PART IV
QUANTITATIVE MRI IN RELATION TO BODY FLUID BIOMARKERS
CHAPTER 8
CSF NEUROFILAMENT AND N-ACETYLASPARTATE RELATED BRAIN CHANGES IN CLINICALLY ISOLATED SYNDROME

Michael Khalil\textsuperscript{1,2}, Christian Enzinger\textsuperscript{1,3}, Christian Langkammer\textsuperscript{1}, Stefan Ropele\textsuperscript{1}, Arthur Mader\textsuperscript{1}, Alessandro Trentini\textsuperscript{2}, Marissa L.G. Vane\textsuperscript{2}, Mirja Wallner-Blazek\textsuperscript{1}, Gerhard Bachmaier\textsuperscript{4}, Juan-Jose Archelos\textsuperscript{1}, Marleen J.A. Koel-Simmelink\textsuperscript{2}, Marinus A. Blankenstein\textsuperscript{2}, Siegrid Fuchs\textsuperscript{1}, Franz Fazekas\textsuperscript{1}
and Charlotte E. Teunissen\textsuperscript{2}

\textsuperscript{1} Department of Neurology, Medical University of Graz, Austria, \textsuperscript{2} NUBIN, Department of Clinical Chemistry, VU University Medical Center Amsterdam, The Netherlands, \textsuperscript{3} Division of Neuroradiology, Department of Radiology, Medical University of Graz, Austria, \textsuperscript{4} Institute for Medical Informatics, Statistics and Documentation, Medical University Graz, Austria

\textit{Multiple Sclerosis Journal}, 2012; in revision.
ABSTRACT

OBJECTIVE: Axonal damage is considered a major cause of disability in multiple sclerosis (MS) and may start early in the disease. Specific biomarkers for this process are of great interest. We aimed to study if body fluid biomarkers for axonal damage reflect and predict disease progression already in the earliest stages of the disease, i.e. in clinically isolated syndrome (CIS).

METHODS: We assessed CSF levels of neurofilament heavy (NfH), neurofilament light (NfL) and N-acetyl aspartic acid (NAA) in 67 CIS patients and 18 controls with neurological diseases of non-inflammatory aetiology (NC). CIS patients underwent a baseline MRI at 3T to assess T2 lesion load and normalized regional and whole brain volumes, and a follow-up MRI after one year was obtained in 28 of them.

RESULTS: Compared to NC, CIS patients had higher NfL (p<0.001) and NfH (p=0.05) levels. No significant group differences were found for NAA. In NC, NfH (r=0.713, p<0.005) and NfL (r=0.929, p<0.001) levels correlated with age. In CIS this correlation was less strong for NfH (r=0.325, p<0.01) and absent for NfL levels. Patients’ NfH levels correlated with physical disability (r=0.304, p<0.05) and with change in brain volume over 1 year of follow-up (r=−0.518, p<0.01) but not with change in T2 lesion load.

CONCLUSION: Our results confirm increased neurofilament levels already in the earliest stage of MS being related to the level of physical disability. The association of NfH levels with brain volume but not lesion volume changes supports the association of these markers with axonal damage.
INTRODUCTION


Assessment of cerebrospinal fluid (CSF) neurofilaments (NF) and N-acetyl-aspartic acid (NAA) provide promising tools to indicate the degree of axonal damage (Teunissen, et al. 2005, Teunissen, et al. 2009a). The presence of robust and validated assays for NF heavy chain protein (Kuhle, et al. 2010), NF light chain protein (Norgren, et al. 2003) and NAA (Jasperse, et al. 2007) may help to pave the way for their clinical application. Whereas two recent studies provide evidence that CSF neurofilament levels are altered already in early phases of the disease (Kuhle, et al. 2011, Teunissen, et al. 2009a), studies on CSF NAA suggest that this may rather be a marker for axonal damage in progressive forms of MS (Teunissen, et al. 2009a). NF heavy levels have been shown to correlate with relapses and disability in MS (Kuhle, et al. 2011, Teunissen, et al. 2009a).

No information exists so far whether markers of axonal damage and neurodegeneration are related to morphologic brain changes as measured by 3T MRI at the earliest clinical stage of MS, i.e. in clinically isolated syndrome (CIS). Moreover, it is also unclear if these markers are predictive of the evolution of subsequent brain tissue damage. With this study we therefore aimed to confirm previous findings on CSF levels of neurofilament light and heavy chain and N-acetylaspartate in CIS and to explore if they could also serve as predictive markers for disease progression in CIS, i.e. to analyse if their concentrations might indeed predict accumulating tissue damage as indicated by MRI.

SUBJECTS/MATERIALS AND METHODS

Patients and Controls

For this study, we recruited 67 consecutive patients with a clinically isolated syndrome suggestive of MS (Miller, et al. 2008) from our MS outpatient clinic (Table 1). Patients underwent diagnostic lumbar puncture for CSF analysis, detailed clinical examination and a 3T MRI. Assessment of demographical and clinical data included age, age at disease onset, disease duration, Expanded Disability Status Scale score (Kurtzke 1983), occurrence of relapses and MS therapy. A relapse was defined as the appearance or reappearance of at least one neurological symptom or the worsening of an old symptom attributed to MS that lasted for at least 24 hours and which was preceded by a relatively stable or improving neurological state of at least 30 days. Clinical follow-up data were available in 46 CIS patients (Table 1). None of the CIS patients had received any MS specific therapy at the time of the lumbar puncture.

We included 18 patients with other neuropsychiatric diseases of non-inflammatory aetiology and a normal CSF as controls (Table 1). This group consisted of 10 patients with headache, 2 with facial palsy, 1 with depression, 1 with pain, 1 with polyneuropathy, 1 with vertigo, 1 with peripheral nerve palsy, and 1 patient with subjective complains of sensory disturbances. CIS and control patients had similar mean age and gender distribution (Table 1).
CHAPTER 8

Standard protocol approvals, registrations, and patient consent

The study was approved by the ethics committee of the Medical University of Graz. All participants gave written informed consent.

Cerebrospinal fluid analyses

A total volume of 6-10 ml of CSF was obtained by lumbar puncture. After diagnostic work up, excess of CSF was stored at -80 °C until use in agreement with international consensus guidelines (Teunissen, et al. 2009b). All CSF analyses were performed by trained technicians or biochemists blinded to clinical information. In 46 patients (68.7%) lumbar puncture was performed during their first clinical attack, in 21 patients (31.3%) CSF was obtained during remission after their first attack.

Neurofilament heavy (NfH) concentrations were assessed by an in-house developed multiplex assay as previously described (Kester, et al. 2011). All analyses were performed in duplicate and normalized. Inter-assay assay coefficient of variance (CV) was 17.9% and intra-assay CV was 3.9%. The hook effect (Lu, et al. 2011) caused by neurofilament aggregates was avoided by further dilution of the samples to ensure accurate quantification of the immunoassay.

Neurofilament light (NfL) concentrations were measured using a commercial available ELISA kit from Uman-Diagnostics AB (www.umandiagnostics.com). This ELISA kit has recently been validated in a multi-center study (Petzold, et al. 2010a). Due to depletion of some CSF samples, NfL levels were obtained from 47 CIS patients and 15 controls.


Magnetic resonance imaging

Patients underwent MRI at 3 Tesla (Siemens Tim Trio, Siemens Healthcare, Erlangen, Germany) using a phased-array head coil with 12 receiver elements and a consistent imaging protocol as

Table 1. Demographical and clinical data

<table>
<thead>
<tr>
<th></th>
<th>CIS</th>
<th>Controls</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (% female)</td>
<td>67 (70.1)</td>
<td>18 (66.7)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Age at LP (years) *</td>
<td>33.4 (9.9)</td>
<td>34.7 (13.9)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Age at disease onset (years) *</td>
<td>33.3 (9.9)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Disease duration at LP (months) **</td>
<td>0.3 (0.2-1.3)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>EDSS at LP in remission **</td>
<td>1.0 (0.0-2.0)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>N (% female) with clinical FU</td>
<td>46 (69.6)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Clinical FU (yrs) **</td>
<td>2.3 (1.4-3.4)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>EDSS at FU in remission **</td>
<td>1.0 (0.0-2.0)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>N (% female) with follow-up MRI</td>
<td>28 (64.3)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>MRI FU (yrs) **</td>
<td>1.0 (1.0-1.1)</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

N, number of patients/controls; LP, lumbar puncture, EDSS, expanded disability status scale, FU, follow-up; CIS, clinically isolated syndrome; NA, not applicable; values are presented as number (%), * mean (SD) or ** median (interquartile range).
described previously (Khalil, et al. 2009, Khalil, et al. 2011). There was a median time interval of 3.2 (IQR 1.0-6.2) months between the lumbar puncture and brain MRI at 3T. Follow-up MRI at the same magnet using identical protocols after one year was obtained from 28 CIS patients (Table 1).

All image analyses were performed by trained and experienced technicians and MR readers, blinded to clinical information.

Brain tissue volume and hyperintense T2-lesion load were measured as previously described (Enzinger, et al. 2004, Khalil, et al. 2009, Khalil, et al. 2011). In addition, separate estimates of volumes of gray matter, white matter, cortical gray matter and ventricular CSF, normalized for subject head size, were estimated using SIENAX, which is part of FSL (Smith, et al. 2004). For assessing T2 lesion load, masks defining the lesions were created and the total lesion load was calculated by multiplying the area of all masks by the slice thickness.

**Statistics**

Statistical analyses were performed using SPSS 18.0 (SPSS Inc., Chicago, Illinois, USA). The Kolmogorov–Smirnov test assessed normality of data distribution. Groups were compared by Mann–Whitney U-test. Student’s t-test was applied to compare mean age values between groups. Spearman correlations served to calculate the correlation coefficients between CSF, imaging and clinical data. Pearson partial correlations were performed on ranked variables to correct for age.

**RESULTS**

**Group differences of CSF Nf and NAA levels and correlations with demographical data**

Median levels of CSF NfL were 2.3 fold and of NfH 1.5 fold higher in CIS compared to controls. NAA levels were not significantly different (Figures 1 A-C). CSF NfH, NfL and NAA levels did not significantly differ between patients in remission compared to those with an acute relapse at the time of lumbar puncture (data not shown).

In controls, both NfL (r=0.929, p<0.001) and NfH (r =0.713, p<0.005) levels correlated with age. In CIS patients, this correlation was less strong for NfH (r=0.325, p<0.01) and absent for NfL levels (r=0.145, p>0.05). NAA levels did not correlate with age at time of lumbar puncture.

**Correlations of CSF Nf and NAA levels with clinical data**

EDSS score at initial assessment positively correlated with NfH (r=0.362, p<0.01), NfL (r=0.324, p<0.05) and NAA levels (r=0.284, p<0.05). After adjusting for age, this correlation remained significant for NfH (r=0.304, p<0.05; data adjusted for age) and NAA (r=0.308, p<0.05; data adjusted for age).

CSF NfH, NfL and NAA levels were not significantly different in CIS patients who later on converted to MS compared to non-converters (data not shown).

**Correlations of CSF Nf and NAA levels with MRI parameters**

There were no significant correlations of NfL, NfH and NAA levels with baseline normalized volumes of whole brain, gray matter, white matter, cortical gray matter and ventricular CSF (adjusted for age). Higher CSF NfH levels correlated with accelerated global brain volume decrease over a median follow-up of 1.0 years (r=-0.518, p<0.01; data adjusted for age) (Figure 2).
Figure 2. Correlation of CSF NfH levels (ranked variable) with the change of normalized brain tissue volume over time. Higher NfH levels are associated with increased brain tissue loss over time ($r=-0.518$, $p<0.01$; Pearson partial correlation corrected for age).

Figures 1A-C. Group differences of CSF NfH, NFL and NAA between CIS and control (CO) patients. CIS patients had higher levels of NFL (A) and NfH (B). No significant group difference was observed regarding NAA levels (C).
There were no significant correlations between NFL, NfH or NAA levels neither with baseline T2 lesion volume, nor with its change over time during follow-up.

**Correlations among CSF Nf and NAA levels and relation to other CSF parameters**

NfL levels correlated with NfH levels in the total group of patients ($r=0.551$, $p<0.001$; data adjusted for age). NAA levels did not correlate with NFL and NfH levels.

Routine CSF parameters are listed in table 2. NFL ($r=0.646$, $p<0.001$, data adjusted for age) and NfH ($r=0.337$, $p<0.005$, data adjusted for age) levels significantly correlated with CSF cell count. Figure 3 represents the correlation of NFL levels with CSF cell count. NFL levels correlated with albumin quotient ($r=0.348$, $p<0.01$, data adjusted for age) and both NFL ($r=0.456$, $p<0.001$, data adjusted for age) and NfH ($r=0.252$, $p<0.05$, data adjusted for age) levels correlated with the IgG index. No significant correlations between NAA and IgG-index or albumin quotient were present.

![Figure 3. Scatter plot of CSF NFL levels and CSF white cell count variables (unranked and not corrected for age). NFL levels were significantly correlated with CSF white cell count ($r=0.646$, $p<0.001$, Pearson partial correlation corrected for age).](image)

<table>
<thead>
<tr>
<th>Table 2. CSF parameters in CIS patients and controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N</strong></td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>Cell count N/µl (Ref. &lt;4)</td>
</tr>
<tr>
<td>CSF protein mg/dl</td>
</tr>
<tr>
<td>Q Alb</td>
</tr>
<tr>
<td>IgG index (Ref. &lt;0.7)</td>
</tr>
<tr>
<td>OCB positive N (%)</td>
</tr>
</tbody>
</table>

N, number; Q Alb, albumin quotient; OCB, oligoclonal bands; ND, not done; NA, not applicable. Values are given as number, number (%) or as median (interquartile range).
DISCUSSION

The association of NfH levels with subsequent brain tissue damage over a period of one year as defined by MRI is a main and new finding. Our study confirms that CSF NfL and NfH levels are already increased in patients with a CIS suggestive of MS (Kuhle, et al. 2011, Teunissen, et al. 2009a). This preliminary observation suggests that CSF NfH protein levels may predict progressive brain damage specifically caused by axonal damage.

The relationship between CSF Nf and 3T MRI measures of tissue damage in CIS has not been investigated so far. One study reported a correlation between the CSF/serum index of antibodies directed against NfL and brain atrophy, but this study analyzed only patients with relapsing remitting or progressive forms of MS and no relation with neurofilament proteins was observed (Eikelenboom, et al. 2003). None of the CSF markers of axonal damage analyzed in our study correlated with T2 lesion volume at baseline or its change over time. This partly contrasts a previous finding where a correlation of NfL levels with the number of T2 lesions has been described in a combined analysis of CIS and MS patients (Teunissen, et al. 2009a). Another study found an association of CSF NfH levels with the volume of enhancing lesions in patients with MS (Lim, et al. 2005). However, in our CIS cohort, the prevalence of enhancing lesions was too low to allow such an analysis.

Our findings of increased CSF NfL and NfH levels in CIS support earlier suggestions (Kuhle, et al. 2011, Teunissen, et al. 2009a) and argues for axonal damage to occur already in the earliest stage of the disease, which is also corroborated by recent neuropathological findings (Kuhlmann, et al. 2002). Neurofilaments are the major axonal cytoskeletal proteins (Teunissen, et al. 2005). After axonal injury they are released into the extracellular compartment, and subsequently into the CSF and peripheral blood (Petzold. 2005). Thus analysis of CSF/serum neurofilament levels may provide a valuable tool to estimate the extent of axonal damage in MS patients. Potential clinical applications of assessment of neuroaxonal damage markers have been illustrated by two recent studies showing that neurofilament levels may indicate treatment efficacy with the monoclonal antibody natalizumab (Gunnarsson, et al. 2011), and further capture neurotoxic side effects of aggressive treatment regimes (Petzold, et al. 2010b).

Another neuroaxonal marker is NAA (Teunissen, et al. 2005). Neuronally synthesized NAA is believed to be released into the blood via astrocytes (Benarroch. 2008, Moffett, et al. 2007). Evidence for a clinical relevance of NAA comes from several studies, showing that both higher serum and CSF NAA levels correlated with physical disability in relapsing remitting MS patients (Jasperse, et al. 2007, Tortorella, et al. 2011). Compared to controls, CSF NAA levels were not altered in CIS in the current study, supporting previous findings of decreased NAA levels rather in later stages of the diseases such as in secondary progressive MS (Teunissen, et al. 2009a).

In the control group, we observed a close correlation of NfL and NfH levels with age. This correlation was less strong in CIS for NfH and absent for NfL levels. In line with a recent publication (Kuhle, et al. 2011), these findings suggest that age related changes in the levels of CSF Nf may at least partly be caused by ongoing neurodegeneration, which increases with age. In contrast, this age effect may be covered by pathological conditions, where increased brain tissue damage leads to higher CSF Nf levels. Due to the correlation of Nfs with age, statistical analyses require correction for this variable to ensure adequate clinical interpretation (Kuhle, et al. 2011).
After adjusting for age, NfH and NAA levels were correlated with EDSS in remission after the first attack. Although NfH levels have been shown to correlate with EDSS also by several other groups (Kuhle, et al. 2011, Lim, et al. 2005, Petzold, et al. 2006, Petzold, et al. 2010b, Teunissen, et al. 2009a), our results still need to be regarded with caution since the range of the EDSS score in our CIS cohort was relatively small. Previous results on correlations of NfL with EDSS (Norgren, et al. 2004, Teunissen, et al. 2009a) could not be confirmed by our study when correcting for age.

Although we did not observe any group differences for NAA, higher NAA levels correlated with the EDSS score. This argues for a temporal dynamic of NAA levels as previously suggested (Teunissen, et al. 2009a). Whereas in early disease phases CSF NAA levels increase upon axonal damage, decreased levels are observed in advanced stages of the disease (Jasperse, et al. 2007, Teunissen, et al. 2009a).

When comparing levels of Nf and NAA in patients who converted to MS during follow-up compared to non-converters, no significant differences in biomarker levels emerged. Thus, we could not confirm previous reports on altered NfH, NfL or NAA levels in CIS patients who converted to definite MS compared to non-converters (Brettschneider, et al. 2006, Kuhle, et al. 2011, Norgren, et al. 2004, Teunissen, et al. 2009a). In our CIS cohort, we could also not find any differences for NfH, NfL and NAA comparing patients whose CSF was obtained in remission compared to acute patients, as previously reported (Kuhle, et al. 2011, Norgren, et al. 2004, Teunissen, et al. 2009a).

Another interesting finding of our study is that both NfL and NfH but not NAA levels correlated with CSF white cell count, which is in line with a previous report (Norgren, et al. 2004). NfL and NfH may thus be rather related to inflammatory disease activity (Lim, et al. 2005, Lycke, et al. 1998). The correlation of NfL and NfH with the IgG-index further supports this notion, but this correlation has not been found in a recently published study (Kuhle, et al. 2011). Similar to our observation a previous study reported a correlation between NfL-index and the IgG-index (Silber, et al. 2002). However, at this point it can not be completely excluded that correlation of NfH and NfL with CSF cell count might be an epiphenomenon related to the time from the last relapse. We found a correlation of the albumin quotient only with NfL levels, which has not been described in other studies on CSF NfL chain protein (Norgren, et al. 2003, Norgren, et al. 2004). In line with previous reports no such relation was found for NfH levels (Kuhle, et al. 2011) and the NfL and NfH levels were correlated (Teunissen, et al. 2009a).

Our results indicate that analysis of both Nf subunits in the CSF markers is relevant in CIS since their relation to clinical and paraclinical findings are different. Whereas NfL more than NfH is closer related to inflammatory signs such as CSF white cell count and IgG-index, NfH rather indicates permanent axonal damage and neuronal degeneration which is documented by its relation to physical disability and brain volume loss over time already over short-term. Thus, assessment of CSF Nfs in CIS may be of clinical importance to help stratifying patients with more aggressive disease already at the earliest stage of the disease. Although there is good evidence for CSF NAA to serve as a marker for axonal damage in established and progressive MS (Teunissen, et al. 2009a), our results indicate that it is of minor importance in patients with CIS.

**ACKNOWLEDGEMENT**

We thank Mrs. Annemarie Ferstl-Rohrbacher, Mrs. Kerstin Kröll and Mrs. Roberta Bichler for excellent technical assistance. The Austrian Science Fund (FWF) [J2992-B09] is acknowledged for support of MK.
REFERENCES


