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Biomarkers of demyelination and axonal damage are decreased after autologous hematopoietic stem cell transplantation for multiple sclerosis

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Highlights

- Patients treated with AHSCT for MS were followed with lumbar punctures up to five years.
- CSF concentrations of MBP and NFL decreased successively at each follow-up.
- CSF concentrations of GFAp were unchanged.
- At end of follow-up a majority of patients had normal values of MBP and NFL.
- Demyelination and axonal damage can be halted by AHSCT for MS.

Abstract

Background

Autologous hematopoietic stem cell transplantation (aHSCT) has seen increased use for relapsing-remitting multiple sclerosis (RRMS) in recent years. It is considered one of the most effective treatments for RRMS and has been associated with improvement in disability and prolonged remission. This suggests that the tissue-injuring disease process may have been altered by aHSCT. To assess whether this hypothesis is correct, we performed a study of three commonly used cerebrospinal fluid biomarkers of tissue damage.

Methods

In this single center study, 63 patients treated with aHSCT at Uppsala University Hospital between January 1st 2012 – January 31st 2019 were screened for participation. A control group consisting of volunteers without neurologic disease were included as a reference. Cerebrospinal fluid concentrations of neurofilament light (NFL), myelin basic protein (MBP) and glial acidic fibrillary protein (GFAp) were determined using ELISA and a multiplex proteomics platform from Meso Scale Discovery.

Results

Forty-three patients with a mean age of 31 and a median follow-up time of 3.9 years were included. Their median baseline expanded disability status scale (EDSS) score was 3.5 and the annualized relapse rate in the year preceding aHSCT was 1.6. At baseline the proportion of patients with values above the upper limit of normal was 67% for NFL, 63% for MBP and 16% for GFAp. At 5-year follow-up, the proportion of patients with values above the upper limit of normal was 12% for NFL, 12% for MBP and 25% for GFAp. The mean concentration of NFL decreased from 920 pg/mL at baseline to 270 pg/mL at 5-year follow-up (p < 0.001); MBP decreased from 1500 to 680 pg/mL (p < 0.001); whereas the mean concentration of GFAp was unchanged.
Conclusion

In a majority of patients, biomarkers of demyelination and axonal damage reached normal values within five years from treatment with aHSCT.

Keywords

multiple sclerosis, autologous hematopoietic stem cell transplantation, demyelination, neurofilament light chain, myelin basic protein, glial fibrillary acidic protein
1. Introduction

Multiple sclerosis (MS) is an inflammatory disease targeting the oligodendrocyte, leading to demyelination and secondary axonal damage in the central nervous system (CNS) [1, 2]. Inflammation in MS is a tissue-damaging process, [3] which impairs brain and spinal cord function and is the origin of disability in individual patients. When the tissue is damaged the injured cells release intracellular proteins into the cerebrospinal fluid, which can be measured in order to assess the extent of the tissue damage. Several biomarkers have been used for this purpose, among them neurofilament light (NFL), myelin basic protein (MBP) and glial acidic fibrillary protein (GFAP).

Neurofilaments are 8–10 nm heteropolymers with three major subunits: the light, intermediate and heavy chain. They constitute the predominant cytoskeletal component in large-diameter myelinated axons, but are scarcely expressed in the neural soma [4]. With axonal damage, neurofilaments are degraded, releasing NFL fragments into CSF [3, 5, 6]. In several studies (reviewed in [7]) CSF concentrations of NFL were affected by therapeutic intervention. MBP is an oligodendrocyte protein, positioned at the intracellular surface of myelin membranes, and via interactions with acidic lipid moieties involved in maintaining the structure of compact myelin [8]. MBP was put forward early as a potential biomarker of relapses in MS [9, 10] and later confirmed to be associated with gadolinium enhancing lesions on MRI [3]. In a clinical trial of natalizumab for progressive MS, CSF concentrations of MBP were decreased, suggesting that demyelination may be affected by therapeutic intervention. GFAP is the principal intermediate filament in mature CNS astrocytes. GFAP is thought to be important in modulating astrocyte motility and shape by providing structural stability to astrocytic processes. Following tissue injury, astrocytes become reactive and respond with rapid synthesis of GFAP [11]. Several studies (reviewed in [12]) have reported moderately elevated CSF concentrations of GFAP in patients with progressive MS. As far as we know, there has been no report of GFAP being affected by therapeutic intervention for MS.

Autologous hematopoietic stem cell transplantation (aHSCT) is a one-time therapeutic intervention for MS. The purpose of the intervention is to ablate the immune system with biologics and cytotoxic drugs and rebuild a novel immune system with the aid of autologous hematopoietic stem cells. It was first performed for MS more than two decades ago [13], but it took twenty years before a randomized controlled trial was concluded. In 2015 the results of a phase II trial comparing aHSCT and mitoxantrone were reported [14]. Patients treated with aHSCT had fewer new MRI lesions and lower annualized relapse rate. A few years later, the outcome of a phase III study comparing aHSCT with approved disease modifying drugs (DMDs) was reported [15]. The primary endpoint was disease progression, which occurred in 5.8% of patients treated with aHSCT and 67% of patients treated with regular DMDs. The secondary outcomes included MRI T2-lesion volume and self-reported quality of life and were also highly in favor of aHSCT. The promising results led to the approval of the Swedish Board of Health and Welfare of aHSCT as a therapeutic option for patients with active relapsing-remitting MS (RRMS) [16].
The aim of this study was to investigate how intervention with aHSCT affected CSF concentrations of NFL, MBP and GFAp in a cohort of RRMS patients, treated at a single centre.

2. Material and methods

2.1 Ethical approval

The study was approved by the Regional Ethical Board of Uppsala (Dnr 2008/182 and 2012/080/1). All patients provided informed and written consent in accordance with the Declaration of Helsinki.

2.2 Subjects

Patients with relapsing-remitting MS according to the revised McDonald criteria [17] treated with aHSCT at Uppsala University Hospital in the time period January 1st 2012 – January 31st 2019 were considered for the study. Patients were included if: i) they had been conditioned with a cyclophosphamide-based regimen (c.f. procedures) and ii) CSF samples from baseline and at least one follow-up were available. A total of 63 patients was screened, 19 were excluded because no CSF samples were available and one patient was excluded due to treatment with another conditioning regimen. The remaining 43 patients were included. A control group consisting of volunteers without neurologic disease were included as a reference.

2.3 Follow-up and collection of clinical data

Follow-up visits were made at least once per year with recording of clinical relapses, expanded disability status scale (EDSS) [18] score and assessment with MRI of the brain and spinal cord. Clinical data were extracted from the Swedish Multiple Sclerosis Register (SMSreg) [19] on June 30th 2020 and electronic health records were vetted to ensure accuracy of data.

2.4 Lumbar punctures and collection of CSF samples

All patients treated with aHSCT were offered to undergo lumbar puncture at baseline and follow-up at 1, 2 and 5 years. The controls underwent one lumbar puncture at a single timepoint. CSF samples were handled according to previously published consensus guidelines [20] and snap frozen at −80 °C in 1 mL aliquots.

2.5 Procedures

Autologous hematopoietic stem cells were mobilised with a single dose of 2 g/m$^2$ cyclophosphamide followed by filgrastim 5–10 µg/kg/day for 6–7 days and then harvested approximately 10 days after the start of the mobilization regimen. No ex-vivo graft manipulation was performed. Patients were conditioned with a combination of cyclophosphamide and rabbit anti-thymocyte globulin (cyclophosphamide 200 mg/kg; rATG
6 mg/kg). Prophylaxis for fungal, viral and bacterial infection was administrated during neutropenia. Prophylaxis for herpes viruses and *Pneumocystis jiroveci* continued for a minimum of 3 months.

2.6 Analysis of biomarkers

NFL was analysed at an accredited clinical laboratory (Sahlgrenska University Hospital) using the NF-light® ELISA (UmanDiagnostics AB, Umeå, Sweden), which is a CE certified method approved for in vitro diagnostics. The lower limit of quantification was 50 pg/ml. Normal values of NFL in different age groups have been established at the laboratory: age below 20 years < 387 pg/mL; age below 30 years < 525 pg/mL; age below 40 years < 713 pg/mL; age below 50 years < 967 pg/mL. The upper limit of normal has also been described as a continuous function of age (CSF NFL = 210.22 × 1.031^age pg/mL) [21].

MBP was analysed in-house with a Human MBP DuoSet Elisa kit (R&D Systems, Minneapolis, MN, United States. Cat. No. DY4228). The plates were read with a spectrophotometer with a microplate reader and SoftMax Pro 7 software (Molecular Devices, San Jose, CA, USA). For the purpose of this study, normal values for MBP were calculated as the mean value of the controls ±2SD.

GFAP was analysed at the Science for Life facility, using a multiplex proteomics platform: the Meso Scale Discovery (MSD) U-PLEX™ Metabolic Group 1 Assay (Meso Scale Discovery, Gaithersburg, MD, USA. Cat. No. K15280K). For the purpose of this study, normal values for GFAP were calculated as the mean value of the controls ±2SD.

The pooled intra-assay coefficients of variation were below 10% for all analytes.

2.7 Definition of clinical endpoints

A clinical relapse was defined as a period of acute worsening of neurological function lasting ≥24 hours not attributable to an external cause such as increased body temperature or acute infection. Confirmed disability improvement (CDI) was defined as a decrease in EDSS score with at least one point from baseline sustained between two follow-up visits separated in time by no less than 6 months (0.5 points if the baseline EDSS ≥6). Confirmed disability worsening (CDW) was defined as an increase in EDSS score with at least one point from baseline sustained between two follow-up visits separated in time by no less than 6 months (1.5 point if EDSS at baseline was 0, 0.5 points if the baseline EDSS ≥5.5). An MRI event was defined as the appearance of any T2 lesion >3 mm or gadolinium enhancing lesion in the brain or spinal cord not present on the baseline scan. No evidence of disease activity (NEDA-3) was defined as absence of clinical relapses, CDW and MRI events. Patients who did not maintain NEDA-3 were considered to have evidence of disease activity (EDA).

2.8 Statistical analyses

Statistical analyses were performed using R version 4.0.3.[22]. Details on the R packages used are available in the Appendix. D'Agostino’s normality test was used to assess distributions of variables. Data were log-transformed in order to reduce skewness. Student’s
t-test was used when comparing biomarker concentrations in patients versus controls. To assess concentration differences compared to baseline, mixed multilevel linear regression models were fitted with gender and age as covariates using the lme4 package. Post-hoc comparisons to baseline were performed using the estimated marginal means using the R package emmeans. Spearman’s ranked correlation analysis was used to determine correlations between biomarkers and between biomarkers and EDSS. A two-tailed p value < 0.050 was considered significant.

3. Results

3.1 Patient characteristics

The characteristics of the subjects are summarized in Table 1. Seventy-nine percent of the patients had been treated with DMDs prior to aHSCT – the median number of DMDs was 2 [IQR 1 – 3]. Nine were treatment naïve; 7 were treated with dimethyl fumarate; 10 with fingolimod; 9 with glatiramer acetate; 23 with interferons; 3 with intravenous IgG; 2 with mitoxantrone; 17 with natalizumab; 8 with rituximab; and 1 with teriflunomide. The annualized relapse rate one year prior to treatment was 1.6 (±1.5). Baseline samples were available from 43 subjects, 1-year follow-up samples from 43, 2-year follow-up samples from 26 and 5-year follow-up samples from 8 subjects. The average number of days between baseline CSF sampling and mobilization was 5.8 days (±15), baseline and stem cell transplant 45 days (±16), and baseline and MRI investigation was 1.6 days (±18).

3.2 Patient outcomes

The median follow-up was 3.9 years [IQR 2.2 – 4.3]. Fifteen patients were stable in EDSS during the follow-up period, 23 improved in EDSS with CDI and 5 patients got worse with CDW. Nine out of 43 patients exhibited EDA during the follow-up period. Four of these patients had a clinical relapse, five had new MRI lesions and five experienced CDW. Two patients with clinical relapses started rituximab treatment approximately 2 years after aHSCT. The other patients did not receive any additional treatment.

3.3 NFL

Normal values of NFL had been established at the reference laboratory and the upper limit of normal was calculated as $210.22 \times 1.031^{\text{age}}$ pg/mL. At baseline, 67% of patients had CSF concentrations of NFL above the upper limit of normal. Of the patients with gadolinium enhancing lesions, 12/12 (100%) had CSF concentrations of NFL above the upper limit of normal, whereas 16/29 (55%) of patients without gadolinium enhancing lesions had CSF concentrations of NFL above the upper limit of normal (Supplementary table 1). Patients with gadolinium enhancing lesions at baseline had higher levels of NFL the 29 patients without (2700 vs 590 pg/mL, p = < 0.01).

The age-adjusted NFL concentrations decreased after aHSCT (Figure 1) and the proportion of patients with normal values increased with each year of follow-up reaching 88% at five years (Table 2). Patients who maintained NEDA-3 over the follow-up period had numerically lower
baseline median CSF NFL than patients with EDA post-AHSCT, but the difference was not statistically significant (720 vs 2100 pg/mL, p = 0.13) (Table 3). During follow-up, 1/14 (7%) of patients with normal NFL values at baseline had EDA and 8/29 (28%) of patients with pathological NFL values had EDA. Thus, 93% of patients remained in NEDA-3 if they had normal NFL values at baseline. Higher baseline levels of NFL were observed in patients with CDW (p-value = 0.049) as well as patients with CDI (p-value = 0.04) in comparison with patients who were stable in EDSS during follow-up (Table 4).

3.4 MBP

The upper limit of normal was set at 990 pg/mL. At baseline, 63% of patients had CSF concentrations of MBP above this limit. Of the patients with gadolinium enhancing lesions, 12/12 (100%) had CSF concentrations of MBP above the upper limit of normal, whereas 14/29 (48%) of patients without gadolinium enhancing lesions had CSF concentrations of MBP above the upper limit of normal (Supplementary table 1). Patients with gadolinium enhancing lesions at baseline had higher levels of MBP than those without (3200 vs 850 pg/mL, p < 0.001).

The age-adjusted MBP levels decreased after aHSCT (Figure 2) and the proportion of patients with normal values increased with each year of follow-up reaching 88% at five years (Table 2). Patients who maintained NEDA-3 over the follow-up period had lower baseline median CSF MBP concentration than patients with EDA post-AHSCT (1200 vs 2400 pg/mL, p = 0.03) (Table 2). During follow-up, 1/16 (6%) of patients with normal MBP values at baseline had EDA and 8/27 (30%) of patients with MBP values above the upper limit of normal had EDA. Thus, 94% of patients remained in NEDA-3 if they had normal MBP values at baseline. Higher baseline levels of MBP were observed in patients with CDW (p-value = 0.03) as well as patients with CDI (p-value = 0.01) in comparison with patients who were stable in EDSS during follow-up (Table 4).

3.5 GFAp

The upper limit of normal was set at 36 ng/mL. At baseline, 16% of patients had GFAp values above this limit. Of the patients with gadolinium enhancing lesions, 3/12 (25%) had CSF concentrations of GFAp above the upper limit of normal, whereas 3/29 (10%) of patients without gadolinium enhancing lesions had CSF concentrations above the upper limit of normal (Supplementary table 1). Patients with gadolinium enhancing lesions at baseline had numerically higher levels than those without (26 vs 20 ng/mL, p=0.17).

The age-adjusted GFAp levels did not decrease after aHSCT (Figure 3). The proportion of patients with normal values decreased somewhat with each year of follow-up reaching 75% at five years (Table 2a). Patients who maintained NEDA-3 had the same baseline median CSF GFAp concentration as patients with EDA post-AHSCT (23 vs 23 ng/mL, p =0.82) (Table 3). During follow-up, 7/36 (19%) of patients with normal GFAp values at baseline had EDA and 2/7 (29%) of patients with GFAp values above the upper limit of normal had EDA. Thus, 81% of patients remained in NEDA-3 if they had normal GFAp values at baseline. Numerically higher baseline levels of GFAp were observed in patients with CDW as well as patients with
CDI in comparison with patients who were stable in EDSS during follow-up (Table 4), but the difference was not statistically significant.

3.6 Correlation analyses

We first determined how the concentrations of NFL, MBP and GFAP at baseline correlated with each other. NFL and MBP correlated highly with Spearman $r=0.84$ ($p < 0.0001$), GFAP less so. At 1 year, a correlation between GFAP and MBP was no longer present, while the strength of the correlations of NFL and MBP as well as NFL and GFAP were weaker. From 2 years and onwards no correlations between NFL, MBP and GFAP were present (Supplementary table 2). Then we determined how NFL, MBP and GFAP correlated with EDSS. At baseline, they correlated moderately to the EDSS with Spearman $r = 0.36 – 0.41$. Over time, this correlation disappeared and when NFL, MBP and GFAP at 2-year follow-up were analysed in relation to EDSS at 2-year follow-up, none of the biomarkers correlated with EDSS anymore (Table 5).

4. Discussion

In recent years, the use of aHSCT for MS has steadily increased [23] and it has been approved for patients with active RRMS in Sweden [24]. It was also the subject of recently published guidelines from the U.S. National Multiple Sclerosis Society, in which it was concluded that aHSCT is a treatment option for people with RRMS and breakthrough disease activity despite treatment with high-efficacy DMDs [25]. aHSCT is typically associated with a 5-year rate of NEDA-3 of about 70% [15, 26], but how aHSCT affects tissue damage has been considerably less studied. We used a biomarker approach and focused on three of the most widely used biomarkers for tissue damage in MS, namely NFL, MBP and GFAP. Before treatment with aHSCT was initiated, the CSF concentrations of NFL and MBP was pathologically increased in about two-thirds of patients. The concentrations decreased after aHSCT and at the first follow-up, one year after aHSCT, the proportion of patients with pathological values had decreased to about one-third. This decrease continued and by five years only 1/8 patients had a pathological concentration. This stands in contrast to GFAP, where only 16% of patients had pathological values before aHSCT and we could not detect any statistically significant effect of aHSCT on the GFAP concentrations.

NFL is now widely used as a biomarker for axonal injury in neurological disease, reflecting its utility [27]. All of the patients with gadolinium enhancing lesions had NFL values above the upper limit of normal, and more than half of those without. Patients with active disease usually have increased levels of NFL [3], and the high proportion of patients with increased concentrations of NFL likely reflects the fact that patients selected for aHSCT have active disease. Following aHSCT, CSF NFL concentrations were decreased successively at each follow-up, consistent with previous reports from the Canadian Bone Marrow Transplantation group [28] as well as our own group [29].

MBP is released into the CSF during demyelination and was initially investigated as a potential biomarker for MS nearly fifty years ago, in the mid-1970s [9, 10]. Despite promising results, MBP has not been used as much as NFL in studies of MS; one contributing factor may be that the half-life of MBP is relatively short [30]. During clinical attacks, increased MBP CSF
concentrations can be seen in approximately 80% of patients, but only in a minority of patients with stable disease [31]. As far as we know, the effect of aHSCT on CSF MBP concentrations have not been investigated before. At baseline MBP correlated highly with NFL and in similarity to NFL, MBP was pathologically increased in all patients with gadolinium enhancing lesions and about half in those without. MBP concentrations also decreased successively at each follow-up.

GFAp is highly expressed in astrocytes and although astrocytes are not believed to be targeted by MS, a large body of literature exist on GFAP as a biomarker in MS. It has consistently been reported that MS patients have increased levels of GFAP in CSF. [12] Several reports have also claimed that GFAP is a biomarker for progressive MS, but this is likely confounded by the strong correlation between GFAP and age [32]. The CSF concentrations of GFAP were somewhat higher in MS patients in comparison to the controls, most likely reflecting reactive astrocytes and astrogliosis rather than tissue damage. The GFAP levels were essentially unchanged after aHSCT, which should be expected if GFAP reflects astrogliosis. Similar results were also seen in a previous study investigating serum GFAP concentrations before and after aHSCT [33].

Patients with stable EDSS throughout follow-up tended to have lower values of biomarkers than those that worsened or improved. This likely reflects that patients with more severe disease are at higher risk of worsening, but also have more to gain from aHSCT. Patients with EDA during follow-up had higher MBP concentrations at baseline, but this did not translate to higher levels of MBP during follow-up, as those who maintained NEDA and those exhibiting EDA had comparable concentrations. At baseline all biomarkers were associated with each other and EDSS. However, this association disappeared with time, suggesting that the injurious process leading to disability had been halted.

4.1 Limitations

All patients who were treated with aHSCT were offered to undergo lumbar puncture and participate in the study. About one-third declined. We did not collect any data on the reason for declining, but it is possible that it may have led to a sampling bias towards patients with more active disease during follow-up. Only a minority of patients was followed for the entire 5 years. This means that the long-term effects of aHSCT must be interpreted with caution and further studies are needed to ascertain the results on the time scale 5–10 years after aHSCT. We relied on samples from healthy controls to assess normal levels of MBP and GFAP, but these were slightly mismatched in terms of age and sex. There is little indication of that sex has a large impact on the CSF concentrations of these proteins, but GFAP clearly increases with age. This means that our upper limit of normal may have been set too low and that a slightly larger percentage of patients should have been considered as having normal values.

4.2 Conclusions

In this study, we investigated if therapeutic intervention with aHSCT could halt the injurious process leading to tissue damage in MS. Our main finding is that a majority of patients reach that goal with time, regardless of subsequent disease activity.
5. Author statement

Christina Zjukovskaja: Data curation, data analysis, visualization of data, writing- original draft preparation.

Anders Larsson: supervision, writing- reviewing and editing.

Honar Cherif: was part of the team that performed autologous hematopoietic stem cell transplantation, writing- reviewing and editing.

Kim Kultima: methodology, supervision, writing- reviewing and editing.

Joachim Burman: Follow-up of patients, conceptualization, methodology, supervision, writing- original draft preparation, reviewing and editing.

6. Disclosures

Declarations of interest: none.

7. Funding

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8. Acknowledgements

The authors would like to thank Asma Al-Grety, who assisted with the ELISA of MBP.

Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
9. References


10. Tables

**Table 1. Baseline demographical and clinical data**

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Baseline before aHSCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>31</td>
<td>43</td>
</tr>
<tr>
<td>Female/Male</td>
<td>14/17</td>
<td>28/15</td>
</tr>
<tr>
<td>Mean age (SD)</td>
<td>25 (7.3)</td>
<td>31 (6.5)</td>
</tr>
<tr>
<td>Mean disease duration (SD)</td>
<td>6.3 (5.7)</td>
<td></td>
</tr>
<tr>
<td>Mean annualized relapse rate (SD)</td>
<td>1.6 (1.5)</td>
<td></td>
</tr>
<tr>
<td>Median EDSS at baseline (Q1 – Q3)</td>
<td>3.5 (2.5 – 4.0)</td>
<td></td>
</tr>
<tr>
<td>Median previous treatments (Q1 – Q3)</td>
<td>2 (1 – 3)</td>
<td></td>
</tr>
<tr>
<td>Gadolinium enhancing lesions at baseline</td>
<td>12/29*</td>
<td></td>
</tr>
</tbody>
</table>

aHSCT, autologous hematopoietic stem cell transplantation; EDSS, expanded disability status scale. *Two patients underwent MRI without gadolinium contrast at baseline.

**Table 2. Cerebrospinal fluid concentrations of NFL, MBP and GFAp at baseline and follow-up.**

<table>
<thead>
<tr>
<th></th>
<th>NFL (pg/mL)</th>
<th>MBP (pg/mL)</th>
<th>GFAp (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>n/a</td>
<td>Median (IQR)</td>
</tr>
<tr>
<td>Control</td>
<td>31</td>
<td>n/a</td>
<td>430 (290 – 730)</td>
</tr>
<tr>
<td>Baseline</td>
<td>43</td>
<td>920 (420 – 2700)</td>
<td>33%</td>
</tr>
<tr>
<td>1 year</td>
<td>43</td>
<td>440 (300 – 540)</td>
<td>65%</td>
</tr>
<tr>
<td>2 years</td>
<td>26</td>
<td>350 (270 – 430)</td>
<td>81%</td>
</tr>
<tr>
<td>5 years</td>
<td>8</td>
<td>270 (210 – 350)</td>
<td>88%</td>
</tr>
</tbody>
</table>

Median values with interquartile range (IQR) for neurofilament light (NFL), myelin basic protein (MBP) and glial acidic fibrillary protein (GFAp). Normal values of NFL had been established at the reference laboratory and the upper limit of normal was calculated as $210.22 \times 1.031^{\text{age}}$ pg/mL. For MBP and GFAp the normal values were calculated as ±2SD from the mean of the controls.
Table 3. Cerebrospinal fluid concentrations of NFL, MBP and GFAp at baseline and follow-up divided into NEDA and EDA.

<table>
<thead>
<tr>
<th>N</th>
<th>NEDA Median (IQR)</th>
<th>EDA Median (IQR)</th>
<th>P-value</th>
<th>NEDA Median (IQR)</th>
<th>EDA Median (IQR)</th>
<th>P-value</th>
<th>NEDA Median (IQR)</th>
<th>EDA Median (IQR)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>34/9</td>
<td>720 (340 – 2300)</td>
<td>ns</td>
<td>1200 (620 – 2700)</td>
<td>2400 (2400 – 9200)</td>
<td>0.03</td>
<td>23 (17 – 30)</td>
<td>23 (18 – 30)</td>
<td>ns</td>
</tr>
<tr>
<td>1</td>
<td>34/9</td>
<td>430 (290 – 540)</td>
<td>ns</td>
<td>630 (490 – 1000)</td>
<td>1100 (670 – 1500)</td>
<td>0.05</td>
<td>23 (18 – 32)</td>
<td>23 (19 – 25)</td>
<td>ns</td>
</tr>
<tr>
<td>2</td>
<td>19/7</td>
<td>330 (250 – 420)</td>
<td>ns</td>
<td>700 (460 – 1000)</td>
<td>970 (600 – 1100)</td>
<td>ns</td>
<td>25 (18 – 36)</td>
<td>21 (19 – 23)</td>
<td>ns</td>
</tr>
<tr>
<td>5</td>
<td>4/4</td>
<td>270 (200 – 350)</td>
<td>ns</td>
<td>590 (550 – 680)</td>
<td>710 (690 – 800)</td>
<td>ns</td>
<td>31 (27 – 35)</td>
<td>24 (23 – 33)</td>
<td>ns</td>
</tr>
</tbody>
</table>

*: B – Baseline, 1 – 1 year, 2 – 2 years, 5 – 5 years

Median values with interquartile range (IQR) for neurofilament light (NFL), myelin basic protein (MBP) and glial acidic fibrillary protein (GFAp). Normal values of NFL had been established at the reference laboratory and the upper limit of normal was calculated as $210.22 \times 1.031^{\text{age}}$ pg/mL. For MBP and GFAp the normal values were calculated as ±2SD from the mean of the controls.

Table 4. Levels of biomarkers at baseline based on whether EDSS status showed improvement, stability or worsening.

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<tr>
<th></th>
<th>Baseline NFL (pg/mL) Median (IQR)</th>
<th>Baseline MBP (pg/mL) Median (IQR)</th>
<th>Baseline GFAP (ng/mL) Median (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDW (n = 5)</td>
<td>2100 (660 – 8300)</td>
<td>2400 (2400 – 9200)</td>
<td>30 (23 – 41)</td>
</tr>
<tr>
<td>Stable (n = 15)</td>
<td>450 (320 – 1400)</td>
<td>760 (480 – 1900)</td>
<td>18 (16 – 23)</td>
</tr>
<tr>
<td>CDI (n = 23)</td>
<td>1500 (540 – 4600)</td>
<td>1800 (810 – 4200)</td>
<td>25 (19 – 33)</td>
</tr>
</tbody>
</table>

NFL, neurofilament light; MBP, myelin basic protein; GFAP, glial fibrillary acidic protein; EDSS, expanded disability status scale; CDW, confirmed disability worsening; CDI, confirmed disability improvement.
Table 5. Correlations between biomarkers and EDSS over time

<table>
<thead>
<tr>
<th></th>
<th>NFL</th>
<th></th>
<th>MBP</th>
<th></th>
<th>GFAp</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spearman r P-value</td>
<td>Spearman r P-value</td>
<td>Spearman r P-value</td>
<td>Spearman r P-value</td>
<td>Spearman r P-value</td>
<td>Spearman r P-value</td>
</tr>
<tr>
<td>Baseline EDSS</td>
<td>0.40 0.0075</td>
<td>0.41 0.0058</td>
<td>0.36 0.018</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1 year EDSS</td>
<td>0.22 0.15</td>
<td>0.32 0.037</td>
<td>0.020 0.90</td>
<td></td>
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<tr>
<td>2 year EDSS</td>
<td>0.2 0.33</td>
<td>0.22 0.27</td>
<td>-0.18 0.37</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 year EDSS</td>
<td>0.53 0.18</td>
<td>0.09 0.83</td>
<td>0.41 0.32</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Statistically significant correlations are shown in bold. NFL, neurofilament light; MBP, myelin basic protein; GFAp, glial fibrillary acidic protein; EDSS, expanded disability status scale.
Figure 1. Neurofilament light levels before and after autologous hematopoietic stem cell transplantation.

Log₂ of cerebrospinal fluid neurofilament light (NFL) concentrations before and after aHSCT. A multilevel linear regression analysis of NFL with gender and age as covariates was made. In this model, the concentrations of NFL decreased after aHSCT with every year, eventually approaching 88% normal values at five years. Patients with no evidence of disease activity (NEDA-3) during follow-up are shown as black circles, whereas patients with evidence of disease activity (EDA) are shown as red triangle. Patients with EDA had numerically higher NFL concentrations at baseline (2100 pg/mL) than patients with NEDA-3 (720 pg/mL), but the difference was not statistically significant (p=0.13).

*** p-value < 0.0001
RRMS patients were compared with a control group of volunteers without neurologic disease. MS patients had almost four times higher concentrations of MBP than the controls (p-value < 0.0001). A multilevel linear regression analysis of MBP with gender and age as covariates was made. In this model, the concentrations of MBP decreased after aHSCT with every year, eventually approaching 88% normal values (±2SD from the mean value of the controls) at five years. Patients with no evidence of disease activity (NEDA-3) during follow-up are shown as black circles, whereas patients with evidence of disease activity (EDA) are shown as red triangles. Patients with EDA had higher concentrations of MBP at baseline than patients with NEDA-3 (2400 pg/mL vs 1200 pg/mL, p = 0.03).

B=Baseline; HC=Healthy controls. * p-value < 0.01, *** p-value < 0.0001
RRMS patients were compared with a control group of volunteers without neurologic disease. MS patients at baseline had higher concentrations of GFAP than the controls (p < 0.01). A multilevel linear regression analysis of GFAP with gender and age as covariates was performed along with a post-hoc comparison using estimated marginal means after model fitting. In this model, the concentrations of GFAP after aHSCT did not significantly alter every year, eventually dropping to 75% normal values (±2SD from the mean value of the controls) at five years. Patients with no evidence of disease activity (NEDA-3) during follow-up are shown as black circles, whereas patients with evidence of disease (EDA) activity are shown as red triangles. Patients with NEDA-3 had similar baseline concentrations as patients with EDA (23 ng/mL vs 23 ng/mL, p=0.82).

B=Baseline; HC=Healthy controls. * p-value < 0.01