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**Establishment and Applications of a
Multiple Sclerosis Biobank**
Analysis of Biomarkers and Therapeutic Complications in MS

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For my mother

ABSTRACT

Biomarkers for disease activity and prognosis in multiple sclerosis (MS) are lacking. We established a MS biobank consisting of paired cerebrospinal fluid samples (CSF) and peripheral blood samples from MS patients and controls. A standardized procedure for sampling and data collection was developed when forming the European network for MS biomarker research (BioMS) and applied when processing samples and data. The MS biobank was then used for the following studies:

In paper I, we analyzed the basal viral load of JC virus (JCV) DNA in untreated MS patients and controls in aspects of PML risk. In our system, a low JCV DNA replication could occasionally be found in CSF or plasma in MS patients and controls without clinical signs of PML. We concluded that JCV analysis, in untreated MS patients, is not useful for identification of patients with higher risk of PML.

In paper II a comparison of N-acetylaspartate (NAA) and neurofilament subunits in CSF as biomarkers for axonal damage in MS was performed. We observed that CSF-NAA might serve as a biomarker for axonal damage during later stages of disease. Increased levels of CSF neurofilament light chain (NfL) correlated to acute axonal damage associated with inflammation whereas a high level of CSF neurofilament heavy chain (NfH) was indicative of irreversible axonal damage. The combined analysis of all biomarkers identified a higher number of MS patients with an abnormal biomarker panel compared to analysis of a single biomarker.

In paper III we used a new assay for detection of anti-myelin antibodies in CSF and report that approximately 50% of the MS patients had increased anti-myelin antibody reactivity in CSF and that their levels correlated to the number of MRI lesions in MS patients.

In paper IV, we analyzed the levels of vascular endothelial growth factor A (VEGF-A) in CSF cells and peripheral blood mononuclear cells (PBMCs) from MS patients and controls and demonstrated that both relapsing-remitting MS patients (RRMS) and secondary progressive MS patients (SPMS) had decreased levels of VEGF-A in CSF. In addition, SPMS also displayed a significant decrease of VEGF-A in PBMCs which was associated with an altered peripheral blood monocyte phenotype. We conclude that decreased PBMCs VEGF-A may be a new biomarker for SPMS.

In paper V we performed a large genetic association study in MS patients and controls and demonstrated that IL7R gene variants influence the risk of MS. Functional analysis showed that MS patients had an increase of IL7R and IL7 mRNA expression in CSF cells compared to controls. These findings suggest that IL7-IL7R signalling might be involved in the dysregulated immune response in MS.

In summary, we present an example of a genetic biomarker for risk of disease. We related different markers for neurodegeneration to disease stage, explored two possible immune related markers, and analyzed a treatment related complication in MS.

The papers included in this thesis are examples of the value of quality biobanking for clinical research.

LIST OF PUBLICATIONS

- I. ELLEN IACOBÆUS, C Ryschkewitsch, M Gravell, M Khademi, E Wallström, T Olsson, L Brundin and E.O Major. **Analysis of cerebrospinal fluid and cerebrospinal fluid cells from patients with multiple sclerosis for detection of JC virus DNA.** *Multiple Sclerosis* 2009;15: 28-35

- II. C.E Teunissen , ELLEN IACOBÆUS, M Khademi, L Brundin, N Norgren, M.J.A Koel-Simmelink, M Scheppens, F Bouwman, H.A.M Twaalfhoven, HJ Blom, C Jakobs, C.D Dijkstra. **Combination of CSF N-acetylaspartate and neurofilaments in multiple sclerosis.** *Neurology* 2009;72:1322-1329

- III. M Vogt, C.E Teunissen, ELLEN IACOBÆUS, P Heijnen, E Breij, T Olsson, L Brundin, J Killestein, C D Dijkstra. **CSF anti-myelin antibodies are related to MR measures of disease activity in multiple sclerosis.** *J Neurology Neurosurgery and Psychiatry* 2009;80:1110-1115

- IV. ELLEN IACOBÆUS, P Amoudruz, M Ström, M Khademi, L Brundin, J Hillert, I Kockum, V Malmström, T Olsson, E Tham and F Piehl. **Altered monocyte phenotype with decreased vascular endothelial growth factor-A in progressive multiple sclerosis.** *Manuscript*

- V. F Lundmark, K Duvefelt, ELLEN IACOBÆUS, I Kockum, E Wallstrom, M Khademi, A Oturai, L.P Ryder, J Saarela, H.F Harbo, E.G Celius, H Salter, T Olsson, Jan Hillert. **Variation in interleukin 7 receptor a chain (IL7R) influences risk of multiple sclerosis.** *Nature Genetics* 2007; 39: 1108-1113

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LIST OF ABBREVIATIONS

Ab	Antibody
BACE1	β -site APP-Cleaving Enzyme 1
BBB	Blood Brain Barrier
BDNF	Brain Derived Neurotrophic Factor
CIS	Clinically Isolated Syndrome Suggestive of MS
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
CSF	Cerebrospinal Fluid
EAE	Experimental Autoimmune Encephalomyelitis
EDSS	Expanded Disability Status Scale
Gd	Gadolinium
GFAP	Glial Fibrillary Acidic Protein
HLA	Human Leukocyte Antigen
I-CAM1	Inter Cellular Adhesion Molecule 1
IgG	Immunoglobulin G
IL	Interleukin
INF- β	Interferon-beta
JCV	JC virus
MBP	Myelin Basic Protein
MMP	Matrix-metalloproteinase
MOG	Myelin Oligodendrocyte Glycoprotein
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
MRS	Magnetic Resonance Spectroscopy
MSFC	MS Functional Composite Measure
MSSS	MS Severity Score
NAA	N-acetylaspartate
Nabs	Neutralizing Antibodies
NAWM	Normal Appearing White Matter
N-CAM	Neuronal Cell Adhesion Molecule
NfH	Neurofilament heavy chain
NfL	Neurofilament light chain
NO	Nitric Oxide
OB	Oligoclonal Bands
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PPMS	Primary Progressive MS
PML	Progressive Multifocal Leukoencephalopathy
RIS	Radiologically Isolated Syndrome Suggestive of MS
RRMS	Relapsing-Remitting MS
SNP	Single Nucleotide Polymorphism
SPMS	Secondary Progressive MS
VEGF	Vascular Endothelial Growth Factor

INTRODUCTION

This thesis is on the establishment and application of a Multiple Sclerosis (MS) biobank and corresponding database. Our MS biobank contains cerebrospinal fluid (CSF) and blood samples collected mainly from patients visiting the neurology clinic, Karolinska Hospital between 2001 and 2009. The procedure and work performed during sample handling and data collection will be discussed and I will present examples of how the biobank and database was used in my research projects.

1 MULTIPLE SCLEROSIS

MS is a disease characterized by chronic inflammation in the central nervous system (CNS), demyelination and axonal damage. It is the most common, non-traumatic, cause of neurological disability in young adults of the Caucasian population (1) and associated with a considerably reduced quality of life compared to the general population (2). Most patients develop the disease between 20 and 40 years and it affects women twice as often as men (3).

1.1 EPIDEMIOLOGY

The worldwide incidence of MS has a clear geographic heterogeneity which roughly can be divided in three frequency zones. The area with highest frequency are (prevalence of more than 30/100 000) most of Europe, Israel, Canada, northern US, southeastern Australia and New Zealand. The medium frequency areas (prevalence of 10-30/100 000) comprise southern US, most of Australia, South Africa and Russia while the lowest frequency areas (prevalence rates under 5/100 000) are found in northern South America, Africa and most of Asia (4). Observations from two large studies estimated the incidence of MS in Sweden to be approximately 4-5/100 000/year (5, 6). Recent data from the Swedish MS registry suggest that the prevalence of MS is increasing, and is at the moment close to 140/100 000 in Sweden (7).

1.2 THE DIAGNOSIS OF MS

No single test or clinical sign is sufficient for the diagnosis of MS why the diagnostic criteria of MS include a combination of disease history, clinical and paraclinical features first defined by Poser *et al.* in 1983 (8) and rewritten in 2001 by McDonald *et al.* (9). The hallmarks are that evidence of two clinical episodes suggestive of MS, separated in space and time, must be present in order to diagnose relapsing-remitting MS (RRMS) (9). In addition, differential diagnoses should have been ruled out. This definition mean that individuals presenting with only one clinical episode indicative of MS, can not obtain the diagnosis RRMS, if another clinical episode is not recalled. This group should rather be diagnosed as patients with a “clinically isolated syndrome suggestive of MS”, CIS (10). The CIS patients

are usually considered as a subgroup of MS since the majority of them will convert to RRMS within an average of 1, 7 years, and initiation of MS disease-modifying treatments is highly recommended in this group (10-12). In order to facilitate the diagnosis of RRMS, a revision of the McDonald criteria was carried out in 2005 which put forward that magnetic resonance imaging, (MRI) findings of a new lesion can contribute to demonstration of dissemination in time or space and substitute for a clinical exacerbation (13). The presence of CSF oligoclonal bands, (OB) and/or elevated IgG index and delayed visual evoked potentials, (VEP) supports the diagnosis but is not specific for MS. In summary, the revised criteria have met the need for early, accurate diagnosis of MS (14-17).

According to Lublin and Reingold the subdiagnosis secondary progressive MS (SPMS) can be ascertained when there has been at least six months of steadily increasing neurological impairment, with or without superimposed relapses, following an initial relapsing-remitting phase (18).

The criterion for establishing the diagnosis primary progressive MS, (PPMS), is at least one year of progressive neurological dysfunction from onset and evidence for dissemination in space determined by MRI, presence of OB/increased IgG index in CSF and VEP (13).

Recently a new subgroup of MS has been defined namely individuals with incidentally discovered MRI lesions fulfilling criteria for MS but reporting absence of objective clinical symptoms suggestive of the disease. This group is now referred to as “radiological isolated syndrome suggestive of MS” (RIS) and have an increased risk for conversion to RRMS compared to controls (19).

1.3 DISEASE COURSE AND NEUROPATHOBIOLOGY

1.3.1 Relapsing – remitting MS

Approximately 85% of all MS patients initially present with a relapsing-remitting MS disease course characterized by recurrent episodes of neurological symptoms followed by a varying degree of recovery and a stable course between the attacks (20). Early in the disease, most patients have an average of one to two relapses yearly while the number of attacks decrease over time (21). Dependent on the anatomical localization of the MS lesions, a large variety of symptoms can appear during a relapse, but the most common symptoms are sensory loss, optic neuritis, muscle weakness, ataxia and balance disturbance (22).

The RRMS phase appears to be largely driven by inflammation. A relapse is primarily a consequence of disturbed conduction block due to inflammatory mediators (23, 24) and demyelination (25). The pathological hallmarks of MS lesions are multifocal inflammation, including T-cells, B-cells, plasma-cells, macrophages and monocytes, demyelination, oligodendrocyte loss, reactive gliosis and axonal degeneration (26-28) and remyelination to a varying degree (29).

Clinical exacerbations are frequently visualized on MR imaging as new or enlarging T2 lesions and occasionally as gadolinium-enhancing (Gd) T1 lesions which is a reflection of a profound inflammation and breakdown of the blood-brain-barrier (BBB)(30). However conventional MRI does not detect all new inflammatory activity, cortical plaques are also common and not easily detected with this technique.

Remission is considered to occur when the inflammation ceases and the conduction is restored as a consequence of resolved inflammation, axolemmal plasticity, adaptive synaptic changes and remyelination (31). Even though a remission is clinically evident, it has been demonstrated that axonal loss occurs already at early stages in MS (32, 33). The absence of symptoms is most likely due to the plasticity of the CNS, compensating for axonal loss until a threshold level is reached. Functional MRI studies have shown evidence for compensatory activity in the cerebral cortex in MS patients with axonal damage (34).

1.3.2 Progressive MS

With time, the relapsing-remitting disease course eventually converts into a phase with stable progression of neurological deficits; SPMS (18). There are large inter-individual differences but on a group level approximately half of all RRMS patients have entered the SPMS phase 10 years after disease onset (21). Absence of remission reflects a failed recovery process usually due to repeated attacks of inflammatory demyelination of the same nerve, failed remyelination and irreversible axonal damage (33, 35).

In 15% of the MS patients, the disease is progressive from onset with a steady accumulation of neurological impairment without recovery; PPMS (36). It is characterized by absence of clinically evident relapses, less prominent inflammation by MRI and often spinal cord symptoms (22, 37). Older age of onset and lack of female preponderance also distinguish PPMS from RRMS (38). Studies on the natural history of all MS subgroups demonstrate that PPMS has the worst prognosis

although the clinical course of PPMS is found to be similar to that of SPMS when only the progressive phases are compared (39).

Histopathologically, lesions in progressive phases of MS are sharply defined, display a lesser amount of cellular infiltrates, extensive myelin loss, prominent fibrillary gliosis and oligodendrocyte and axonal loss (26). A comparative study of MS lesions in PPMS and SPMS demonstrated that presence of less inflammatory cells, more pronounced oligodendrocyte loss and axonal reduction differentiate PPMS patients from SPMS patients (40, 41).

The axonal degeneration can be detected as chronic hypointense so called T1 “black holes” and brain atrophy on MRI (42-45).

Axonal loss has been accepted as the major cause of irreversible disability in MS patients (33, 46) and transition from RRMS to SPMS occurs when the CNS can no longer compensate for the neuronal loss (33). The mechanism behind neurodegeneration is not yet clarified but several mechanisms have been proposed; Axonal transection has been demonstrated to correlate with macrophages and microglia suggesting that these cells together with inflammatory mediators such as cytokines, proteolytic enzymes and nitric oxide can mediate both myelin and axonal damage (33) (47). Axonal injury might also be secondary to demyelination, CD8+ mediated cytotoxicity and/or excitotoxic mechanisms (48, 49). Other suggested hypotheses for the basis of axonal damage are mitochondrial failure or a failed energy supply due to an increased energy demand when restoring impulse conduction in damaged axons (50).

CNS damage in all MS phases is not only restricted to the white matter, demyelinating lesions in the grey matter and diffuse inflammation, gliosis, and axonal loss is a common feature in both the “normal appearing white matter” (NAWM) and the grey matter which might be visualized as generalized atrophy on conventional MRI (51, 52).

1.4 WHAT CAUSES MS?

1.4.1 Environmental factors

It has long been proposed that various environmental factors might play a role in MS causation. Migration studies have shown that persons migrating from a low prevalence area to a high prevalence area after adolescence will retain their low risk

of MS, whereas persons migrating before adolescence seem to acquire the higher risk of MS in the new area which support the role of the environment in MS (53, 54).

The involvement of common childhood infections in the development of MS has been suggested. Infection with morbilli, rubella and Epstein-Barr virus (EBV) at a later age than at early childhood (55) was observed to associate with an increased risk of MS. On the other hand, a Swedish cohort demonstrated that the introduction of vaccination against measles, morbilli and rubella not changed the incidence of MS in Sweden (56). From Umeå, Sundström *et al.* performed a prospective study and reported that the individuals who developed MS displayed an altered immune response against an EBV-antigen (57).

Low sunlight exposure or vitamin D deficiency (58, 59) and cigarette smoking (60, 61) are other compelling environmental risk factors for MS.

1.4.2 Genetics

The observation of the uneven distribution of MS and that some races are more susceptible to MS support a genetic etiology of MS. Twin studies report a concordance rate of 25-30% in monozygotic twins and 2-5% in dizygotic twins which clearly demonstrate that genetic sharing are related to the incidence of MS (62, 63). A positive family history is found in approximately 15-20% of all MS patients (64). It has been questioned whether the familial aggregation of MS is due to joint environmental factors rather than genetic factors. However, studies report that the increased MS risk in family members of an affected individual is correlated to the degree of genetic sharing, and the children of parents both suffering from MS, have a higher risk for MS compared to children with only one affected parent (63, 64).

The first gene region that was reported to influence MS was the human leukocyte antigen (HLA) gene complex located on chromosome 6p21 (65). A large number of studies have thereafter confirmed and specified the HLA class II haplotype DR15-DQ6 as the strongest genetic risk factor, conferring a three fold increased risk of MS (66). In Northern Europe, 60% of the MS population carries this risk haplotype compared to 30% of the controls (67). More recently, haplotypes of the HLA class I was also confirmed to associate with MS (68). In 2007 our group and the group of Gregory were able to present data on the IL7R gene as the first non-HLA gene affecting MS risk (Paper V) (69). Several later studies have now

confirmed our finding including the first published MS Genome Wide Association Scan (GWAS) performed by the International MS Genetics Consortium (IMSGC) (70-72). The development of the GWAS technique has allowed a major change in the ability to discover MS risk genes and today several non-HLA MS susceptible genes, most of them coding for immunoregulatory proteins, have been discovered (71, 73, 74). In addition, a large GWA study across several human diseases revealed that some of the MS risk genes were shared by other autoimmune diseases which supports that MS has an autoimmune origin (74). At the moment, a “second generation GWA study” is ongoing which includes genotyping of up to one million SNP markers in 20 000 MS patients and controls and is expected to uncover the complete list of MS genes.

The non-MS genes discovered so far have only a modest effect on susceptibility to MS with odds ratio ranging from 1.1-1.2 (75, 76) which however commonly is the case in complex diseases where both genetic and environmental factors are required for development of disease.

Taken together, a large body of evidence suggests that MS triggering factors include interactions between several genes, epigenetic factors and interactions between environmental agents and genes. Therefore, all these factors should be included when performing reliable models for prediction of MS disease (77).

1.4.3 Immunopathogenesis

MS is usually referred to as an autoimmune disease because of the abnormal presence of immune cells and immune mediators at the sites of injury within the CNS. However, this observation does not necessarily declare that the immune system is responsible for triggering MS onset (78). On the other hand, several observations in MS are indeed consistent with autoimmune mechanisms:

1) The genetic association with the HLA complex, which is known to regulate immunity (66) **2)** the association of CNS myelin destruction and infiltration of T-cells, B-cells and macrophages (33, 79) **3)** the increased T-cell and B-cell response to several myelin proteins in CSF and peripheral blood of MS compared to controls (80-82), **4)** the existence of increased levels of myelin-specific autoantibodies in CSF (83, 84) and **5)** that the animal model for MS, experimental autoimmune encephalomyelitis (EAE) can be induced either by immunization with myelin-antigens or by transfer of myelin- reactive T-cells (85).

The most common hypothesis is that MS is initiated by a peripheral dysregulated immune response; autoreactive myelin-specific T-cells gets activated and thereby up-regulate their expression of adhesion molecules which enable them transit through the BBB. (86). Within the CNS, the autoreactive T-cells becomes reactivated upon recognition of their antigen and resident microglia (87, 88) followed by secretion of cytokines and chemokines and further recruitment of T-cells, monocytes/macrophages, B-cells and plasma cells. In addition, autoreactive antibodies and inflammatory mediators, released by macrophages and microglia, such as nitric oxide, glutamate and proteolytic enzymes contribute to a profound inflammation, demyelination and axonal damage.

The traditional view has been that axonal damage is a consequence of chronic inflammation and demyelination. However, the neurological decline in absence of relapses, the radiological signs of a lesser amount of inflammation, and the lack of response to traditional treatment during progressive MS phases, suggest that also other non-inflammatory disease mechanisms might be operative (89-91).

An alternative view of MS immunopathogenesis is that MS rather is a primary CNS degenerative disease with secondary inflammatory demyelination (91). Failure to regulate such immune response could result in superimposed CNS-directed inflammatory injury with increased neurodegeneration. A quantitative neuropathologic study investigated axonal injury in the NAWM from different MS subgroups and revealed evidence for ongoing acute axonal damage in sites remote from active demyelination which supports this theory (92). Moreover, extensive axonal loss in areas not affected by demyelination or inflammation have been found (93) and the development of brain atrophy independent of focal lesions is also in accordance with this hypothesis (94) In addition, histopathologic findings, suggest that an oligodendrogliopathy may be an early event in the MS disease process and apoptosis of oligodendrocytes has been described as an event preceding macrophage invasion in acute MS lesions (95).

Interestingly, the common stated relationship between inflammation and neurodegeneration in MS, as occurring in series, has recently been challenged by findings of common molecular pathways being able to bring inflammation and neurodegeneration together. These results suggests that the two processes might be parallel events rather than occurring one after the other (96) .

2 CEREBROSPINAL FLUID

CSF surrounds, permeates, flows and forms an integral component of the brain and spinal cord. It is produced from arterial blood by the plexus choroideus, in the lateral, third and fourth ventricles and to a minor degree by the ependyma and in the perivascular spaces and involves a regulated process of diffusion, pinocytosis and active transfer (97, 98). The total volume of CSF in the adult is about 140 ml located in the ventricular system, subarachnoid space, brain parenchyma and spinal canal and is turned over 3-4 times /day (99, 100). The major functions of CSF is, besides mechanical protection, to provide circulation of metabolites, toxins, nutrients and signaling substances and to compensate for changes in the intracranial pressure (101). In addition, CSF circulation can be regarded as the functional equivalent of the lymphatic system since there are no lymphatic vessels in the CNS (102).

Consensus on CSF circulation and absorption has recently been revised. According to the old model, “the bulk flow model” there is a consistent net flow of CSF from the production site i.e. plexus choroideus to the absorption site which here refers to the arachnoids villi that extend into the cerebral venous sinuses (103-105). Greitz *et al.* more recently presented a new theory; “the hydrodynamic flow model” which proposed that the main absorption of CSF occurs through the brain capillaries in the subarachnoidal space and that circulation is maintained by intracranial arterial pulsations (106).

2.1 CSF ANALYSIS IN HEALTHY PERSONS

The performance of lumbar puncture and CSF analysis was introduced by Cotugno who already in the early eighteen century removed CSF from dead human bodies (107). In 1891, Quinke was the first to develop a technique of spinal puncture, which has changed only little since then, and introduced the procedure into clinical practice (101).

Normal CSF mainly consists of water, glucose and protein (primarily albumin), immunoglobulins and a very low number of mononuclear cells. CSF proteins originates to 80% from serum proteins and to 20% from proteins synthesized in the plexus choroideus by the leptomenigeal cells (108). The concentration of these proteins gradually increases from the ventricular spaces to the lumbar CSF due to

the gradual influx of proteins during its way from the plexus choroideus to the lumbar spine. In addition, CSF also contains a low level (less than 1%) of brain specific proteins such as S100b and neurofilaments and in contrast to the other proteins, their concentration are equal or decreased in the lumbar region compared to the ventricular spaces (109). Under normal conditions, there is no intrathecal production of immunoglobulins and all CSF immunoglobulins are derived from the blood (110).

2.1.1 The cellular compartment of healthy CSF

Normal CSF contains a very low number of mononuclear cells, (reference: 1-5 cell/ mm³) which mainly represent lymphocytes apart from a few monocytes. Most of the lymphocytes correspond to memory CD4+ cells or, to a lesser amount, memory CD8+ cells which both have a high expression of several adhesion molecules (111, 112). B-cells are rarely found in healthy persons (113).

The leukocytes derive from the peripheral blood and enter the CSF via the choroid plexus or via extravasation in Virchow-Robin perivascular spaces (114).

2.2 BARRIERS BETWEEN THE BRAIN, BLOOD VESSELS AND CSF

Many large molecules are unable to pass from the blood into the CSF or into the interstitial fluids of the brain even though the same molecules pass easily into the usual interstitial fluids of the body (115).

The BBB is an endothelial barrier between cerebral capillaries and neuronal tissue (115). The endothelial cells here, lack fenestration and are connected by unique tight junctions which inhibits any unspecific passage or paracellular diffusion across it, maintaining the homeostasis within the CNS (115). The BBB is also composed of the capillary basement membrane, astrocyte end-feet ensheating the blood vessels and pericytes embedded within the base membrane (116). Specific transport systems mediate the directed transport between the blood and CNS which therefore also disable the transfer of many drugs into the CNS (117).

The blood-CSF-barrier is the epithelial layer of the plexus choroideus in which the blood is filtered and secreted to form the CSF (97, 98). Disintegration of this barrier can be detected as an increased level of albumin and immunoglobulin concentration in the CSF (118). Reiber was the first to describe that the CSF-albumin/serum-albumin quotient can be used as a measure of blood-CSF barrier dysfunction since albumin is only synthesized in the liver (118) and not in the brain.

The barriers limit immune cell trafficking into the brain, under normal conditions leukocytes enter the brain across the choroid plexus or via extravasation of meningeal vessels into the subarachnoid space and the Virchow-Robin perivascular spaces. During neuroinflammation as in MS, the properties of the BBB are changed and the upregulation of adhesion molecules on leukocytes make it possible for them to transit directly cross the BBB (116, 119, 120).

2.3 CSF ANALYSIS IN MS PATIENTS

Lumbar puncture and CSF analysis of MS patients has been recommended as standard analysis in the diagnosis of MS (121) although the information thereby provided, is only supportive and not itself diagnostic. MS patients have a normal or slightly elevated CSF albumin level although large spinal cord lesions might result in an extreme elevation ($> 2, 5$ mg/l). Further, a modest mononuclear pleocytosis is usually found (rarely more than 50 cells/mm³), especially if the sample is obtained during a relapse. The cells represent mainly CD4+ lymphocytes. An increased ratio of CD4+/CD8+ compared to peripheral blood and to healthy CSF has been demonstrated. In addition, MS-CSF also contain a small number of monocytes, macrophages, B-cells and plasma cells (122). Similar to healthy persons, the majority of CD4+cells are memory cells. Increased expression of activation and adhesion molecules as well as secretion of pro-inflammatory cytokines and an increased number of myelin-antigen reactive T-cells have been detected in the CSF from MS patients (123-125). Since healthy CSF also comprises T-cells with upregulated expression of adhesion molecules (although not as extensive as in MS-CSF), it has been suggested that these cells are selectively recruited to the CNS compartment under both pathological and healthy conditions (112).

2.3.1 CSF oligoclonal bands

Intrathecal production of immunoglobulins, visualized as OB or raised IgG index in CSF is a diagnostic hallmark of MS and OB can be found in 90-95% of all MS patients while a raised IgG index usually is found in approximately 70% of MS patients (126). The oligoclonal bands represents antibodies produced by clonally expanded memory B-cells and plasma blasts/cells which antigen specificity, however, still is largely unknown (127). Detection of OB is considered as a more sensitive test for the diagnosis of MS, compared to a raised IgG index (128) though

the specificity for MS is low and presence of OB can be seen in a wide variety of inflammatory and non-inflammatory disorders (129).

3 BIOMARKERS IN MS

3.1 WHAT IS A BIOMARKER?

A *biological marker* or *biomarker*, is “a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (130). A *clinical end-point* is a “characteristic or variable that reflects how a patient feels, functions or survives” and a *surrogate end-point* is “a biomarker intended to substitute for a clinical end-point and expected to predict clinical benefit (or harm, or lack of benefit) based on epidemiologic, therapeutic, pathophysiologic or other scientific evidence” (130). The ideal biomarker should be reproducible, reliable, simple to use and non-invasive (131). Biomarkers can be used for several purposes including diagnosis, screening, evaluation of risk/predisposition of a disease, prediction of prognosis and for monitoring disease and response to treatment.

3.2 WHY IS IT IMPORTANT TO FIND BIOMARKERS IN MS?

The most important goal in MS research and treatment is to avoid future disability in each patient or even better, to be able to predict who is at risk for severe disease and in the long run be able to completely prevent disease. MS patients have a highly heterogeneous clinical and neuropathologic picture and therapeutic response. Since MS is usually diagnosed in young persons, the uncertainty of disease progression might influence personal and professional decisions of the patients why prognosis prediction is important. At present we have only limited methods to measure disease activity and to foresee the prognosis. It is therefore of utmost importance to find biomarkers for monitoring of the clinical course and response to disease-modifying therapies and to better predict and prevent development of exacerbations and disability. In addition, biomarkers reflecting ongoing pathological processes within the CNS are needed for a better understanding of the pathological mechanism behind the disease. Also, reliable surrogate end-point markers could provide information in a shorter time than would be needed by following the clinical course of the disease (130) which is important in clinical trials where determination of the therapeutic response requires a relatively long clinical follow up.

3.3 CURRENT CLINICAL AND PARACLINICAL MEASURES OF DISEASE ACTIVITY

Today common measures of disease activity in MS are a) clinical evaluation including number of relapses/ patient year, clinical scales i.e. the Kurtzke Expanded Disability Status Scale, (EDSS) (132), the MS Functional Composite Scale (MSFC) (133), and the MS Severity Scale (MSSS) or disease progression index (134) and b) paraclinical indicators including number of MRI lesions and measurements of brain atrophy (135). However none of these measures have proven optimal as clinical endpoints (136,137). Measuring the frequency of relapses/ year is merely a partial reflection of disease activity since only lesions occurring in articulate areas of the brain give rise to clinical symptoms. MRI studies have reported that for each clinical episode, approximately ten new clinically silent MS lesions had been developed. (138,139).

The non-linear EDSS scale has several disadvantages (140) functioning as an impairment scale in the lower end while the upper end rather represents a disability scale or ambulation index. In addition, EDSS has only limited assessment of upper limb function and cognitive deficits (140). Other limitations with clinical scoring scales are that they claim follow up during longer periods to identify changes, that they are based on the subjective assessment by the physician, and that they are labor intensive.

Measurements of brain atrophy and the number of T1 lesions on MR imaging can be used as a rough surrogate marker for neurodegeneration (42, 94, 141) although smaller axonal damages are not visible and MRI activity do not necessarily correlate with disability (142, 143).

3.4 PROGNOSTIC FACTORS OBTAINED FROM CLINICAL OBSERVATIONS

The clinical course of MS is highly variable and difficult or impossible to predict for an individual patient. However, several factors have been established to predict a shorter time to reach more advanced levels of impairment (EDSS 6) such as male gender, older age at onset (>40 years), motor, cerebellar or sphincter symptoms at initial presentation, poly-symptomatic disease at onset, frequent relapses within the first five year, short time to reach an EDSS level of 4 and a progressive course (144-147). Runmarker *et al.* followed a large cohort of Swedish CIS and MS patients for 25 years and observed that presence of efferent tract lesions

and incomplete recovery after relapses early in the disease course where early predictors of an increased risk for development of secondary progressive course and a high degree of disability (148). All factors that predict progression or disability over time appear to be operative only until the EDSS score 4 is achieved. Thereafter the course is independent of baseline predictive factors in the majority of patients (149).

3.5 BODYFLUID BIOMARKERS IN MS

3.5.1 Cerebrospinal fluid

CSF is considered the most adequate body fluid for biomarker studies in MS because of its close proximity to the CNS which yields an accurate reflection of ongoing pathology of the brain, spinal cord and meninges. MS lesions are typically located in the periventricular white matter of the brain and in superficial areas of the spinal cord with close relationship to the CSF space.

A limitation with CSF collection is the invasiveness of lumbar puncture which makes longitudinal studies with selection of samples at several time-points difficult. Many inflammatory and neurodegenerative biomarkers in MS are only released intermittently (150) and the optimal strategy would therefore be repeated CSF sampling and analysis. The sensitivity to detect CSF biomarkers derived from MS lesions depends on the topographical distance between the lesions and the CSF compartment (151). Moreover, CSF is not produced at a constant rate, the maximum production rate occurs at midnight and falls until noon (152). The volume of sampled CSF and a rostrocaudal concentration gradient might also influence the concentration of CSF proteins (153). Brain derived CSF proteins are however not influenced by changes of blood-CSF permeability, which is in contrast to proteins synthesized also outside the CNS, for which changes from normal blood-CSF permeability must be taken into account during analysis (118). If measuring proteins that potentially originate from both blood and brain compartments, both CSF and peripheral blood should be analyzed.

3.5.2 Peripheral blood

Sampling of peripheral blood is less invasive compared to CSF and facilitates longitudinally studies and frequent sampling with short intervals. It is also much easier and more ethically correct to collect blood from healthy individuals for

control samples. In addition such a control group is much more homogenous than current used control groups in biomarkers studies, which usually consists of patients with other non-inflammatory neurological disease. Another advantage of using peripheral blood is that sampling (in most countries) can be performed by both nurses and physicians of all sub-specialties while lumbar puncture is usually only performed by a subgroup of physicians. However the major disadvantage with peripheral blood analysis in MS is that the disease pathology is restricted to the CNS which is separated from the periphery by the BBB. In addition immunological markers in the peripheral blood may lack specificity since they might be altered by systemic events.

3.6 CLASSIFICATION OF BIOMARKERS

There are several ways to classify biomarkers and one common approach is to characterize them according to their pathophysiological mechanisms (135) which usually separates them into markers for 1) immune system alterations 2) blood-brain-barrier disruption 3) demyelination 4) oxidative stress and excitotoxicity 5) axonal/ neuronal damage 6) gliosis and 7) remyelination and repair.

Other researchers (137) have a more clinical approach and subgroup them into 1) disease predictive biomarkers, 2) diagnostic biomarkers, 3) biomarkers associated with disease activity and 4) biomarkers for therapeutic efficacy. The latter is the classification that will be used in the discussion below.

3.6.1 Disease predictive biomarkers

Genetic markers, i.e. certain variants of a specific gene or gene region, that associates to increased risk of MS can be regarded as disease predictive biomarkers although they are not biomarkers in the strict definition of the term (137). The strongest MS risk loci is at present, the HLA class II haplotype DR15-DRQ6 followed by the HLA class I haplotype HLA-A (154). Some of the most convincing MS risk genes outside the HLA-complex are alleles for the IL7R, IL2RA, CD58, CLEC16A, KIF1B, CD6, IRF8 and TNFRSF1A genes (71, 73, 155-157).

It is essential to explore what biological effects these genes have and if the allelic risk variant causes any biological difference of the protein it encodes and if this has any consequence for disease mechanisms in MS. An example is the risk IL7R allele associated to MS which produces a difference in the proportion of soluble/non soluble IL7R protein (further discussed in the “Results and discussion”

part). Several attempts to find an association between the HLA risk variant and different clinical MS phenotypes have mostly been unsuccessful. Both HLA-DR1*B15 and HLA-A*02 have similar frequency in benign or severe MS and in PPMS versus RRMS (68, 76, 158). In contrast, it has been reported that MS patients carrying the DRB1*15 allele had earlier age at disease onset compared to non-carriers (76, 158).

Risk evaluations based on genetic variants should also take environmental factors into account and possibly also include paraclinical data such as MRI findings, presence of OB in CSF and the immunological phenotype in order to increase the power of prognosis prediction in MS (159).

Numerous studies show that early initiation of disease-modifying therapeutics in CIS patients delay conversion to MS (160, 161). Therefore, if well characterized disease predictive risk markers can be defined we could possibly provide a better clinical outcome for the patients. Such markers would be specifically useful in patients with a higher risk to develop MS. Studies on CIS patients have shown that presence of OB in their CSF, doubles the risk for development of definite MS, independently of MRI findings at onset (162).

Proteomic analysis of MS-CSF is a relatively new approach to discover new candidate biomarkers in MS using mass-spectrometry methods. Several proteins have been identified exclusively in MS-CSF compared to normal CSF but these finding must be validated with other methods in order to estimate their value as biomarkers for MS disease activity. Tumani *et al.* reported up-regulation of a protein (serineprotease inhibitor) and downregulation of nine proteins (i.e. Fetuin-A, apolipoprotein A4 and haptoglobin) in CSF from CIS patients converting to clinically definite MS within two years compared to nonconverters (163).

3.6.2 Diagnostic biomarkers

There is currently no diagnostic marker for MS although presence of oligoclonal bands in CSF is highly characteristic for MS (126, 164). Lennon *et al.* recently identified an autoantibody, (NMO-IgG) binding to aquaporin 4 (AQP4), in sera and CSF of patients with neuromyelitis optica (NMO) (165). Detection of this antibody can be used to distinguish this rare diagnosis from MS and might be considered the first biomarker defining a previous subgroup of MS-like patients as a separate entity (165).

Elevated levels of kappa free light chains (KFLCs) in MS-CSF has been observed and Presslauer *et al.* have reported that presence of KFLCs in CSF has a sensitivity of 96% and specificity of 86% for the diagnosis of MS (166, 167).

Neuropathological classifications of different subtypes of MS patients indicates that antibodies play a more important role in a subset of MS patients (29). This group of patients might be distinguished by a presence of a higher level of anti-myelin antibodies in their CSF (Paper III) and is important to recognize since they might have a better response to therapeutics targeting B cells.

Differences in microRNAs (miRNAs) profiles in peripheral blood cells was recently found to differentiate RRMS patients from healthy persons with high accuracy suggesting that such analysis might be a potential diagnostic biomarker for RRMS (168).

3.6.3 Biomarkers associated with disease activity

3.6.3.1 Inflammation

Inflammatory mediators such as interleukins, adhesion molecules, chemokines, selectins, nitric oxide metabolites and antibodies have all been investigated for its potential as biomarkers for disease activity in MS (169). Up-regulation of the pro-inflammatory cytokines IL-12 and IL-6 and down-regulation of the anti-inflammatory cytokines IL-4 and IL-10 and the chemokines CCL5 and CCL2 (137) have been found in CSF and plasma during relapses. Recently, Sellebjerg *et al.* demonstrated that the B-cell chemokine CXCL13 was increased in CSF in MS patients and CIS patients compared to controls (170). Osteopontin is a proinflammatory cytokine highly expressed in MS lesions and involved in recruitment of proinflammatory cytokines to the CNS and induction of relapses (171). Plasma and CSF levels of osteopontin were found to be increased in active MS but did not correlate with disability in MS patients (172-174).

Nitric oxide (NO) is a free radical which is recognized as part of the innate immune system. Excessive NO production has been discovered in numerous inflammatory conditions (175-177). Inducible nitric oxide synthase converts arginine to citrulline and nitric oxide (178) and this enzyme is upregulated in MS lesions (47, 179). In fluids, NO is rapidly oxidized to nitrite and nitrate and in the absence of heme, such as in CSF where the oxidation is slower, the nitrite/nitrate levels can serve as indicator of ongoing inflammation. Svenningsson *et al.* and Brundin *et al.* analyzed nitric oxide metabolites in MS patients and found increased

nitrite levels which correlated to clinical disease activity (180, 181). Measurements of nitric oxidation products as a biomarker for inflammation, is of clinical interest, the limiting step today is however, the fast oxidation which calls for extremely fast handling of the samples.

Expanded CD8+ cell clones have been observed in CSF and blood of MS patients and are frequent cells in active demyelinating MS lesions. CD8+ cells mediate cytotoxicity partially by granzyme release and it has been shown that levels of granzyme-A and B are elevated in the CSF of RRMS patients and correlates to neurofilament light chain levels during relapse, which indicates their value as inflammatory biomarkers (182).

A number of studies have investigated the presence of different myelin protein-specific antibodies in serum and CSF and their correlation to disease activity. The group of Berger(183) demonstrated that detectable serum antibodies against myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP) in CIS patients predicted a rapid conversion to clinically definite MS. However repeated analysis failed to replicate these findings(184) (185).

Potential biomarkers for an increased permeability of the endothelial BBB are the muscle protein dysferlin and the matrix-metalloproteinase MMP-9. Both markers have been shown to correlate with disease activity (186, 187). Up-regulation of the soluble intercellular adhesion molecule ICAM-1 in MS-CSF have been found in MS patients sampled during relapses and correlated with MRI activity and dysfunction of the BBB (188).

Vascular endothelial growth factor A (VEGF-A) is an important regulator of vascular permeability and increased levels in peripheral blood in MS patients during relapse has been reported (189, 190). VEGF-A also exerts important neuroprotective and neurotrophic effects and, as discussed in Paper IV, we have observed decreased levels of VEGF-A in secondary progressive MS in both CSF and peripheral blood, and suggest that decreased levels of VEGF-A is a new peripheral blood biomarker for SPMS(191).(See Paper IV for further discussion).

Glia cells are activated early during inflammation in MS, Glial Fibrillary Acidic Protein, (GFAP) is an astrocyte-specific structural protein and the main protein constituent of chronic MS plaques. Analysis of CSF GFAP demonstrated an increased level of GFAP in MS-CSF and a correlation to clinical deficits and disease progression (192, 193). The calcium binding protein S100b is another glia cell

specific protein, found in astrocytes and schwann cells, which have been found to be elevated in MS-CSF particularly during relapses(194).

3.6.3.2 Demyelination

The myelin basic protein is found in the inner layer of myelin and during demyelination, MBP is released into the CSF. Its concentration has been shown to correlate with the degree of active demyelination (195) and CSF-MBP levels was found to increase during relapses (196). Barkhof *et al.* demonstrated that MBP levels correlated with MRI Gd-lesions and that corticosteroid therapy reduced MBP levels (197). Presence of anti-MOG antibodies in serum in CIS and MS patients have been found and was suggested to serve as markers for detection of early inflammation and/or CNS autoimmune demyelination (198).

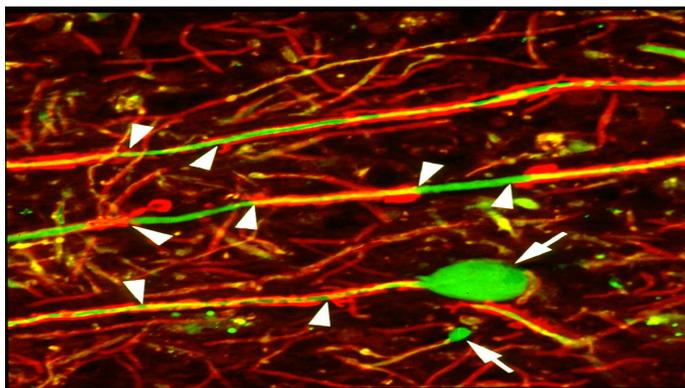
3.6.3.3 Neurodegeneration

Biomarkers for axonal damage in MS would be useful as indicators of overall disease burden and could potentially predict future disability. Well studied candidate CSF markers for neurodegeneration are the cytoskeleton protein neurofilaments and tau protein. Neurofilaments are intermediate filaments and form an important component of the axoskeleton and compose three subunits: a light (NfL), intermediate (NfM) and heavy chain (NfH). Trapp *et al.* performed pathological studies on MS lesions and demonstrated different stages of axonal damage, with the use of antibodies directed against neurofilaments (33) (Fig 1). Several studies have demonstrated increased NfL and NfH levels in MS-CSF. (Paper II), (199-202). Increased levels of NfL have been found to correlate with relapse rate and the degree of disability (192, 199). Interestingly, Norgren *et al.* investigated the prognostic value of CSF NfL levels and observed that high NfL levels correlated with clinical progression (203). CSF studies on the neurofilament heavy chain have given evidence for it as a marker for permanent disability and neuroaxonal loss (204) (Paper II). In addition elevated levels of antibodies against neurofilament proteins in CSF have been detected in MS patients (205). Other important components of the axonal cytoskeleton are the microtubule proteins. Tau protein is a microtubule-associated protein and increased levels in both RRMS and progressive MS has been found (206) One research group reported correlation of tau protein and poor outcome in RRMS patients (207) while other studies report similar levels in MS patients and controls. Other microtubule associated proteins are the actin and tubulin

proteins which were reported to be increased in SPMS compared to RRMS and to correlate with EDSS (202). These markers might therefore be used as indicators of disease progression. The neuron-specific amino acid N-acetylaspartate (NAA) has been widely used in Magnetic Resonance Spectroscopy (MRS) as a marker for axonal damage (208) and recently it was shown that SPMS have decreased levels of CSF-NAA compared to RRMS and controls and that the decrease of CSF-NAA correlated to clinical disability and MRI measures of disease (209).

Cholesterol is the main lipid in cellular membranes including the axonal membranes. The metabolite 24S-hydroxycholesterol is specifically produced in the brain by a neuron-specific enzyme. Neurodegeneration with a significant loss of neurons and thereby loss of this enzyme, are associated with a decrease of 24S-hydroxycholesterol in the plasma since the vast majority of this metabolite is excreted through the BBB into the circulation. Consequently, decreased levels of plasma 24S-hydroxycholesterol has been observed and associated with neurodegeneration in MS (210). By contrast, in parallel, CSF levels of 24S-hydroxycholesterol are increased since a minor level of it is secreted into the CSF and appears to reflect neuronal damage rather than total number of metabolically active neuronal cells (210). Interestingly, increased CSF levels of 24S-hydroxycholesterol has been proposed as a highly sensitive marker for cognitive impairment in MS (210).

Figure 1 Confocal microscopic image of the pathology in an active MS lesion. Biomarker research in MS attempts to measure these events in CSF or plasma.



The image shows three large axons undergoing MS pathology; both active demyelination and axonal damage. Staining of nonphosphorylated neurofilaments (green) demonstrates demyelinated axons, one axon ending in a large terminal ovoid indicating its transection. The red stain indicates myelin (Trapp B. NEJM 1998). *This picture was reprinted with kind permission of B. Trapp and NEJM.*

3.6.3.4 Remyelination and repair

Immune cells in inflammatory lesions release neurotrophic factors which can stimulate regeneration and promote repair in MS lesions. Promising markers for remyelination are the neuronal cell adhesion molecule (N-CAM), growth factor ciliary neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF). CNTF is a factor that promotes oligodendrocyte survival and was together with N-CAM found to be increased in MS-CSF following a relapse with a positive correlation to clinical improvement (188, 211). On the other hand, BDNF seem to be a marker for secondary progression since reduced levels have been reported in SPMS patients compared to RRMS and controls (212). Mattsson *et al.* recently demonstrated that MS patients had decreased activity of the β -site APP-cleaving enzyme 1 (BACE1) in CSF (213). BACE1 is a gene coding for an integral membrane protease involved in myelination (213). It was observed that the activity of BACE1 also decreased over ten years in the RRMS group while the SPMS group displayed constantly low BACE1 activity (213).

3.6.4 Biomarkers for therapeutic efficacy

The increasing number of available therapies for MS, their associated risk of side-effects and/ or severe adverse reactions and the risk of treatment failure in certain patients has increased the importance of more individualized treatment strategies.

Established biomarkers for treatment efficacy are today the neutralizing antibodies (Nabs) to Interferon-beta (IFN- β) and natalizumab which have been reported to associate with reduced clinical effects (214, 215). Repeated analysis of Nabs during IFN- β and natalizumab-treatment is consensus in clinical practice (216). Expression analysis of the myxovirus-resistance protein A (MxA) is also a sensitive measure of the biologic response to IFN- β . It has been observed that IFN- β Nab-positive MS patients have undetectable levels of MxA expression and associates with reduced biologic effect of IFN- β (217). Recently it was also reported that absence of MxA expression in Nab-positive MS patients predict the risk of new relapses (218) and the authors of this study, recommended measurements of MxA mRNA prior to Nab-analysis for monitoring of IFN- β treatment due to its slightly stronger prognostic significance and easier method for analysis (218).

Khademi *et al.* reported increased levels of proinflammatory cytokines in the peripheral blood in MS patients treated with natalizumab suggesting their role as biomarkers for natalizumab treatment response (219). Natalizumab treatment is associated with reactivation of JC virus and development of PML (220). Further discussion on risk markers for PML development is discussed below (Paper I, Results and discussion).

Individual genetic and molecular profiling with the aim to predict the response or toxicity to a drug, i.e. pharmacogenomics, is a growing field with the goal to facilitate individualized drug therapy regimens. Recent results from a genome-wide pharmacogenomic study demonstrated that a certain polymorphism in a gene coding for the glutamate receptor (GRIA3) was associated with the response to IFN- β treatment (221).

At last, it is important to point out that it seems unlikely that a single biomarker will be identified that fulfills all of a surrogate endpoint in MS. In the future, individual MS patients might be screened for a panel of biomarkers, which together can give information on the ongoing grade of inflammation, neurodegeneration and repair, in that specific patient (Table 1). Such information could hopefully optimize individual treatment choice and prevent disability

Table 1 Selected potential biomarkers in MS are summarized below.

MS risk	Inflammation	Neurodegeneration	Repair	Demyelination
<i>Genes:</i>	IL-12, IL-6	Neurofilaments	N-CAM	MBP protein
HLA class II	IL-4, IL-10	NAA	BDNF	Anti-myelin ab:
HLA class I	CCL5, CCL2	Anti-neurofilament ab	C-NTF	MOG, MBP
IL7R	CXCL13	Actin	BACE1	whole myelin
IL2R	Osteopontin	Tubulin		
CLEC16A	NO	Tau		
CD58	MMP-9	24S-hydroxycholesterol		
KIF1B	Dysferlin	VEGF-A		
CD6	Granzyme A, B			
IRF8	Anti-myelin ab:			
TNFRSF1A	MOG, MBP			
	whole myelin			
	ICAM-1			
	GFAP			
	S100b			

4 NATALIZUMAB, JC-VIRUS AND PML IN MS

One of the new MS treatments, natalizumab, is a monoclonal antibody that binds to integrin molecules ($\alpha 4\beta 1$ and $\alpha 4\beta 7$) on lymphocytes and plasma cells which prevents their extravasation into the brain or other tissues with a consequent reduced inflammatory response (222). It has proven to be more potent compared to traditional MS drugs (223). Follow-up studies on the efficacy and safety of natalizumab have demonstrated a reduced annualized relapse rate of 68%, a reduction of brain lesions of 83% and also a modest effect on disability progression (224). Even so, shortly after the first introduction of natalizumab, the optimism concerning this drug was dampened when it appeared that 3 out of 3000 patients treated with natalizumab had developed the fatal disease progressive multifocal leukoencephalopathy, (PML) (225-227).

PML, is a demyelinating disease of the CNS first identified by Åström *et al.* (228) and it is caused by the polyomavirus, John Cunningham virus (JCV) (229). Infection with JC virus usually occurs asymptotically in childhood, in the majority of the normal population (230). After infection the virus establish latency in the kidneys, bone marrow and lymphoid tissues (231-233) and can be found in the urine in approximately one third of both healthy and immunosuppressed adults (234). Some studies have reported that healthy individuals can have low levels of JCV in their peripheral blood while other studies report contrasting findings with absence of JCV DNA in healthy persons (235-238). Under conditions of immunosuppression, the virus can reactivate and enter the brain, where it infects oligodendrocytes establishing a demyelinating disease known as PML (239).

Studies on the reactivated neurotrophic JC viral strain, isolated from PML brains, demonstrate, that this strain has remarkable rearrangements in the regulatory regions compared to the archetypal strain, found latently in the kidney and urine (240). It is therefore assumed that the archetypal strain is altered during reactivation forming a neurotrophic rearranged strain (240, 241).

The most common clinical presentations of PML are visual disturbances, limb weakness and cognitive impairments (242). The onset is usually insidious with slower progression of clinical symptoms compared to relapses in MS where symptoms develop over a shorter time period. Conclusions from a study on clinical and MRI features distinguishing PML from RRMS patients demonstrate that mono-

symptomatic presentations were more common in MS than PML while PML patients more often developed hemiparesis and altered mentation. Spinal cord and optic neuritis were not seen in PML patients in contrast to RRMS patients. Brain MRI scans revealed that PML lesions were localized at the border of the grey and white matter and more frequently in the deep gray matter in PML patients. In addition, gadolinium-enhancement is less common in PML lesions, (Fig. 2) (243).

Throughout history, PML was an extremely rare disease until the HIV-era appeared with concomitant increased incidence of PML (229, 244). AIDS is still the most common PML associated immunosuppressive disease. Other common diseases associated with PML are lymphoproliferative malignancies, immunosuppressive conditions after transplantation, solid tumors and rheumatologic diseases (244-246). Lately it was also reported that PML can develop in patients with minimal or occult immunosuppression (247) which is in sharp contrast to the general notion that PML always is associated with profound immunosuppression.

PML is a fatal disease with a mean survival time of 3-6 months (229). No specific antiviral therapy is today available. In natalizumab treated patients with PML, plasma exchange has been partly successful in treating these patients. Also, Mefloquine, a drug approved for malaria therapy, has recently been shown to have an effect on PML although other researchers doubts its efficacy (248).

The common diagnostic test for PML is detection of JCV DNA in CSF but diagnosis based on brain biopsy material provides higher specificity and sensitivity (249). Routine CSF analysis is usually normal with absence of pleocytosis (250).

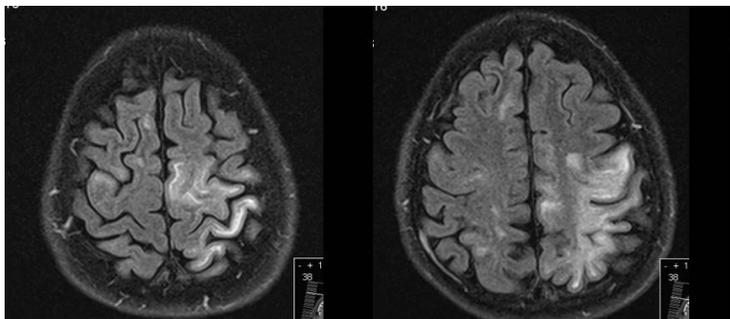
Until the appearance of PML in natalizumab treated MS patients, the disease had never before been reported in MS (251). However JCV has been suggested to possibly be involved in MS pathogenesis, due to its latency, persistence and tropism for oligodendrocytes although no such association has been verified (242, 252).

Prescription of natalizumab for MS is increasing, and at present approximately 64 000 patients are on natalizumab (personal communication, Biogen Idec) which however also have led to an increase of PML cases. At present, January 2010, 31 MS patients (personal communication, Biogen Idec) have so far developed PML after 12-35 infusions of natalizumab which corresponds to an incidence of approximately 1/2000. At our clinic, PML has occurred in one out of 150 MS patients treated with natalizumab. However the worldwide incidence seems to stabilize and it has been estimated that the final incidence will be around 1/1000.

PML development has also been associated to other new biological immunomodulatory treatments including the monoclonal antibodies rituximab, efalizumab and infliximam (229, 253-255). These findings suggest that the total incidence of PML will increase.

The mechanism behind natalizumab mediated reactivation of JCV is not known. Stuve *et al* have demonstrated that natalizumab causes a significant decreased immunosurveillance of the brain which can result in a defect control of JCV replication (256). Interestingly, some studies have reported presence of JCV even in the CNS of healthy immunocompetent individuals (236, 238) which would support this hypothesis. Natalizumab has also effects on the bone-marrow which as well is a major reservoir for latent JC virus. It has been suggested that natalizumab, by inhibiting vascular-cell binding, stimulates the release of hematopoietic stem cells and immature B-cells from the bone-marrow (257, 258). Indeed it was reported that natalizumab treated patients have increased levels of CD34+ stem cells in the peripheral blood (259). Moreover, it has been discovered that immature B-cells contains transcription factors intervening with the regulatory region of JC virus with subsequent transactivation of the virus. These findings have led to the hypothesis that the increased immature B-cell population could increase the amount of reactivated JC virus which might be brought to the CNS and lead to PML(258). Another important observation was that a conserved part of the JC viral strain contains an NFk-binding site to which intracellular cytokines can bind which results in viral replication. Since many cytokines are up-regulated in MS brains, this might also be part of the process leading to PML development (241).

Figure 2



Brain MR imaging of typical PML lesions in a MS patient developing PML during natalizumab treatment.

5 AIMS

The major aims of this thesis were:

- 1 To establish a multiple sclerosis biobank and corresponding database.
- 2 To characterize the MS patients and controls according to clinical and paraclinical parameters and to continuously validate the data.
- 3 To use the biobank and database for studies on different biomarkers in MS.
- 4 To use the biobank and database for investigations of treatment complications in MS.

6 METHODOLOGICAL CONSIDERATIONS

The methodological part of my thesis can be divided in two major parts: First, I have during the years continuously been working with the biobank for updating of the database. Secondly I have performed laboratory work, as part of the biomarker studies. Selected methodological principles and considerations will be discussed.

6.1 ESTABLISHMENT OF A BIOBANK AND DATABASE

A biobank is a well-organized stored collection of biological samples with associated clinical and paraclinical data. The samples can include any kind of tissue, fluid or other material that can be obtained from an individual and should be acquired by informed consent. The collection of data for the biobank is conducted as a parallel process to the sample collection. The value of banked biomaterial is much dependent on the quality of the clinical data. Biobanks are essential for research aiming at characterizing diseases from epidemiological, diagnostic and prognostic views.

6.1.1 Collection of body fluid samples

We started in 2001 to collect CSF and peripheral blood samples from patients with suspicion of MS, definite MS and patients with signs of, or definite diagnosis of other neurological disease. Most samples were obtained when the persons underwent lumbar puncture and blood sampling as part of routine neurological diagnostic assessment at the neurological clinic, Karolinska University Hospital, Solna. A few samples have also been collected from patients at the neurological clinics at Huddinge and Danderyds Hospital. Informed consent was achieved from all patients and the biobanking was approved by the local ethical committee.

As standard procedure we collected 20 ml of peripheral blood and 20 ml of CSF obtained by lumbar puncture between the L4/L5 or L3/L4 intervertebral space. The samples were processed within one hour, with a few exceptions of two hours. We prepared peripheral blood mononuclear cells (PBMCs), plasma, CSF cells and cell-free CSF samples in coded tubes which were stored at -80°C until use.

6.1.2 Characterization of patients - creation of a database

The creation of a database started at year 2001 but was intensively updated in details from spring 2006. All patients were characterized according to the data listed in table 2. The clinical and paraclinical data were obtained from paper and computerized medical reports. Laboratory data was collected via the medical reports or directly from the laboratory of clinical chemistry at Karolinska Hospital or Danderyds Hospital. MRI data was obtained mainly from written interpretations of Neuroradiologists or by my interpretation of MRI scans. The work with the biobank and database is continuously proceeding. We collect CSF and blood samples from 4-8 new patients every week.

Table 2 Parameters registered for each patient in the database

Diagnosis/subdiagnosis at sampling and follow up
Age at sampling
Gender
CSF analysis: cells, albumin, OB and IgG index
EDSS at sampling and follow up
Relapse/remission at sampling
MR imaging, preferable at sampling
Treatment, ongoing and previous
Ethnicity
Heredity
HLA class II genotype

6.1.3 Standardization of CSF biomarker research

Several reviews on CSF biomarker research in MS show that this field lacks adequate power mainly due to insufficient numbers of CSF samples obtained in single-centre studies (135). Therefore, collaboration between research groups is important for establishing larger biobanks of well defined samples. Standardization of collection protocols is crucial to set up such a uniform biobank in order to be able to compare the samples from different locations within one study. Our research group is a member of the European network for Biomarkers in MS (BioMS, www.bioMS.eu). This is an association of 14 research groups in Europe aiming for standardization of sample and data collection and provides collaboration between members.

We have together developed a consensus protocol (128) regarding CSF procedures and data processing which points out that:

- At least 12 mL CSF should be obtained and the location (lumbar or ventricles) should be documented since both factors can influence the concentration of the biomarker.

- CSF samples with an erythrocyte count of more than 500 /mL should not be used for biomarkers studies since the content of peripheral blood can lead to false results.

- The time delay between CSF withdrawal and freezing should be maximum 1,5 hours although CSF processing is not as sensitive as blood. By contrast, samples of CSF cells should be processed as soon as possible since the cell numbers decrease quickly.

- The CSF samples should be stored at -80° C in small aliquots since repeated freeze/thaw cycles can influence the biomarker concentration.

- The protocol must allow for exchange of samples and relevant patient information and consent.

Our consensus protocol is expected to facilitate large biomarker studies and collaborations between research groups. Inappropriate sampling, transport, handling, and storage of samples as well as errors in data entry, recording, and withdrawal may all cause results that are irreproducible and, more importantly, unreliable. Through this network we established collaboration with the group of Dijkstra/Teunissen in Amsterdam which resulted in a study about CSF- NAA and cytoskeleton biomarkers (Paper II) and another study on CSF anti-myelin antibodies (Paper III).

6.2 LABORATORY PROCEDURES

6.2.1 Real-time RT-PCR

Real-time RT-PCR is an established method used for quantification of a total gene product (DNA or RNA) in tissues or cell extracts using the PCR method. It is highly sensitive and allows high-quality reproduction and quantification even of genes with low mRNA expression. In this thesis, real-time RT-PCR was used for quantification of JC virus DNA (Paper I) and expression analysis of VEGF-A and monocytemarker mRNA levels (Paper IV) and IL7/ IL7R mRNA levels (Paper V) in MS patients and controls.

RT-PCR is quantitative and measures the amount of fluorescent PCR product continuously throughout the reaction, i.e. “in real time”. For analysis of mRNA, the RNA is first transcribed into the more stable cDNA. The cDNA is amplified with PCR, with specific primers and the fluorescent dye SYBR Green or fluorescently labeled sequence specific probes e.g. TaqMan probe.

SYBR Green is a general dye that binds to double-stranded DNA and easy to optimize and use for different genes at lower cost. However contamination of DNA must be avoided and high PCR specificity is required since nonspecific PCR products and primer-dimers can contribute to the fluorescent signal. Also the primers must be designed to be highly specific.

The TaqMan probes are sequence specific oligonucleotide probes that carry a fluorophore (reporter) and a quencher dye. The fluorophore is attached at the 5' end of the probe and the quencher dye is located at the 3' end. When the probe binds to the target the reporter is cleaved off by the Taq DNA polymerase and emits its fluorescent signal. The specificity of the TaqMan probe is higher compared to SYBR Green as three different unique sequences need to bind to the target in order for fluorescence to be detected (primers and probe). A drawback of this method is that specific probes have to be designed and produced for every target to be detected.

During the PCR reaction, every time the template is copied, the fluorescence signal doubles. Each sample has a specific threshold value which is equal to the PCR cycle where the cDNA has been amplified in enough amounts to yield detectable fluorescence above a defined background of fluorescence. The PCR cycle number at the threshold level (CT value) of each sample is determined and then translated into amount of starting cDNA with absolute or relative quantification. The fluorescence signal is detected by a camera detector within the PCR machine (ABI sequencing system, Applied Biosystems).

6.2.1.1 Absolute quantification

Absolute quantification was used in Paper I. A standard curve was produced using serial dilutions of a JCV viral-plasmid positive control with a known concentration of JC virus DNA. The serial dilutions of the JCV standard were run on the same PCR plate as the patient samples. A standard curve was then generated by plotting the CT values, of the different dilution series, against log of the amount of the standard which gives a linear curve. The CT values of the samples with

unknown JCV DNA where then plotted onto the standard curve and the absolute copy number of JCV DNA in each sample was determined.

The validated TaqMan RT-PCR analysis used for JCV detection was performed at the National Institute of Health (NIH), Maryland, USA, and has a sensitivity of 95% and a specificity of nearly 100% (260). Two sets of primer pairs and probes were used, specific for the reactivated neurotrophic JCV Mad-1 strain, earlier found in PML brains (260, 261). Our detection limit of 25 copies/mL can be considered as sufficient compared to other studies using 1-1000 copies/mL as threshold (249, 262-265). Until 2008, our national lab for JCV analysis, Swedish Institute for Infectious Disease Control (SMI) utilized a detection threshold of 200 copies/mL, this limit has now been lowered to 50 copies/mL. A sample was only defined as positive if JCV copies were found in duplicates. Studies from the near past on patients with PML have reported on a false-negative detection rate for JC virus of 15-25% but virtually no false-positive results (265).

Failure to detect JCV DNA in CSF can depend on laboratory procedures such as selection of primer pairs, the method for DNA extraction which both can differ between different laboratories. It has also been reported that a false negative result can be due to inhibitory factors in CSF which however can be avoided by the use of a JCV DNA positive control in each test, which also was applied in our experiments (266). JCV analysis in CSF performed at early stages of PML disease can also provide false negative results (267). False positive results might appear if there is contamination of peripheral blood in the CSF

6.2.1.2 Relative quantification

Relative quantification (used in Paper IV and Paper V) was based on the standard curve method which means that the ratio between the amount of the target gene and an endogenous reference/ housekeeping gene is determined in each sample. We used GAPDH as endogenous control which is expressed and degraded in constant levels in samples from MS patients and controls. Relating the amount of target cDNA to a housekeeping gene will compensate for variations in sample size, the efficiency of cDNA synthesis and the degradation of cDNA. In Paper V, relative quantification of IL7 and IL7R was also calculated with the comparative delta CT method which relies on direct comparison of CT values and not by a standard curve.

6.2.2 ELISA

The analysis of the relative mRNA expression level has limitations since the mRNA level not always correlate to the protein level why it is valuable to perform complementary protein analysis so as to confirm mRNA results. Enzyme-Linked Immunosorbent Assay (ELISA) was used for protein quantification of neurofilament subunits and tau protein in CSF (Paper II) and VEGF-A protein in peripheral blood (Paper IV) from MS patients and controls. In these assays a monoclonal antibody, for the protein of interest, was pre-coated onto a 96-well plate. Samples of CSF/plasma and a serial dilution of a standard with known concentration of the protein were added into the wells. The protein will then bind to the monoclonal antibody and any unbound substances are washed away. Next, an enzyme-linked polyclonal antibody specific for the protein is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of protein bound in the initial step. The color development is stopped and the intensity of the color is measured.

6.2.3 Genetic association studies

Complex diseases are difficult to study since the genetic and environmental factors do not act equally or independently on the induction of the disease in different cases.

One of the most common methods for genetic studies on complex diseases are case-control association studies which also was employed in the genetic studies in this thesis (268). Association studies aims at unravel gene variants with a higher frequency in cases (persons with the disease) compared to controls, and thereby associated with the disease (268). These studies were until recently mostly performed on specific candidate genes. However lately a new approach was developed, “the genome wide association approach” (GWA). In the GWAs, hundreds of thousands of single nucleotide polymorphisms (SNPs) spread across the entire genome are genotyped in order to screen for risk genes associated with disease (269). A SNP is a DNA sequence variation occurring when a single nucleotide in the sequence differs. SNPs can be obtained from a SNP map of the human genome, performed as part of the “International HapMap Project” providing informaton of allele frequencies in different ethnic groups. In this thesis several SNPs covering the VEGF-A gene (Paper IV) and the IL7R gene (Paper V) respectively was genotyped in a large group of MS patients and matced controls.

Genotyping was performed with two different methods; TaqMan SNP genotyping assay (Paper IV) in which two differentially fluorescence labelled probes are used for discrimination of the two alleles in the PCR reaction. In Paper V, genotyping was performed by the Mutation Analysis Facility (MAF) at Karolinska Institutet using MALDI-TOF mass-spectrometry.

A disadvantage with case-control studies is that genotype and haplotype frequencies vary between ethnic or geographic populations. It is crucial to match the cases and controls in order to get reliable results. Another issue, when performing genetic studies, is the importance of sufficient sample size and power. It has been recommended that genetic studies of 1000 cases and 1000 controls might be a “threshold” for a minimum sample size in order to achieve reasonable power to detect associations in both MS and several other disease (269).

6.2.4 Myelin Flow Cytometry Assay

Detection of anti-myelin antibodies in CSF was performed with a relatively new myelin flow cytometry assay developed by the group of Dijkstra (270) (Paper III). Human CNS myelin solved in an aqueous solution and CSF from MS/CIS patients and controls were incubated in 96-well plates. Unbound CSF proteins were removed by washing. The myelin particles were then incubated with a biotinylated goat-anti-human IgG antibody. After washing the samples were incubated with Alexa 488/594-conjugated streptavidin and subsequently washed again and taken up in FACS tubes. Myelin immunoreactivity in each sample was measured by FACS Calibur (B&D Biosciences, Franklin Lakes NJ, US).

Anti-myelin antibody levels were expressed as the mean fluorescence intensity (MFI). This method enables detection of native posttranslationally modified antigens i.e. with non-linear epitopes and also lipid antigens. It has therefore advantages over previous assays for detection of myelin antibodies that uses peptides or recombinant myelin proteins which all have different post-translational processing compared to *in vivo* systems.

6.2.5 Statistical analysis

Differences in relative mRNA, protein levels and antibody levels were tested for significance with the nonparametric Kruskal-Wallis and Dunn's post test. For two group comparisons, the Mann Whitney U test was used. Correlations between biomarkers and quantitative measures were analyzed with Spearman's rank test and non-linear regression for curve fit. Correlations between biomarkers and qualitative data were performed with the Mann-Whitney test. (GraphPad Prism 3.0, San Diego, CA). In Paper IV, the genetic association was analyzed using the package SNPassoc and Haplowiev 4.0. In Paper V, genetic association was analyzed by single point analysis based on a X^2 test and then logistic regression was performed for single SNPs. Power calculation for detecting association was performed for detecting an OR at least 1.5 (Paper IV) 1.3 (Paper V). Haploview software was used for haplotype analysis (Paper IV, V).

7 RESULTS AND DISCUSSION

7.1 THE BIOBANK

There are nearly 13 000 patients diagnosed with MS in Sweden today and approximately 11 000 of them are registered in the Swedish MS registry. According to the registry, the neurology clinic, Karolinska University Hospital, Solna, is caregivers of 1400 of these patients which represents 44% of all MS patients in Stockholm. The biobank and corresponding database is based on these patients and at present, January 2010, it comprises samples and data from approximately 500 MS patients which roughly represents 16% of all persons diagnosed with MS in Stockholm. In addition we have approximately 200 CIS patients and 700 controls (exact numbers can not be given since some of the diagnosis from 2009 have not been validated yet).

Table 3 No of patients sampled every year.

	Total	RRMS	SPMS	PPMS	CIS	OND	OND.INF
2001	16	1	11	1	0	3	0
2002	124	42	1	0	26	50	5
2003	126	56	5	1	25	35	4
2004	147	47	2	3	22	19	54
2005	186	32	35	3	30	26	60
2006	171	64	1	0	24	36	46
2007	167	80	2	4	14	34	33
2008	115	34	4	0	18	21	37
2009	150	Not completely validated yet					

All patients sampled from 2001 until today are listed according to their diagnosis.

In Table 3 all patients sampled from 2001 until today are listed according to their diagnosis. In 2001 only 16 patients were sampled since that was the year this project started. We have sampled 115-186 samples every year and 40-50% of them are MS or CIS patients. The CIS patients have been followed and until today, 85% of them have converted to MS within a median of 3 years. 83 % of the MS patients were diagnosed as RRMS, 14% as SPMS and 3% as PPMS, (table 4). The majority of RRMS and PPMS represent newly diagnosed MS patients since the samples were collected during

diagnostic work-up. In rare cases, we have CSF or blood samples from several time points, mainly from CIS or MS patients. The reason for re-sampling CSF has mostly been due to diagnostic difficulties or when the patients were included in a clinical trial requiring repeated CSF sampling.

The majority of the samples were collected in an early stage when the classification of disease was not definite but has later, during time, been clarified. It has therefore been important to continuously update all the parameters of the database. Gender, age, disease activity at sampling (relapse/remission) and EDSS score for the patients is given in the table below.

Table 4 Characteristics of the patients sampled for the biobank, Jan. 2010.

	CIS	RRMS	SPMS	PPMS	OND	OND.INF
Female/male	120/39	217/139	38/20	4/8	146/75	162/77
Mean age*	28	33	55	51	41	37
Relapse/remission	32/127	73/283	-	-	-	-
EDSS*	1.5	2.5	5.5	3.9	-	-

* Median, at sampling

It was important to scrutinize medical reports thoroughly especially when looking for time of symptom onset. In several cases the patients did not announce or remember the first episode of neurological symptoms when asked. Eventually, when the question was repeated, the patient had had time to think about this and could recall earlier episodes of symptoms. Other difficulties with the data collection have been to define whether and when a MS patient has entered secondary progression. It seems that we sometimes delay or forget to state in the medical reports that the patient actually is in secondary progression. Moreover, if it exceeds more than one year between the visits, it can be difficult to determine the correct year for conversion to SPMS.

The purpose of the biobank was originally to use the samples for laboratory analysis including studies on biomarkers and genetic analysis and was not intended for epidemiological research. The samples can however not be used without the clinical data and the work with the database is and has been very important for phenotyping of the patients. A descriptive study of the MS patients in the database today would not give any new information since there are several studies published on this.

However, we have planned to analyze the data on all newly diagnosed MS patients and CIS patients and to compare them to clinical follow-up data after 10 years. It could also be interesting to compare these data to other patient-cohorts from neurological clinics elsewhere.

7.1.1. The control patients

The control patients are patients that presented at our clinic with suspicion of neurological disease, mostly due to their symptoms but also sometimes because of abnormal MRI findings. In our studies we have divided the control patients in two groups. The largest subgroup was patients “non inflammatory” disease or symptoms. These patients either presented symptoms such as sensory disturbances, headache, vertigo or pain suggestive of neurological disease or were diagnosed with a disease which not involved extensive immune-activation such as normal pressure hydrocephalus, cerebrovascular disease or ALS. In Paper I, IV and V this group is referred to as OND, whereas in Paper II this group was subdivided into NIND (non inflammatory neurological disease) and ND (here patients without neurological disease) i.e. patients with suspicion of neurological disease but appeared normal after diagnostic work-up.

The other control group was patients with inflammatory diseases (OND. INF in Paper I, IND in Paper II and III) in which the most common diagnoses were Systemic Lupus Erythematosus (SLE), postpolio syndrome and herpes encephalitis. We also had access to a small group of healthy controls. The work of following these patients to determine and validate their diagnosis and fate was as extensive as following the MS group.

7.2 APPLICATIONS OF THE BIOBANK

7.2.1 Analysis of CSF and CSF cells from patients with MS for detection of JC virus DNA (Paper I)

This study was undertaken in 2004, just after the first announcement that three out of 3000 MS patients treated with natalizumab had developed PML. The report illustrates an example of how the biobank can be used for the analysis of treatment-related side effects.

The objective of this study was to analyze the basal viral load of JCV in CSF and peripheral blood in treatment naïve MS patients and controls. We speculated that the aberrant immune system in MS could lead to higher levels of reactivated JC virus in MS patients compared to controls. We also wanted to investigate if some individuals with MS had an increased JC virus replication and therefore would be at risk for PML if natalizumab treatment was initiated.

CSF and plasma samples from 217 MS patients, 86 CIS patients and 212 controls were analyzed for presence of JCV DNA. In addition, CSF cells and peripheral blood mononuclear cells were analyzed in a subgroup of MS patients and controls. None of the MS patients included in the study had at that time received natalizumab and the majority of patients were treatment naïve.

We found two MS patients with positive JCV in their CSF. One RRMS patient had 25 JCV copies/ mL in CSF cells and one SPMS patient had 103 JCV copies/ mL in cell-free CSF but none of them had detectable virus in their blood. In addition two of the control patients, diagnosed with SLE, had low detectable level of JCV DNA in their plasma. Previous studies on detection of JCV DNA in MS-CSF are discordant, Ferrante *et al.* found 11 / 121 MS patients with detectable JC virus in their CSF but no quantitative analysis was performed (271). Another research group reported positive JCV DNA in 2/42 MS with a mean viral load of 2.1 and 6.7 copies/mL in their CSF (264). By contrast others have failed to detect JCV DNA in CSF from treatment naïve MS patients (262, 272). There are also contrasting results regarding detection of JCV in peripheral blood from MS patients. Some studies report detection of JCV in comparable levels to controls (273, 274) while other researchers have failed to detect JC virus in both MS patients and controls (272). In our study both the JCV positive MS patients and the control patients have been followed clinically without any signs of PML development.

The findings of JCV DNA in two untreated MS patients were surprising but we believe it is less likely that it represents viral persistence in the CNS. We rather suggest that the JCV DNA originates from blood-borne lymphocytes carrying JCV when trafficking the CNS as part of normal physiology. However, if starting the RRMS patient on natalizumab, repeated sampling and JCV analysis should be performed.

Our results suggest that a low copy number of JCV can be found in patients without PML. Consequently, screening of JCV in CSF in untreated MS patients as a measurable risk for PML is not useful. Our conclusion was also based on the

numbers of detectable JCV copies which was considered to be very low compared to levels of viral load in earlier confirmed PML cases (275, 276). However, more recently, analysis on PML cases associated with natalizumab treatment, have demonstrated much lower JCV copy numbers in CSF compared to levels in confirmed PML cases described in older studies (267). One reason for this could be that the earlier patient group, associated with PML, was more immunocompromised compared to MS patients, which allowed the virus to replicate much faster. Moreover, our two Swedish MS patients developing PML during natalizumab treatment, had early in the disease, a negative CSF JCV analysis despite typical clinical and radiological findings of PML (267). In addition, Landry *et al.* have reported on a case with repeatedly false negative JCV detection in CSF despite confirmed PML and a high viral load in CSF (277). This alarming finding was due to JCV genome variability's escaping detection by standard JCV PCR analysis (277).

Repeated analysis of JCV DNA in CSF as a measurable risk for PML, in natalizumab treated MS patients, has been suggested which however not yet has been taken into clinical praxis (278). Chen *et al.* recently suggested that analysis of JCV in the urine could be a screening method for PML risk in these patients (279). They reported that increased levels of JCV DNA in urine was correlated to increased levels in the peripheral blood and decreased activity of the specific immune response against JC virus which thereby could indicate an increased risk for PML development (279). However several other studies on large material of natalizumab treated patients have all reported constant low levels of JCV in the urine and peripheral blood (280). Also it is known that up to 40% of the normal population excrete JC virus DNA in the urine which indicates that JC viraemia is not a reliable predictive marker for PML (251).

Major *et al.* have suggested that examination of plasma for elevated levels of CD34+ stem cells (since they may harbour JCV) and viral JCV DNA might be a better way to identify patients with high risk of PML (281).

In summary, in the future, clinicians will not only have to balance between the more effective but potentially very riskful treatments (natalizumab or other upcoming potent immunosuppressive therapies) and the older safer but less effective agents, in addition we will have to search for adequate methods for treatment monitoring. At present, this monitoring includes frequent clinical and radiological evaluation and repeated CSF JCV analysis if the slightest clinical or

radiological suspicion of JCV infection appears. However, in the future, repeated CSF/ plasma analysis of JCV (perhaps with different sets of primers), peripheral blood analysis of the specific JCV immune response, levels of immature B-cells/CD34+ stem cells in blood and analysis of JCV transcription factors might be part of a safer risk evaluation panel for PML development (Paper I) (220).

At last, one should not forget that the performance of brain biopsies for JCV analysis might in some difficult cases be the fastest way to detect PML.

Further knowledge on the reactivation of JCV and PML development is of great concern. We are currently performing a descriptive study on the characteristics of Swedish PML cases. We have used the national patient registry and identified 133 cases of PML since 1987. This new study might possibly increase the knowledge about how patients on immunosuppressive treatment should be monitored in order to avoid development of PML.

7.2.2 Combination of CSF N-acetylaspartate and neurofilaments in MS (Paper II)

As mentioned previously N-acetylaspartate (NAA) is a neuron specific amino acid and decreased levels of NAA in MRS analysis has been used as a marker for axonal damage in MS (208). The group of Dijkstra/Teunissen, which is co-authors of Paper II, has previously reported that SPMS was associated with decreased levels of CSF NAA compared to RRMS. The group demonstrated a correlation between CSF NAA levels and clinical functioning and MRI measures of disease (209). The present study was a continuation of that work.

We aimed at analyzing if measures of CSF NAA can be used as a biomarker for early axonal injury in MS. In addition we explored the value of CSF NAA as a neurodegenerative biomarker compared to other well characterized markers for axonal damage including neurofilament subunits and tau protein.

CSF samples from the biobank, including 38 CIS, 42 RRMS, 28 SPMS and 6 PPMS were obtained for this study. The controls consisted of three groups: 18 patients with neurological diseases without intrathecal inflammation (NIND), 40 patients with inflammatory neurological diseases (IND), defined as presence of intrathecally or systemic inflammation. The third group included 28 patients without neurological disease (ND) which were persons who presented with symptoms

suggestive of neurological disease but appeared to be normal after diagnostic workup.

We observed decreased levels of CSF NAA in SPMS patients compared to RRMS and a positive correlation between CSF NAA levels and EDSS which confirmed previous published results (209). Since the levels of CSF NAA were comparable in RRMS, CIS and ND patients we conclude that CSF NAA is not a useful marker for axonal damage early in the disease course of MS. This result contrast from some MRS studies demonstrating decreased levels early in the disease course of MS (282). However, MRS measures a local region in the brain while CSF analysis may reflect the pathology of larger CNS areas. In addition, our control group included patients classified as “no neurological disease” (ND) which comprised a very heterogeneous group. All of the ND had been sent to us, for neurological examination, from their family doctors, which indicates that their complaints sustained for a longer period and might have been a reflection of some diffuse structural brain injury, not visualized by MRI.

The decrease of CSF NAA and its correlation with EDSS and the MRS findings (209) (46) may suggest evidence for its value as a biomarker for later stages of axonal damage. In contrast, we found that a few RRMS patients had increased levels of CSF NAA. Since CSF-NAA is produced in neuronal mitochondria, we believe that this finding reflects an augmented mitochondrial activity associated with an increased demand of energy supply in damaged axons during earlier stages of neurodegeneration (33, 208). This is also supported by our observation of an extreme high level of CSF NAA in acute meningitis. It would be of valuable to perform a similar study as this but with healthy persons as controls. Moreover, a follow-up study correlating present CSF NAA levels with EDSS scores after 5-10 years might add information on the prognostic value of CSF NAA.

The most important finding of the CSF neurofilament analysis was that NfL levels were increased in CIS patients compared to ND and that NfL levels were highest in CIS patients who converted to RRMS within 3 years compared to non-converters. This suggest that NfL may be a early and prognostic marker which is in agreement with a previous study by Norgren *et al.* (201). We also confirmed that NfL is higher in RRMS during relapses and in patients with OB as earlier shown (192, 199, 203). The levels of NfH were, in contrast to NfL, highest in SPMS patients and correlated with EDSS indicating its value as biomarkers for irreversible axonal damage, consistent with earlier studies (283).

All biomarkers displayed its specific pattern during MS disease progression with absence of correlation between neurofilament and NAA levels. When combining analysis of all biomarkers in individual patients, a higher number of MS patients with an abnormal axonal damage biomarker level were found, compared to analysis of each biomarker on its own. Sixty six percent of the RRMS patients had an abnormal combined axonal biomarker value compared to 27 % with abnormal levels of NAA and 51 % with abnormal levels of neurofilaments. In the SPMS group, 71% had an abnormal combined axonal biomarker value compared to 43 % with abnormal NAA levels and 50% of abnormal neurofilament levels. These results emphasize the use of combined analysis of these markers in order to better define ongoing axonal damage in individual MS patients.

7.2.3 CSF anti-myelin-antibodies are related to MR measures of disease activity in MS (Paper III)

Earlier studies on the presence of anti-myelin protein antibodies in MS and their relation to disease activity have demonstrated conflicting results. The objective of this study was to take advantage of a newly developed cytometry based assay for determination of myelin specific antibodies in CSF from MS patients and controls. The work was performed in collaboration with the research group in Amsterdam. They had earlier demonstrated that approximately 50% of MS patients had enhanced anti-myelin antibodies in their sera (270).

CSF samples from our biobank were obtained from 37 CIS, 36 RRMS and 29 SPMS patients. The control groups were subdivided in patients with other neurological diseases (OND, n= 17), patients with inflammatory neurological diseases (IND, n= 33) and 22 healthy subjects (HC).

We detected anti-myelin antibodies in CSF from all patients except in the OND group and only 5% of the healthy subjects had detectable anti-myelin antibodies. We defined the threshold for increased anti-myelin antibody reactivity as all values exceeding the average of HC +3 SD which revealed that 46% of CIS, 56% of RRMS, 55% of SPMS and 21% of the IND patients had increased levels.

The findings of an approximately 50% frequency of CSF anti-myelin antibodies in MS patients can be considered as a low value and thereby diminish its value as a biomarker for MS. These levels are however, higher than levels for single myelin

protein analysis reported in several earlier studies (284-286). MOG-specific antibodies have been detected in MS patients in variable frequencies from nearly undetectable to 38% of the patients (284-287). By contrast, using mass-spectroscopy analysis, the presence of serum anti-MBP antibodies was found in 88 % of RRMS patients compared to

9 % in neurological controls (288). However, we believe that our method probably might be more favorable when investigating anti-myelin antibodies in early stages of disease compared to antibodies targeting proteins located deeper in the myelin sheet, such as MBP, since it requires a substantial part of myelin destruction to expose the epitopes to the immune system.

A correlation between anti-myelin antibodies and clinical measures of MS was negative which was not surprising since EDSS is a reflection of axonal damage rather than demyelination. A significant higher number of CIS patients had increased anti-myelin antibodies in comparison to all control groups which suggest that they distinguish CIS from other neurological disease and/or that demyelination occurs early. Both MS and CIS patients with presence of CSF oligoclonal bands had higher levels of anti-myelin antibody reactivity compared to patients without OB.

Interestingly we found that the three patients with herpes simplex encephalitis, in the IND group, had highly increased anti-myelin IgG levels. We do not consider this as a MS diagnostic problem since herpes encephalitis is no major differential diagnosis of MS and the finding may just reflect the general lyses of myelin in this serious infection. One can however hypothesize about its biological significance. Previous studies have reported presence of HSV-1 DNA and mRNA in peripheral blood from MS patients in relapse (289) and herpes virus has been implicated in MS pathogenesis (290-292). Our finding may support the hypothesis that infection with herpes virus might trigger an immunological response targeting myelin which might trigger onset of MS in persons with a MS susceptible genotype.

A positive correlation between number of T2 lesions and gadolinium-enhancing T1 lesions, on MRI scans and anti-myelin antibodies was found which suggests that measures of CSF anti-myelin antibodies can be used as biomarkers for ongoing inflammatory activity. Only a few earlier studies have investigated the relation between MRI lesion burden and anti-myelin antibodies. Lim *et al.* reported no association between levels of anti-MOG and anti-MBP antibodies and MRI findings in CIS and MS patients (185). Another study has reported that CIS patients with

MOG and MBP antibodies had a higher lesion load in the white matter compared to patients without these antibodies (293) although most patients with ≤ 9 lesions where antibody negative while the majority of patients with comparable lesion number in our study were antibody positive which also further concludes that our method may detect myelin autoantibodies early in the disease course. A weakness of this study is that MR imaging was performed up to 2 months before or after CSF sampling since the lesions load could have changed during this time.

7.2.4 Altered monocyte phenotype with decreased VEGF-A in progressive MS (Paper IV)

Vascular endothelial growth factor A (VEGF-A) is a central regulator of vascular permeability and angiogenesis (294, 295) and has important neuroprotective and neurotrophic effects (191). In addition it can function as a pro-inflammatory factor recruiting inflammatory cells and upregulating adhesion molecules (296, 297). All together it is clearly proven that VEGF-A have multiple important effects within the CNS.

Since our group earlier reported a downregulation of VEGF-A mRNA expression in the spinal cord of an animal model of MS, (EAE), and reduced levels of VEGF-A in CSF from MS patients (298), we sought to confirm these results in a larger group of RRMS (n=65), SPMS (n=35) and controls with other neurological disease (n=68). Further we explored the biological significance behind changes in VEGF-A expression in MS patients with different clinical courses.

We confirmed our previous findings and report a three- and eight-fold decrease of CSF VEGF-A in RRMS and SPMS, respectively, compared to controls. SPMS displayed a significant reduction of VEGF-A also in peripheral blood mononuclear cells (PBMCs) while RRMS patients had similar levels as controls. Analysis of VEGF-A protein in plasma revealed comparable levels as VEGF-A mRNA expression in PBMCs. In order to explore if VEGF-A production were subjected to genetic regulation we performed a large genetic association study on 1114 MS patients and 1234 controls analyzing six different SNPs in the VEGF-A gene, located on the chromosome 6p12. However we could not observe any association between VEGF-A gene variants and MS or VEGF-A mRNA expression. These results contrast to studies on the neurodegenerative diseases Amyloid Lateral

Sclerosis (ALS) and Alzheimers disease to which association in the VEGF-A gene has been found which also correlated to decreased levels of VEGF-A mRNA expression (299-301).

Since the MS patients included in the genetic study only consisted of 6,8 % PPMS we were not able to perform meaningful statistical analysis in order to clarify if VEGF-A gene polymorphisms were associated with different subgroups of MS.

FACS sorting on PBMCs from MS patients, followed by expression analysis of VEGF-A in the sorted cell populations revealed that the major source of VEGF-A mRNA was monocytes and to a minor level B-cells and CD8+ cells. The reduced level of PBMCs VEGF-A in SPMS was not a consequence of changes in the peripheral cellular composition, instead we observed that SPMS had an altered peripheral blood monocyte phenotype characterized by decreased expression of the monocytemarkers CD14, CD11b and CD163 which also correlated to VEGF-A expression. Measures of disease severity as EDSS, MSSS, disease duration and number of MRI lesions did not correlate to VEGF-A mRNA levels in PBMCs or CSF accordingly to another study (190).

Two studies have earlier reported upregulation of VEGF-A in peripheral blood sampled from MS patients during relapse (190, 302) which also has been found in other autoimmune diseases during active disease phases (303). These results are in line with our results finding a trend of increased PBMCs VEGF-A in relapse compared to remission. This finding might reflect the well-known proinflammatory effect of VEGF-A, being able to activate proinflammatory cells and increase the permeability of the blood-brain barrier and hypothetically, increased levels of PBMCs VEGF-A might be part of the initiation of a clinical exacerbation (189, 296, 297). In contrast, the observation of a prominent reduction in SPMS suggests that reduced levels of VEGF-A in PBMCs may serve as a biomarker for progressive MS.

Regarding the CSF analysis, both RRMS and SPMS had decreased levels of VEGF-A in their CSF compared to controls which contrast to Proeshold *et al.* who reported upregulated VEGF-A mRNA expression in MS lesions from post-mortem brain (304). However that study only included ten plaques and since it is known that there is a large heterogeneity of MS plaques (305), VEGF-A expression might be different in plaques with different pattern. Also, since CSF measures represent a larger area of the brain and the fact that we repeatedly have found decreased levels of CSF VEGF-A in MS patients support our results.

Based on the evidence for VEGF-A as a neuroprotective and neurotrophic factor, it might be speculated that reduced levels of CSF VEGF-A may, as part of MS pathogenesis, slow down the restoration of injured neurons or contribute to the failed remyelination process (191, 306). Since SPMS is characterized by a prominent neurodegeneration and decreased levels of VEGF-A also has been found in the neurodegenerative diseases ALS and Alzheimer (299, 307) we suggest that this reflects neurodegeneration and not inflammation which also was supported by our observation that VEGF-A did not correlate to the expression of the pro-inflammatory cytokines IFN- γ or IL-23.

In summary, our study suggests that the involvement of VEGF-A in MS is complex. However reduced VEGF-A in PBMCs could serve as a biomarker for SPMS. In addition our findings indicate that monocytes or other mediators of the innate immune system may be investigated as targets for future therapeutics in progressive MS. In order to better elucidate the role of monocytes and innate immune mechanisms in MS we are planning to perform a more detailed characterization on sorted monocyte cell populations from different subgroups of MS patients.

7.2.5 Variation in the IL7R α chain (IL7R) influences risk of MS (Paper V)

This study was performed in collaboration with the group of Hillert. They had earlier reported an association of the IL7R gene, located on chromosome 5p13, and MS susceptibility in a smaller association study which was interesting since the IL7R gene also was identified as one of the candidate genes in the first whole-genome scan in a mouse model of MS, experimental autoimmune encephalitis (EAE)(308). The IL7R gene codes for IL7R α (CD127) which is a subunit of two different receptor complexes, the IL7R and the thymic stromal lymphopoietin receptor (TSLPR). The IL7R is expressed on T and B-cells and IL7-IL7R signaling is crucial for survival and proliferation of T-cells (309). TSLPR is expressed on thymic stromal cells to which TSLP binds with subsequent effects on the maturation and activation of dendritic cells (310). This knowledge warranted us to further analyze the potential of IL7R as a candidate gene in MS and assessed an independent case-control association study on a large material of MS patients and controls.

The IL7R gene was first genotyped with the three SNPs, that earlier had been associated with MS, in a Nordic material consisting of 1820 MS patients and 2634 healthy controls and confirmed their association with MS. Subsequently the whole gene was fine mapped with 15 SNPs in 1210 MS patients and 1234 controls of Swedish origin. We found three SNPs with significant association with MS but only one SNP, located in exon 6 (rs6897932), survived statistical analysis on its independent association with MS. We estimated an etiologic fraction of 12% for IL7R in MS, based on the risk genotype of this SNP (rs6897932).

Next we used the biobank for functional studies and performed mRNA expression analysis of the IL7R and its ligand IL7 in CSF and PBMCs from 75 MS patients and 48 controls consisting of patients with other neurological disease (OND). In addition 20 healthy controls were analyzed for IL7R and IL7 mRNA expression in PBMCs. We found a significant increase in expression of both IL7R and IL7 mRNA in CSF of MS patients compared to controls. The levels of IL7R and IL7 mRNA expression in PBMCs were comparable in both groups. No correlation between mRNA expression levels and the risk genotype of IL7R was observed which was in agreement with another study (311). The result from the expression analysis is important since it reflects a functional difference between MS patients and controls and thereby increases the validity of the IL7R gene as a true MS gene.

When this paper was published in 2007 our findings were confirmed by an accompanying paper of Gregory *et al.* and was at that time the first discovered convincing non-HLA gene associated with MS (69). These results have been confirmed in additional studies including a recent meta-analysis which demonstrated that the marker rs6987932 is one of the top marker for the IL7R gene and was estimated to confer a relative risk for MS of 1.20 ($p=10^{-17}$) (73).

Gregory *et al.* found that the risk IL7R genotype causes an alternative splicing which lead to increased production of the soluble form of IL7R in relation to the membrane bound IL7R (69). The soluble form of IL7R can bind IL7 but lacks the transmembrane domain and the biological function of this complex is not known. It can however be speculated to cause an impaired survival and proliferation of T-cells which might lead to a disturbed T-cell population and accumulation of autoreactive T-cells (312-314). In addition, the IL7R is important for development of regulatory T-cells (Tregs) which are important in suppression of autoimmune diseases (315).

Since it has been shown that peripheral Tregs are impaired in MS it might be hypothesized that this is a consequence of an impaired IL7-IL7R signaling (316). By contrast other researchers have reported that the soluble form of IL7R was over-expressed in progressive MS compared to RRMS irrespective of the genotype (311, 317). These results might be supported by another study reporting that Treg functioning is more affected in RRMS compared to SPMS (318). Nonetheless, additional expression studies in different subgroups of MS patients are warranted in order to better elucidate this matter.

The association of IL7R gene variants and changed expression levels of IL7R and IL7 in MS supports that MS is an autoimmune disease and targeting the IL7/IL7R signaling pathway has recently been suggested as a potential therapeutic for transplantation which perhaps could be considered for MS (319).

8 CONCLUDING REMARKS

We have established a validated MS biobank with clinical and paraclinical data of approximately 500 MS patients, 200 CIS patients and 700 controls. The sampling and data processing was performed according to a standardized procedure which was developed together with members of the European network for Biomarkers in MS, BioMS. This consistency will facilitate reliable results and collaborations between different MS labs.

I applied the biobank for investigation of treatment complications in MS and for studies on biomarkers.

The prescription of natalizumab is expected to increase in coming years, and the parallel introduction of other potent new up-coming MS treatments might anticipate an increase of PML cases to occur. The identification of screening methods for PML development before, or during potent immunomodulatory treatment is of great concern and was attempted in Paper I. This study demonstrated that CSF analysis of JCV in treatment naive MS patients is not sufficient as a method for identifying risk patients, however, repeated analysis might be valuable during natalizumab treatment as a screening process.

There is an urgent need to get trustworthy MS biomarkers in order to determine disease activity and predict neurological disability with the intention of optimizing and individualize treatment. Large well-characterized MS biobanks are essential for biomarker studies.

Our results from Paper II suggests that decreased CSF NAA can be used as a measure of late neurodegeneration, i.e. burnt-out stages in MS but is less efficient for detection of axonal damage during early stages of disease. We also propose that NfL is a marker for early neurodegeneration correlated to inflammation and that NfH reflects late irreversible axonal injuries. Additionally, we conclude that panels of biomarkers increase the possibility to find abnormal levels in MS patients. This result emphasizes the use of multiple biomarker analysis in MS patients for more information on disease activity compared to single biomarker analysis.

In Paper III we demonstrated that detection of anti-myelin antibodies in MS patients can be facilitated by using methods that detects native myelin antibodies which has advantages particularly early in disease when the myelin is less affected. We observed that MS patients had increased anti-myelin antibody reactivity in CSF

and that the levels correlated to MRI measures of MS. These findings suggest that increased CSF anti-myelin antibodies reflect inflammatory demyelinating activity and might assist identification of MS patients more prone to respond to therapeutics targeting B-cells.

In Paper IV we put forward that VEGF-A may serve as a potential new biomarker for SPMS. Decreased levels of VEGF-A, distinguished SPMS from RRMS and controls in peripheral blood mononuclear cells. The downregulation of VEGF-A was found to be a consequence of an altered monocyte phenotype which supports the hypothesis that progressive MS is dominated by innate immune mechanisms and indicates a new target area for future MS treatments. In addition, in relation to the multiple properties that VEGF-A display in the CNS, our findings of robust changes in VEGF-A levels in both peripheral blood and CSF in MS patients, support that VEGF-A might be involved in the pathogenesis of MS and merits further investigations.

Identification of genetic markers, i.e. genes associated with the risk of MS can add knowledge on important pathophysiological mechanisms in MS. In Paper V, we discovered a new MS gene, the IL7R gene which can be considered as a breakthrough in the search for MS genes since it was the first convincing non-HLA MS gene identified. Together with our functional studies, showing increased levels of IL7 and IL7R mRNA expression in CSF in MS patients, and the knowledge that IL7-IL7R signaling has crucial effects on T-cells maintenance, this study also present evidence that MS is an autoimmune disease.

In summary, the outcome of my research work is a large source of body fluid samples and data from MS patients and controls which opens up for a vast number of research studies in a wide range of aspects of MS. Besides this, we contributed to knowledge about the JCV risk and PML in MS patients. Furthermore, several MS biomarkers have been characterized which may increase understanding of disease mechanisms in MS. For clinical praxis, this type of studies may provide information on disease activity and prediction of disability and may facilitate treatment decisions and monitoration in individual MS patients.

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