PART V
GENERAL DISCUSSION
CHAPTER 10
GENERAL DISCUSSION, RECOMMENDATIONS FOR FUTURE RESEARCH AND CONCLUDING STATEMENTS

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GENERAL DISCUSSION

General Objective
The overall aim of this thesis was to provide more insight into the value of quantitative MRI and body fluid biomarkers to explain disease evolution in MS, with a special focus on CIS. This has been approached using several quantitative MRI measures and body fluid biomarkers in relation to patients’ clinical variables. In this thesis we aimed:

To explore the role of increased iron deposition in CIS and MS by MRI quantitation of brain iron using R2* relaxometry.

To investigate the extent of cognitive deficits and their relation to MRI metrics, including MT transfer imaging, in CIS compared to RRMS.

To explore the clinical value of serum anti-MOG antibodies to explain disease activity and progression in MS.

To assess the clinical value of CSF biomarkers for axonal damage, including Nfs and NAA, and markers for neuronal repair, i.e. NCAM, in relation to quantitative MRI-metrics in CIS and MS.

Quantitative MRI biomarkers
Iron deposition in MS
In chapter 2 and chapter 3 we quantitatively assessed brain iron deposition in CIS patients compared to MS using R2* relaxometry. We found increased iron deposition in MS compared to CIS and HC. R2* levels were correlated with age, disease duration, EDSS, and the z-values of mental processing speed. Stepwise linear regression analysis identified gray matter atrophy as the strongest independent predictor of basal ganglia R2* levels, followed by age and T2 lesion load (Chapter 2 and 3).

Increased iron deposition has been described in a variety of chronic neurological disorders, including MS (Stankiewicz, et al. 2007). More than two decades ago, Drayer et al. were the first to describe abnormal signal reduction in the thalamus and putamen on T2 weighted images, which was believed to result from increased iron deposition (Drayer, et al. 1987a, Drayer, et al. 1987b). Further studies followed investigating the clinical implication if increased iron deposition in MS, reviewed in (Khalil, et al. 2011). Increased deep gray matter T2 hypointensities have been associated with disease duration (Bakshi, et al. 2000, Bakshi, et al. 2002), physical disability (Bakshi, et al. 2000, Ceccarelli, et al. 2009, Tjoa, et al. 2005, Zhang, et al. 2010) and cognitive impairment (Brass, et al. 2006). Another finding, which has been consistently reported, is that deep gray matter T2 hypointensities, suggestive of increased iron content, are correlated to brain atrophy (Bakshi, et al. 2002, Bermel, et al. 2005). While most studies investigated patients with definite MS, less information existed on iron deposition and its impact on clinical findings in patients with CIS, reviewed in (Khalil, et al. 2011). The extent of deep gray matter T2 hypointensities, suggestive of higher iron content was comparable in CIS and healthy controls, and they were further not associated with conversion to definite MS (Ceccarelli, et al. 2010).

However, the studies described above suffered from methodological drawbacks from deducing iron deposition by the signal reduction on T2 weighted images (Stankiewicz, et al. 2007). In recent years technical development allowed to assess brain iron levels in a quantitative manner (Ropele, et al. 2011). Several methodological approaches are currently used to assess brain iron levels, including relaxation time mapping, phase imaging, susceptibility-weighted imaging
(SWI), quantitative susceptibility mapping, magnetic field correlation (MFC) and direct saturation imaging, reviewed in (Ropele, et al. 2011). R2* relaxometry, a relaxation time mapping method, is based on a spoiled gradient echo sequence with multiple echoes. By voxel-wise fitting of a single-exponential decay function into the signal intensities provided by the individual echoes one can obtain R2* maps within a clinically reasonable scan time. These maps can show artefacts from macroscopic susceptibility effects, i.e. macroscopic distortions of the magnetic fields, which are caused by local differences in the magnetic susceptibility. However, susceptibility artefacts can be reduced by increasing the image resolution and by applying higher order shimming (=homogenization of the magnetic field prior to scanning with shimming gradients). By using a sufficient number of echoes and adequate handling of macroscopic susceptibility effects, which are magnetic field distortions due to different susceptibility of distinct tissue compartments, robust R2* maps can be obtained within a clinically acceptable acquisition time (Chapter 2) and (Ropele, et al. 2011). We thus decided to use this R2* technique to quantitatively determine brain iron levels in a larger series of CIS patients compared to MS and to reassess suggested association of iron deposition with clinical and morphologic findings in MS. R2* relaxometry has recently been validated in a post-mortem study (Langkammer, et al. 2010).

Using this method we found increased brain iron levels in deep gray matter areas in advancing MS, including RRMS and SPMS patients, which confirmed earlier reports (Bakshi, et al. 2002, Drayer, et al. 1987b, Ge, et al. 2007, Hammond, et al. 2008) (Chapter 2 and 3). We observed increased iron deposition in basal ganglia structures, including the putamen, globus pallidus and caudate nuclei in RRMS compared to CIS. The extent of brain iron deposition was associated with disease duration, which confirms earlier findings either based on T2 hypointensity measures (Bakshi, et al. 2000), or by a recently performed study using MR phase at 7T (Hammond, et al. 2008).

Another interesting finding of our studies on iron deposition was the association of iron deposition with brain atrophy measures, which has also been suggested in previous reports (Bakshi, et al. 2001, Bakshi, et al. 2002, Bermel, et al. 2005). The quantitative approach used in this study identified age as an important factor on iron deposition in deep gray matter areas. Hallgren and Sourander have described the effect of age on brain iron levels already in 1958 (Hallgren and Sourander. 1958). Therefore, to allow adequate interpretation it is important to consider age as a covariate in analysis on brain iron deposition (Chapter 2 and 3).

We identified gray matter atrophy as an independent predictor of brain iron deposition, which supports the notion that accumulation of iron may occur in parallel to neurodegeneration (Zecca, et al. 2004). Although earlier studies have reported an association of brain iron deposition and physical disability (Bakshi, et al. 2000, Bakshi, et al. 2002, Tjoa, et al. 2005), we could not confirm these findings. Our results corroborate another study performed at 7T (Hammond, et al. 2008).

In extension to the work presented in chapter 2, in chapter 3 we aimed to investigate main determinants of brain iron deposition in MS in a larger cohort of patients in comparison to healthy controls (HC). Towards this end brain iron deposition was assessed by R2* relaxometry at 3T in defined cerebral structures in 113 consecutive patients (35 CIS, 78 MS) compared to 35 HC. Besides a detailed neurologic examination, patients further underwent neuropsychological testing to allow for a more comprehensive assessment of the clinical impact of brain iron levels in MS.

We found increased iron deposition in basal ganglia structures in MS compared to CIS and HC. The absence of increased iron levels in CIS patients indicates that iron accumulation does
not precede the development of MS. Our findings provide strong arguments against a recently generated hypothesis claiming that iron deposition, caused by a reduction of venous outflow, might be implicated in the aetiology of MS (Zamboni, 2006, Zivadinov, et al. 2010). The existence of a vascular factor in MS, termed chronic cerebrospinal venous insufficiency (CCSVI) is at the moment controversial and debated (Khan, et al. 2010) and there is growing body of evidence in the medical literature, challenging this hypothesis (Doepp, et al. 2010, Sundstrom, et al. 2010, Wattjes, et al. 2010, Worthington, et al. 2010). Nevertheless at this point one should mention that a possible association of MS with vascular abnormalities, which might in part be caused by MS, is currently being discussed (D’haeseleer, et al. 2011). This includes findings from epidemiological studies suggesting a relation between MS and the risk of ischaemic events, and observations on global cerebral hypoperfusion in MS patients, reviewed in (D’haeseleer, et al. 2011).

The underlying mechanism by which iron accumulates in the brain and whether it is involved in MS pathology is not completely understood. It is speculated that iron induced oxidative stress may enhance the extent of neurodegeneration (Salvador. 2010, Zecca, et al. 2004). It is also believed that iron accumulation occurs as a by-product of inflammatory processes during the disease (Craelius, et al. 1982, Levine and Chakrabarty. 2004, Williams, et al. 2011).

When performing univariate correlation analysis we found basal ganglia iron levels to be correlated with age, disease duration, physical disability and decreased mental processing speed performance (Chapter 3). However, subsequent regression analysis identified besides age only gray matter atrophy and T2 lesion load as independent predictors of basal ganglia iron levels. Other clinical variables, including cognitive performance, then did no longer remain significantly associated with basal ganglia R2* rates, indicating that the extent of deep gray matter iron deposition is not a major determinant of cognitive decline. We did also not find a significant correlation of mental processing speed with thalamic R2* rates.

Taking together the results on studies of iron deposition in MS (Chapter 2 and 3) we can conclude that basal ganglia iron accumulation occurs with advancing disease and is related to the extent of morphologic brain damage rather than clinical variables, including cognitive performance, which argues for iron deposition as an epiphenomenon of the disease. The observation that brain iron levels are not increased in CIS compared to healthy controls indicates that iron accumulation does not precede the development of MS, nor that it is an initiating event.

Cognitive impairment in MS

Using the Brief Repeatable Battery of Neuropsychological Tests (BRB-N) (Rao. 1990) for assessment of cognitive performance, we found a comparable frequency and pattern of cognitive impairment (CI) in CIS and RRMS (Chapter 3 and 4). Up to now only little information has been available regarding the comparison of the pattern and frequency of CI in different MS stages (Potagas, et al. 2008). In line with our study Potagas et al. found a similar pattern of CI throughout different MS subtypes including CIS (Potagas, et al. 2008). We found deficits in at least one cognitive domain in 18.2% of CIS patients, which is lower than in some earlier reports on CI in CIS ranging between 27.3% and 80% (Achiron and Barak. 2003, Feuillet, et al. 2007, Kocer, et al. 2008, Potagas, et al. 2008). One reason for these discrepancies might be use of different cut-off levels and reference values for the BRB-N (Achiron and Barak. 2003, Feuillet, et al. 2007, Zipoli, et al. 2010). In both studies presented in this theses restrictive cut-off levels were used, considering a test result as abnormal if z-values were less than or equal to -1.68, which equates the performance of the lowest 5th percentile of healthy controls in a German speaking population as in ours (Scherer, et al. 2004).
We observed deficits predominantly in the cognitive domain of mental processing speed, which is in line with previous reports (Achiron and Barak. 2003, Feuillet, et al. 2007, Potagas, et al. 2008). In CIS, we did not find any correlation between clinical data and cognitive performance, suggesting that cognitive deficits develop independently from clinical variables, including physical disability and relapse rate. In line with these results, other studies could also not demonstrate a relation of cognitive deficits and physical disability in a CIS cohort (Feuillet, et al. 2007) and in patients with CIS or newly diagnosed MS (Glanz, et al. 2007). In RRMS, we found only a weak to moderate correlation of EDSS scores with reduced mental processing speed. Previous studies have also shown that both the EDSS and time from disease onset are poor predictors of reduced cognitive performance in RRMS (Rao, et al. 1991, Ron, et al. 1991).

We then investigated the relation of cognitive performance to quantitative MRI-metrics, including magnetization transfer imaging (MTI). As expected, the T2-lesion load was higher and brain volume measures and MTR values were lower in RRMS compared to CIS. Using stepwise linear regression analyses, the strongest predictor for decreased mental processing speed was normalized cortex volume followed by T2-lesion load in RRMS, whereas cortical MT ratio was the only MRI parameter associated with decreased mental processing speed in CIS. These findings suggest that structural cortical changes are the predominant morphological correlate to cognitive deficits in early phases of the disease as seen in CIS. In contrast to that, with advancing disease stages other signs of brain tissue damage, including atrophy and T2 lesion load gain importance, which may mask this primary association. At this stage structural imaging as assessed by MTI may no longer add independent information in regard to cognitive deficits, while cortical atrophy and T2-lesion load become its strongest predictors. Our results support the notion that cognitive dysfunction develops independently from focal disease activity in early phases of MS. Recent studies showing that CI was not associated with routine MRI measures for focal disease activity support this conclusion (Achiron and Barak. 2003, Glanz, et al. 2007).

As already described earlier, in several studies increased brain iron deposition was linked to cognitive deficits (Brass, et al. 2006, Ge, et al. 2007, Pujol, et al. 1992). In the study presented in chapter 3, we could not confirm such association, when assessing deep gray matter iron levels by R2* relaxometry (Chapter 3).

Altogether, the results deduced from the studies in chapter 3 and 4 provide evidence that cognitive impairment in CIS may develop in advance of gross morphological abnormalities detectable by routine MRI. Increased brain iron deposition provides no independent factor of reduced cognitive performance in early phases of the disease.

**Body fluid biomarkers**

We analyzed blood biomarkers in relation to clinical status and progression of MS, including antibodies directed against the CNS component, myelin oligodendrocyte glycoprotein (MOG) (Chapter 5 and chapter 6) and first validation results of a potential novel biomarker, termed secretogranin III (SGIII) (Chapter 7). A main aim of this thesis was to relate body fluid to MRI biomarker results. We explored whether CSF markers for axonal damage, including neurofilament light and heavy protein and N-acetylaspartic acid, are related to measures of brain tissue damage as indicated with 3T MRI (Chapter 8). We further questioned whether neural cell adhesion molecule (NCAM), a candidate marker for reparative and regenerative mechanisms, might be related to clinical data and 3T MRI-metric in CIS and MS (Chapter 9).
**MOG antibodies**

In experimental autoimmune encephalomyelitis (EAE), the animal model of MS, antibodies directed against the extracellular domain of the CNS specific myelin oligodendrocyte glycoprotein (MOG) cause widespread demyelination (Linnington, et al. 1988), comparable to that seen in MS. Due to its pathogenic properties in EAE and the description of anti-MOG antibodies in MS lesions (Genain, et al. 1999), much interest had been generated in analyzing serum and CSF anti-MOG antibodies in MS patients.

We studied the epitope specificity of serum antibodies directed against the extracellular domain of MOG in patients with RRMS compared to healthy controls (Chapter 5). We found immunodominant antibody responses against two overlapping epitopes aa37–48 and aa42–53 in MS-patients. In line with this, recent crystallization studies of MOG revealed that the residues aa41-46 are surface exposed in the native protein structure, thus available to be bound by antibodies (Breithaupt, et al. 2003, Clements, et al. 2003). A prerequisite of antibodies with pathogenic properties is their capability to recognize epitopes on the native protein structure, which are predominantly conformation dependent (Marta, et al. 2005, Mathey, et al. 2004, Menge, et al. 2007, von Budingen, et al. 2004). For example, it has been shown that the demyelinating anti-MOG antibody 8-18C5 recognizes one dominant conformational epitope region in rodents (Breithaupt, et al. 2008). Thus it is important that adequate test systems are being developed to allow dissection of potential pathologically relevant versus non-relevant antibodies (Reindl, et al. 2010). It is hypothesized that antibody responses against cryptic linear MOG epitopes may arise upon tissue destruction, when myelin debris are being released into the blood circulation. These antibodies even though pathologically irrelevant may however serve as an indirect marker reflecting the extent of tissue damage in MS. Our results point in that direction showing that anti-MOG peptide antibody responses were more pronounced in patients who experienced a recent relapse within 1–6 months before study entry compared to those patients whose last relapse before study entry dated more than 6 months back (Chapter 5). Epitope spreading during ongoing immune responses against CNS myelin may explain these findings (Robinson, et al. 2003, Vanderlugt and Miller. 2002). In our study, disease-modifying drugs, including interferon-beta and glatiramer-acetate had no major impact on the anti-MOG epitope antibody response after one year of treatment. Similarly, other groups reported that one year of interferon-beta treatment did not induce significant changes of anti-MOG antibody levels (Angelucci, et al. 2005, Bitsch, et al. 2004).

In chapter 6 we aimed to investigate ELISA IgM antibody responses directed against a native refolded MOG antigen preparation to indicate treatment response of acute MS relapses. Patients who showed complete remission from acute MS relapse had a significant increase of anti-MOG IgM antibody levels comparing baseline and acute relapse values. Using this detection system our results indicate that responsiveness to standard corticoid treatment of acute relapses may depend on the dynamic of serum anti MOG IgM antibodies directed against native MOG. This might be important for future decisions in patients with acute relapses, especially decisions regarding either dose/duration of corticoid or escalating therapies.

A variety of studies on MOG antibodies in MS have been published but their results are controversial with prevalence of MOG antibodies reported ranging between 0%-80% in MS patients and 0%-60% in healthy controls (Reindl, et al. 2006, Reindl, et al. 2010). Differences in detection systems and antigen preparations used may hamper direct comparisons of...
these studies (Reindl, et al. 2010). The value of anti-MOG and anti-myelin basic protein (MBP) antibodies to predict conversion to clinically definite MS has been studied in several CIS cohorts, reviewed in (Reindl, et al. 2010). While some groups found highly significant correlations (Berger, et al. 2003, Greeve, et al. 2007, Tomassini, et al. 2007), partial significant results were reported from sub-analyses (Kuhle, et al. 2007a, Rauer, et al. 2006) and others did not find any significant correlation at all (Kuhle, et al. 2007b, Lim, et al. 2005b, Pelayo, et al. 2007). It is important to state that the above-mentioned studies were performed with the same detection system for anti myelin antibodies based on immunoblotting (Reindl, et al. 2010). Differences in study cohorts and pre-analytical sample handling may in part be the reason for these discrepancies.

In recent years antibody research was successful in other disease conditions, such as the NMO-IgG antibody in patients with neuromyelitis optica (NMO) (Lennon, et al. 2004, Wingerchuk, et al. 2006). Regarding MOG antibody assays, refined detection systems enabling to assess antibodies directed against membrane bound, glycosylated native MOG have brought new insights into potential clinical applicability of anti MOG antibodies (Reindl, et al. 2010). It has been shown that anti-MOG antibodies were present in approximately 40% of children with acute disseminated encephalomyelitis (ADEM), while these antibodies were rarely detected in CIS or MS (Di Pauli, et al. 2010, Lalive, et al. 2011), which is consistent with other reports (Brilot, et al. 2009, McLaughlin, et al. 2009, O’Connor, et al. 2007, Selter, et al. 2010). Just recently, it has been shown that anti-MOG antibodies are transient in childhood ADEM, while they persist in patients eventually diagnosed with childhood MS (Probstel, et al. 2011). Altogether, these results provide good evidence that they may serve as a biomarker for the diagnosis and prognosis of ADEM and childhood MS.

If MOG antibodies are of pathogenic relevance in a subset of MS still needs to be elucidated, although a recent report on serum IgG antibodies directed against conformational MOG epitopes in MS patients supports this notion (Menge, et al. 2011).

**Novel biomarkers: Secretogranin III**

A recently performed proteomics based biomarker discovery study identified several candidate markers in MS (Teunissen, et al. 2011). Among the interesting ones was SGIII fragment, a polypeptide belonging to the granin family (Bartolomucci, et al. 2010).

The granin family consists of single poly-peptide chains (Taupenot, et al. 2003) and exert both intracellular and extracellular functions (Helle. 2004). While intracellular they are important for protein trafficking (Taupenot, et al. 2003), extracellular they may serve as pro-hormones for putative regulatory peptides either locally or distinct from the site of secretion (Helle. 2004). However, up to now only little is known about functional properties of SGIII derived peptides (Helle. 2004). SGIII is synthesized as an N-glycosylated protein and cleaved proteolytically in secretory vesicles (Bartolomucci, et al. 2011, Holthuis, et al. 1996). No biologically active peptides derived from SGIII have been described so far (Bartolomucci, et al. 2011).

We here present first validation results of serum SGIII as a biomarker for discrimination of MS patients from controls and different subtypes of MS (Chapter 7). We found a distinct pattern of SGIII peptides using Western Blot analysis; some of them were differently expressed in MS compared to controls. In addition, our results provide evidence that specific isoforms of SGIII may serve as a marker for physical disability in MS. Moreover, different Western Blot band intensities were observed in treated compared to untreated patients, indicating that immunomodulatory treatments influence SGIII expression.
In contrast to SGIII, other granins had already been studied in MS. Decreased levels of chromogranin B and secretogranin II in MS have recently been reported by a mass spectrometry based study (Mattsson, et al. 2007). Differentially expression of chromogranin A has been reported in two other proteomic studies (Stoop, et al. 2008, Stoop, et al. 2009). Another study investigated the proteolytic processing of chromogranin A and B and secretogranin II in CSF of different neurological disorders, including MS and observed only limited changes in biosynthesis compared to controls (Eder, et al. 1998).

SGIII has not been studied in MS so far. Our results in conjunction with the initial proteomic study (Teunissen, et al. 2011) suggest that immunopathologic processes during the course of MS may lead to alterations in SGIII peptide secretion. Along with this, the finding that immunomodulatory treatment impacts serum SGIII isoform patterns also may lead to the conclusion that serum SGIII peptides levels might be closely related to immunologic conditions. However, this certainly needs further confirmation. If so, this would be a very welcome tool, as serum is obtained by minimally invasive procedures and is therefore suitable for repeated sampling.

The next step of validation and verification (Rifai, et al. 2006) of the candidate biomarker SGIII will comprise development of a clinical suitable test to allow analysis of large sample numbers in a quantitative manner. Future studies should aim at the establishment of luminex multiparameter detection system in order to enable assessment of several markers at the same time, including the assessment of various SGIII isoforms in MS.

**Body fluid biomarkers in relation to quantitative MRI**

**CSF markers for axonal damage**

The main finding of the study on CSF biomarkers for axonal damage in relation to 3T MRI in CIS was that neurofilaments are increased already in the earliest stage of MS and that the neurofilament heavy protein (NfH) is associated with increased brain volume loss already over short term. In this CIS cohort NAA levels did not add significant information on axonal damage (Chapter 8).

The relation of increased NfH levels in CSF with brain atrophy in CIS patients has not been reported so far. However, supporting evidence on the relations of increased Nf and brain atrophy comes from a recently published study on secondary progressive MS patients receiving chemotherapy as preconditioning for bone marrow transplant (Petzold, et al. 2010). Following this treatment, serum NfH protein immediately increased and persisting high levels were associated with disability progression and brain atrophy, indicating that neurotoxic side effects arise following aggressive treatment regimes (Petzold, et al. 2010). The potential value of using Nf as markers to monitor treatment effects has further been demonstrated in patients receiving natalizumab therapy (Gunnarsson, et al. 2011). CSF NFL levels were markedly decreased in RRMS patients treated with the monoclonal antibody natalizumab, indicating that highly effective anti-inflammatory therapy may indeed reduce the extent of axonal damage (Gunnarsson, et al. 2011). This observation is of great importance since axonal loss is the major contributor of permanent physical disability in MS (Bjartmar, et al. 2000).

Antibodies as markers for pathophysiological processes in MS have largely focused on myelin components as target antigens (Reindl, et al. 2006, Reindl, et al. 2010), among them, myelin oligodendrocyte glycoprotein has extensively been studied (Chapter 5 and 6). However, only some information exists on antibodies directed against axonal cytoskeletal components.
On the one hand these antibodies may arise reactive to axonal damage when tissue debris are released to the periphery following an autoimmune attack. On the other hand, animal studies provide evidence that autoimmune responses directed against axonal and neuronal components, including cytoskeletal proteins, may directly induce axonal damage (Huizinga, et al. 2008). One particular study found NfL chain antibodies to be correlated with brain atrophy, but they did not include patients with CIS (Eikelenboom, et al. 2003). NfL antibodies have been reported to be increased in progressive forms of MS (Ehling, et al. 2004, Silber, et al. 2002), although this has not been confirmed by others (Bartos, et al. 2007). While some found an association of NfL antibodies with clinical data, including disease duration and physical disability (Silber, et al. 2002), others did not (Bartos, et al. 2007, Ehling, et al. 2004, Eikelenboom, et al. 2003). These controversial findings may in part be explained by the usage of different antigen preparations and different study cohorts investigated.


Well-validated detection systems with increased sensitivity and specificity (Kuhle, et al. 2010, Norgren, et al. 2003) (Chapter 8) may facilitate the use of NF proteins in clinical practice. During immunoassay development several important technical issues need to be taken into account. One frequently observed problem of Nf assays is the occurrence of the so-called “hook effect” (Lu, et al. 2011), which is caused by the formation of neurofilament protein aggregates, rendering parallelism of sample and standard dilutions (Lu, et al. 2011). It has recently been shown that the Nf “hook effect” can be overcome by pre-incubation of the samples with a specific urea-calcium chelator-enriched buffer (Lu, et al. 2011). The NfH assay used in the study presented in chapter 8 avoided the “hook effect” by further dilution of the samples to ensure linearity of sample and standard material (Chapter 8), since usage of specific buffers did not eliminate the “hook effect” in our test system. Stoichiometric analysis revealed that Nfs have a distinct stable in vivo protein structure (Kim, et al. 2011). However, care should be taken on pre-analytical factors, like storage temperature until use, which may impact test results but are not fully established yet for Nfs (Teunissen, et al. 2009b). Protein stability in vitro is a prerequisite to ensure its use as a biomarker (Petzold. 2005, Teunissen, et al. 2005). In particular NfL lacked stability when stored at room temperature or at 4 °C as detected by Western Blot (Koel-Simmelink, et al. 2011).

We additionally explored the role of N-acetylaspartic acid (NAA) in CSF as a marker for axonal damage in CIS patients and whether it might indicate disease progression and brain tissue damage as indicated by 3T MRI (Chapter 8). Although CSF NAA levels did not significantly differ between patients and controls, higher NAA levels were correlated with physical disability in CIS (Chapter 8). This finding supports the notion that NAA underlies a temporal dynamic as previously suggested (Teunissen, et al. 2009b). While increased levels may be observed in early disease phases of MS, decreased levels occur in advanced disease stages (Jasperse, et al. 2007, Teunissen, et al. 2009a). However, we did not find any correlation of NAA levels with...
brain atrophy measures or T2 lesion load in CIS (Chapter 8). NAA is synthesized in neuronal mitochondria and then transported to oligodendrocytes for further processing (Benarroch, 2008). It is released into the blood circulation via astrocyte end feet (Benarroch, 2008, Moffett, et al. 2007). Increased CSF but also serum NAA levels have recently been reported to correlate with physical disability in relapsing remitting MS patients (Teunissen, et al. 2009a, Tortorella, et al. 2011). Because of its mitochondrial origin, NAA may serve as a marker of integrity for the neuronal mitochondrial metabolism (Moffett, et al. 2007). From several magnetic resonance spectroscopy (MRS) studies evidence exists that NAA levels are reduced in MS brains (Filippi and Rocca, 2011). However, it is not yet clear if CSF and serum NAA measures are related to MRS NAA levels in MS. This should be addressed in future studies in order to investigate whether the combined assessment of body fluid and imaging NAA as a marker for axonal loss may yield more information on disease progression in MS or whether one is more specific than the other.

Altogether, our results on CSF neurofilaments in relation to quantitative 3T MRI, presented in chapter 8, confirm increased Nf levels already in the earliest stage of MS with a correlation to physical disability. The association of NfH levels with increased brain volume changes in CIS patients already over short-term supports the notion that these markers might be associated with ongoing brain tissue damage and have predictive value. This is relevant for treatment decision, as those patients with high Nf levels would benefit from axonoprotective therapies. Accurate test systems and sample handling are important requirements for Nfs to be used as a biomarker in clinical practice. However, before being introduced into the clinic, current tests still need to be compared and validated among different centers in international multicenter studies, also by using a set of several quality controls and including several control groups. Compared to Nfs, NAA may be less relevant in early phases of the disease, but its association with progressive forms of MS underscores its potential as a marker for axonal damage. Whether CSF and/or serum NAA may comprise predictive information on disease evolution in established MS still needs to be determined.

**CSF markers for repair and regeneration**

After an immunological attack, the demyelinated plaque can partially or completely be remyelinated (Lassmann, et al. 2007). A completely remyelinated plaque, the so-called “shadow plaque”, is characterized by reduced myelin density and relatively thin myelin sheaths (Lassmann, et al. 2007). In a subgroup of MS patients, including relapsing remitting and progressive MS, remyelination can be extensive (Albert, et al. 2007, Patani, et al. 2007, Patrikios, et al. 2006). Efficient remyelination may be one of the most important factors to prevent neurodegeneration (Dutta and Trapp, 2011, Lubetzki, et al. 2005). However, the reason why complete remyelination occurs in some patients, while it fails in others is not yet completely understood (Lubetzki, et al. 2005). Besides myelin repair, several other factors may contribute to neuronal protection and regeneration, including immunomodulatory molecules, inducible NO synthase inhibitors, free radical scavengers, neurotrophic factors and factors promoting axonal outgrowth (McDonald, et al. 2011, Seifert, et al. 2007).

Up to now there are no tools available which allow adequate assessment of neuronal repair and regeneration in vivo. Finding a marker for regeneration seems to be even much more challenging than assessing tissue destruction.

Several CSF markers for regeneration have been investigated in MS, including neuronal cell adhesion molecule (NCAM) and neurotrophic factors, such as ciliary neurotrophic

Reduced CSF NCAM levels have been described in MS (Gnanapavan, et al. 2010, Massaro, 2002) and a continuously increase of CSF NCAM in acute MS patients who clinically improved after treatment of the attack. This suggests that NCAM may be involved in recovery processes of the brain (Massaro, 2002).

Our study confirmed the occurrence of altered CSF NCAM levels in patients with MS (Chapter 9). We found reduced CSF NCAM levels in MS compared to CIS and in clinically active compared to non-active patients. A major finding of our study was that higher CSF NCAM levels correlated with MRI measures for brain tissue loss over time regarding normalized volumes for whole brain, gray matter and cortex, but not with the change in T2 lesion load. At a first glance, this finding seems counter-intuitive, since previous studies associated higher CSF NCAM levels with clinical improvement (Massaro, 1998, Massaro, 2002). In that study, patients were followed for up to 5 weeks and serial CSF samples were analyzed. Whereas higher CSF NCAM levels may be associated with clinical improvement over short-term, our results demonstrate that over a longer period, e.g. one year, higher CSF NCAM levels could be associated with brain atrophy, in particular the gray matter. Subsequent to an immunological attack, CSF NCAM could increase upon regenerative processes, which however may limit brain tissue damage for only a short interval. Indirectly, however, this could be evidence for a more active and tissue damaging disease process. Our finding of increased CSF NCAM levels in patients in whom CSF was sampled close to a clinical attack compared to sampling during clinically inactive disease would support this hypothesis. Another explanation for our findings could be that higher CSF NCAM levels may also exert deleterious effects. From in vitro and in vivo studies evidence exists, that high levels of soluble NCAM may indeed lead to impaired synaptic connectivity, possibly via formation of toxic aggregates (Pillai-Nair, et al. 2005, Secher, 2010). Thus, soluble CSF NCAM could rather act as double-edged sword.

Altogether, our results from chapter 9 showing large overlap between controls and CIS patients, support the notion that CSF NCAM may not be used as a single marker for regeneration in early phases of MS. The use of combined approaches to define markers for regenerative processes in MS seems to be fundamental. This should include combined biochemical analyses of CSF and serum, MRI, MTI together with improved clinical scales (Lubetzki, et al. 2005).

REFERENCES


CHAPTER 10


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RECOMMENDATIONS FOR FUTURE RESEARCH AND CONCLUDING STATEMENTS

Recommendations for future research

In this thesis we aimed to provide more insight into the value of quantitative MRI and body fluid biomarkers to explain disease evolution in MS, with a special focus on CIS. The data generated on the role of iron deposition in MS based on R2* relaxometry using 3T MRI provide new evidence that changes in basal ganglia iron accumulation occur with advanced disease stages, i.e. rather in MS than in CIS, and that it is related to the extent of morphologic brain damage. Furthermore, we were able to show that basal ganglia iron deposition is not increased in CIS indicating that iron accumulation does not precede the development of MS. Since R2* relaxometry has been validated in a post-mortem study and also due to its availability on common modern MRI scanners, this method is currently been viewed as a focus of MR research and a promising marker of neurodegeneration in MS (Rovira and Montalban. 2011). In the near future the present results should be reproduced in other MS cohorts reflecting patients with a wide range of physical disability, thus also including a larger proportion of SPMS patients. Furthermore, the value of R2* relaxometry to assess iron levels in MS lesions, normal appearing white matter and the cortex still needs to be determined, as we focused mainly on deep gray matter structures. In addition its reproducibility across multiple sites and in longitudinal studies should be investigated (Rovira and Montalban. 2011). In this respect it would be interesting to investigate whether increased deep gray matter iron levels, as indicated by R2* relaxometry, may indeed predict the evolution of brain atrophy. So far only one study has been published showing that baseline brain iron levels predict longitudinal brain atrophy, but in this study assessment of brain iron accumulation was based on the signal reduction on T2 weighted images (Bermel, et al. 2005). It is not completely clear how iron accumulates within the brain and whether increased iron deposition contributes to MS pathology. Only a few studies investigated CSF and serum levels of iron and iron related proteins, reviewed in (Khalil, et al. 2011). Combining clinical disease status, CSF/serum iron and iron-related proteins (as well as iron regulator proteins) in conjunction with quantitative MRI techniques sensitive for iron may provide further insight on the implication of increased iron deposition in MS (Khalil, et al. 2011).

Our studies on cognitive impairment demonstrate that neuropsychological deficits are present already in CIS in a pattern similar to MS, with impaired mental processing speed being most prevalent. Cortical structural changes as indicated by MTI may be an early sign for tissue damage related to impaired mental processing speed in CIS while this association shifts to increased signs of cortical atrophy and lesion load in advanced stages of the disease. Remarkably, basal ganglia iron accumulation was not associated with cognitive impairment in any stage of MS, when performing linear regression analyses. Two recent reports underscore the importance of neuropsychological assessment already in early disease phases showing that cognitive impairment is related to short term disease evolution (Portaccio, et al. 2009, Zipoli, et al. 2010). It would be interesting to investigate whether in CIS neuropsychological assessment together with MTI may yield more information on the risk of disease progression. In future studies this should be analyzed in a clinically and MRI follow-up setting.

Recent developments on MOG antibody research revealed that antibodies directed against the native, membrane bound and properly glycosylated form of MOG are increased in children...
with acute disseminated encephalomyelitis (ADEM) (Di Pauli, et al. 2010, Lalive, et al. 2011). Although conformation-dependent anti-MOG antibodies have been shown to be pathogenic in experimental settings (Brehm, et al. 1999, Breithaupt, et al. 2008), in humans this has not been proven so far. Recent findings suggest a pathogenic role of NMO-IgG antibodies in NMO (Bradl, et al. 2009, Roemer, et al. 2007). The question whether native MOG antibodies imply a pathogenic potential should be addressed in the future using a similar approach.

The present thesis provides first validation results of a new biomarker for disease progression in MS, termed SGIII fragment. The results generated so far are based on a Western Blot technique. Distinct SGIII patterns have been observed in MS compared to control sera and SGIII intensities were furthermore correlated to physical disability. Although we performed quantification of SGIII band intensities a clinical convenient test system needs to be developed (Rifai, et al. 2006). Thus, much effort is currently being put on the development of a quantitative luminex detection system. This shall ultimately serve to determine SGIII together with a set of other biomarkers to prove their potential value in clinical practice of MS.

Our results on biomarkers for axonal damage in relation to MRI findings underscore their potential value to indicate the extent of axonal injury. Assessment of these markers could be included in future randomized clinical trials to determine their value as a further outcome measures for monitoring treatment efficacy. Particular care should be taken on pre-analytical factors (Koel-Simmelink, et al. 2011, Teunissen, et al. 2009) and analytical conditions (Lu, et al. 2011) to ensure adequate analysis and interpretation of the data.

In contrast to assessment of axonal damage, evaluation of neuronal repair and regeneration seems to be much more challenging. Our results on CSF NCAM in MS do not support the notion that this marker can be used as a simple tool to indicate neuro-regenerative processes in MS. Moreover the association of higher CSF NCAM levels with brain tissue loss over time may suggest that CSF NCAM could increase upon regenerative processes, which however may limit brain tissue damage for only a short time interval. Indirectly, however, this could be evidence for a more active disease process. To address the important issue of evaluating tissue repair, combining several candidate biomarkers for neuronal repair (Tumani, et al. 2009) together with quantitative MRI (Barkhof, et al. 2009) and improved clinical and cognitive outcome measures seems to be inevitable (Lubetzki, et al. 2005).

Altogether, a combined approach of biomarker research in MS assessing both imaging and body fluid markers may be superior in identifying factors for disease progression in MS. Towards this end in the first step comparison of patients with established or progressive MS in relation to defined control groups may facilitate elucidating markers for disease progression, by comparing patients cohorts with clinical extremes. In the second step identified candidate biomarkers then need to be investigated in patients with CIS, to prove their clinical value for indication of disease progression already at the earliest stage of the disease.

The following table summarises main findings of the studies presented in this thesis comparing CIS and MS patients. As can be seen some candidate markers may add significant information in CIS patients while others might be relevant either in both disease stages or only in established MS. However, not all markers have yet been analysed in both disease stages. This should be addressed in future studies not only by comparing MRI and body fluid markers between CIS and MS patients and controls but also by analysing them intra-individually in clinical, imaging and body fluid biomarker follow up studies.
**General concluding statements**

Brain iron in the basal ganglia accumulates with advancing disease and is closer related to tissue destruction rather than clinical and neuropsychological findings, which argues for iron deposition to occur as an epiphenomenon of the disease.

Cortical magnetization transfer imaging changes may be an early sign of structural tissue alterations related to impaired mental processing speed.

Serum antibodies against MOG peptides and native refolded MOG may indicate disease activity and progression in MS, while antibodies directed against the native, membrane bound and glycosylated form of MOG are more relevant in children with acute disseminated encephalomyelitis.

Novel identified candidate biomarkers, including serum secretogranin III, to indicate disease progression in a subset of MS patients are currently being validated in large MS cohorts.

Neurofilaments are reliable markers to indicate the extent of axonal damage in MS and may thus be helpful to monitor clinical status and treatment efficacy in future studies.

Combined approaches of biochemical and quantitative MRI measures together with improved clinical outcome measures are fundamental to establish assessment tools for axonal damage and/or neuronal repair in MS.

### REFERENCES


### TABLE

<table>
<thead>
<tr>
<th>Main finding</th>
<th>CIS</th>
<th>MS</th>
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</thead>
<tbody>
<tr>
<td>Increased brain iron by R2* relaxometry</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Cognitive impairment</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R2* relaxometry related to cognitive impairment</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cortical MT ratio related to cognitive impairment</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Gray matter atrophy and T2 lesion load related to cognitive impairment</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Serum anti MOG peptide antibodies increased compared to controls</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>Serum anti native MOG IgM antibodies related to corticoid treatment response</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>Specific serum SGIII isoforms changed compared to controls</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>CSF NfH and NfL increased compared to controls</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>Relation of NfH with evolution of brain atrophy</td>
<td>+</td>
<td>?</td>
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<tr>
<td>CSF NCAM levels decreased compared to controls</td>
<td>-</td>
<td>+/-</td>
</tr>
</tbody>
</table>

CIS, clinically isolated syndrome; MS, multiple sclerosis; MT imaging, magnetization transfer imaging; MOG, myelin oligodendrocyte glycoprotein; SGIII, secretogranin III; CSF, cerebrospinal fluid; NfH, neurofilament heavy; NfL, neurofilament light; NCAM, neural cell adhesion molecule; ?, not yet analyzed.


