen et al., 2009; Mesaros et al., 2009; Roosendaal et al., 2009). In agreement with postmortem findings, histogram-derived DTI metrics in the cerebral normal-appearing gray matter (NAGM) were found with greater diffusivity in SPMS patients than those with RRMS or healthy controls. NAGM abnormalities have been detected in even the earliest stages of the disease (CIS stage) (Henry et al., 2009; Shiee et al., 2012). Recently studies have demonstrated the consistent involvement of thalamus from the earliest stages of MS and short-term accrual of thalamus damage is associated with accumulation of disability in PPMS patients (Batista et al., 2012; Mesaros et al., 2011; Tao et al., 2009; Tovar-Moll et al., 2009). Also, caudate atrophy has been associated with disability, lesion load, and fatigue in MS (Hasan et al., 2009; Jafari, Kreft, Flach, Janssens, & Hintzen, 2009; Tao et al., 2009). The DTI derived metrics could serve as potential quantitative radiological markers of MS pathology.

3 AIMS OF THE STUDY

The studies in this thesis were aimed to investigate whether the melatonin pathway genes, major adipocytokines, and some exploratory immune molecules are useful as biomarkers of MS. For this purpose these molecules were analysed in different MS subtypes and their association with clinical measures and volumetric MRI measures reflecting the focal changes of MS lesions was evaluated. The usefulness of DTI indices from NAWM and NADGM in diagnostics of MS was also explored.

Our specific aims were:

I. To investigate whether the melatonin pathway genes are associated with the disability progression in relapsing and progressive subtypes of MS (Study I).

II. To investigate whether the adipocytokines are differentially expressed in the plasma of patients with different MS subtypes and their association with disease activity, neurological disability and MRI volumes during 2 year follow-up (Study II).

III. To explore whether the levels of sCD26 and sCD30 are associated with clinical phenotypes, disease activity, and disability progression in MS (Study III).

IV. To assess whether DTI indices of NAWM and NADGM in different subtypes of MS and CIS are associated with clinical parameters and candidate immune biomarkers in sera (Study IV).

4 PATIENTS AND METHODS

4.1 Patient characteristics and ethical considerations

In all the studies, the patients were recruited and observed at the MS outpatient department at Tampere University Hospital. Patients underwent neurological and MRI examinations as well as blood sampling on the same day. The diagnosis of MS was based on the revised McDonald criteria and CIS patients were defined as patients who had their first clinical episode suggestive of MS (Polman et al., 2005). All RRMS and SPMS patients were in remission. We excluded the patients who were pregnant or suffering from any other clinically significant disease or treated with immunosuppressive drugs at least eight weeks before entering the study. All patients gave their informed consent. The studies were approved by the Ethics Committee of Tampere University Hospital.

Neurological evaluation included determination of Expanded Disability Status Scale score (EDSS) (Kurtzke, 1983) and progression index (PI) at baseline (Studies I, II, III and IV) and during follow-up (Study II). Clinical disease activity was determined by calculating the number of relapses during the preceding two years (Study I, III and IV), baseline and during 2-year follow-up (Study II).

MRI evaluation of disease activity included the measurements by volumes of T1 and FLAIR lesions at the baseline and in the follow-up (Study II). The diffusion abnormalities in eight different regions from NAWM and NAGM regions were analyzed by FA and ADC indices by DTI analyses (Study IV). A summary of patient demographics and clinical characteristics is presented in Table 3.

In study I, a case-control analysis was used to analyze SNPs in melatonin pathway genes and their association to neurological disability. Study II was a 2-year longitudinal follow-up of MS patients and CIS that reports the levels of adipokines and their association to disease activity and neurological disability. Study III was a cross-sectional design which included CIS, RR and SPMS patients to analyze the levels of sCD26 and sCD30 and their association to disease activity and EDSS. Study IV was also a cross-sectional study which included MS patients and CIS patients that determines the association between immune biomarkers and DTI indices. Overall, 47% of RRMS, 53% of SPMS and 53% of PPMS patients were found to be similar between study I with other three studies. CIS patients were not included in study I, but across the other three studies (Study II to IV), 63% of CIS patients were found to be same.

Table 3. Clinical characteristics of patients and healthy controls in Studies I to IV

	CIS	RRMS	SPMS	PPMS	Controls
Study I – Melatonin Pathway genes in MS (n=590)					
No. of Patients[a]	-	109	51	33	397
Gender (M/F)[a]	-	28/81	21/30	18/15	216/181
Age[b]	-	37.3±10.1	47.4±9.0	51.8±10.3	44.3±11.1
Disease duration[b]	-	6.1±5.2	12.8±7.0	8.4±7.1	NA
EDSS[b]		2.0 ±2.0	5.3 ±1.4	4.8 ±2.3	NA
Study II – Adipokines in MS – follow-up Study (n=77)					
No. of Patients[a]	15	34	15	16	-
Gender (M/F)[a]	2/13	10/24	5/10	7/9	-
Age[b]	35.6 ±7.9	37.6±9.2	49.3 ±10.0	58.1 ±8.5	-
Disease duration from diagnosis[b]	NA	4.2 ±4.1	12.9±9.0	13.1±8.4	-
BMI (kg/m^2)[b]	25.3 ±3.2	24.9 ±4.0	26.2 ±5.0	24.6±3.4	-
EDSS at baseline[b]	0.1 ±0.3	1.4±1.5	5.2 ±1.6	4.7 ±2.2	-
EDSS at the end of follow-up[b]	0.1 ±0.4	1.5±1.6	5.5 ±1.6	4.8 ±2.1	
Prestudy disease activity	0.7±0.6	1.2 ±1.4	0.2 ±0.6	NA	-
Number of relapses during the follow-up	0.1±0.3	0.6±1.1	0.4 ±0.7	NA	
Treatment (NT/IFN/GA/OT)[a]	15/0/0/0	12/18/2/1	15/0/0/0	16/0/0/0	-
Study III – sCD26 and sCD30 in MS (n=197) – Article III					
No. of Patients[a]	19	39	19	-	60 (sCD30) 58 (sCD26)
Gender (M/F)[a]	3/20	11/24	8/13	-	25/33; 21/39
Age[b]	35.0 ± 9.0	37.4 ± 8.7	49.1 ± 8.6	-	30.1 ± 9.4; 34± 11.8
Disease duration[b]	NA	3.9 ± 3.8	11.7 ± 9.7	-	NA
Relapses (0/1/2 to 5)[b,c]	4/13/2	12/12/15	15/2/2	-	NA
EDSS[b]	0.1 ± 0.3	1.6 ± 1.3	4.8 ± 1.8	-	NA
Treatment (NT/IFN/GA)[a]	19/0/0	19/18/2	19/0/0	-	NA
Study IV – Levels of other Inflammatory Biomarkers in MS (n=110) – Article IV					
No. of Patients[a]	24	36	19	21	10
Gender (M/F)[a]	11/25	7/12	9/12	3/21	4/6
Age[b]	34.3 ± 9.5	36.6 ± 8.4	49.5 ± 8.2	57.0 ± 9.2	39.8 ± 12.9
Disease duration[b]	NA	3.9±3.9	11.3±9.3	11.9±8.4	NA
EDSS[b]	0.0±0.2	1.7±1.6	4.7±1.7	4.8±2.0	NA
Pre-study disease activity[b,c]	0.8±0.6	1.6±1.4	0.2±0.5	NA	NA
Treatment (NT/IFN/GA)[a]	24/0/0	13/20/3	18/1/0	21/0/0	NA

CIS: clinically isolated syndrome; RRMS: relapsing-remitting MS; SPMS: secondary progressive MS; PPMS: primary progressive MS; EDSS: expanded disability status scale; NT: no treatment; IFN: interferon-β; GA: glatiramer acetate; OT Other treatments; BMI Body mass index

[a] Number of patients

[b] Mean (± SD)

[c] Number of relapses 2 years before study entry

4.2 Molecular biology methods

4.2.1 Blood (Studies I–IV)

The venous blood was collected and allowed to clot for at least 30 minutes before separation. The sera was separated from the blood by centrifuging for 15 minutes at 1600 x g then aliquoted and stored at -70C. The whole blood was centrifuged at 2500 x g for 10 minutes at room temperature (15–25°C) after which, three different fractions were distinguishable: the upper clear layer was plasma; the intermediate layer buffy coat, and the bottom layer contained concentrated erythrocytes. The plasma and buffy coat were then aliquoted and stored at -70C. Sera were then used for ELISA and Luminex analysis of immune molecules, plasma for adipocytokine analysis and buffy coat for DNA isolation and genotyping.

4.2.2 DNA isolation (Study I)

Genomic DNA was extracted from buffy coats using the QIAamp DNA Blood Minikit and automated biorobot M48 extraction (Qiagen, Hilden, Germany) according to the manufacture's recommendations. The automated extraction method consists of cell lysis using chaotropic reagents, binding of the DNA to silica coated magnetic particles, followed by washing steps and the elution of the pure nucleic acid samples. Two hundred microlitres of the buffy coat sample is transferred by pipetting to a 1.5-ml tube placed on the instrument stage. DNA isolation and purification procedure was initiated on the fully automated GenoMTM-48 Robotic Workstation with the "DNA from blood" protocol according to the manufacturer's recommendations. DNA was eluted in 75 ml volumes of bi-distilled water (Braun, Melsungen, Germany). Prior to and during analysis the DNA samples were stored at 4°–8°C in the refrigerator and at 20°–8°C after finishing casework. After each working day, the workstation was UV sterilized at least for 1 h for decontamination. Purity is determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. Pure DNA has an A260/A280 ratio of 1.7–1.9.

4.2.3 Genotyping (Article I)

Four genes including those involved in melatonin biosynthesis and function were genotyped. The following SNPs: TPH1 (rs1800532 and 10488982), TPH2 (rs10506645 and rs4570625), AANAT rs12942767) and MTNR1B (rs4753426 and rs10830963) were chosen (II, S Table 1). Genotyping was performed using Taqman SNP Genotyping Assays C_8940793_10, C___2645675_10, C___30277975_20, C___226207_10, C___31556625_20, C___289583_10, C___3256858_10 and the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, California, USA). The

PCR was optimized to 35 cycles and the annealing temperature was set at 63°C. Random duplicates and control samples were run in parallel with unknown DNA samples. An ABI 7900HT instrument was used for genotyping, and to visually analyze the genotype clustering.

A. Haplotype analyses (Study IV)

Haploview program (http://www.broadinstitute.org/scientificcommunity/science/programs/medical-and-population-genetics/haploview/haploview) was used to examine linkage disequilibrium patterns and likely haplotype frequencies. The linkage disequilibrium (LD) at each of the three loci (TPH1, TPH2, MTNR1B) was studied using data from the Hapmap phase III from Feb09 (CEU population, assembly build36, found at http://hapmap.ncbi.nlm.nih.gov). Visual inspection of loci gave an idea of the overall LD patterns, and individual D prime (D') values between the two loci were obtained by analyzing the Hapmap data in Haploview 4.2. The two markers within TPH1 (rs1800532, rs10488682), TPH2 (rs4570625, rs10506645) and MTNR1B (rs4753426, rs10830963) were each in strong linkage disequilibrium (D'>0.5, data not shown) and were used in haplotype analyses. Haplotype association analysis was performed in different subtypes and groups including all MS patients and controls. The association of haplotypes with gender and EDSS scores was also determined.

4.2.4 Enzyme-linked immunosorbent assay (Articles II, III and IV)

A. Determination of adipokines levels (Article II)

Plasma levels of adipocytokines (resistin, leptin, adiponectin, adipsin) were determined by enzyme-immuno-assay (EIA) by using commercial reagents (DuoSet ELISA, R&D Systems Europe Ltd, Abindgon, U.K). As plasma adipokine levels are dependent on the amount of adipose tissue, adipocytokine levels were adjusted for BMI by dividing the measured concentration by BMI. The detection limits and inter-assay coefficients of variation, respectively, were 15.6 ng/l and 4.0% for resistin, 15.6 ng/l and 3.9% for leptin, 15.6 ng/l and 2.0% for adiponectin, 4.0 ng/l and 3.8% for adipsin.

B. Determination of sCD26 and sCD30 levels (Article III)

sCD26 and cCD30 levels of serum samples were analyzed as single samples with ELISA (Human sCD26 Platinum ELISA BMS235CE and Human sCD30 instant ELISA BMS240INSTCE, eBioscience, Bender MedSystems GmbH, Austria). Analyses were performed according to manufacturer's instructions. Concentrations of analytes were determined by absorbance reader (Labsystems Multiskan® MCC/340) using 450 nm as

the primary wave length and 620 nm as the reference wave length. A standard curve for each run was generated using four parameter 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 evaluated using pooled plasma sample (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).

C. Determination of TRAIL levels (Study IV):

Levels of sTRAIL in undiluted sera were quantified by a Diaclone solid phase sandwich ELISA kit (# 850.770.096; Diaclone, Besancon Cedex, France). Detection limits were 64 pg/ml. TRAIL absorbancies were read with a Multiskan MS version 4.0 spectrophotometer (Labsystems, Helsinki, Finland) at wavelength 450 nm.

4.2.5 Luminex (Article IV)

The levels of sFas, sFasL and MIF were measured with a Human sepsis/apoptosis LINCOplex Kit (Linco Research, St. Charles, Missouri, USA), CCL2, CCL3, CCL4 and CXCL10 with a Human cytokine LINCOplex kit (Linco Research) and IL-2, IL-6, IL-10, IL-12p70, IFN-γ and TNF-α with High Sensitivity Human cytokine LINCOplex kit (Linco Research). All data were collected and analyzed using Bio-Plex suspension array system and Bio-Plex Manager software 4.1 (Bio-Rad laboratories, California, USA). A five-parameter regression formula was used to calculate the sample concentration from the Human sepsis/apoptosis LINCOplex Kit and the High Sensitivity Human cytokine LINCOplex kit, and a four-parameter regression formula was used for the Human cytokine LINCOplex kit. All 96 well plates included samples from all disease subtypes and controls to minimize inter-assay variation. The same batch of monoclonal antibodies for the Bio-Plex Cytokine Assay System was used throughout the experiments; the inter-assay and intra-assay values are reported by the manufacturer to be less than 15%. The percentage recovery of standards ranged from the 90% to 110% and used as a detection limit for each protein. The lower detection limits were as follows: 12.2 pg/ml for sFas, sFasL and MIF, 16.0 pg/ml for CXCL10, CCL3 and CCL4, 3.2 pg/ml for CCL2, 0.13 pg/ml for IL-10, TNF-a, IL-6, IL-12p70, IL-2 and IFN-g.

4.2.6 Magnetic Resonance Imaging (Studies II and IV)

A. Volumetric Analysis (Study II)

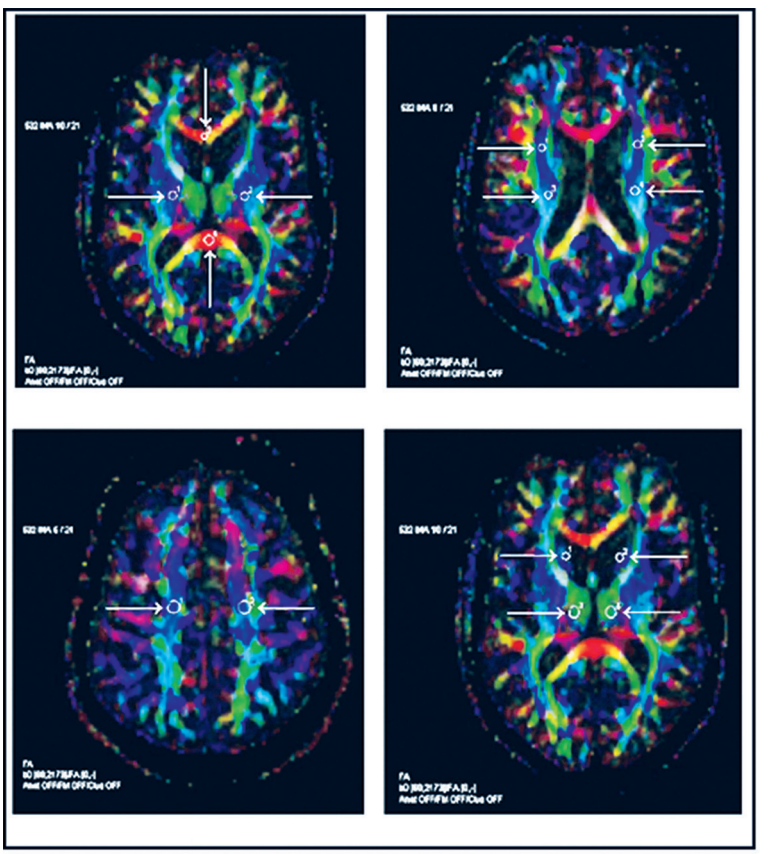
MR Image Acquisition: MR imaging was acquired using a 1.5-TeslaMR scanner (Siemens Avanto, Erlangen, Germany). All subjects were examined using the same MRI protocol, which included a T1 weighted header followed by an axial T1-weighted magnetization prepared rapid gradient echo (MP-RAGE), and a T2-weighted turbo spin-echo (TSE), fluid attenuation inversion recovery (FLAIR), magnetization transfer contrasts (MTC), diffusion weighted imaging (DWI), and gadolinium enhanced T1 weighted MP-RAGE sequences. In this study, 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; in-plane resolution = 0.45*0.45 mm. In FLAIR, the parameters used in this sequence are TR = 8500ms; TE = 100ms; TI = 2500ms; slice thickness = 5.0 mm; in-plane resolution = 0.45*0.45 mm. The total scanning time was approximately 30 minutes. The volumetric segmentation of plaques in the brain was performed using semiautomatic software Anatomatic™ operating in a PC/Windows 95 environment (Heinonen et al., 1998; Heinonen, Dastidar, Kauppinen, Malmivuo, & Eskola, 1998) and the images were analyzed blindly.

The DTI data were collected by a single shot spin-echo-based echo-planar diffusion-weighted imaging (EPI) sequence with the following parameters: repetition time 3500milliseconds (ms), echo time (TE) 96ms, slice thickness 5 mm, interslice gap 1.5 mm, field of view (FOV) 230 mm, matrix 128 × 128 (in-plane resolution = 1.8 × 1.8mm^2), *b* values 0 and 1000 s/mm^2, number of excitations 3, and with 12 diffusion gradient

B. DTI Analysis (Study IV)

The DTI analysis was performed by an experienced radiologist together with a physicist who was blinded to the clinical details of the study subjects. The analysis was performed as previously described by (Hakulinen et al., 2012) using the commercial software Neuro 3D (Siemens Healthcare, Malvern, PA, USA) on an offline workstation. In every individual, circular regions of interest (ROIs) of approximately 6 to 106mm^2 (depending on the anatomical regions) were manually placed simultaneously in exactly the same location on B0 images (T2WI) from which the ADC and FA maps are obtained. The ROIs were placed bilaterally (except for the corpus callosum) at the following anatomical locations: the posterior limb of the internal capsule, the centrum semiovale anterior, the posterior corona radiata anterior and posterior, the splenium and the genu of the corpus callosum, the thalamus, and the caudate nucleus (Figure 15). The ROIs were centered in the structure of interest in the most homogenous area, avoiding border areas to avoid partial volume effects. The size of the ROI was reduced if a lesion was identified in the predefined ROI.

ROIs of the same size were drawn in images of the healthy control subjects at the same anatomic locations as those of the patients.



The posterior limb of the internal capsule (1, 2), the splenium (3), and the genu (4) of the corpus callosum (a); the posterior corona radiata anterior (1, 2) and posterior (3, 4) (b); the centrum semiovale anterior (c); and the caudate nucleus (1, 2) and the thalamus (3, 4) (d). Colors indicate the directions of fiber tracts (red, transverse; blue, craniocaudal; green, anterior-posterior).The circular ROIs were transferred from the corresponding *B* 0 image, and their sizes adjusted to avoid any visible lesions. The size of the ROIs ranged from 2 to 33 pixels (6–106.5mm^2; pixel size 1.8 * 1.8mm^2) depending on the size of the brain structure. This figure is a representative analysis from an SPMS patient. Adapted from (Natarajan et al., 2013).

Figure 7. Region-of-interest (ROI) placement on axial FA colour maps.

4.3 Statistical analysis

Statistical analyses in all studies were performed using PASW version 18.0 for Windows (SPSS Inc., Chicago, Illinois, USA).

In ***Study I*** Allele and haplotype associations in controls and in different subtypes of MS were analyzed using Chi-square ($\chi 2$) analysis, implemented in the PASW (version 18.0, SPSS Inc., Chicago, Illinois, USA) and the Haploview program. The association between alleles and the progression index in the inflammatory or progressive subtypes of MS was analyzed with Mann-Whitney U test. A p value of less than 0.05 was considered significant.

In ***Study II***, the comparison of the adipocytokine levels in different subtypes was analyzed by repeated measures of ANOVA followed by Bonferroni correction for multiple comparisons. For each outcome, the analyses were also adjusted for age and gender. The differences in clinical parameters, MRI volumes between the subtypes and adipocytokine levels between genders were analyzed by Mann-Whitney U test. Wilcoxon signed-rank test was used to analyze the intra-individual changes in the volumes of MRI at each time point. Pearson's correlation coefficient was used to explore the relationship between the levels of adipocytokines with BMI or age. The associations of adipsin levels with EDSS scores, the volumes of T1 weighted and FLAIR lesions were studied by linear regression model adjusting for age, gender and disease subtype. Logistic regression model was used to study the association between adipokines and disease activity. A p value < 0.05 was considered significant in all analyses.

In ***Study III***, the difference in levels of sCD26 and sCD30 levels and clinical parameters between the subtypes was analyzed by Mann-Whitney U test. Spearman's correlation coefficient was used to explore the relationship between the levels of sCD26 and sCD30 with clinical characteristics of patients. A p value of less than 0.05 was considered significant.

In ***Study IV***, the differences in DTI indices between and within groups were assessed using the univariate analysis of variance with age as covariate followed by a post hoc multiple pairwise comparisons with Bonferroni correction. Comparisons were considered to be statistically significant if the p value was smaller than 0.005 (n= 10; 5 groups) or 0.0006 (n = 80; 5 groups and 8 brain regions) after Bonferroni correction. Comparison of serum immune molecules was tested by non-parametric analyses: the Kruskal-Wallis test and Mann-Whitney U test for comparison of more than two unpaired groups. Correlations between variables were tested by two-tailed non-parametric Spearman correlation analysis and a p value of less than 0.01 was considered significant.

5 RESULTS

5.1 Melatonin Pathway genes in MS – candidate markers for neurological disability (Study I)

In this study it was observed that in TPH2 gene, an overrepresentation of T allele frequency of SNP rs4570625 in the transcriptional control region was associated with an increased risk of MS in the progressive MS subtypes ($p<0.05$) [Odds ratio (95% confidence interval): 1.665 (1.023–2.709)]. There was no allelic association between the two SNPs and disability or disease progression was found. Analysis of haplotypes revealed that the haplotype rs4570625–rs10506645TT of TPH2 gene was associated with the risk of severe disability in PPMS ($p<0.05$), while haplotype rs4570625–rs10506645TC appeared to be protective against disability in SPMS ($p<0.05$) (I, Table 4). Moreover, analysis of gender-specific haplotype association showed that the rs4570625–rs10506645 TC haplotype was associated with an increased risk of MS in males with progressive MS ($p<0.05$) and this effect was mainly due to the males of the SPMS group ($p<0.05$) (I, Table 3).

In the MTNR1B gene, allelic associations of the SNPs rs4753426 and rs10830963 with MS or any of its phenotypes were not detected. However, a tendency of association was found between decreased C allele frequency in rs10830963 with the progressive MS group (I, Table 3). Further analysis of haplotypes with disease susceptibility and disability status was performed. The haplotype rs10830963–rs4753426GC was associated with the risk of SPMS ($p<0.05$), whereas another haplotype rs10830963–rs4753426GT tended to associate with the risk of PPMS ($p=0.057$) (I, Table 4). Analyses of gender-based association of the rs10830963–rs4753426GT haplotype in the progressive MS group revealed an association of this haplotype with the risk of MS in males with SPMS ($p<0.05$). No allelic association between the two MTNR1B SNPs with disability status or disease progression index was observed. No other significant difference in genotype distribution between the subtypes was noticed (I, Table 3).

Table 4. Association of haplotypes of polymorphisms in TPH2 and MTNR1B genes with disability status in progressive MS group and its subtypes

	Progressive MS[a] (n=84)		SPMS (n=51)		PPMS (n=33)	
Haplotypes	Low/High EDSS frequency	p-value	Low/High EDSS frequency	p-value	Low/High EDSS frequency	p-value
TPH2/rs4570625 (G/T) – rs10506645 (C/T)						
GC	0.717/0.772	0.476	0.755/0.699	0.608	0.642/ 0.833	0.099
TT	0.233/0.136	0.173	0.194/0.199	0.958	0.308/ 0.083	**0.035****
GT	0.025/0.069	0.187	0.038/0.001	0.388	0.049/ 0.042	0.901
TC	0.025/0.023	0.942	0.013/0.101	**0.040****	0.001/ 0.042	0.202
MTNR1B/rs10830963 (C/G) – rs4753426 (C/T)						
CT	0.340/0.396	0.505	0.401/0.395	0.963	0.364/ 0.471	0.396
GC	0.366/0.282	0.317	0.328/0.295	0.781	0.279/ 0.238	0.713
CC	0.239/0.218	0.772	0.221/0.205	0.874	0.292/ 0.179	0.308
GT	0.055/0.104	0.269	0.050/0.105	0.362	0.064/ 0.113	0.491

MS Multiple Sclerosis, SPMS Secondary Progressive Multiple Sclerosis, PPMS Primary Progressive Multiple Sclerosis, EDSS Expanded disability Status Scale
Low EDSS group is mildly disabled (EDSS<4)
High EDSS group is more severely disabled (EDSS>4)
[a] Progressive MS group (SPMS and PPMS subtypes)
** p value < 0.05, * p value < 0.10 (tendency of association), significant values are in bold.

5.2 Adipokines in MS (Study II)

5.2.1 Levels of adipokines in MS subtypes and CIS during 2 year follow-up (Study II)

Comparison between the subtypes showed lower levels of adipsin (adjusted for age and BMI) in the RRMS than the PPMS group from baseline throughout the follow-up period (p<0.05). No other differences in the levels of adipocytokines were found between the MS groups and/or including converted and non-converted CIS patients throughout the study. Also, the levels of BMI-adjusted adipsin in treated and untreated RRMS patients were decreased in comparison to PPMS, but no differences were found between these RRMS groups.

The influence of gender on secretion of adipokines was studied by comparing the baseline levels in men and women. It appeared that in CDMS group the levels of leptin [869.6 (536.9–1504.9) vs. 242.3 (152.9–441.3) pg × m^2/ml × kg, p<0.001] and adiponectin [5540.9 (4197.4–8036.1) vs. 3808.0 (3178.7–5545.4) ng/ml, p=0.01, median (interquartile range)] were higher in women.

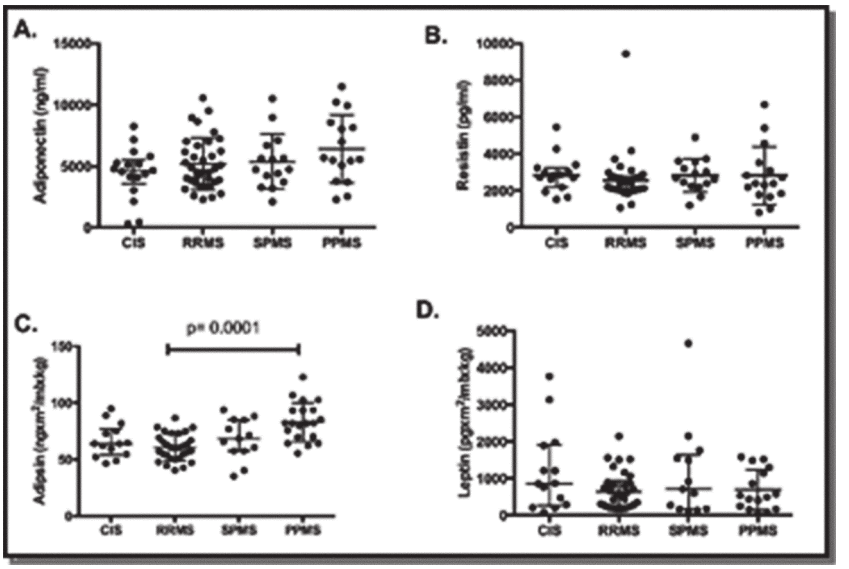


Figure 8. The baseline levels of Adiponectin (A), Resistin (B), BMI-adjusted Adipsin (C) and BMI-adjusted Leptin (D) in MS and CIS (median (IQR)). (Adapted from Natarajan et al., 2015).

5.2.2 Adipokines association with disease activity and neurological disability (Article II)

At study entry, the presence of higher clinical disease activity (n=13, at least 2 relapses/2 years before baseline) was associated with higher levels of BMI-adjusted adipsin than in patients with stable disease course (n=21, 0–1 relapses/ 2 years before baseline) (II, Figure 3). However, no associations were found during follow-up. Based on MRI, half of the patients (18/34) were found to be active according to defined criteria (presence of Gd-enhancing lesion or new T2 lesion over the follow-up period), but no association between adipokines levels and MRI activity was found.

In the CDMS group, the levels of adipokines were not associated with the volumes of FLAIR- or T1-weighted lesions or the change in their volumes during follow-up ($p > 0.05$). However, according to subgroup analysis, in RRMS correlations were found between the baseline levels of BMI adjusted adipsin and the volumes of T1-weighted ($p < 0.05$) (III, Fig 2(b)) and FLAIR ($p = 0.0001$) (II, Fig 2(b)) lesions or the changes of T1 lesion volumes during follow-up ($p < 0.005$) (II, Fig 2(d)).

Association of adipokines levels to neurological disability was observed by a positive correlation between BMI-adjusted adipsin levels and EDSS score adjusting for age/gender/disease subtype separately or in combination in CDMS group ($p < 0.05$) and more strongly in RRMS (adjusted for age or age and gender) ($p \leq 0.002$) (II, Fig 2). No correlations were found with any other molecule with other subtypes.

5.3 Soluble CD26 and CD30 in MS (Study III)

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 to RRMS and SPMS subtypes. The SPMS group had higher EDSS scores than patients with RRMS (p<0.05). Two years before enrolment, 12/39 (31%) RRMS patients were relapse-free, another 12 patients had one relapse, and 15/39 (38%) patients had 2–5 relapses. CIS patients were followed-up clinically for four years. During that time, 10 out of 19 patients (53%) converted to RRMS.

5.3.1 Serum sCD26 and sCD30 levels and correlation with clinical parameters

Serum sCD26 and sCD30 levels were significantly higher in MS patients and CIS than controls (p<0.05). Among the CIS patients, converted patients (n=10, 53%) showed higher levels of sCD30 compared to the unconverted patients (mean±SD; 23.0±6.4 ng/ml vs. 16.3±7.4 ng/ml; p=0.009) (Fig 9a). The levels of sCD30 in treated RRMS patients was higher than in untreated patients (mean±SD; 26.4±8.0 ng/ml vs. 20.5±9.4 ng/ml; p=0.01) (Fig 9b). No significant correlations between the levels of sCD26 and sCD30 and disease activity or disability status were observed in the 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), (IV, Fig 2).

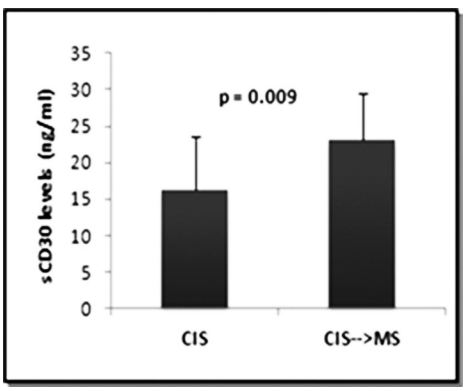


Figure 9a. Levels of sCD30 in unconverted and converted CIS patients.

Graphical representation of significant comparison between Unconverted CIS, vs converted CIS patients using Mann-Whitney U test p<0.05.

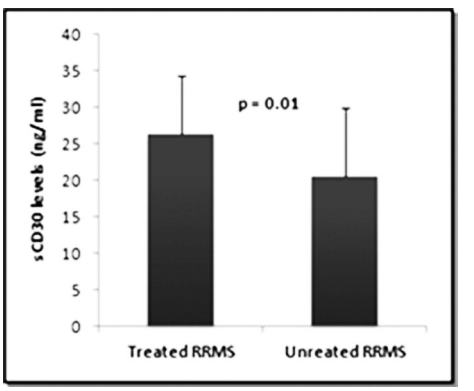


Figure 9b. Levels of sCD30 in treated and Untreated RRMS patients.

Graphical representation of significant comparison between treated and Untreated RRMS patients using Mann-Whitney U test p<0.05. MS multiple sclerosis, CIS clinically isolated syndrome, RRMS relapsing remitting MS.

5.4 Candidate inflammatory biomarkers in MS subtypes and their association with DTI indices (Study IV)

5.4.1 Levels of candidate inflammatory biomarkers in sera

Comparison of levels of inflammatory biomarkers between the respective subtypes and controls showed a decreased MIF in RRMS (138.2 vs. 378.5 pg/ml, $p<0.05$) and increased sFas, TNF-α and CCL2 levels in PPMS (sFas: 6787.6 vs. 4824.9 pg/ml, $p<0.05$; TNF-α: 6.4 vs. 3.7 pg/ml, $p<0.05$; CCL2: 288.4 vs. 152.8 pg/ml, $p<0.05$). Further comparison between MS subtypes revealed higher MIF, TNF-α and sFas in PPMS than in RRMS (MIF: 290.7 vs. 138.2 pg/ml, $p<0.05$; TNF-α: 6.4 vs. 4.5 pg/ml, $p<0.05$; sFas: 6787.6 vs. 4612.8 pg/ml, $p<0.05$) (Table 5). The levels of other molecules did not differ between the groups ($p>0.01$) (IV, S1).

Table 5. The levels (pg/ml) of soluble molecules in different subtypes of MS and controls (median (25–75th percentiles))

Molecule	RRMS (N=33)	SPMS (N=18)	PPMS (N=20)	CIS (N=15)	CON (N=21)
MIF	138.2 (50.0-229.4)^{A,C}	225.8 (134.0-317.3)	290.7 (189.3-449.3)^{C}	167.3 (97.8-268.2)	378.5 (236.4-535.9)^{A}
sFas	4612.8 (4058.0-5940.7)^{C}	5572.4 (4388.3-6845.4)	6787.6 (5393.9-7709.2)^{B,C}	4318.8 (3320.0-5697.9)	4824.9 (3405.1-5638.1)^{B}
sTNF-a	4.5 (3.4-6.5)^{C}	4.1 (3.3-5.8)	6.4 (4.4-8.9)^{B,C}	4.6 (3.5-6.6)	3.7 (2.4-4.6)^{B}
CCL2	177.0 (140.4-271.8)	218.8 (157.0-331.0)	288.4 (201.3-461.6)^{B}	248.3 (165.7-332.0)	152.8 (122.6-273.1)^{B}

(Modified from Natarajan et al., 2013). RRMS, relapsing remitting MS; SPMS, secondary progressive MS; PPMS, Primary progressive MS; CIS, clinically isolated syndrome; CON, controls. Analyses were performed for all four subtypes versus control, as well as between the subtypes. The results from the Mann-Whitney U Test are shown as the Bonferroni-corrected p-values ($p<0.05$).
A Comparison between the RRMS and HC groups
B Comparison between the PPMS and HC groups
C Comparison between the PPMS and RRMS groups

5.4.2 DTI abnormalities in MS subtypes, CIS, and controls

ADC and FA values of eight different anatomical brain regions were analysed from patients with different subtypes of MS, CIS, and healthy controls. Compared to controls, increased ADC values were detected in 6/8 regions in SPMS (internal capsule, corona radiata anterior and posterior, centrum semiovale, and splenium and genu of the corpus callosum), 4/8 regions in both RRMS or PPMS (internal capsule, corona radiata anterior and posterior, and centrum semiovale), and 2/8 regions (internal capsule and centrum semiovale) in CIS (IV, Fig 2(a)). The corresponding comparison between FA indices showed significantly

lowerFA values in 2/8 regions (genu and splenium of corpus callosum) in SPMS, 1/8 regions in both RRMS and PPMS (genu of corpus callosum), and 1/8 regions (caudate nucleus) in CIS (IV, Fig 2(b)).

5.4.3 Correlation between inflammatory biomarkers with DTI

The correlations between the immune biomarkers and DTI indices were restricted to the chronic progressive groups and CIS. In SPMS, the ADC values of the corona radiata posterior correlated with the levels of MIF ($r = 0.614$, $p = 0.007$) while in PPMS the ADC values of the thalamus correlated with sTNF-α ($r = 0.616$, $p = 0.003$). In CIS, the ADC values of internal capsule correlated with the levels of sFas ($r = 0.650$, $p = 0.009$) (I, Fig 3).

6 DISCUSSION

This study is the first study to address the interplay between adipocytokines and melatonin interaction and their association to neurological disability in MS. The studies in this thesis were aimed to explore the expression of melatonin pathway genes, the levels of adipokines including adiponectin, adipsin, leptin, resistin, and serum soluble immune molecules including, CD26 and CD30 in different MS subtypes and assessed their association with disease activity, neurological worsening, MRI volumes and DTI indices in MS patients.

6.1 Melatonin Pathway Genes – Candidate markers for Neurological Disability

An association of polymorphisms in the TPH2 and MTNR1B genes with the disability progression in progressive subtypes of MS was noticed. The two markers selected within TPH1 (rs1800532, rs10488682), TPH2 (rs4570625, rs10506645), and MTNR1B (rs4753426, rs10830963) genes were each in strong linkage disequilibrium (D'>0.5). We identified an increased frequency of T allele of the SNP rs4570625 (–703 G/T) in of TPH2 gene in progressive MS subtypes. The SNP rs4570625) is in the promoter region of TPH2 gene that may alter the gene function and serotonin biosynthesis. Furthermore, haplotype analysis of both the SNPs of TH2 gene showed an association with the risk of severe disability. The haplotype rs4570625–rs10506645TT of TPH2 gene was associated with the risk of severe disability in PPMS, while haplotype rs4570625-rs10506645TC appeared to be protective against disability in SPMS. This clearly shows that the T allele of rs4570625 is the risk allele of neurological disability, while the C/T allele of rs10506645 seems to contribute to the disease type in modulating its effect.

MTNR1B was the other gene that was associated with the progressive subtypes of MS. The SNPs rs10830963 and rs4753426 analyzed in this study have been shown to be associated with the risk of other diseases such as diabetes, insulin resistance, obesity, and autoimmune disorders (Li, Shi, You, Wang, & Chen, 2011; Lyssenko et al., 2009; Qiu et al., 2007). We observed a significant association of rs10830963–rs4753426 haplotype GT with the progressive MS group including both SPMS and PPMS, while the SPMS possessed haplotype rs10830963–rs4753426GC. Thus, the G allele of rs10830963 seems to be a risk allele for the progressive forms of MS. On the other hand, it has been shown that the C allele of rs4753426 is associated with sunlight exposure (Ji et al., 2010). Consistent with

the above phenomenon we consider that the increased frequency of C allele seen in our patients suggests their adaptability to the prevailing dark environmental conditions. In line with our observation, Ji et al., have suggested that the selection of derived rs4753426C allele may occur in order to adapt individuals to environmental pressure and may also increase the immunostimulatory effect of melatonin (Ji et al., 2010). Recently, many studies have analyzed the melatonin levels and their function in serum, urine and salivary samples and have shown the dysregulation in melatonin pathway in MS disease (Farhadi, Oryan, & Nabiuni, 2014; Gholipour et al., 2015; Ghorbani et al., 2013; Golan et al., 2013; Melamud et al., 2012). Taken together, these observations suggest that there could be at least two SNP variants in each melatonin genes that interact in predisposing to the risk of accumulation of disability in MS disease.

6.2 Adipokine Adipsin – Candidate Biomarker for Neurological Disability

The role of adipokines in the pathogenesis of MS has not been studied before, but the involvement of other complement proteins has been demonstrated within lesions and adjacent to white matter in immunohistochemical studies (Ingram et al., 2014). Adipsin (complement factor D) is a key enzyme involved in the activation alternative pathway of complement activation that is primarily secreted from adipocytes and monocytes/macrophages in human subjects (White et al., 1992; Alonso & Hernan, 2008). The presence of decreased adipsin in RRMS patients in comparison to those with PPMS is most likely related to different pathological mechanisms in these MS subtypes. The early phase of RRMS is characterized predominantly by inflammatory events initiated by activation and differentiation of myelin specific $CD4^+$ T cells into Th1 and Th17 cells and their transmigration from periphery to CNS eventually resulting in demyelination and axonal loss (Yadav et al., 2015). During the transition to more advanced stages like SPMS, BBB becomes less permeable leading to diminished entry of peripheral immune cells and their products into CNS (Revesz, Kidd, Thompson, Barnard, & McDonald, 1994). Recent pathologic studies have showed that progressive subtypes are characterized by the widespread diffuse inflammation with slowly expanding lesions, abundant cortical lesions, and lymphocyte infiltration and microglia activation in the NAWM (Haugen, Frederiksen, & Degn, 2014). Thus the elevated levels of adipsin in our PPMS patients most likely reflect peripheral immune activation.

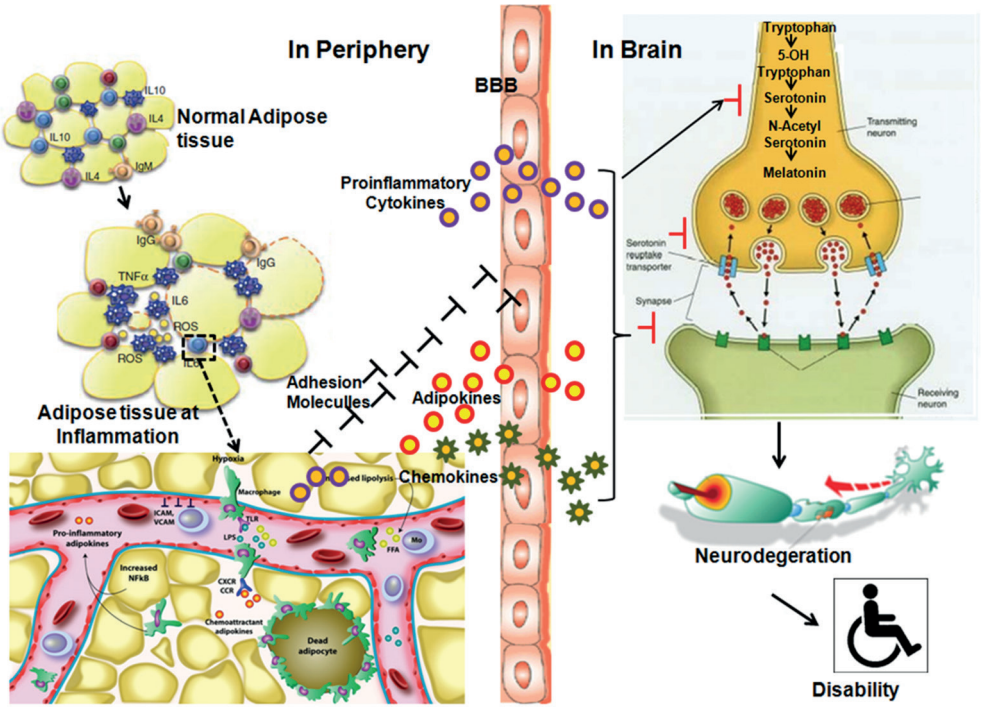
The presence of higher levels of leptin and adiponectin in women indicates the presence of a gender-specific association with the secretion of these adipokines. Similar results in MS and healthy subjects have also been reported by others (Evangelopoulos, Koutsis, & Markianos, 2014; Rotondi et al., 2013; Saad et al., 1997).

The observed association of baseline adipsin with baseline EDSS scores reflecting the neurological disability in CDMS and RRMS cohorts suggests a role of adipsin in accumulation of neurological disability. Moreover, in RRMS at baseline an association

between the adipsin and the volumes of T1-weighted lesions as well as their increase during follow-up suggests the predictive potential of adipsin as a biomarker of neurodegeneration irrespective of age and gender. The absence of evolution of adipokines levels during follow-up is most likely explained by relatively stable clinical disease course in most of our patients. However, an increase in the volumes of T1 and FLAIR lesions seen by MRI is consistent with exacerbation of MS even during the relatively short follow-up in this study. In parallel, the presence of higher adipsin in a subgroup of patients with more active RRMS (≥2 relapses/2 years before baseline) together with a positive correlation between the baseline adipsin and the volumes of FLAIR lesions in the whole RRMS group suggests an involvement of adipsin also in inflammatory disease activity. It is noteworthy that the inflammation-promoting activity of the alternative complement pathway on adaptive immune responses has recently also been reported by other investigators (Ingram et al., 2009; Kwan, van der Touw, & Heeger, 2012). According to these studies, anaphylatoxins, especially those produced during the activation of alternative pathway, may trigger inflammation and chemotaxis (Ingram et al., 2009). Taken together, according to our observations, adipsin is a neuroinflammation-promoting molecule that facilitates neurological deterioration and underlying neurodegeneration.

6.3 Interplay between Adipose tissue and Melatonin in MS pathogenesis

The interplay between adipokines and melatonin has not so far been studied. Our studies (mainly study III and IV) clearly indicate the proinflammatory environment that drives the inflammation and subsequent degeneration. Moreover, the results obtained from our studies I and II indicate a possible interplay between both of these molecules in MS. We therefore hypothesize that in MS the peripheral immune activation creates an inflammatory environment that causes the adipose tissue to secrete more adipokines, proinflammatory cytokines, chemokines, and adhesion molecules, leading to further inflammation and promoting BBB damage. Once reaching the brain, these adipokines along with the proinflammatory cytokines and chemokines, may influence the synthesis and release of serotonin and melatonin. One possible mechanism by which this could be achieved is by increasing Indoleamine 2, 3-dioxygenase (IDO) activity, which decreases the levels of serotonin, NAS, and melatonin by shifting the tryptophan metabolism towards the kyneurinine pathway (Anuradha, Rakh, Ishaq, Murthy, & Valluri, 2008; Oxenkrug, 2010; Raitala, Pertovaara, Karjalainen, Oja, & Hurme, 2005). Thus, decreased melatonin production together with neuronal atrophy and degeneration may ultimately lead to irreversible disability (Figure 10). Studies are therefore needed to elucidate the interplay between adipokines and melatonin in MS disease.



Schematic diagram representing our hypothesis regarding the interaction of adipose tissue, immune cells, and melatonin in promoting inflammation and neurodegenration.

Figure 10. Possible Interplay between Adipose tissue and melatonin in MS pathogenesis.

6.4 sCD26 and sCD30 levels in MS

In this study, a constant rise in the levels of sCD26 and sCD30 in CIS and MS subtypes was observed thus indicating the coexistence of Th1 and Th2 immune responses in the entire course of MS. In MS, CD80/CD86-CD28/CTLA4 are the most important and best known costimulatory signals, but several other costimulatory molecules, such as CD26 and CD30, are known to mediate the activation of T cells (Del Prete et al., 1995; Slavik, Hutchcroft, & Bierer, 1999; Tanaka, Kameoka, Yaron, Schlossman, & Morimoto, 1993). CD26 (dipeptidyl peptidase IV, DPPIV) is an 110kD glycoprotein and is a modulator of Th1 type immune response. Its role in Th17- type immune responses was recently reported (Bengsch et al., 2012; Morimoto & Schlossman, 1998). Several studies have reported a higher expression of CD26 on T cells in blood obtained from RRMS patients and progressive MS (Khoury et al., 2000), whereas contradictory results have been reported for its soluble form (Narikawa et al., 2006; Tejera-Alhambra et al., 2014). The observation of increased levels of sCD26 in MS seems to reflect the stable phase of the disease because our

RRMS patients had relatively inactive disease based on their number of relapses in the two years preceding the study. These results are consistent with those of other studies reporting higher levels of sCD26 in all MS patients and also specifically in patients in remission compared to patients in relapse, which may indicate the regulatory role of this molecule (Tejera-Alhambra et al., 2014).

CD30 (TNFRSF8) is a 120kD membrane bound glycoprotein that belongs to the tumor necrosis factor/nerve growth factor receptor superfamily (Durkop et al., 1992) (Del Prete et al., 1995). It is an activation marker of T-cell clones that produce regulatory Th2-related cytokine pattern (Del Prete et al., 1995). Our MS patients had a relatively stable disease course; the increased level of sCD30 seems to be consistent with the predominance of Th2 type responses. This concept is in line with the results of McMillan et al., who reported increased levels of sCD30 in RRMS patients in clinical remission compared to patients in relapse (McMillan, McDonnell, Douglas, Droogan, & Hawkins, 1998). The regulatory role of sCD30 was further supported by our observation of increased levels of sCD30 in RRMS patients treated with immunomodulatory therapy than compared to untreated patients. This observation may be due to the effect of IFN-β therapy that induces the Th2-type immune response by increasing cytokines in IL10 and IL4 (Sega, Wraber, Mesec, Horvat, & Ihan, 2004). Furthermore, the positive correlation detected between the levels of IL-10 and sCD30 in the MS group is consistent with this concept. It is well known that CD30-positive T cells produce high levels of IL10 and perform its immunoregulatory role in synergy with IL4 (Gerli et al., 2000; Gerli et al., 2001). Thus, sCD30 may be a marker for regulatory immune responses in MS.

Prognostic biomarkers are used to identify individuals at risk of developing MS. At present, the validated predictive biomarkers are the OCBs, GWAS genes, EBNA and Vitamin D levels. The study also evaluated the predictive potential of sCD26 and sCD30 co-stimulatory molecules to reveal the conversion from CIS stage to MS. It appeared that the levels of sCD30 were higher among those patients who converted to definite MS. However, since only a limited number of patients could be included in this exploratory study, the data obtained should be interpreted with caution. These preliminary interesting findings need to be confirmed by larger patient series with longer follow-up periods.

6.5 DTI – Candidate Biomarker for disease progression

According to this study, increased ADC values in NAWM regions are already present at the CIS stage, and these changes become most prevalent in SPMS. In earlier studies, DTI abnormalities have been detected in all MS subtypes and CIS, although the degree of damage differs between subtypes (Cercignani, Bozzali, Iannucci, Comi, & Filippi, 2002; Gallo et al., 2005; Hannoun et al., 2012; Preziosa et al., 2011; Roosendaal et al., 2009; Tortorella et al., 2006; Vrenken, Pouwels et al., 2006; Yu et al., 2008). In line with this data, Preziosa et al. reported increased ADC values in all NAWM regions studied already in

the CIS stage, while the most pronounced microstructural damage was detected in SPMS (Preziosa et al., 2011). We found that FA abnormality was detected only in the corpus callosum regions, and it was most apparent in the SPMS phase. Similar results have also been reported by other investigators (Cercignani et al., 2002; Hannoun, Durand-Dubief et al., 2012; Preziosa et al., 2011). Cercignani et al. detected lower FA and higher ADC values in the corpus callosum of patients with SPMS compared to patients with RRMS and PPMS (Cercignani et al., 2002). Another study showed increased ADC values in the genu of the corpus callosum in SPMS when compared to controls and RRMS, but the FA values showed no significant differences (Vrenken, Pouwels et al., 2006). Thus, the data generated in this study and by others suggest that the greater increase in diffusivity is consistent with the more advanced phases of the disease, in which degenerative changes prevail over inflammation. However, diffuse inflammatory damage in NAWM and NAGM together with cortical demyelination is the hallmark of chronic progressive subtypes (Kutzelnigg et al., 2005). According to this study diffusivity changes are also present in PPMS, but the damage in this subtype appears to be less severe than in SPMS. In respect to methodology, the data from our work and others' suggest that ADC indices are more sensitive than FA measures in detecting microstructural changes in the NAWM in MS.

The study also detected a strong association between the FA values of thalamus and neurological disability expressed by EDSS score in RRMS patients. This is in line with earlier studies reporting a positive correlation between the FA and ADC values of the thalamus and caudate nucleus and neurological disability in RRMS and SPMS (Hannoun et al., 2012; Tovar-Moll et al., 2009). These studies have additionally reported higher ADC and FA values in the caudate nucleus and thalamus in these subtypes than in healthy controls, although in the present study, such differences between the subtypes and controls could not be found. It is suggested that increased FA in gray matter may indicate microglial activation or other inflammatory events (Calabrese et al., 2011).

Conventional MRI studies have shown the presence of atrophy of the caudate nucleus and thalamus even in the earliest stages of the disease (Henry et al., 2009; Shiee et al., 2012). Such thalamic damage in MS patients has been associated with physical disability and cognitive impairment (Horakova et al., 2012; Mesaros et al., 2011). In light of these data, the DTI indices of NADGM may be useful indicators of disability accumulation in early MS. It should be taken into account that the present study applied a ROI-based approach, while other studies have mainly used histogram analyses detecting whole-brain damage (Vrenken et al., 2006; Yu et al., 2008) and voxel-wise analysis detecting regional abnormalities (Onu et al., 2012; Preziosa et al., 2011; Raz et al., 2010; Roosendaal et al., 2009) in parallel. However, In comparison to other techniques the ROI approach has yielded more regional specific information. Other techniques require more complex modeling and image processing while ROI utilizes manual delineation which is closer to the original data. Also, ROI analyses are less dependent on parameter settings than tractography and voxel-based analysis (Garin-Muga & Borro, 2014). Thus, we consider that

DTI may be useful in detecting pathological processes where/which tissue and which stage of the disease in MS and might prove valuable in clinical practice.

6.5.1 Correlation between Inflammatory biomarkers and DTI

The involvement of pro- and anti-inflammatory cytokines/chemokines/apoptotic molecules has been extensively investigated in MS. Among these biomarkers, several molecules have been studied by us and others and some have been associated with disease activity, neurological disability, or therapeutic responses (Bielekova et al., 2012; Comabella & Montalban, 2014; Hagman et al., 2011; Harris & Sadiq, 2009; Katsavos & Anagnostouli, 2013a; Khademi et al., 2011; Lutterotti et al., 2007; Martinez et al., 2015; Modvig et al., 2013; Tumani et al., 2009; Witkowska et al., 2016). The data on candidate immune biomarkers used in this study have been previously reported by our group (Hagman et al., 2011). In this study, we noticed higher levels of sFas, TNF-α and MIF in PPMS patients than RRMS patients. Thus, the robust increase in these molecules in PPMS patients further supports the possibility of the presence of inflammatory activity in this subtype.

In this study, a significant association between DTI findings and immunebiomarkers such as, MIF, TNF-α and sFas was observed that could be useful in evaluating disease progression. Neuropathological studies have shown that mild inflammation, along with microglial activation, gliosis, diffuse axonal injury, and nerve fiber degeneration, is also present in NAWM and NAGM (Lassmann et al., 2007). However, the correlations between candidate immune markers and lesion volumes quantified by conventional MRI have been relatively weak (Giovannoni et al., 2001; Kraus et al., 2002). Our studies reveal that, in patients with chronic progressive subtypes and CIS, these molecules (MIF, TNF-α and sFas) correlated with diffusivity changes in the corona radiata posterior and internal capsule of NAWM and the thalamus of NADGM. It is generally considered that immunological markers in blood at least partially reflect the inflammatory activity within the CNS (Sospedra & Martin, 2005). CSF is absolutely better for these purposes, but there are well-known limitations to its availability, and thus biomarker research is ususally based in blood samples (Rajasekharan et al., 2012). Thus, these observations and existing studies suggest that the levels of MIF, TNF-a, and sFas which reflect inflammatory disease activity are associated with the development of microstructural changes in MS. These results are also in line with the understanding that diffuse inflammatory damage in NAWM and NAGM are characteristic of chronic progressive subtypes.

6.6 Strengths, limitations and future aspects

This thesis has several strengths. The studies in this thesis have explored the role of new molecules such as, melatonin and adipokines as biomarkers in MS. This study is first of its

kind to addresses the relationship between melatonin pathway and MS disease by analyzing the SNPs in melatonin pathway genes and their association to neurological disability in Finnish patients. The study also indirectly points the role of etiological factors like latitude and sunlight in MS. Currently very little is known about the impact of adipokines on MS. The longitudinal data were collected prospectively which included clinical parameters like BMI, disease activity and disability status and also volumetric MRI. Thus, the ability of best known adipokines to discriminate between MS subtypes and their potential to depict inflammatory activity and neurological deterioration in MS has been assessed. To highlight the inflammatory milieu of the patients and their correlation to MS disease, various immune biomarkers including sCD26, sCD30 have been analyzed in MS patients and healthy controls. The use of ROI approach in DTI analyses has yielded more regional specific information in MS. This may be useful in detecting pathological processes where/ which tissue and which stage of the disease in MS and might prove valuable in clinical practice. The correlation between DTI indices and immunological molecules that is used in this study is a new approach to identify biomarkers for MS.

The studies were limited with a relatively low sample size, but the results of this study could be considered as preliminary results for further research. The statistical power of the study was relatively weak due to less number of patients to determine the gene analyses. However, despite of this limitation the SNPs were correlating with disability status of progressive patients. Methodological variations in analyzing the adipokines and other immune biomarkers are a major factor that impacts the results. DTI with ROI based approach has its own limitations with respect to the area selected since it is made manually by the radiologist. This has the highest impact due to intra-observer and inter-observer differences.

In future, studies on biomarkers in MS must take into account the complexity of processes and the possibility of a high degree of false positive results due to methodology. Procedures entitled for validation of biomarkers should be used. The use of longitudinal data with satisfactory numbers of patients from all subtypes should be encouraged. The preliminary results from these studies suggest the possible interplay of adipokines and melatonin leading to neurological disability in MS. Analysis of SNPs in melatonin pathway might be of great value in underscoring the role played by melatonin in neurological disability in MS. Also, analysis of melatonin and other IDO metabolites together with adipokines in same cohort of RRand PPMS patients can reflect the shift in tryptophan metabolism in MS. This may be the converging point for gene-environment interaction. In this regard, the studies indicate that these molecules separately or in combination might serve as good candidate biomarkers for predicting the neurological disability that underlie the neurodegenration in MS.

7 SUMMARY

In reference to the main findings of the study, the following conclusions may be drawn:

1. The association of alleles and haplotypes of the promoter SNP in the TPH2 (rs4570625) gene and the SNPs rs4753426 and rs10830963 in the MTNR1B gene with the progressive subtypes of MS and disability implies dysregulation in melatonin pathway in these progressive patients, which may facilitate the accumulation of neurological disability.

2. The relapsing-remitting patients with progressive phase of the disease were found to have higher levels of adipsin which further correlated with the volumes of T1-W and FLAIR lesions as well as with the changes of T1-W lesion volumes over the follow-up period. This implicates the role of adipsin in inflammation and the accumulation of neurological disability. The data also suggest the predictive potential of adipsin as a biomarker of neurodegeneration.

3. The increased levels sCD30 among patients converting from CIS to MS indicate the potential of this molecule as a prognostic biomarker for the disease. The positive correlation between IL-10 and sCD30 might suggest the role of sCD30 as a marker of regulatory immune responses in MS.

4. The association between disability and the thalamic FA indices suggests that DTI could be a useful indicator of neurological disability. The association between the DTI indices in both NADGM and NAWM and the levels of MIF, sFas, and sTNF-α suggests the involvement of these molecules in promoting such microstructural changes in the CNS.

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Melatonin pathway genes are associated with progressive subtypes and disability status in multiple sclerosis among Finnish patients

Renuka Natarajan[a], Elisabet Einarsdottir[b], Asko Riutta[c], Sanna Hagman[a], Minna Raunio[d,e], Nina Mononen[f], Terho Lehtimäki[f], Irina Elovaara[a,d,*]

[a] Neuroimmunology Unit, School of Medicine, University of Tampere, Tampere, Finland
[b] Department of Medical Genetics and Research Program for Molecular Medicine, Biomedicum Helsinki, University of Helsinki, Helsinki, Finland
[c] Department of Pharmacological Sciences, School of Medicine, University of Tampere, Tampere, Finland
[d] Department of Neurology, Tampere University Hospital, Tampere, Finland
[e] Department of Neurology, Kanta-Häme Central Hospital, Hämeenlinna, Finland
[f] Fimlab Laboratories, Tampere University Hospital and Department of Clinical Chemistry, School of Medicine, University of Tampere, Tampere, Finland

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ABSTRACT

In this study we investigated the relationship between melatonin pathway and multiple sclerosis (MS) in a high-risk Finnish population by studying the single nucleotide polymorphisms (SNPs) in the genes coding for critical enzymes and receptors involved in the melatonin pathway. A total of 590 subjects (193 MS patients and 397 healthy controls) were genotyped for seven SNPs in four genes including tryptophan hydroxylases (TPH)1 and 2, arylalkylamine N-acetyltransferase (AANAT) and melatonin receptor 1B (MTNR1B). An overrepresentation of T allele carriers of a functional polymorphism (G-703T, rs4570625) in the promoter region of TPH2 gene was observed in the progressive MS subtypes. The haplotype rs4570625-rs10506645TT of TPH2 gene was associated with the risk of severe disability in primary progressive MS (PPMS), while haplotype rs4570625-rs10506645TC appeared to be protective against disability in secondary progressive MS (SPMS). In the MTNR1B gene, the haplotype rs10830963-rs4753426GC was associated with the risk of SPMS, whereas another haplotype rs10830963-rs4753426GT showed an association with the risk of PPMS. These data showing the association of polymorphisms in the TPH2 and MTNR1B genes with the progressive subtypes of MS and disability suggest dysregulation in melatonin pathway. Melatonin pathway seems to be involved in disease progression, and therefore its potential effects in overcoming MS-related neurodegeneration may be worth evaluating in future clinical trials.

1. Introduction

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system (CNS) that develops in genetically susceptible individuals and may require additional environmental triggers causing immune dysregulation leading to neuroinflammation and degeneration (Hauser and Oksenberg, 2006). MS has an increasing prevalence with increasing latitudes and the disease affects approximately 0.1% of the population of the northern hemisphere (Hafler, 2004). Among environmental factors, insufficient exposure to sunlight has been shown to be associated with the development of MS. Prior research has focused on the ultraviolet radiation of sunlight, its ability to influence melatonin synthesis, and an association with vitamin D metabolism (Smolders et al., 2008; Mehta, 2010). Sunlight has been reported to have both direct and indirect effects on melatonin biosynthesis (Schwarz, 2005; Hauser and Oksenberg, 2006). Since Finland is located at high latitude with a prolonged period of darkness during the winter and has one of the highest rates of MS in the world (Sumelahti et al., 2001), it is appropriate to investigate the relationship between the melatonin pathway and MS in such a high-risk Finnish population.

Melatonin, a hormone contributing to a wide array of physiological functions, is secreted by the pineal gland specifically at night (Simonneaux and Ribelayga, 2003). The biosynthesis of melatonin begins with the hydroxylation of L-tryptophan into serotonin by tryptophan hydroxylases (TPH) 1 and 2. Subsequently, serotonin is N-acetylated by arylalkylamine N-acetyltransferase (AANAT) to N-acetylserotonin, which is converted to melatonin by hydroxyindole-O-methyltransferase (Chattoraj et al., 2009). Melatonin partly exerts its physiological effects by acting on its G-protein coupled membrane receptors (MT1 and MT2) or by the nuclear receptors belonging to the RZR/ROR orphan receptor subfamily (Dubocovich et al., 2003). In recent years, the antioxidant property of melatonin via free radical scavenging mechanism has been considered to protect against neuronal cell death. These studies provide a rationale for

* Corresponding author at: University of Tampere, School of Medicine, Neuroimmunology Unit and Department of Neurology, Lääkärinkatu 1, Arvo Building, Room b322, 33014, Finland.
E-mail address: irina.elovaara@uta.fi (I. Elovaara).

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trials evaluating the possibility of beneficial effects of melatonin in neurodegenerative disorders which may also be adapted for MS (Sharma et al., 2006; Reiter et al., 2009).

In MS, the degree of motor disability and chronic progressive course of the disease seems to correlate with the depletion of serotonergic function (Sandyk, 1998; Markianos et al., 2009). Additionally, it has also been shown that the production of melatonin is lowered and phase-shifted in MS patients with depressive symptoms that have been explained by pineal gland calcification and hypothalamic dysfunction (Sandyk and Awerbuch, 1993; Akpinar et al., 2008). Furthermore, the inverse association detected between nocturnal melatonin secretion and duration of clinical symptoms suggests that the function of the pineal gland declines with the progression of MS (Sandyk and Awerbuch, 1994). The association detected between melatonin pathway genes with various other disorders has been studied (see supplementary Table 1), but until now no single study has addressed their association with MS.

In this exploratory study based on a high-risk Finnish population, we examined the polymorphisms in the genes coding for critical enzymes and receptors involved in melatonin biosynthesis and analyzed whether these polymorphisms constitute risks for the MS disease and the development of progressive disability. For this purpose, we selected four genes, namely tryptophan hydroxylase (TPH I and II) aryl-alkylamine N-acetyltransferase (AANAT) and melatonin receptor 1B (MTNR1B) essential for the synthesis and function of melatonin and studied their single nucleotide polymorphisms (SNPs) that have been shown to affect the melatonin pathway in other diseases (Supplementary Table 1). We analyzed allele, genotype and haplotype association to inflammatory (RRMS) and degenerative subtypes (SPMS and PPMS) of MS and neurological disability.

2. Patients and methods

2.1. Study population

This case–control genotyping study comprised a total of 590 subjects. One hundred and ninety three patients (126 females, 67 males), including 109 relapsing remitting MS (RRMS), 51 secondary progressive MS (SPMS) and 33 primary progressive MS (PPMS) patients took part in this study. The diagnosis of MS was based on McDonald's Criteria (McDonald et al., 2001; Polman et al., 2005). As inflammatory changes are characteristic for RRMS and degenerative changes for SPMS and PPMS, we termed the RRMS group the inflammatory MS group (n = 109) and combined SPMS and PPMS groups as progressive MS group (n = 84). Three hundred and ninety seven (397) healthy subjects with similar range of age were included as controls. All MS patients underwent neurological evaluation including the determination of Expanded Disability Status Scale (EDSS) score (Kurtzke, 1983) (Table 2). The progression index was calculated as: disability score divided by the duration of the disease. The study was approved by the Ethics Committee of the Tampere University Hospital and all subjects gave their informed consent. The patient groups were divided on the basis of their EDSS scores to assess the association of alleles and haplotypes with neurological disability in both inflammatory and progressive MS: those with EDSS scores less than 4 were considered mildly disabled, while those with EDSS scores higher than 4 were more severely disabled.

2.2. Genotyping

Three genes (TPH1, TPH2, and AANAT) in melatonin biosynthesis pathway as well as a membrane receptor gene (Melatonin receptor 1B, MTNR1B) essential for the function of melatonin were genotyped. The following SNPs: TPH1 (rs1800532 and 10488982), TPH2 (rs10506645 and rs4570625), AANAT (rs12942767) and MTNR1B (rs4753426 and rs10830963) were chosen based on prior evidence of their possible role in the melatonin pathway (Supplementary Table 1). Genomic DNA was extracted from buffy coats using the QIAamp DNA Blood Minikit and automated biorobot M48 extraction (Qiagen, Hilden, Germany). Genotyping was performed using Taqman SNP Genotyping Assays C_8940793_10, C___2645675_10_, C___30277975_20, C___226207_10, C___31556625_20, C___289583_10, C___3256858_10 and the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, California, USA). Random duplicates and control samples were run in parallel with unknown DNA samples.

2.3. Association of alleles with MS subtypes and disability status

Allele association of the genotyped markers in patients with inflammatory MS, progressive MS (including SPMS and PPMS) and the whole CDMS group (group including all MS patients) was assessed. The association of alleles with neurological disability expressed by EDSS scores and progression index was analyzed in both inflammatory and progressive MS groups.

2.4. Association of haplotypes with MS subtypes and disability

Haploview program (http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview) was used to examine linkage disequilibrium patterns and likely haplotype frequencies. The linkage disequilibrium (LD) at each of the three loci (TPH1, TPH2, MTNR1B) was studied using data from the Hapmap phase III from Feb09 (CEU population, assembly build36, found at http://hapmap.ncbi.nlm.nih.gov). Visual inspection of loci gave an idea of the overall LD patterns, and individual D' values were obtained by analyzing the Hapmap data in Haploview 4.2. The two markers within TPH1 (rs1800532, rs10488682), TPH2 (rs4570625, rs10506645) and MTNR1B (rs4753426, rs10830963) were each in strong linkage disequilibrium ($D' > 0.5$, data not shown) and were used in haplotype analyses.

Haplotype association analysis was performed in different subtypes, group including all MS patients and controls. The association of haplotypes with gender and EDSS scores was also determined.

2.5. Statistical analyses

Among the seven SNPs studied, six SNPs (rs1800532, 10488982, rs10506645, rs4570625, rs4753426 and rs10830963) had a minor allele frequency (MAF) above 2%, while one SNP, rs12942767 had a very low MAF and most individuals were homozygous for the G allele. Allele and haplotype associations in controls and in different subtypes of MS patients were analyzed using Chi-square (χ^2) analysis, implemented in the PASW (version 18.0, SPSS Inc., Chicago, Illinois, USA) and the Haploview program. The association between alleles and the progression index in the inflammatory or progressive subtypes of MS was analyzed with Mann–Whitney *U* test. Due to the explorative nature of this study, $p < 0.05$ was considered significant.

3. Results

The demographic and clinical characteristics of the subjects including age, gender, disease duration, EDSS scores and progression index are summarized in Table 1. As expected, the patients in the SPMS and PPMS groups were older and had longer disease duration (DD) and higher EDSS scores than the patients in the RRMS [DD years (mean ± SD): SPMS (12.8 ± 7.0), PPMS (8.4 ± 7.1) vs RRMS (6.1 ± 5.2); EDSS (mean ± SD): SPMS (5.3 ± 1.4), PPMS (4.8 ± 2.3) vs RRMS (2.0 ± 2.0)].

Table 1
Demographic and clinical characteristics of MS patients and controls.

Characteristics	Inflammatory MS[a]	Progressive MS[b]		Controls
	RRMS	SPMS	PPMS	
Number of patients[c]	109	51	33	397
Sex (male/female)[c]	28/81	21/30	18/15	216/181
Age (years)[d]	37.3(±10.1)	47.4(±9.0)	51.8(±10.3)	44.3(±11.1)
Duration of disease (DD) (years)[d]	6.1(±5.2)	12.8(±7.0)	8.4(±7.1)	–
EDSS[d]	2.0 (±2.0)	5.3 (±1.4)	4.8 (±2.3)	–
Progression Index (PI)[d] (EDSS/DD)	0.63(±0.92)	0.57(±0.40)	0.75(±1.00)	–

MS Multiple Sclerosis, RRMS Relapsing Remitting Multiple Sclerosis, SPMS Secondary Progressive Multiple Sclerosis, PPMS Primary Progressive Multiple Sclerosis.
[a] Inflammatory MS group (RRMS subtype).
[b] Progressive MS group (Progressive subtypes, SPMS and PPMS).
[c] Number of patients.
[d] Mean ± SD.

3.1. Association of TPH2 markers with MS subtypes and neurological disability

As indicated in Table 3, the increased T allele frequency of SNP rs4570625 in the transcriptional control region of the TPH2 gene was associated with an increased risk of MS in the progressive MS subtypes ($p<0.05$) [Odds ratio (95% confidence interval): 1.665 (1.023–2.709)]. There was no significant difference in genotype distribution between the subtypes (data not shown). Likewise, no allelic association between the two TPH2 SNPs and disability or disease progression was found.

The association of TPH2 marker haplotypes with the disease subtypes and disability status was studied further (Tables 2–4). In males with progressive MS, the rs4570625-rs10506645 T-C haplotype was associated with an increased risk of MS ($p<0.05$), and this effect was mainly due to the males of the SPMS group ($p<0.05$). When looking at the association of TPH2 haplotypes with EDSS scores, it appeared that in PPMS, the T-T haplotype was significantly associated with the risk of disability in more severely disabled group (EDSS score >4) ($p<0.05$), while the T-C haplotype was associated with milder disability in SPMS group (EDSS<4) ($p<0.05$).

3.2. Association of MTNR1B markers with MS subtypes and disability

Significant allelic associations of the SNPs rs4753426 and rs10830963 in MTNR1B gene with MS or any of its phenotypes were not detected, but the decreased C allele frequency tended to associate with rs10830963 in the progressive MS group (Table 3). Significant differences in genotype distribution between the subtypes were not seen. Likewise, there was no allelic association between the two MTNR1B SNPs with disability status or disease progression index (data not shown).

Association of MTNR1B haplotypes with disease susceptibility and disability status was analyzed further (Tables 2–4). The rs10830963-rs4753426 G-T haplotype was associated with the risk of MS in the progressive MS group ($p<0.05$). Among the progressive subtypes, this haplotype tended to associate with disease risk in PPMS ($p=0.057$). However, in the SPMS group, another haplotype, rs10830963-rs4753426 G-C was associated with the risk of disease ($p<0.05$). Analyses of gender-based association of the rs10830963-rs4753426 G-T haplotype in the progressive MS group, revealed an association of this haplotype with the risk of MS in males with SPMS ($p<0.05$).

No association was found between the TPH1 markers (rs1800532 and 10488982) or AANAT marker (rs12942767) and the risk of MS in any of the phenotypes studied.

4. Discussion

In this exploratory study we addressed the relationship between melatonin pathway and MS disease by analyzing the polymorphisms of melatonin pathway genes in different subtypes of MS and determined their association with neurological disability. Our results showed that the SNPs in the TPH2 and MTNR1B genes involved in melatonin biosynthesis and function are associated with the risk of MS and more severe disability among patients with progressive subtypes of MS.

In the present study we were able to show an association of the SNPs, rs4570625 and rs10506645, with the risk of disability status in the progressive MS subtypes. These SNPs have been shown to be associated with cognitive decline and psychiatric disorders (Mossner et al., 2006; Campos et al., 2011). In the present study, we identified an increased T allele frequency of the SNP rs4570625 (−703 G/T) in the promoter region of TPH2 gene in progressive MS subtypes. Earlier studies have also noticed the increased T allele frequency of this SNP in patients with executive dysfunction (Reuter et al., 2007; Osinsky et al., 2009) and attention deficits (Leppanen et al., 2011). Additionally, in the progressive MS subtypes we observed that the haplotypes of the TPH2 SNPs were associated with the risk of severe disability. The haplotype rs4570625-rs10506645TT of TPH2 gene was associated with the risk of severe

Table 2
Haplotype association of polymorphisms within the TPH2 and MTNR1B genes in progressive MS group and its subtypes compared to healthy controls.

	Progressive MS[a] (n = 84)			SPMS (n = 51)			PPMS (n = 33)		
Haplotypes	Case/control frequency	$\chi 2$	p-value	Case/control frequency	$\chi 2$	p-value	Case/control frequency	$\chi 2$	p-value
TPH2/rs4570625 (G/T) – rs10506645 (C/T)									
GC	0.735/0.786	2.191	0.139	0.749/0.786	0.773	0.379	0.711/0.786	2.019	0.155
TT	0.206/0.149	3.446	0.063*	0.194/0.149	1.452	0.228	0.226/0.149	2.772	0.096*
GT	0.035/0.047	0.413	0.520	0.029/0.047	0.718	0.397	0.047/0.047	0.002	1.0
TC	0.024/0.018	0.255	0.613	0.029/0.018	0.548	0.459	0.016/0.018	0.011	0.915
MTNR1B/rs10830963 (C/G) – rs4753426 (C/T)									
CT	0.366/0.411	1.19	0.275	0.335/0.411	2.28	0.131	0.417/0.411	0.0090	0.924
GC	0.355/0.311	1.265	0.261	0.409/0.311	4.125	**0.042****	0.268/0.311	0.543	0.461
CC	0.219/0.251	0.783	0.376	0.202/0.251	1.237	0.266	0.247/0.251	0.0050	0.945
GT	0.059/0.026	4.875	**0.027****	0.054/0.026	2.5	0.114	0.068/0.026	3.627	**0.057***

MS Multiple Sclerosis, SPMS Secondary Progressive Multiple Sclerosis, PPMS Primary Progressive Multiple Sclerosis.
Significant values are in bold.
[a] Progressive MS group (Progressive subtypes, SPMS and PPMS subtypes).
** p value<0.05.
* p value<0.10 (tendency of association).

Table 3
Association of haplotypes of polymorphisms in TPH2 and MTNR1B genes in males and females with progressive MS group and its subtypes compared to controls.

	Progressive MS[a] (n = 84)			SPMS (n = 51)			PPMS (n = 33)		
Haplotypes	Case/control frequency	$\chi 2$	p-value	Case/control frequency	$\chi 2$	p-value	Case/control frequency	$\chi 2$	p-value
Females: TPH2/rs4570625 (G/T) – rs10506645 (C/T)									
GC	0.759/0.781	0.189	0.664	0.780/0.781	0.0	0.99	0.712/0.781	0.691	0.406
TT	0.216/0.139	3.264	0.071*	0.202/0.139	1.67	0.196	0.248/0.139	2.45	0.117
GT	0.023/0.059	1.908	0.167	0.017/0.059	1.922	0.166	0.038/0.059	0.222	0.638
TC	0.002/0.021	1.67	0.196	0.001/0.021	1.188	0.276	0.002/0.021	0.499	0.48
Males: TPH2/rs4570625 (G/T) – rs10506645 (C/T)									
GC	0.724/0.792	1.758	0.185	0.738/0.792	0.657	0.417	0.710/0.792	1.373	0.241
TT	0.187/0.157	0.439	0.507	0.166/0.157	0.024	0.877	0.210/0.157	0.713	0.398
GT	0.038/0.036	0.01	0.921	0.024/0.036	0.152	0.696	0.053/0.036	0.299	0.585
TC	0.051/0.016	3.403	**0.047****	0.072/0.016	5.922	**0.015****	0.027/0.016	0.279	0.5971
Females: MTNR1B/rs10830963 (C/G) – rs4753426 (C/T)									
CT	0.354/0.438	2.075	0.150	0.322/0.438	2.94	0.086	0.427/0.438	0.012	0.912
GC	0.356/0.318	0.474	0.491	0.432/0.318	3.117	0.077*	0.184/0.318	2.21	0.137
CC	0.242/0.217	0.258	0.611	0.209/0.217	0.02	0.886	0.316/0.217	1.479	0.224
GT	0.048/0.027	1.025	0.311	0.037/0.027	0.181	0.670	0.073/0.027	1.849	0.174
Males: MTNR1B/rs10830963 (C/G) – rs4753426 (C/T)									
CT	0.376/0.388	0.044	0.834	0.346/0.388	0.288	0.591	0.408/0.388	0.061	0.805
GC	0.363/0.305	1.052	0.305	0.393/0.305	1.389	0.239	0.330/0.305	0.1	0.752
CC	0.187/0.281	3.053	0.081*	0.178/0.281	2.052	0.152	0.197/0.281	1.248	0.264
GT	0.074/0.026	4.858	**0.027****	0.083/0.026	4.107	**0.043****	0.065/0.026	1.906	0.167

MS Multiple Sclerosis, SPMS Secondary Progressive Multiple Sclerosis, PPMS Primary Progressive Multiple Sclerosis.
Significant values are in bold.
[a] Progressive MS group (SPMS and PPMS subtypes).
** p-value < 0.05.
* p-value < 0.10 (tendency of association).

disability in primary progressive MS (PPMS), while haplotype rs4570625-rs10506645TC appeared to be protective against disability in secondary progressive MS (SPMS). This clearly shows that the T allele of rs4570625 is the risk allele of neurological disability, while the C/T allele of rs10506645 seems to contribute to the disease type in modulating its effect. Taken together, these observations suggest that there could be at least two TPH2 SNP variants that interact together in modulating the risk towards disability in MS. It may be that the presence of this polymorphism rs4570625 (−703 G/T) in the promoter region affects the serotonin biosynthesis in melatonin pathway of progressive MS patients thereby influencing the neurological functions in such individuals.

MTNR1B gene in chromosome 11q21-22 encodes for a receptor which mediates several functions of melatonin (Dubocovich et al., 2003). The haplotype analysis in this study revealed a significant association of rs10830963-rs4753426 haplotype G-T with the progressive MS group including both SPMS and PPMS, while the SPMS possessed haplotype rs10830963-rs4753426 G-C. This suggests that the G allele of rs10830963 is a risk allele for the progressive forms of MS. However, the involvement of T/C allele of rs4753426 in the risk of progressive disease should also be accounted for. Recently it has been shown that the C allele of rs4753426 is associated with decreased sunshine duration per day (Ji et al., 2010). Although we did not observe any striking difference between the alleles of rs4753426, the increased frequency of C allele seen in our patients suggests their adaptability to existing dark environmental conditions. Consistent with our observation, the selection of derived rs4753426C allele is believed to occur in order to adapt individuals to environmental pressure and may also increase the immunostimulatory effect of melatonin (Ji et al., 2010).

Table 4
Association of haplotypes of polymorphisms in TPH2 and MTNR1B genes with disability status in progressive MS group and its subtypes.

	Progressive MS[a] (n = 84)		SPMS (n = 51)		PPMS (n = 33)	
Haplotypes	Low/High EDSS frequency	p-value	Low/High EDSS frequency	p-value	Low/High EDSS frequency	p-value
TPH2/rs4570625 (G/T) – rs10506645 (C/T)						
GC	0.717/0.772	0.476	0.755/0.699	0.608	0.642/ 0.833	0.099
TT	0.233/0.136	0.173	0.194/0.199	0.958	0.308/ 0.083	**0.035****
GT	0.025/0.069	0.187	0.038/0.001	0.388	0.049/ 0.042	0.901
TC	0.025/0.023	0.942	0.013/0.101	**0.040****	0.001/ 0.042	0.202
MTNR1B/rs10830963 (C/G) – rs4753426 (C/T)						
CT	0.340/0.396	0.505	0.401/0.395	0.963	0.364/ 0.471	0.396
GC	0.366/0.282	0.317	0.328/0.295	0.781	0.279/ 0.238	0.713
CC	0.239/0.218	0.772	0.221/0.205	0.874	0.292/ 0.179	0.308
GT	0.055/0.104	0.269	0.050/0.105	0.362	0.064/ 0.113	0.491

MS Multiple Sclerosis, SPMS Secondary Progressive Multiple Sclerosis, PPMS Primary Progressive Multiple Sclerosis, EDSS Expanded disability Status Scale.
Low EDSS group is mildly disabled (EDSS < 4).
High EDSS group is more severely disabled (EDSS > 4).
Significant values are in bold.
[a] Progressive MS group (SPMS and PPMS subtypes).
** p-value < 0.05.

In conclusion, this exploratory study suggests an involvement of melatonin pathway in the MS. The polymorphisms in TPH2 and MTNR1B genes may interact together in modulating the risk of the disease and accumulation of disability in the progressive subtypes of MS. The association of alleles and haplotypes of the promoter SNP in the TPH2 (rs4570625) gene and the SNPs rs4753426 and rs10830963 in the MTNR1B gene implies the dysregulation in melatonin pathway in these progressive patients which may facilitate the accumulation of neurological disability. In order to confirm these preliminary data, additional studies with larger sample size and functional experiments covering the entire gene variability of the studied genes are needed. Our data suggesting the contribution of melatonin to MS susceptibility and disability could provide a rationale for future clinical trials on the effects of melatonin in overcoming neurodegeneration in MS.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jneuroim.2012.05.014.

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Supplementary Table 1: Studied gene polymorphisms of melatonin pathway.

Gene	Chromosome	Function	SNP	Position	Alleles	MAF (%)	Previous studies
TPH1	11p15.3--> 14	Serotonin biosynthesis in peripheral tissues	rs1800532	Intron	G/T	0.470	(Gizatullin et al., 2006); (Porter et al., 2008)
TPH1	11p15.3--> 14		rs10488682	Promoter	T/A	0.274	(Xu et al., 2011); (Wang et al., 2008)
TPH2	12q21.1	Serotonin biosynthesis in brain	rs4570625	Promoter	G/T	0.166	(Marziniak et al., 2009); (Brown et al., 2005); (Canli et al., 2005); (Canli et al., 2008); (Baehne et al., 2009); (Serretti et al., 2011); (Yoon and Kim, 2009); (Leppanen et al., 2011); (Osinsky et al., 2009)
TPH2	12q21.1		rs10506645	Intron	C/T	0.196	(Campos et al., 2011); (Campos et al., 2010); (Roche and McKeon, 2009)
AANAT	17q25	Melatonin biosynthesis from serotonin	rs12942767	Intron	G/A	0.009	-
MTNR1B	11q21-22	Downstream signaling	rs10830963	Intron	C/G	0.338	(Lyssenko et al., 2009); (Sparso et al., 2009); (Prokopenko et al., 2009); (Bouatia-Naji et al., 2009)
MTNR1B	11q21-22		rs4753426	Promoter	C/T	0.435	(Ji et al., 2010); (Qiu et al., 2007)

SNP Single Nucleotide Polymorphisms, MAF Minor Allele Frequency, TPH1 Tryptophan Hydroxylase 1, TPH2 Tryptophan Hydroxylase 2, AANAT Aryl alkylamine N-acetyltransferase, MTNR1B Melatonin receptor 1B

Supplementary Table 2: Allele frequencies of studied polymorphisms in MS subtypes and healthy controls (Odds ratio with 95% CI).

	CDMS group[A] (n=193)	Controls (n=397)	Odds Ratio (*95% CI*) p-value	Inflammatory MS[B](n=109)	Controls (n=397)	Odds Ratio (*95% CI*)	Progressive MS[C] (n=84)	Controls (n=397)	Odds Ratio (*95% CI*) p-value
TPH1 rs1800532									
T	137(71%)	275(72.6%)	1.081 (0.736-1.587) *0.692*	78(71.6%)	275(72.6%)	1.051(0.655-1.687) *0.837*	59(70.2%)	275(72.6%)	0.893(0.531-1.500) *0.668*
G	147 (76.2%)	299 (78.9%)	1.170(0.774-1.767) *0.457*	87(79.8%)	299(78.9%)	0.945(0.557-1.604) *0.834*	60(71.4%)	299(78.9%)	0.669(0.392-1.141) *0.138*
rs10488682									
T	177(92.2%)	354(92.7%)	1.071(0.558-2.058) *0.836*	101(93.5%)	354(92.7%)	0.876(0.372-2.065) *0.763*	76(90.5%)	354(92.7%)	0.751(0.330-1.713) *0.495*
A	77(83.7%)	154(84.6%)	1.071(0.541-2.124) *0.843*	43(86 %)	154(84.6%)	0.895(0.366-2.190) *0.809*	34(81%)	154(84.6%)	0.773(0.324-1.843) *0.560*
TPH2 rs10506645									
C	184(95.8%)	369(96.6%)	1.234(0.503-3.030) *0.646*	105(97.2%)	369(96.6%)	0.811(0.227-2.899) *0.747*	79(94%)	369(96.6%)	0.557(0.193-1.606) *0.272*
T	81(42.2%)	135(35.3%)	0.749(0.525-1.068) *0.111*	44(40.7%)	135(35.3%)	0.795(0.513-1.231) *0.304*	37(44%)	135(35.3%)	1.440(0.892-2.326) *0.134*
rs4570625									
G	186(96.9%)	361(96.8%)	0.970(0.358-2.627) *0.953*	106(98.1%)	361(96.8%)	0.568(0.125-2.576) *0.463*	80(95.2%)	361(96.8%)	0.665(0.209-2.115) *0.487*
T	71(37%)	112(30%)	***0.731(0.507-1.056)* 0.094****	36(33.3%)	112(30.1%)	0.858(0.543-1.356) *0.512*	35(41.7%)	112(30%)	***1.665(1.023-2.709)** 0.039*****
MTNR1B rs4753426									
C	153(79.3%)	298(79.3%)	0.999(0.651-1.533) *0.996*	84(77.1%)	298(79.3%)	1.137(0.682-1.896) *0.623*	69(82.1%)	298(79.3%)	1.204(0.653-2.219) *0.551*
T	131(67.9%)	249(66.2%)	0.928(0.641-1.344) *0.692*	76(69.7%)	249(66.2%)	0.851(0.537-1.350) *0.494*	55(65.5%)	249(66.2%)	0.967(0.588-1.592) *0.896*
rs10830963									
C	162(85.3%)	341(89.7%)	1.511(0.898-2.543) *0.120*	93(86.9%)	341(89.7%)	1.316(0.686-2.527) *0.409*	69(83.1%)	341(89.7%)	***0.564(0.290-1.094)* 0.087****
G	117(61.6%)	217(57.1%)	0.831(0.582-1.186) *0.307*	63(58.9%)	217(57.1%)	0.930(0.602-1.437) *0.743*	54(65.1%)	217(57.1%)	1.399(0.853-2.294) *0.183*
AANAT rs12942767									
A	3(1.6%)	7(1.8%)	1.158(0.296-5.526) *0.833*	0(0%)	7(1.8%)	4.598(0.000-) *0.999*	3(3.6%)	7(1.8%)	2.026(0.513-8.003) *0.304*
G	193(100%)	388(99.5%)	0.000(0.000-) *0.999*	109(100%)	388(99.5%)	1.615(0.000-) *0.999*	84(100%)	388(99.5%)	3.497(0.000-) *0.511*

MS Multiple Sclerosis, CDMS Clinically Definite Multiple Sclerosis, RRMS Relapsing Remitting Multiple Sclerosis, SPMS Secondary Progressive Multiple Sclerosis, PPMS Primary Progressive Multiple Sclerosis, CI Confidence interval

A CDMS group (RRMS, SPMS and PPMS subtypes)

B Inflammatory MS group (RRMS subtype)

C Progressive MS group (SPMS and PPMS subtypes)

** *p* value < 0.05, * *p* value < 0.10 (tendency of association), significant/borderline values are in bold.

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Research Article

Adipsin Is Associated with Multiple Sclerosis: A Follow-Up Study of Adipokines

Renuka Natarajan,[1] Sanna Hagman,[1] Mari Hämälainen,[2] Tiina Leppänen,[2] Prasun Dastidar,[3] Eeva Moilanen,[2] and Irina Elovaara[1,4]

[1]*Neuroimmunology Unit, Medical School, University of Tampere, Tampere University Hospital, Biokatu 10, 33520 Tampere, Finland*
[2]*The Immunopharmacology Research Group, University of Tampere, School of Medicine and Tampere University Hospital, Medisiinarinkatu 3, 33520 Tampere, Finland*
[3]*Department of Radiology, Medical Imaging Centre, Tampere University Hospital, Teiskontie 35, 33520 Tampere, Finland*
[4]*Department of Neurology, Tampere University Hospital, Teiskontie 35, 33520 Tampere, Finland*

Correspondence should be addressed to Irina Elovaara; irina.elovaara@uta.fi

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Background and Objective. The role of adipokines in regulation of immune responses has been recognized, but very little is known about their impact on multiple sclerosis (MS). In this study, we analysed whether the major adipokines are differentially expressed in plasma of patients with different MS subtypes and clinically isolated syndrome (CIS) and explored their association with major disease characteristics. *Methods*. The levels of adiponectin, adipsin, leptin, and resistin in the plasma of 80 patients with different subtypes of MS and CIS were followed up annually over the two years. The data obtained were correlated with disease activity, EDSS and volumes of T1-weighted lesions (T1-LV), and fluid attenuation inversion recovery lesions (FLAIR-LV) on MRI. *Results*. In MS group, a correlation was found between the level of adipsin and EDSS score at baseline ($r = 0.506$, $p < 0.001$). In RRMS, the levels of adipsin correlated with EDSS scores ($r = 0.542$, $p = 0.002$), T1-LV ($r = 0.410$, $p = 0.034$), and FLAIR-LV ($r = 0.601$, $p = 0.0001$) at baseline and an increase in the T1-LV over the follow-up ($r = 0.582$, $p = 0.003$). Associations with other adipokines were not detected. *Conclusion*. Our exploratory study provides novel insights on the impact of adipokines in MS and suggests that adipsin exerts predictive potential as a biomarker of neurodegeneration.

1. Introduction

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system (CNS) mediated by the transendothelial migration of activated T helper 1 (Th1) and Th17 lymphocytes into the brain tissue where they trigger the destructive inflammatory cascade resulting in the accumulation of inflammatory infiltrates, demyelination, axonal loss, and gliosis [1, 2]. The damage to neural tissue is induced by various effector mechanisms and substances such as macrophage phagocytosis, secretion of inflammatory cytokines, chemokines and antibodies, complement activation, mitochondrial dysfunction, release of cytotoxic proteases, and products of oxidative stress and excitotoxicity that all together contribute to the development of neurological worsening [1].

Increased risk of MS in subjects with obesity during adolescence and early adulthood has been recently reported [3, 4]. It has been considered that such risk is explained by modulatory effect of adipose tissue on inflammatory responses in obese subjects. Indeed, adipose tissue is recognized as an endocrine organ that secretes multiple cytokine-like hormones, adipokines that are involved in regulation of multiple physiological functions including inflammation [5, 6]. Although dysregulation of adipokines during obesity and in autoimmune diseases has been recognized, only very little is known about their role in MS [5]. The best-known adipokines are the proinflammatory leptin, adipsin,

resistin and visfatin, and the anti-inflammatory adiponectin, omentin-1, and apelin [5, 7]. Up to now, most studies in MS have been focused on leptin that was found to be increased in blood and cerebrospinal fluid (CSF) of MS patients, but contrasting results have also been reported [8–11]. In these studies, leptin correlated negatively with the number of regulatory T cells [11], but associations to clinical parameters were not reported. The levels of visfatin and resistin in sera of MS patients were found to be increased, while the levels of adiponectin were downregulated [9, 12–14]. Until now only one study analysed several adipokines in a cohort of patients including different subtypes of MS [14]. According to this study, elevated levels of visfatin and decreased levels of leptin were found in patients with relapsing-remitting MS (RRMS), but association with clinical parameters was not detected.

Due to the sparse knowledge on the association of adipokines with clinical characteristics of MS, the purpose of this two-year prospective follow-up study was to assess whether the levels of adiponectin, adipsin, leptin, and resistin in plasma of MS patients are associated with clinical phenotypes, inflammatory disease activity, neurological disability, and the volumes of T1-weighted and fluid attenuation inversion recovery (FLAIR) lesions on magnetic resonance imaging (MRI).

2. Patients and Methods

2.1. Subjects. This two-year prospective follow-up study included altogether 80 subjects of whom 65 had clinically definite MS (CDMS) according to the revised McDonald Criteria [15] and 15 had clinically isolated syndrome (CIS) [16]. The CDMS group included 34 patients with RRMS, 15 patients with secondary progressive MS (SPMS), and 16 subjects with primary progressive MS (PPMS). CIS patients were defined as patients who had had their first demyelinating neurologic event suggestive of MS [16]. All patients underwent annual neurological examinations from baseline up to two years. The blood was drawn on the same day as the neurological examination. The clinical evaluation included the determination of body mass index (BMI, kg/m^2), prestudy disease activity (number of relapses two years before the study), number of relapses over the two-year follow-up, and Expanded Disability Status Scale (EDSS) score [17] at the baseline and the end of the follow-up as summarized in Table 1. Patients who were pregnant or suffering from any other clinically significant diseases were excluded. The study was approved by the Ethics Committee of Tampere University Hospital and all subjects gave informed consent.

2.2. MRI Image Segmentation and Volumetric Analysis. All patients underwent MRI examination at baseline and at the end of follow-up period. All examinations were performed on a 1.5 Tesla MRI Unit (Siemens Avanto, Erlangen, Germany). The MRI protocol included a T1-weighted header followed by axial T1-weighted magnetization prepared rapid gradient echo (MP-RAGE) and T2-weighted turbo spin-echo (TSE), FLAIR, magnetization 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; in-plane resolution = 0.45 ∗ 0.45 mm. In FLAIR images, the following parameters were used: TR = 8500 ms; TE = 100 ms; TI = 2500 ms; slice thickness = 5.0 mm; 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; in-plane resolution = 0.90 ∗ 0.90 mm. Volumetric segmentation of plaques in the brain was performed using semiautomatic software Anatomatic operating in a PC/Window 95 environment [18, 19] and the images were analysed blind.

2.3. Determination of Adipokines. Venous blood was collected for the assessment of plasma levels of adiponectin, adipsin, leptin, and resistin. Blood containing tubes were centrifuged for 15 min at 1600 ×g. Plasma was separated from the blood, aliquoted, and stored at −70°C until use. Adipokines were determined by enzyme-linked immunoassay (ELISA) using commercial reagents according to the manufacturers' instructions (DuoSet ELISA, R&D Systems Europe Ltd., Abingdon, UK). The respective detection limits and interassay coefficients of variation were 15.6 ng/L and 2.0% for adiponectin, 4.0 ng/mL and 3.8% for adipsin, 15.6 ng/L and 3.9% for leptin, and 15.6 ng/L and 4.0% for resistin.

2.4. Statistical Analysis. Statistical analyses were performed with SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). A p value less than 0.05 was considered significant in all analyses. Mann-Whitney U test was used to analyse the differences in clinical parameters and MRI volumes between the subtypes. Wilcoxon signed-rank test was used to analyse the intraindividual changes in the volumes of MRI at each time point.

For comparison of the adipokines levels in different subtypes, repeated measures of ANOVA followed by Bonferroni correction for multiple comparisons were used. For each outcome, the analyses were also adjusted for age and gender. Pearson's correlation coefficient was used to explore the relationship between the levels of adipokines with BMI or age. The associations of adipsin levels with EDSS scores and the volumes of T1-weighted and FLAIR lesions were studied by linear regression model by adjusting for age, gender, and disease subtype. Logistic regression model was used to study the association between adipokines and disease activity. The differences in the adipokines levels between genders were studied by Mann-Whitney U test.

3. Results

3.1. Clinical and MRI Follow-Up

3.1.1. Clinical Data. The demographic and two-year clinical follow-up data of the subjects are summarized in Table 1. As expected, the patients in the SPMS and PPMS groups had

Table 1: Demographic and clinical characteristics of patients with different MS phenotypes.

	RRMS $n = 34$	SPMS $n = 15$	PPMS $n = 16$	CIS $n = 15$
Gender F/M[a]	24/10	10/5	9/7	13/2
Age (years)[b]	37.6 ± 9.2 (20–51)	49.3 ± 10.0 (32–63)	58.1 ± 8.5 (40–70)	35.6 ± 7.9 (24–51)
BMI (kg/m^2)[b]	24.9 ± 4.0 (19.7–35.4)	26.2 ± 5.0 (15.8–33.3)	24.6 ± 3.4 (19.8–31.5)	25.3 ± 3.2 (21.6–31.2)
Disease duration from first symptoms (years)[b]	8.2 ± 7.2 (0.7–29.6)	19.8 ± 7.8 (6.3–34.3)	18.9 ± 9.6 (2.4–43.0)	3.0 ± 2.5 (0.5–8.9)
Disease duration from diagnosis (years)[b]	4.2 ± 4.1 (0.0–13.7)	12.9 ± 9.0 (2.2–32.4)	13.1 ± 8.4 (1.5–27.2)	NA
EDSS at baseline[b]	1.4 ± 1.5 (0.0–6.0)	5.2 ± 1.6 (2.5–7.5)	4.7 ± 2.2 (1.0–7.0)	0.1 ± 0.3 (0.0–1.0)
EDSS at end of the follow-up[b]	1.5 ± 1.6 (0.0–6.0)	5.5 ± 1.6 (2.5–8.0)	4.8 ± 2.1 (1.5–7.0)	0.1 ± 0.4 (0.0–1.0)
EDSS worsening during follow-up[c]	7 (21%)	6 (40%)	4 (25%)	1 (7%)
Prestudy disease activity[b,d]	1.2 ± 1.4 (0–5)	0.2 ± 0.6 (0–2)	0.0 ± 0.0 (0-0)	0.7 ± 0.6 (0–2)
Number of relapses over the follow-up[b]	0.6 ± 1.1 (0–5)	0.4 ± 0.7 (0–2)	0.0 ± 0.0 (0-0)	0.1 ± 0.3 (0-1)
Treatment (NT/IFN/CO/MX)[a]	12/18/3/1	15/0/0/0	16/0/0/0	15/0/0/0

RRMS: relapsing-remitting MS, SPMS: secondary progressive MS, PPMS: primary progressive MS, CIS: clinically isolated syndrome, BMI: body mass index, EDSS: expanded disability status scale, NT: no treatment, IFN: interferon, CO: copaxone, MX: mitoxantrone, and NA: not applicable.
[a]Number of patients.
[b]Mean ± SD (range).
[c]Number of patients (percent).
[d]Number of relapses in the 2 years before baseline.

Table 2: Volumes of T1 and FLAIR lesions at baseline and follow-up (median (interquartile range)).

	T1 lesions (cm^3)			FLAIR lesions (cm^3)		
	BL	1-YR	p value[a]	BL	1-YR	p value[a]
CIS	0.4 (0.0–0.6)	0.3 (0.2–0.8)	0.046	1.0 (0.3–2.2)	1.3 (0.5–3.1)	0.001
RRMS	1.4 (0.5–3.7)$^{*,\#}$	3.0 (0.8–6.0)	0.001	6.3 (2.6–17.5)$^{*,\#}$	14.0 (6.0–24.8)	0.00002
SPMS	5.8 (2.2–9.4)*	6.5 (3.7–18.6)	0.041	18.4 (11.6–30.1)*	29.3 (24.8–40.2)	0.004
PPMS	0.8 (0.6–2.8)$^{*,\#}$	2.0 (0.9–4.7)	0.004	5.3 (3.1–10.4)$^{*,\#}$	9.7 (6.8–15.1)	0.0004

RRMS: relapsing-remitting MS, SPMS: secondary progressive MS, PPMS: primary progressive MS, CIS: clinically isolated syndrome, and BL: baseline.
[a]The intraindividual changes in the volumes of MRI over the follow-up period, Wilcoxon signed-rank test.
[*]Compared to CIS group, Mann-Whitney U test, $p < 0.01$.
[#]Compared to SPMS, Mann-Whitney U test, $p < 0.05$.

longer disease duration and were older than the patients with RRMS or CIS ($p < 0.05$). The EDSS scores were lower in CIS and RRMS than in other MS subtypes ($p < 0.05$), while no differences were found between SPMS and PPMS. There were no differences in BMI between any of the MS subtypes and CIS ($p > 0.05$).

At the end of the follow-up, the EDSS score was increased in 27% ($n = 17/65$) of CDMS patients: (21% RRMS, 40% SPMS, and 25% PPMS). Two years before study entry, half of RRMS patients were relapse-free, 12% had one relapse, and the remaining 38% of subjects had 2–5 relapses. At the end of the follow-up, 68% of RRMS patients were relapse-free, 15% of patients had one relapse, and the remaining 17% of subjects had 2–5 relapses. The majority of RRMS patients were treated with immunomodulatory drugs (53% interferon-beta (IFN-β), 6% glatiramer acetate, and 3% mitoxantrone). At the end of follow-up, 35% of patients were treated with IFN-β, 26% of patients with copaxone, and 3% of patients with natalizumab.

The baseline EDSS score of CIS patients was 0 except for two subjects having score 1. Over the two-year period, 7 out of 15 CIS patients converted to CDMS. All converted patients had elevated IgG index and OCBs in their CSF.

3.1.2. Volumes of T1-Weighted and FLAIR Lesions. The volumes of MS plaques were determined in the 75 MS and CIS patients at the baseline and after one year (Table 2). As expected, the baseline volumes of T1-weighted and FLAIR lesions were lowest in the CIS group ($p < 0.01$). Baseline comparison between the MS subtypes showed higher FLAIR

Table 3: The levels of adipokines in MS subtypes and CIS over follow-up period (median (interquartile range)).

	Baseline	One year	Two years
	Adiponectin (ng/mL)		
CIS	4720.6 (4124.4–5407.7)	5131.0 (4239.4–5880.3)	5149.7 (4789.0–6812.0)
RRMS	4517.3 (3380.6–6417.2)	4695.7 (3452.4–7270.5)	5012.7 (3222.8–7043.1)
SPMS	4748.4 (3977.9–6379.9)	5314.8 (4289.4–7494.9)	6062.3 (4706.5–7871.2)
PPMS	5618.7 (4111.5–8467.1)	5855.8 (4622.6–9948.3)	5278.5 (3761.6–8779.1)
	Adipsin (ng × m^2/mL × kg)[a]		
CIS	63.9 (53.9–76.8)	66.4 (56.9–83.5)	66.7 (59.7–74.4)
RRMS	60.3 (52.3–71.2)[*,#]	62.4 (55.1–71.3)[*,#]	61.5 (56.2–69.6)[*,#]
SPMS	68.4 (57.4–85.0)	70.3 (63.3–81.7)	74.1 (57.7–89.6)
PPMS	80.8 (69.5–93.6)	75.9 (67.1–94.6)	82.5 (66.1–95.0)
	Leptin (pg × m^2/mL × kg)[b]		
CIS	861.0 (269.8–1908.4)	694.9 (240.1–1238.7)	754.5 (424.2–1346.5)
RRMS	662.4 (288.1–981.4)	572.4 (286.7–1159.3)	572.0 (321.4–1032.9)
SPMS	711.1 (166.8–1649.7)	420.0 (157.5–1496.2)	953.2 (287.9–1822.7)
PPMS	515.2 (182.6–1260.2)	603.3 (203.5–1092.2)	479.3 (255.6–776.1)
	Resistin (pg/mL)		
CIS	2851.5 (2509.1–3280.5)	2946.6 (2367.6–3093.8)	2769.5 (2299.2–3072.8)
RRMS	2505.2 (2085.0–2751.0)	2402.1 (2064.9–2869.3)	2315.9 (1946.9–2796.2)
SPMS	2605.4 (2216.0–3388.0)	2649.9 (2358.5–3146.4)	2469.0 (2284.6–2975.3)
PPMS	2392.5 (1794.9–3411.5)	2371.2 (1663.5–3354.6)	2445.4 (1711.1–3349.5)

[a]BMI-adjusted adipsin levels (ng × m^2/mL × kg).
[b]BMI-adjusted leptin levels (pg × m^2/mL × kg).
[*]Comparison of PPMS $p < 0.01$.
[#]Comparison of PPMS after adjusting for age $p < 0.05$.

and T1 lesion volumes in SPMS than in PPMS or RRMS ($p < 0.05$). Over the follow-up, the volumes of these lesions increased in all studied groups ($p < 0.05$).

3.2. Levels of Adipokines in MS Subtypes during the Two-Year Follow-Up. Correlation analyses assessing associations of adipokines with BMI in CDMS group showed correlations with levels of adipsin ($r = 0.277$, $p = 0.018$) and leptin ($r = 0.491$, $p < 0.0001$) but not with adiponectin ($r = -0.132$, $p = 0.267$) or resistin ($r = -0.071$, $p = 0.551$). Due to observed correlations with adipsin and leptin, these adipokines were adjusted by dividing their concentrations by BMI. To assess the differences in the adipokines levels between different MS subtypes, repeated measures of ANOVA adjusting for age and gender were used. It appeared that over the two years the levels of adipokines in different groups remained stable (Table 3). The levels of BMI-adjusted adipsin in RRMS patients were lower than those in subjects with PPMS throughout the whole follow-up period (Table 3) ($p = 0.002$). After controlling for age alone, the difference in the adipsin levels between the groups was still statistically significant ($p = 0.037$ adjusted), while after adjusting simultaneously for age and gender only a trend toward statistical significance was found ($p = 0.057$). Other adipokines levels did not differ between the subtypes. Figure 1 illustrates the baseline distribution of the adipokines levels in patients with different subtypes. Notably, the levels of BMI-adjusted adipsin in treated and untreated RRMS patients were decreased in comparison to PPMS, but no differences were found between these RRMS groups (Supplementary Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/371734). Likewise, the levels of adipokines were of the similar magnitude in converted and nonconverted patients with CIS.

The influence of gender on secretion of adipokines was studied by comparing the baseline levels in men and women. It appeared that in CDMS group the levels of leptin (869.6 (536.9–1504.9) versus 242.3 (152.9–441.3) pg × m^2/mL × kg, $p < 0.001$) and adiponectin (5540.9 (4197.4–8036.1) versus 3808.0 (3178.7–5545.4) ng/mL, $p = 0.010$, median (interquartile range)) were higher in women. In CDMS, also the age correlated with the levels of BMI-adjusted adipsin ($r = 0.491$, $p < 0.001$).

3.3. Association of Adipokines with Clinical and MRI Measures. Association of adipokines levels with baseline EDSS score and the volumes of FLAIR- or T1-weighted lesions as well as the change of their volumes over the follow-up were studied by linear regression model adjusting for age, gender, and disease subtype. In the CDMS group, the analyses among the adipokines showed a positive correlation between the baseline BMI-adjusted adipsin and EDSS scores ($r = 0.506$, $p < 0.001$), and such associations were also observed after adjusting for age alone ($r = 0.387$, $p = 0.003$), for age and gender ($r = 0.376$, $p = 0.004$), or for combination of age, gender, and disease subtype ($r = 0.280$, $p = 0.036$). According to subtype analysis, in RRMS group the correlation was even stronger ($r = 0.542$, $p = 0.002$; Figure 2(a)). Similar associations were observed after adjusting for age ($r = 0.570$,

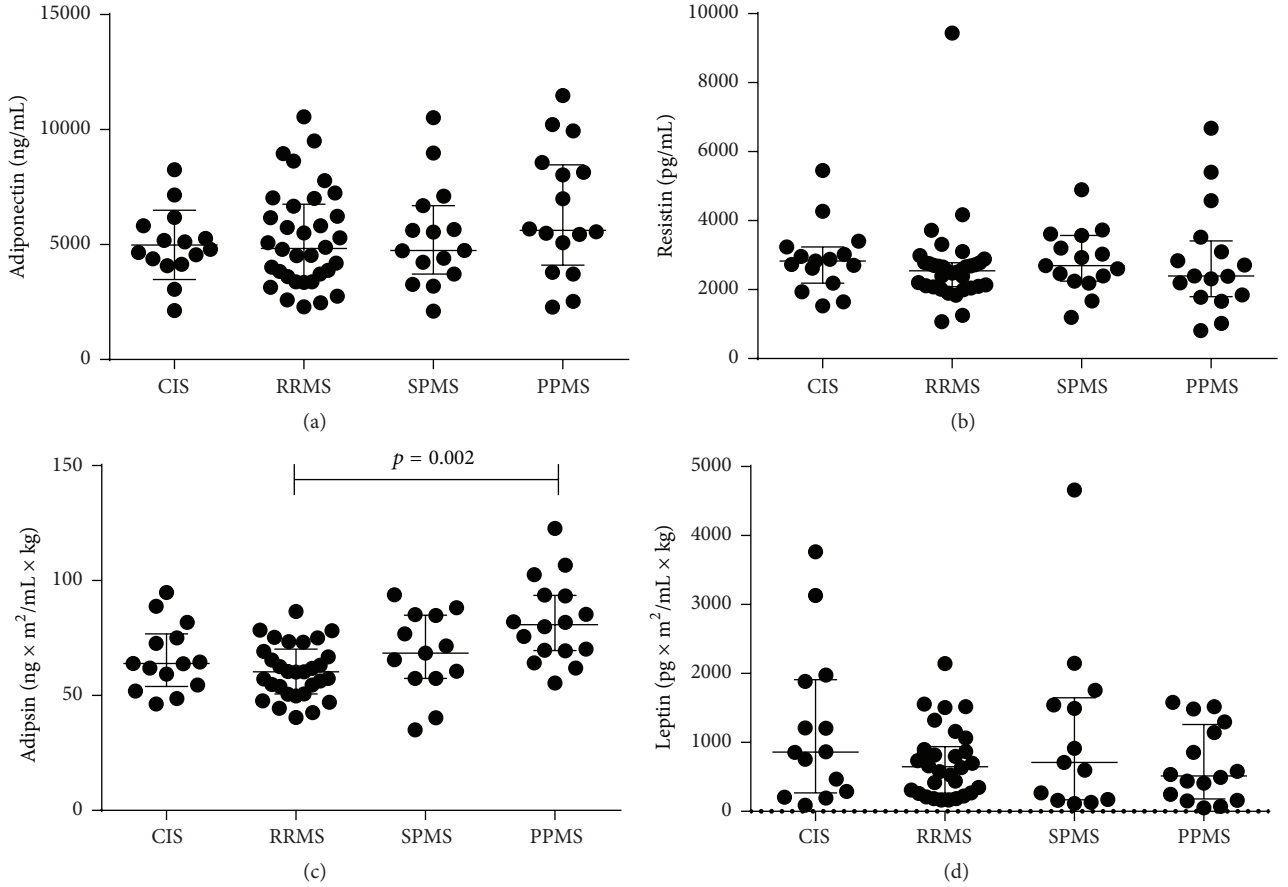

Figure 1: Scatter plot showing the baseline levels of Adiponectin (A), Resistin (B), BMI-adjusted Adipsin (C) and BMI-adjusted Leptin (D) in MS and CIS. The bars indicate the median and interquartile range.

Table 4: Observed associations between the levels of adipsin and clinical and MRI parameters (Pearson's correlation coefficient (p value)).

Parameters		Basic model	Adjusted for age	Adjusted for age and gender	Adjusted for age, gender, and subtype
EDSS	CDMS	0.506 (<0.0001)	0.387 (0.003)	0.376 (0.004)	0.280 (0.036)
	RRMS	0.542 (0.002)	0.570 (0.001)	0.569 (0.002)	—
T1 lesion volume	RRMS	0.410 (0.034)	0.402 (0.042)	0.407 (0.043)	—
FLAIR lesion volume	RRMS	0.601 (0.001)	0.596 (0.001)	0.596 (0.002)	—
ΔT1 lesion volume	RRMS	0.582 (0.003)	0.582 (0.004)	0.583 (0.004)	—

CDMS: clinically definite MS, RRMS: relapsing-remitting MS, EDSS: expanded disability status scale, and FLAIR: fluid attenuation inversion recovery.

$p = 0.001$) or age and gender ($r = 0.569$, $p = 0.002$). Over the two years, the EDSS score increased in 27% of CDMS patients ($n = 17/65$) (21% RRMS, 40% SPMS, and 25% PPMS), but the levels of adipokines did not associate with this change.

In the CDMS group, the levels of adipokines did not associate with the volumes of FLAIR- or T1-weighted lesions or the change of their volumes over the follow-up ($p > 0.05$). However, according to subgroup analysis, in RRMS correlations were found between the baseline levels of BMI-adjusted adipsin and the volumes of T1-weighted ($r = 0.410$, $p = 0.034$; Figure 2(b)) and FLAIR ($r = 0.601$, $p = 0.0001$; Figure 2(c)) lesions or the changes of T1 lesion volumes over the follow-up ($r = 0.582$, $p = 0.003$; Figure 2(d)). After adjusting for age and gender, RRMS group still showed positive correlations in these measures indicating that age and gender did not have an impact on these correlations (Table 4).

We next analysed whether the levels of adipokines are associated with clinical or MRI disease activity before study entry and over the follow-up period. Baseline clinical disease activity was determined by the presence of at least 2 relapses during 2 years before study entry and baseline MRI activity by presence of at least one Gd-enhancing lesion. The disease activity on MRI over the follow-up was assessed based on the presence of at least one Gd-enhancing lesion or new T2 lesion. At the study entry, the presence of higher clinical disease activity ($n = 13$, at least 2 relapses/2 years before baseline) was associated with higher levels of BMI-adjusted adipsin in comparison to patients with stable disease course

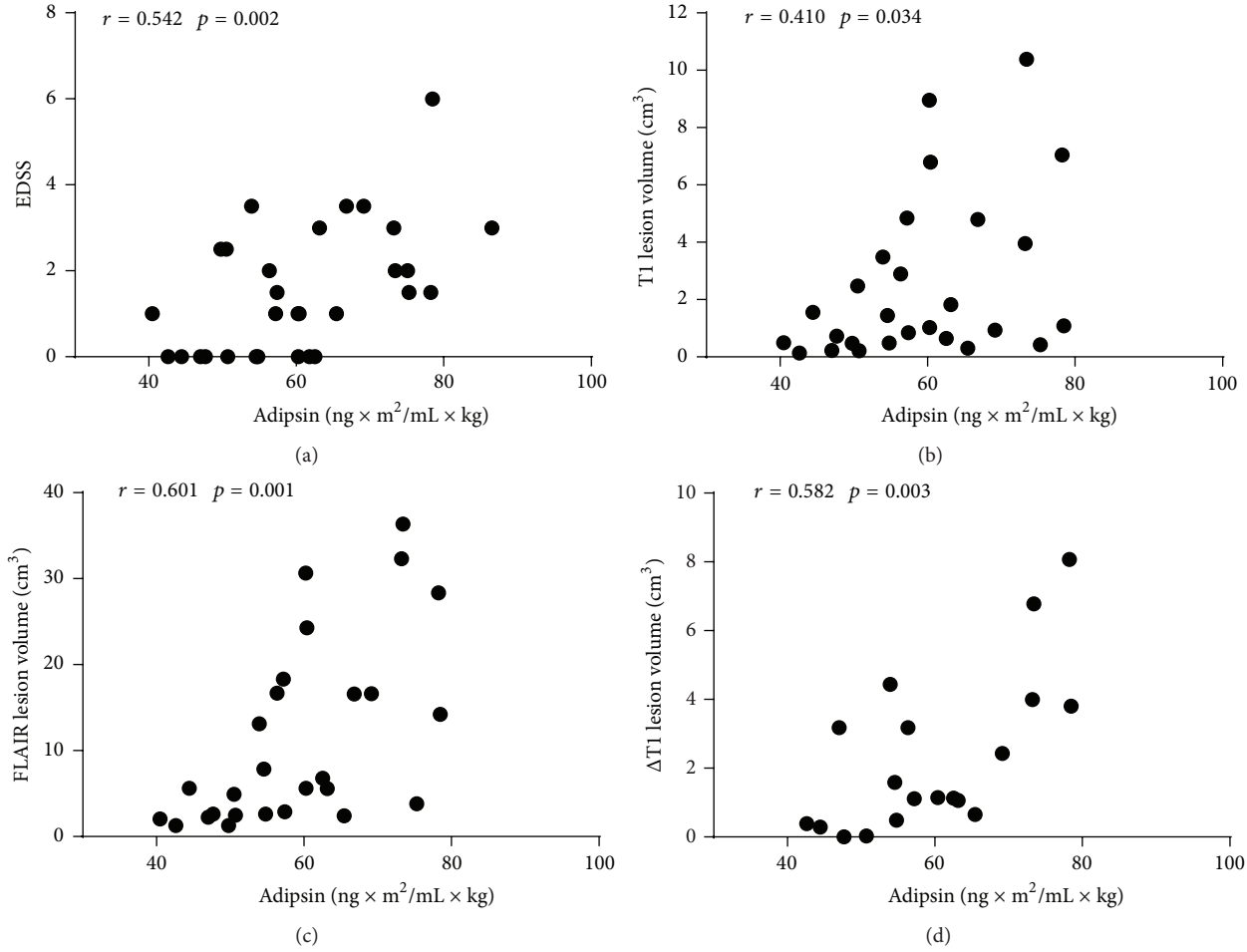

Figure 2: Associations between baseline BMI-adjusted adipsin and EDSS score (a), the volumes of T1 lesions (b), FLAIR lesions (c), and changes of T1 lesion volumes over the follow-up (d) in RRMS patients.

(n = 21, 0-1 relapses/2 years before baseline) (Figure 3). However, no associations were found over the follow-up. Regarding the MRI activity, half of the patients (18/34) showed MRI activity according to defined criteria (presence of Gd-enhancing lesion or new T2 lesion over the follow-up period), but association between adipokines levels and MRI activity was not found.

4. Discussion

Currently very little is known about the impact of adipokines on MS. This exploratory study assessed the ability of best-known adipokines to discriminate between MS subtypes and their potential to depict inflammatory activity and neurological deterioration in MS. A correlation between the baseline levels of adipsin and EDSS scores detected in whole MS and RRMS cohorts suggests an involvement of adipsin in pathophysiology of MS. Such interpretation is further supported by the correlations detected between the baseline adipsin and the volumes of T1-weighted and FLAIR lesions as well as the change of such lesion volumes over the follow-up seen in RRMS group.

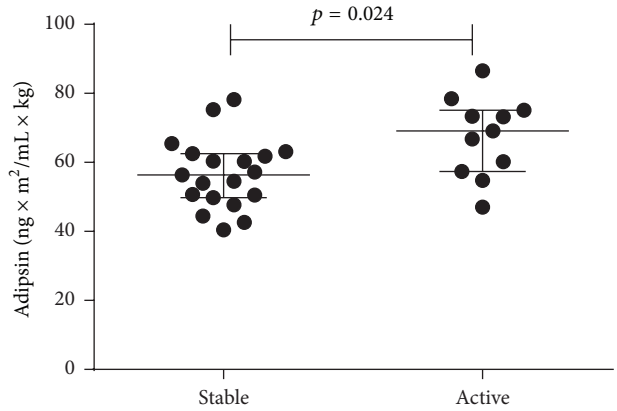

Figure 3: Scatter plot showing the levels of adipsin in stable and active RRMS patients. The bars indicate the median and interquartile range.

Adipsin (complement factor D) is a key enzyme involved in the activation of alternative pathway of complement activation and is primarily secreted from adipocytes and monocytes/macrophages in human subjects [20]. Its role

in the pathogenesis of MS has not been studied, but the immunohistochemical studies have demonstrated the presence of other complement components within the lesion in normal appearing white matter (NAWM) and cortical areas suggesting involvement of complement proteins in MS [21, 22]. Complement components of the classical and alternative pathway including C3, C4, C5, C9, terminals complement complex (TCC), complement receptor, and factors B, I, and H [23–31] have been previously analysed in sera and CSF of MS patients [23–31]. These studies have showed the positive correlation between CSF levels of C3, C9, and TCC and EDSS scores [30, 32, 33].

The observed association of baseline adipsin with neurological disability expressed by EDSS score in whole MS and RRMS cohorts suggests a role of adipsin in accumulation of neurological disability. Moreover, in RRMS at baseline an association between the adipsin and the volumes of T1-weighted lesions as well as their increase over the follow-up suggests predictive potential of adipsin as a biomarker of neurodegeneration. According to statistical analyses, age and gender did not influence these results. The absence of evolution of adipokines levels over the follow-up is most likely explained by relatively stable clinical disease course in most of our patients. However, an increase of the volumes of T1 and FLAIR lesions seen by MRI is consistent with worsening of MS even during the relatively short follow-up in this study. In parallel, the presence of higher adipsin in a subgroup of patients with more active RRMS (≥2 relapses/2 years before baseline) together with a positive correlation between the baseline adipsin and the volumes of FLAIR lesions in whole RRMS group suggests an involvement of adipsin also in inflammatory disease activity. Taken together, according to these observations adipsin is a neuroinflammation-promoting molecule that facilitates neurological deterioration and underlying neurodegeneration. It is noteworthy that inflammation-promoting activity of alternative complement pathway on adaptive immune responses has been recently reported also by other investigators [34, 35]. According to these studies, anaphylatoxins especially produced during the activation of alternative pathway may trigger inflammation and chemotaxis [34], although the role of complement in the adaptive immune responses to induce the T cell activation and proliferation has also been proposed [35].

The presence of decreased adipsin in RRMS patients in comparison to those with PPMS is most likely related to different pathological mechanisms in these MS subtypes. The early phase of RRMS is characterized predominantly by inflammatory events initiated by activation and differentiation of myelin specific CD4+ T cells into Th1 and Th17 cells and their transmigration from periphery to CNS eventually resulting in demyelination and axonal loss [36]. During the transition to more advanced stages like SPMS, BBB becomes less permeable leading to diminished entry of peripheral immune cells and their products into CNS [37]. Recent pathologic studies have showed that progressive subtypes are characterized by the widespread diffuse inflammation with slowly expanding lesions, abundant cortical lesions, and lymphocyte infiltration and microglia activation in the NAWM [38]. The elevated levels of adipsin in our PPMS patients most likely reflect peripheral immune activation and do not associate with ongoing focal CNS changes seen on MRI. Notably, recently the other member of complement pathway, that is, complement factor H, was found to be elevated in sera of patients with progressive MS but not in RRMS or healthy controls [39]. Together the available data suggest that elevated levels of adipsin in patients with progressive MS reflect ongoing peripheral immune activation.

Interestingly, this study revealed positive correlations between the BMI and the levels of leptin and adipsin. These observations support the hypothesis of close interaction between the adipose tissue and immune system in regulation of inflammatory responses [5]. In addition, the presence of higher levels of leptin and adiponectin in women indicates the presence of gender-specific association to secretion of these adipokines. Parallel results in MS and healthy subjects have been reported also by others [8, 40, 41].

5. Conclusions

This study showed an association of adipsin to neurological disability and focal changes on MRI in MS thus suggesting that dysregulation of alternate complement pathway may have an impact on MS disease course. The data suggest that adipsin exerts an inflammation-promoting effect and facilitates the development of neurodegenerative changes. The predictive potential of adipsin as a biomarker of neurodegeneration needs to be evaluated in further studies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Renuka Natarajan and Sanna Hagman contributed equally to the study and they are co-first authors.

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ORIGINAL ARTICLE

Elevated levels of soluble CD26 and CD30 in multiple sclerosis

Pabitra Basnyat,[1] Renuka Natarajan,[1] Julia Vistbakka,[1] Maija Lehtikangas,[2] Laura Airas,[3] Irma Matinlauri,[4] Irina Elovaara[1,5] and Sanna Hagman[1,5]

[1]Neuroimmunology Unit, School of Medicine, University of Tampere, Tampere, Finland, [2]ISLAB Laboratory Centre, Kuopio, Finland, [3]Department of Clinical Neurosciences, Turku University Hospital, Turku, Finland, [4]HUSLAB, Helsinki University Central Hospital, Helsinki, Finland, [5]Department of Neurology, Tampere University Hospital, Tampere, Finland

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Correspondence
Sanna Hagman, PhD, Neuroimmunology Unit, School of Medicine, University of Tampere, Biokatu 10, 33014 Tampere, Finland.
Tel: +358-3-3551-8429
Fax: +358-3-364-0557
Email: sanna.hagman@uta.fi

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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.

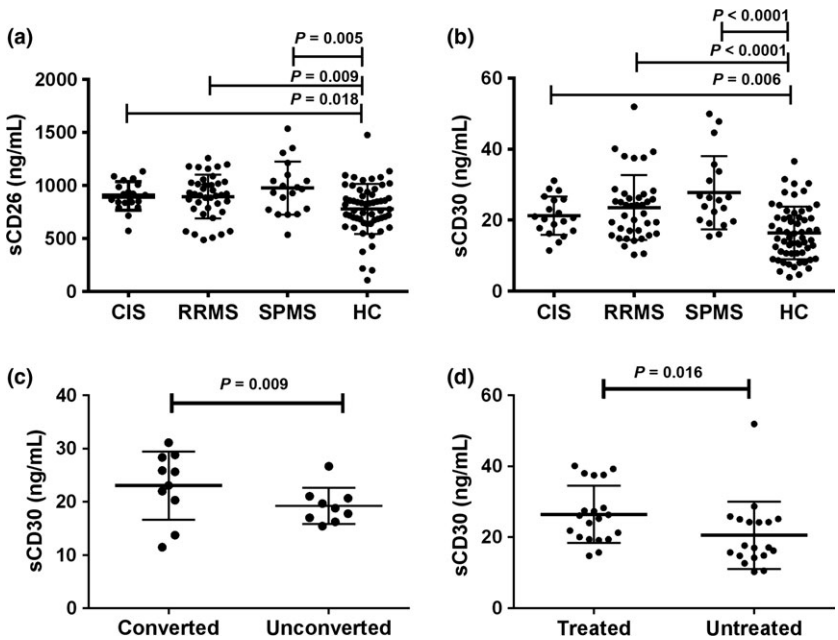

Figure 1 Different levels (mean ± SD) of (a) sCD26 and (b) sCD30 in patients with clinically isolated syndrome (CIS), relapsing–remitting multiple sclerosis (RRMS), secondary progressive MS (SPMS) and healthy controls (HC). (c) sCD30 levels in converted and unconverted CIS patients. (d) sCD30 levels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

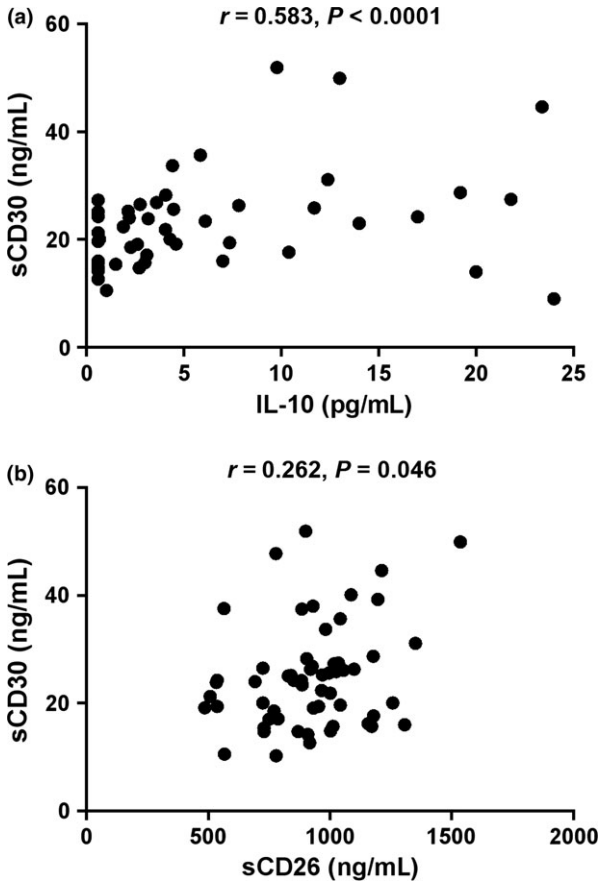

Figure 2 Spearman's correlation analysis between the levels of (a) sCD30 and interleukin (IL)-10 and between the levels of (b) sCD30 and sCD26 in multiple sclerosis (MS) patients.

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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Research Article

Diffusion Tensor Imaging in NAWM and NADGM in MS and CIS: Association with Candidate Biomarkers in Sera

Renuka Natarajan,[1] Sanna Hagman,[1] Xingchen Wu,[1,2] Ullamari Hakulinen,[2,3] Minna Raunio,[1,4] Mika Helminen,[5] Maija Rossi,[2] Prasun Dastidar,[2] and Irina Elovaara[1,6]

[1] *Neuroimmunology Unit, Medical School, University of Tampere, 33520 Tampere, Finland*
[2] *Department of Neurology, Tampere University Hospital, 33520 Tampere, Finland*
[3] *Department of Radiology, Medical Imaging Centre, Tampere University Hospital, 33520 Tampere, Finland*
[4] *Department of Neurology, Seinäjoki Central Hospital, 60220 Seinäjoki, Finland*
[5] *Science Center, Pirkanmaa Hospital District and School of Health Sciences, University of Tampere, 33520 Tampere, Finland*
[6] *Department of Electronics and Communications Engineering, Tampere University of Technology, 33520 Tampere, Finland*

Correspondence should be addressed to Irina Elovaara; irina.elovaara@uta.fi

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The aim of this study was to evaluate diffusion tensor imaging (DTI) indices in the corpus callosum and pyramidal tract in normal-appearing white matter (NAWM) and the caudate nucleus and thalamus in deep grey matter (NADGM) in all MS subtypes and clinically isolated syndrome (CIS). Furthermore, it was determined whether these metrics are associated with clinical measures and the serum levels of candidate immune biomarkers. Apparent diffusion coefficients (ADC) values were significantly higher than in controls in all six studied NAWM regions in SPMS, 4/6 regions in RRMS and PPMS and 2/6 regions in CIS. In contrast, decreased fractional anisotropy (FA) values in comparison to controls were detected in 2/6 NAWM regions in SPMS and 1/6 in RRMS and PPMS. In RRMS, the level of neurological disability correlated with thalamic FA values ($r = 0.479$, $P = 0.004$). In chronic progressive subtypes and CIS, ADC values of NAWM and NADGM were associated with the levels of MIF, sFas, and sTNF-α. Our data indicate that DTI may be useful in detecting pathological changes in NAWM and NADGM in MS patients and that these changes are related to neurological disability.

1. Introduction

Multiple sclerosis (MS) is the most common autoimmune disease of the central nervous system (CNS), and it is characterised by inflammation, demyelination and degenerative changes [1]. The identification of surrogate markers reflecting pathophysiological events in the CNS and correlating with clinical outcomes is highly needed for refining diagnostics and developing therapeutic approaches in patients with MS [2, 3]. Magnetic resonance imaging (MRI) is the most valuable paraclinical tool for monitoring the disease process *in vivo*, but the correlations between clinical and conventional imaging measures detected thus far have been generally suboptimal [4]. This phenomenon is most likely explained by the limitations of expanded disability status scale (EDSS) scoring and the ability of conventional MRI to reflect changes in the CNS consistent with different manifestations of MS [4, 5].

Recent neuropathological studies in MS have shown widespread tissue damage in both normal-appearing white matter (NAWM) and grey matter (NAGM) tissues [6] that are not detected by conventional MRI [7]. Nonconventional MRI approaches such as diffusion tensor imaging (DTI) allow for further examination of brain tissues *in vivo*. DTI utilises the orientation-dependent diffusion property of water molecules within the CNS and provides unique information on the pathological processes that reflect the microstructural damage in brain [8]. Tissue changes studied with DTI are measured by fractional anisotropy (FA) and apparent diffusion coefficient (ADC). Most of the studies on NAWM

in MS have reported increased ADC and reduced FA when compared to corresponding white matter regions in healthy subjects [9–11]. It is believed that changes in these measures reflect axonal damage and demyelination as well as inflammatory processes [12], although the evidence of corresponding pathological alterations is less clear than that in conventional MRI. Recent studies on NAGM emphasize that its damage occurs from the earliest stages of the disease process and may be a major determinant of long-term outcomes in MS [13–15]. However, attempts to correlate DTI indices with clinical measures have provided conflicting results [16–23].

The identification of biomarkers that could indicate pathophysiological processes or responses to a therapeutic intervention in individual MS patients would facilitate both diagnostic approaches and selection of treatments. So far, several candidate biomarkers have been identified in blood and cerebrospinal fluid (CSF) of MS patients [24, 25]. Among these biomarkers, several molecules, including cytokines, chemokines, adhesion molecules, antibodies, and apoptotic proteins, have been studied by us and others, and some have been associated with disease activity, neurological disability, or therapeutic responses [25–29]. However, the correlations between these molecules and conventional MRI findings have been relatively weak [30, 31]. In this study, our aim was to assess whether DTI indices in NAWM and normal-appearing deep grey matter (NADGM) are associated with different subtypes of MS and CIS and to address their association with clinical measures and the levels of candidate immune biomarkers in sera.

2. Patients and Methods

2.1. Study Population. We studied a total of 110 patients (75 females and 35 males), including patients with relapsing remitting MS (RRMS, $n = 36$), secondary progressive MS (SPMS, $n = 19$), primary progressive MS (PPMS, $n = 21$), clinically isolated syndrome (CIS, $n = 24$), and 10 healthy controls. All patients were followed up at the MS outpatient department at Tampere University Hospital and were recruited consecutively to this study. The study was approved by the Ethics Committee of Tampere University Hospital. All patients gave their informed consent. The diagnosis of MS was based on the revised McDonald criteria and CIS patients were defined as patients who had their first clinical episode suggestive of MS [32]. All RRMS and SPMS patients were in remission. We excluded the patients who were pregnant or suffering from any other clinically significant disease or treated with immunosuppressive drugs at least eight weeks before entering the study. Patients underwent neurological and MRI examinations as well as blood sampling on the same day. The determination of neurological disability was based on the EDSS score [33], and the disease activity was based on the number of relapses in the two years before study entry. All the clinical characteristics and immunomodulatory treatments are summarised in Table 1.

2.2. MR Image Acquisition. MR imaging was acquired using a 1.5-Tesla MR scanner (Siemens Avanto, Erlangen, Germany). All of the subjects were examined using the same MRI protocol, which consisted of a sagittal T1-weighted three-dimensional (3D) inversion recovery (IR)prepared gradiente-cho imaging, an axial T2-weighted turbo spin-echo imaging, a conventional axial and a high resolution sagittal fluid attenuation inversion recovery (FLAIR) imaging, an axial $T2^*$-weighted imaging, and an axial susceptibility-weighted imaging (SWI). The DTI data were collected by a single-shot spin-echo-based echo-planar diffusion-weighted imaging (EPI) sequence with the following parameters: repetition time (TR) 3500 milliseconds (ms), echo time (TE) 96 ms, slice thickness 5 mm, interslice gap 1.5 mm, field of view (FOV) 230 mm, matrix 128 × 128 (in-plane resolution = 1.8 × 1.8 mm^2), b values 0 and 1000 s/mm^2, number of excitations 3, and with 12 diffusion gradient orientations. The total scanning time was approximately 30 minutes.

For the volumetric analysis of T2-weighted plaques we used FLAIR sequence. The parameters used in this sequence are TR = 8500 ms; TE = 100 ms; TI = 2500 ms; slice thickness = 5.0 mm; in-plane resolution = 0.45 ∗ 0.45 mm.

2.3. MR Imaging Postprocessing and Analysis

2.3.1. DTI Analysis. The DTI analysis was performed as previously described [34] by an experienced radiologist (PD) together with a physicist (UH) who was blinded to the clinical details of the study subjects. The analysis was performed using the commercial software Neuro 3D (Siemens Healthcare, Malvern, PA, USA) on an offline workstation. In every individual, circular regions of interest (ROIs) of approximately 6 to 106 mm^2 (depending on the anatomical regions) were manually placed simultaneously in exactly the same location on B_0 images, ADC and FA maps. The ROIs were placed bilaterally (except for the corpus callosum) at the following anatomical locations: the posterior limb of the internal capsule, the centrum semiovale anterior, the posterior corona radiata anterior and posterior, the splenium and the genu of the corpus callosum, the thalamus, and the caudate nucleus (Figure 1). The ROIs were centred in the structure of interest in the most homogenous area, avoiding border areas to avoid the partial volume effects. The size of the ROI was reduced if a lesion was identified in the predefined ROI. ROIs of the same size were drawn in images of the healthy control subjects at the same anatomic locations as those of the patients.

2.3.2. Volumetric Analysis. Volumetric segmentation of plaques in the brain was performed using semiautomatic software Anatomatic operating in a PC/Windows 95 environment and the images were analysed blindly.

2.4. Immunological Assay. The levels of cytokines, chemokines, and apoptotic molecules could be determined in 71 patients with MS (33RRMS, 18 SPMS, 20 PPMS), 15 subjects with CIS, and 21 controls in which blood sample was available. Sera were separated from blood and analysed for 14 different molecules: interleukin (IL)-2, IL-6, IL-10, IL-12p70, interferon (IFN)-γ macrophage migration inhibitory factor (MIF), tumor necrosis factor (TNF)-α, TNF-related

Table 1: Clinical characteristics of patients with different subtypes of multiple sclerosis, clinically isolated syndrome, and controls.

Clinical characteristic	Patients with MS and CIS ($n = 100$)				
	RRMS $n = 36$	SPMS $n = 19$	PPMS $n = 21$	CIS $n = 24$	HC $n = 10$
Sex (M/F)[a]	11/25	7/12	9/12	3/21	4/6
Age (years)[b]	36.6 ± 8.4 (18–53)	49.5 ± 8.2[B,C] (35–61)	57.0 ± 9.2[A,B,C,D] (38–73)	34.3 ± 9.5 (20–52)	39.8 ± 12.9 (26–61)[A]
Duration of disease (years)[b]	3.9 ± 3.9 (0.0–12.3)	11.3 ± 9.3[C] (0.2–31.2)	11.9 ± 8.4[C] (0.2–26.2)	NA	NA
EDSS[b]	1.7 ± 1.6[B] (0–6)	4.7 ± 1.7[B,C] (2–7)	4.8 ± 2.0[B,C] (1–8)	0.0 ± 0.2 (0–1)	NA
Number of relapses/ 2 years[b,c]	1.6 ± 1.4 (0–5)	0.2 ± 0.5 (0–2)	NA	0.8 ± 0.6 (0–2)	NA
Treatment (NT/IFN/GA)[a]	13/20/3	18/1/0	21/0/0	24/0/0	NA

MS: multiple sclerosis; CIS: clinically isolated syndrome; RRMS: relapsing-remitting MS; SPMS: secondary progressive MS; PPMS: primary progressive MS; HC: healthy controls; EDSS: expanded disability status scale; NT: no treatment; IFN: interferon-β; GA: glatiramer acetate.
[a]Number of patients.
[b]Mean ± SD (range).
[c]Number of relapses in the two years before study entry.
Analyses were performed for all four subtypes versus control, as well as between the subtypes. The results from the Mann-Whitney U test are indicated as the Bonferroni-corrected P-values ($P < 0.05$).
[A]compared to controls.
[B]compared to CIS.
[C]compared to RRMS.
[D]compared to SPMS.

apoptosis inducing ligand (TRAIL), sFas and Fas ligand (sFasL), and chemokines CXCL10, CCL2, CCL3, and CCL4 as previously described [26]. The levels of the molecules were determined by Luminex (Bio-Plex suspension array system, Bio-Rad laboratories, CA, USA). The levels of TRAIL were determined by ELISA.

2.5. Statistical Analysis. Assessments of clinical, DTI, and immunological data were analysed with PASW Statistics for Windows version 18.0 (SPSS Inc., Chicago, IL, USA). Average DTI values were calculated from the right and left sides to obtain a single value for each region. The differences in DTI indices between and within groups were assessed using the univariate analysis of variance with age as covariate followed by a post hoc multiple pairwise comparisons with Bonferroni's correction. Comparisons were considered to be statistically significant, if the P value was smaller than 0.005 ($n = 10$; 5 groups) or 0.0006 ($n = 80$; 5 groups and 8 brain regions) after Bonferroni's correction. Relationship between the DTI indices and clinical parameter (EDSS, number of relapses, disease duration, age) and immunological molecules MIF, sTNF-α, and sFas was studied with Spearman correlation coefficient. P values of correlation analyses were not corrected for multiple comparisons. In the correlation analyses, if P values smaller than 0.01 were considered to be statistically significant.

3. Results

3.1. Clinical Data. The clinical characteristics including disease duration, its prestudy activity, EDSS scores, and therapies of patients are summarised in Table 1. The RRMS patients had shorter disease duration and lower EDSS scores than patients with SPMS or PPMS. Two years before enrolment, seven of the 36 RRMS patients were relapse-free, 12 other patients had one relapse, and the remaining 17 subjects had 2 to 5 relapses. Twenty-one of 76 MS patients were treated with interferon-beta (IFN-β) and three other patients with glatiramer acetate. In the CIS group, the EDSS score was 0 except for three subjects with a score of 1.

3.2. Volumes of T1 and FLAIR Lesions. The volumes of FLAIR lesions were determined in the 73 patients with MS and 22 subjects with CIS. It appeared that in SPMS the volumes of FLAIR lesions were increased when compared to RRMS and CIS (RRMS: 8.0 ± 9.7; SPMS: 12.6 ± 9.3; PPMS: 9.0 ± 11.4; CIS: 2.1 ± 3.1 cm^3; mean ± SD).

3.3. DTI Indices in Different Subtypes of MS and CIS. ADC and FA values of eight different anatomical brain regions were analysed from patients with different subtypes of MS, CIS, and healthy controls (Figure 2). Compared to controls, increased ADC values were detected in 6/8 regions in SPMS (internal capsule, corona radiata anterior and posterior, centrum semiovale, and splenium and genu of the corpus callosum), 4/8 regions in both RRMS or PPMS (internal capsule, corona radiata anterior and posterior, and centrum semiovale), and 2/8 regions (internal capsule and centrum semiovale) in CIS (Figure 2(a)). The corresponding comparison between FA indices showed significantly lower FA values in 2/8 regions (genu and splenium of corpus callosum) in SPMS, 1/8 regions in both RRMS and PPMS

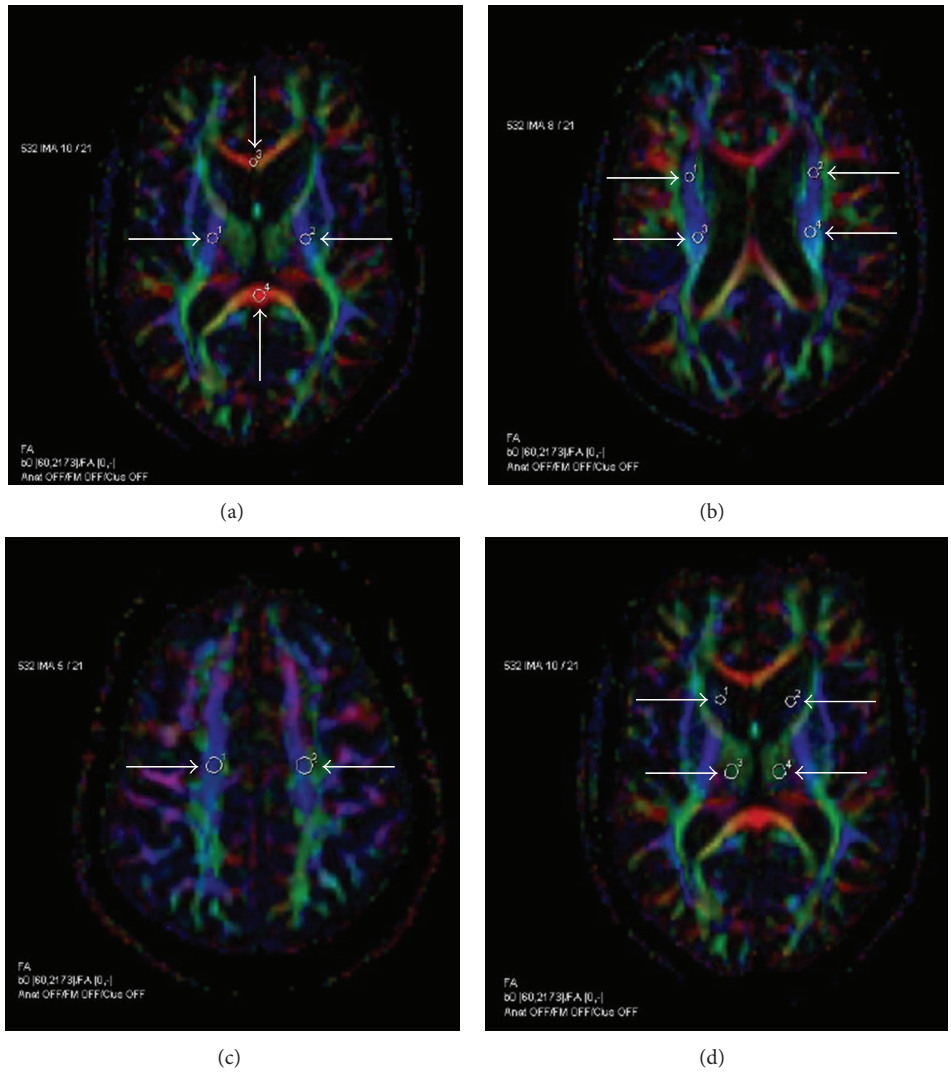
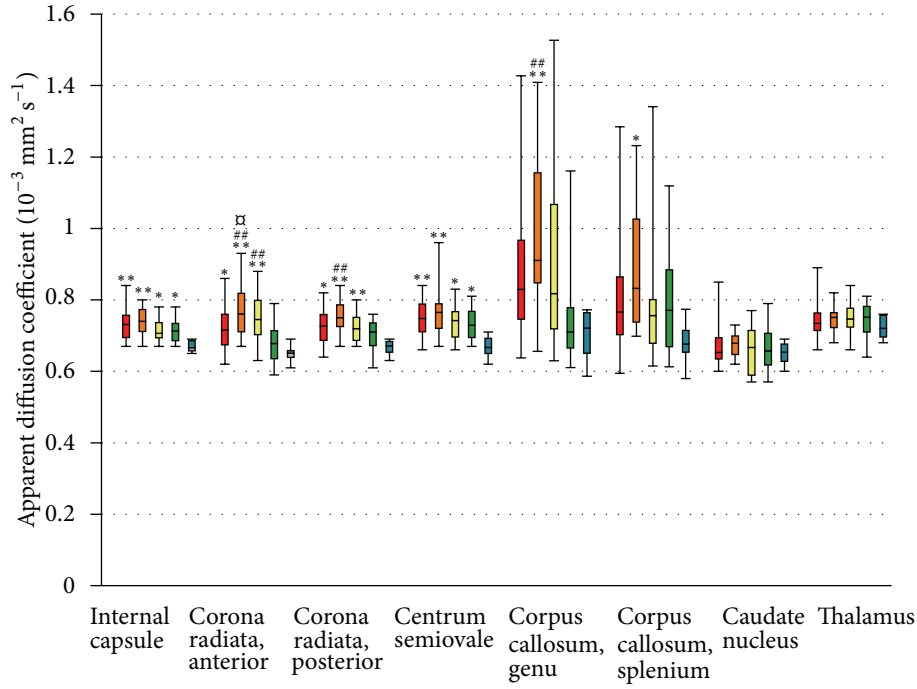


Figure 1: Region-of-interest (ROI) placement on axial FA colour maps. The posterior limb of the internal capsule (1, 2), the splenium (3), and the genu (4) of the corpus callosum (a); the posterior corona radiata anterior (1, 2) and posterior (3, 4) (b); the centrum semiovale anterior (c); and the caudate nucleus (1, 2) and the thalamus (3, 4) (d). Colours indicate the directions of fibre tracts (*red*, transverse; *blue*, craniocaudal; *green*, anterior-posterior). The circular ROIs were transferred from the corresponding B_0 image, and their sizes have been adjusted to avoid any visible lesions. The size of the ROIs ranged from 2 to 33 pixels (6–106.5 mm^2; pixel size $1.8 * 1.8\ mm^2$) depending on the size of the brain structure. This figure is a representative analysis from an SPMS patient.

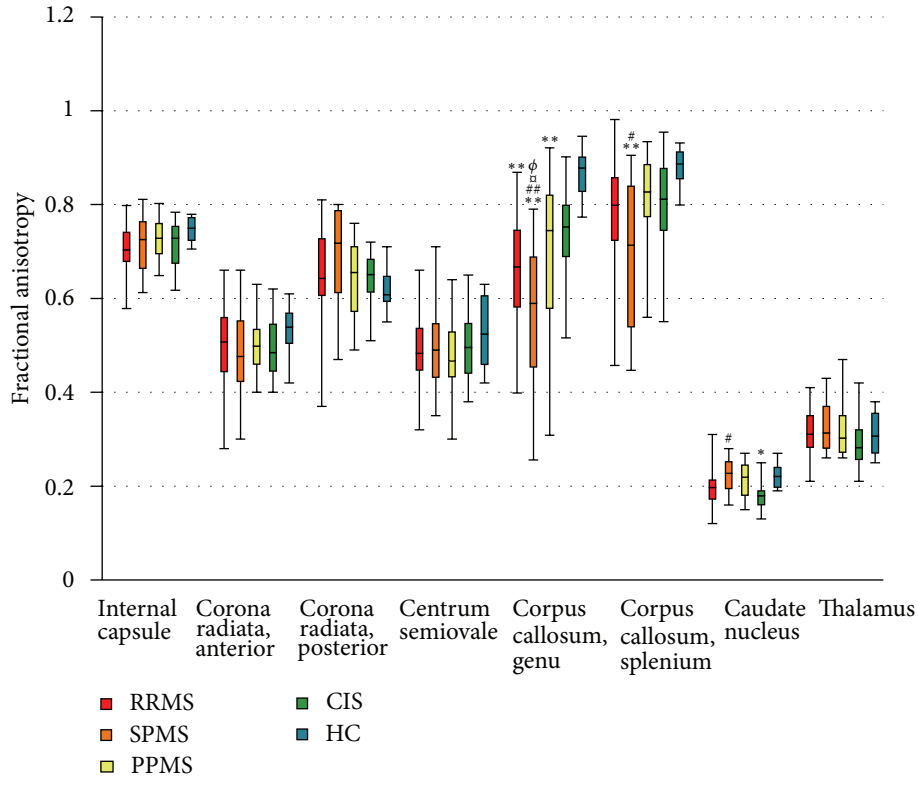
(genu of corpus callosum), and 1/8 regions (caudate nucleus) in CIS (Figure 2(b)).

Comparison between patients with different MS subtypes and CIS revealed higher ADC values in 3/8 regions (corona radiata anterior and posterior and genu of corpus callosum) in SPMS and in 1/8 regions (corona radiata anterior) in PPMS, but no differences were found between RRMS and CIS ($P > 0.005$, Figure 2(a)). Further comparisons between MS subtypes showed increased ADC values in 1/8 regions (corona radiata anterior) in SPMS compared to RRMS, but no differences were seen between RRMS or SPMS and PPMS ($P > 0.005$). Corresponding comparisons between FA values showed significantly lower FA values in 3/8 regions (splenium and genu of corpus callosum and caudate nucleus) of the SPMS group when compared to CIS, but no differences were found between CIS and RRMS or PPMS. (Figure 2(b)). Comparison between MS subtypes showed significantly lower FA values in one region (genu of the corpus callosum) in SPMS. No differences were found in FA between RRMS and PPMS.

3.4. Association between DTI Indices, Clinical Parameters, and Immune Molecules. The ADC and FA values of the eight brain regions were further correlated with clinical parameters, including prestudy disease activity, EDSS scores, disease duration, age, and candidate immune biomarkers (Table 2 and Figure 3). According to clinicoradiological correlation analyses, the EDSS score of RRMS group correlated with its FA values of the thalamus ($r = 0.479$, $P = 0.004$, Figure 3(a)). The disease duration of RRMS group correlated with ADC value of caudate nucleus ($r = -0.427$, $P = 0.009$, Figure 3(b)),



(a)



(b)

Figure 2: ADC and FA values in different brain regions of multiple sclerosis (MS) subtypes, clinically isolated syndrome (CIS), and healthy controls (HC). The length of the box represents the interquartile range, which includes the middle 50% of the values. The line through the middle of each box represents the median. The error bars show the minimum and maximum values (range). RRMS, relapsing remitting MS; SPMS, secondary progressive MS; PPMS, primary progressive MS; ADC, apparent diffusion coefficient; FA, fractional anisotropy. Reported P values were calculated using the univariate analysis of variance with age as covariate followed by post hoc multiple pairwise comparisons with Bonferroni correction. $^{**}P < 0.0006$ ($n = 80$), $^{*}P < 0.005$ ($n = 10$) when compared to HC. $^{\#\#}P < 0.0006$ ($n = 80$), $^{\#}P < 0.005$ ($n = 10$) when compared to CIS. $^{¤}P < 0.005$ ($n = 80$) in comparison to RRMS. $^{\emptyset}P < 0.005$ ($n = 80$) in comparison to PPMS.

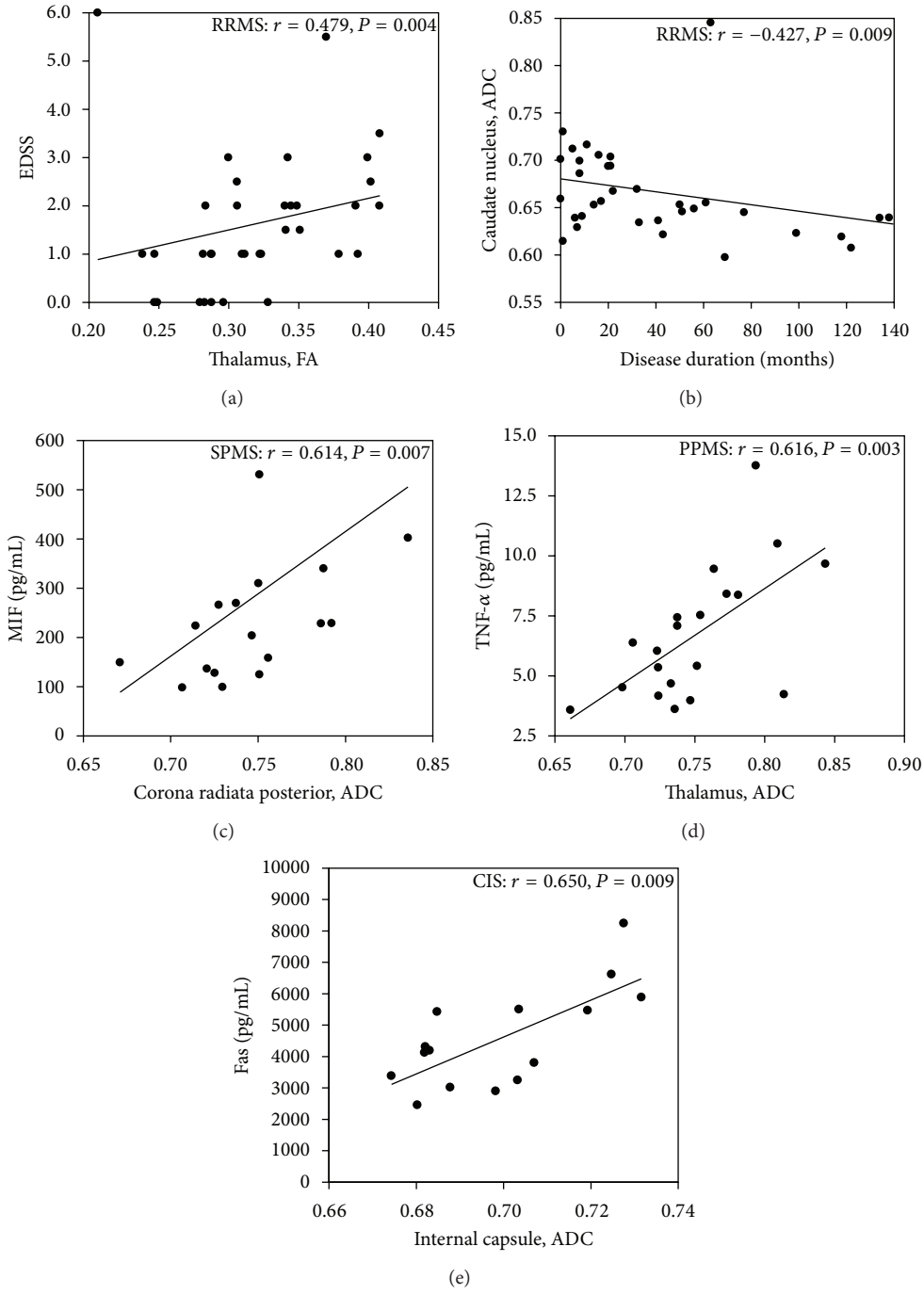


Figure 3: Correlations between DTI indices and clinical and immunological parameters in MS and CIS. Statistically significant correlations were found between the EDSS score and FA values for the thalamus in RRMS (a), disease duration and ADC values for the caudate nucleus in RRMS (b), the levels of MIF and the ADC values for the corona radiata posterior in SPMS (c), the levels of TNF-α and the ADC values for the thalamus in PPMS (d), and the levels of Fas and the ADC values for the internal capsule in CIS (e).

but no associations were found between the prestudy activity, age, and DTI indices in any of the groups.

The data on candidate immune biomarkers used in the correlation analyses of this study have been previously reported by our group (see Supplementary Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2013/265259). In this study, we focused on those molecules (sFas, sTNF-α, and MIF) that appeared to be significant in our previous studies [26, 35] and were therefore considered as candidate biomarkers. The significant correlations between the DTI indices and biomarkers were restricted to the chronic progressive groups and CIS (Figures 3(c)–3(e)). In SPMS, the ADC values of the corona radiata posterior correlated with the levels of MIF ($r = 0.614$, $P = 0.007$, Figure 3(c)), while

Table 2: Clinicoradiological correlations between DTI indices and clinical findings in RRMS patients ($n = 35$).

	EDSS	Relapses[a]	Age	DD
Internal capsule				
ADC	−0.094	−0.149	−0.078	−0.072
FA	0.310	−0.128	−0.143	0.200
Corona radiata, posterior				
ADC	0.099	0.002	0.083	0.080
FA	0.349	−0.032	0.151	0.381
Corona radiata, anterior				
ADC	0.200	−0.177	0.036	0.055
FA	0.359	−0.071	−0.062	0.307
Centrum semiovale				
ADC	0.027	−0.021	0.155	0.149
FA	0.122	0.096	−0.057	0.162
Corpus callosum, genu				
ADC	0.061	−0.277	0.133	−0.002
FA	−0.224	0.153	−0.221	0.002
Corpus callosum, splenium				
ADC	0.028	0.277	−0.368	0.051
FA	−0.115	−0.017	0.175	0.073
Thalamus				
ADC	−0.339	−0.110	−0.323	−0.332
FA	0.479**	−0.304	0.384	0.292
Caudate nucleus				
ADC	−0.182	−0.146	−0.132	−0.427**
FA	0.390	0.058	0.264	0.139

EDSS: expanded disability status scale; DD: disease duration; ADC: apparent diffusion coefficient; FA: fractional anisotropy.
Significant P values: $^{**}P < 0.001$.
[a]Number of relapses in the two years before study entry.

in PPMS, the ADC values of the thalamus correlated with sTNF-α ($r = 0.616$, $P = 0.003$, Figure 3(d)). In CIS, the ADC values of internal capsule correlated with the levels of sFas ($r = 0.650$, $P = 0.009$, Figure 3(e)).

4. Discussion

DTI is a promising technique for detecting demyelination and axonal loss in MS lesions and revealing diffuse microscopic changes in NAWM and NAGM. Until now, most of the previous DTI studies have been focused on particular MS subtypes [17, 23, 36, 37] or combined MS groups [10, 20], while only a few studies have included all MS subtypes and CIS [11, 38, 39]. Therefore, DTI studies covering the whole clinical spectrum of MS and CIS are required for evaluating the applicability of this methodology in clinical practice. In the present study, our purpose was to examine whether the DTI indices in the pyramidal tract and corpus callosum of NAWM and the caudate nucleus and thalamus of NADGM are associated with different subtypes of MS and CIS. Furthermore, we examined whether these measures are associated with neurological dysfunction and disease activity. We also studied the association between DTI indices and candidate immune biomarkers in sera.

According to this study, increased ADC values in NAWM regions are already present at the CIS stage, and these changes become most prevalent in SPMS. In the previous studies, DTI abnormalities have been detected in all MS subtypes and CIS, although the degree of damage differs between the subtypes [10, 11, 38–44]. In line with our data, Preziosa et al. showed increased ADC values in all studied NAWM regions beginning in the CIS stage, while the most pronounced microstructural damage was detected in SPMS [39]. FA abnormality was detected only in the corpus callosum regions, and it was most apparent in the SPMS phase. Similar results have been reported also by other investigators [11, 38, 43]. Cercignani et al. have detected lower FA and higher ADC values in the corpus callosum of patients with SPMS compared to patients with RRMS and PPMS [11]. Another study showed increased ADC values in the genu of the corpus callosum in SPMS when compared to controls and RRMS, but the FA values showed no significant differences [38]. Hannoun et al. measured DTI indices in the centrum semiovale and found both decreased FA values and increased ADC values in SPMS when compared to RRMS [43]. Thus, the data generated by us and others suggest that the greater increase in diffusivity is consistent with the more advanced phases of the disease, in which degenerative changes prevail over inflammation. Neuropathological studies have also shown that white matter is more severely affected in SPMS than in RRMS and PPMS [45]. In these studies, new and active white matter lesions are mainly detected in patients with RRMS, while diffuse inflammatory damage in NAWM and NAGM together with cortical demyelination is the hallmarks of chronic progressive subtypes [6]. Based on our data, it is noteworthy that diffusivity changes are present also in PPMS, but the damage in this subtype appears to be less severe than in SPMS. In respect to methodology, the data from our work and others' suggest that ADC indices are more sensitive than FA measures in detecting microstructural changes in the NAWM in MS. The present study applied an ROI-based approach, while other studies have also used histogram analyses detecting whole-brain damage [9, 40] and voxel-wise analysis detecting regional abnormalities [10, 39, 42, 46] in parallel. We consider that DTI may be useful in detecting pathological processes in MS and might prove valuable in clinical practice.

In RRMS, a strong association was detected between the FA values of thalamus and neurological disability expressed by EDSS score. This is in line with earlier studies reporting a positive correlation between the FA and ADC values of the thalamus and caudate nucleus and neurological disability in RRMS [47] and SPMS [47, 48]. These studies have additionally reported higher ADC and FA values in the caudate nucleus and thalamus in these subtypes compared to healthy controls, although in the present study, such differences between the subtypes and controls could not be found. It is suggested that increased FA in grey matter might indicate microglial activation or other inflammatory events [49]. Moreover, the conventional MRI studies have shown the presence of atrophy of the caudate nucleus and thalamus even

in the earliest stages of the disease [13, 50]. Such thalamic damage in MS patients has been associated with physical disability and cognitive impairment [51, 52]. Thus, based on these data, the DTI indices of NADGM may be useful indicators of disability accumulation in early MS.

Correlation between DTI findings and immunological molecules is a new approach for identifying biomarkers that could be useful both in refining the diagnostics and in selecting and optimising the appropriate MS therapy. Thus far, the correlations between candidate immune markers and lesion volumes quantified by conventional MRI have been relatively weak [30, 31]. Neuropathological studies have shown that mild inflammation, along with microglial activation, gliosis, diffuse axonal injury, and nerve fibres degeneration, is present also in NAWM and NAGM [53]. Therefore, our aim was to explore whether the proinflammatory molecules MIF, sFas, and TNF-α, earlier considered [26, 35] to be important in MS, would be associated with the diffusion and anisotropic changes in NAWM and NADGM. It is generally considered that immunological markers in blood at least partially reflect the inflammatory activity within the blood-brain-barrier compartment [54]. CSF would be better for these purposes, but there are well-known limitations to its availability, and blood is therefore a good alternative for identifying biomarkers [55]. Recently, new and rapidly emerging technologies allowing large-scale identification of potential biomarkers have been developed, and they will provide new opportunities for biomarker discovery [56].

In our previous study, we showed that increased MIF levels are associated with clinical disease activity in RRMS [26, 35], while increased levels of sTNF-α and sFas in sera in PPMS indicate the presence of inflammatory activity in this subtype [26]. MIF is released from its cytoplasmic stores during immune activation, and it promotes the migration of inflammatory cells into the CNS [57]. sTNF-α mediates apoptosis of oligodendrocytes and promotes inflammation [58], while sFas is shown to inhibit Fas-mediated apoptosis [59]. In this study, the levels of blood inflammatory molecules obtained from 86 out of 100 patients were correlated with DTI indices. In patients with chronic progressive subtypes and CIS in the present study, these molecules correlated with diffusivity changes in the corona radiata posterior and internal capsule of NAWM and the thalamus of NADGM (Figures 3(c)–3(e)). Thus, these observations and previous studies suggest that these molecules, which reflect inflammatory disease activity, are associated with development of microstructural changes in MS.

5. Conclusions

The present study showed that the most abnormal DTI indices were present in SPMS, although changes were seen throughout the spectrum of MS disease and CIS. The association between disability and the thalamic FA indices suggests that DTI might be a useful indicator of neurological disability. However, determining the utility of this methodology will require longitudinal studies. The association between the DTI indices in both NADGM and NAWM and the levels of MIF, sFas, and sTNF-α suggests the involvement of these molecules in promoting such microstructural changes in the CNS.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

The authors contributed equally in the study.

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Supplementary table 1. The levels (pg/ml) of soluble molecules in different subtypes of MS and controls (median (25-75th percentiles))

Molecule	RRMS n=33	SPMS n=18	PPMS n=20	CIS n=15	Controls n=21
MIF	138.2 (50.0-229.4) [A,C]	225.8 (134.0-317.3)	290.7 (189.3-449.3)[C]	167.3 (97.8-268.2)	378.5 (236.4-535.9)[A]
sFas	4612.8 (4058.0-5940.7)[C]	5572.4 (4388.3-6845.4)	6787.6 (5393.9-7709.2) [B,C]	4318.8 (3320.0-5697.9) [G]	4824.9 (3405.1-5638.1) [B]
sFasL	90.6 (58.1-161.6)	96.2 (72.8-153.6)	111.2 (71.5-191.9)	83.6(67.1-174.6)	80.4 (56.6-129.1)
sTRAIL	765.9 (577.2-1728.9)	785.5 (587.2-1106.0)	1032.0 (762.8-2036.1)	902.3(593.3-1170.5)	738.7 (523.2-2260.8)
sTNF-α	4.5 (3.4-6.5)[C]	4.1 (3.3-5.8)	6.4 (4.4-8.9) [B. C]	4.6 (3.5-6.6)	3.7 (2.4-4.6) [B]
IFN-γ	0.3 (0.3-3.0)	1.5 (0.3-4.1)	0.6 (0.3-3.3)	2.8 (0.3-25.5)	0.5 (0.3-7.5)
IL-2	1.2 (0.2-3.6)	0.6 (0.2-5.2)	0.4 (0.2-2.4)	1.5 (0.5-6.2)	2.1 (0.2-6.1)
IL-6	4.9 (2.0-21.5)	7.1 (3.2-13.0)	8.8 (1.6-26.7)	3.6 (1.4-17.9)	4.1 (2.0-9.3)
IL-10	2.9 (0.6-5.7)	3.4 (1.8-7.7)	3.6 (2.2-9.2)	4.8 (1.2-25.9)	2.3 (0.2-6.0)
IL-12p70	0.4 (0.1-1.4)	1.3 (0.4-5.4)	0.8 (0.1-4.5)	1.7 (0.3-9.1)	1.6 (0.1-7.9)
CXCL10	95.7 (68.1-155.6)	90.5 (62.8-152.6)	106.9 (74.7-212.9)	65.9 (53.6-178.2)	106.9 (55.1-130.7)
CCL2	177.0 (140.4-271.8)	218.8 (157.0-331.0)	288.4 (201.3-461.6)[B]	248.3 (165.7-332.0)	152.8 (122.6-273.1) [B]
CCL3	37.3 (21.6-96.3)	55.7 (19.2-121.4)	45.4 (27.0-164.7)	72.6 (32.0-186.1)	51.4 (27.7-122.8)
CCL4	126.6 (20.4-668.0)	140.2 (17.0-873.8)	290.7 (39.8-1054.6)	365.1 (144.0-1188.5)	289.1 (16.8-725.0)

RRMS, relapsing remitting MS; SPMS, secondary progressive MS; PPMS, Primary progressive MS; CIS, clinically isolated syndrome. Analyses were performed for all four subtypes versus control, as well as between the subtypes. The results from the Mann-Whitney U Test are shown as the Bonferroni-corrected p-values ($p<0.05$).

A Comparison between the RRMS and HC groups
B Comparison between the PPMS and HC groups
C Comparison between the PPMS and RRMS groups

Supplementary table 2. Clinical characteristics of patients and controls included in serum analyses.

	RRMS n=33	SPMS n=18	PPMS n=20	CIS n=15	HC n=21
Sex (M/F)[a]	10/23	6/12	9/11	2/13	8/13
Age (years) [b]	37.0±8.6 (18-53)	49.5±8.2 (35-61)	56.9±9.2 (38-73)	35.3±9.5 (20-52)	
Duration of disease (years) [b]	4.1±4.0 (0.0-12.3)	11.6±9.5 (0.2-31.2)	12.0±8.6 (0.2-26.2)	NA	
EDSS [b]	1.6±1.5 (0-6.0)	4.8±1.7 (2.0-7.0)	4.7±1.9 (1.0-7.5)	0.1±0.3 (0.0-1.0)	
Number of relapses/ 2years [b,c]	1.6±1.4 (0-5)	0.2±0.5 (0-2)	NA	0.8±0.6 (0-2)	
Treatment (NT/IFN/GA) [a]	12/18/3	17/1/0	20/0/0	15/0/0	

MS, multiple sclerosis; CIS, clinically isolated syndrome; RRMS, relapsing-remitting MS; SPMS, secondary progressive MS; PPMS, primary progressive MS; HC, healthy controls; EDSS, expanded disability status scale; NT, no treatment; IFN, interferon-β; GA, glatiramer acetate.

[a] Number of patients.

[b] Mean ± SD (range).

[c] Number of relapses in the two years before study entry