Plasma neurofilament light chain levels in patients with MS switching from injectable therapies to fingolimod

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Abstract

Background: Neurofilament-light (NFL) is a cerebrospinal fluid (CSF) marker of neuroaxonal damage in MS.

Objective: To determine the correlation of NFL in cerebrospinal fluid (CSF) and serum/plasma, and in plasma after switching from injectable MS therapies to fingolimod.

Methods: A first cohort consisted of MS patients (n=39) and neurological disease controls (n=27) where CSF and plasma/serum had been collected for diagnostic purposes. A second cohort (n=243) consisted of patients from a post-marketing study of fingolimod. NFL was determined with Single Molecule Array (Simoa™) technology (detection threshold 1.95 pg/ml).

Results: Mean NFL pg/ml (SD) was 341 (267) and 1475 (2358) in CSF and 8.2 (3.58) and 17.0 (16.94) in serum from controls and MS, respectively. CSF/serum and plasma/serum levels were highly correlated (n=66, rho=0.667, $p<10^{-9}$ and n=16, rho=0.684, p=0.009, respectively). In patients starting fingolimod (n=243) mean NFL pg/ml (SD) in plasma was reduced between baseline (20.4 (10.7)) and at 12 months (13.5 (7.3) $p< 3\ 10^{-6}$), and levels remained stable at 24 months (13.2 (6.2)).

Conclusion: NFL in serum and CSF are highly correlated and plasma NFL levels decrease after switching to highly effective MS therapy. Serum NFL measurement can be considered as a biomarker for MS therapy response.

Introduction

An increasing body of evidence supports the use of markers for inflammatory activity for assessing disease activity in MS patients, in turn of relevance for treatment decisions. To date only magnetic resonance imaging (MRI) is available in clinical practice, and has shown a high correlation to relapse rates in meta-analyses of several large trials [1]. Still, it is not established how well clinically available MRI measures (new T1/T2 and contrast enhancing lesions) correlate with neuroaxonal damage, in turn a major determinant of permanent disability in MS, see e.g. [2]. In addition, repeated MRI examinations for monitoring purposes are usually restricted to the brain and therefore do not reflect pathology of the spinal cord [3]. The number of candidate biomarkers of possible value in MS is very large [4]. However, in most cases relation to important disease processes, such as neuroaxonal damage, or prognostic value for important outcomes, such as long term disability, is lacking. Among different markers for neuroaxonal damage, neurofilaments, and especially neurofilament light (NFL), has emerged as promising candidates [5, 6 7]. Furthermore, several studies have used cerebrospinal fluid (CSF) NFL as a marker for therapeutic responses [8 9 10 11 12 13]. We have previously shown that CSF NFL levels correlate with several biomarkers of intrathecal inflammation, and that both CSF NFL and inflammatory markers decrease with age in MS [14]. Still, an important obstacle for a wider introduction into clinical practice is the requirement of CSF sampling by lumbar puncture. Disanto and co-workers have reported serum NFL levels in patients with a clinically isolated syndrome (CIS) using an electrochemiluminescence (ECL)-based method [15]. Serum NFL levels were about three-fold higher in CIS patients compared to healthy controls, and within the CIS group NFL correlated with MRI activity and higher disability scores at CIS presentation. Though more sensitive than ordinary enzyme-linked immunosorbent assay (ELISA), the ECL-based method has a reported analytical sensitivity limit above that normally seen in healthy controls and also with a significant overlap with levels seen in MS patients [16]. However, we recently developed an ultrasensitive assay for serum NF-L using Single Molecule Array (Simoa™) technology (ref: Gisslén M et al., EBioMedicine. 2015 Nov 22;3:135-40), which represents a novel immune-based ultrasensitive proteins detection method that has a 25-fold improved analytical sensitivity as compared to the ECL-based method and shows correlations between serum and CSF levels throughout the span of normal to pathological concentrations (ref: Kuhle J et al., Clin Chem Lab Med. 2016 Oct 1;54(10):1655-61). The method has further shown correlations of serum NFL levels with MRI data in two smaller cohorts of early MS patients [17, 18]. We here sought to replicate this finding in a larger cohort

of MS patients and controls, and also determined plasma NFL levels in a large real world cohort of MS patients switching from injectable therapies to fingolimod.

Methods and material

Patients and samples

Two different patient cohorts were used in the study. The first cohort (Cohort 1; n=66 consisted of patients attending the Department of Neurology at the Karolinska University Hospital for MS or psychiatric or other neurological conditions where sampling of blood and CSF for diagnostic purposes had been undertaken. The controls consisted of non-inflammatory neurological disease controls (NINDC) (n=27) patients (56% women, mean age 35.2 years, range 20-56) without signs of intrathecal inflammation on standard CSF tests (Table 1). The group comprised the following diagnoses; new onset psychotic symptoms n=11, sensory symptoms/paresthesia n=10, fatigue n=1, dizziness n=1, headache n=2, visual disturbance n=2. The MS group consisted of 39 patients (54% women, mean age 39.6 years, range 23-70), with the following disease courses; relapsing-remitting MS (RRMS) in remission n=29, relapsing-remitting MS (RRMS) with clinical relapse symptoms with 3 months of sampling n=4, secondary progressive MS (SPMS) n=3, primary progressive MS (PPMS) n=3. CSF and plasma/serum were collected at the same time point using standardized protocols. CSF was centrifuged immediately and the supernatant was frozen at -80 ^{o}C within 1 h.

The second cohort (Cohort 2) consisted of patients enrolled in a post-marketing study of fingolimod (Gilenya®). All clinics in Sweden starting patients on newer MS disease modulatory treatments (DMTs) participate in the Immunomodulation and Multiple Sclerosis Epidemiology (IMSE) study. As of March 31st 2016 a total of 1467 patients had been recruited to the study. All patients are followed over time using the nationwide web-based Swedish Neuroregistry (NeuroReg) and donate a plasma sample at baseline and at 12 and 24 months after treatment initiation. Samples are sent by surface mail in room temperature to a centralized biobanking unit at Karolinska Institutet. From this cohort n=261 individuals were selected based on availability of a baseline sample and at least one sample at either 12 or 24 months, as well as information on prior treatment. We limited the analysis to patients with RRMS at baseline, who had not made treatment breaks for more than 2

weeks, and those which had successful NFL measurements for at least two time points. Thus, n=241 had samples from baseline and 12 months, n= 119 from baseline and 24 months, and n=119 from baseline, 12 and 24 months (Table 2). Twenty-four percent had switched from glatiramer acetate, and 75% from interferons. The remaining 2% had either not been treated for MS previously or had been treated with study-drug or pulsed corticosteroids.

All patients provided written consent and the study was approved by the Regional Ethics Committee of Stockholm (2009/2107-31/2 and 2011/641-31/4, respectively).

Determination of NFL using SIMOA

CSF NFL levels were determined using the NF-Light kit from UmanDiagnostics (UmanDiagnostics, Umeå, Sweden) according to the manufacturer's instructions. Samples were run in duplicate with an intra-assay-variability of 3.5% and a lower detection threshold of 32 pg/ml. Plasma NFL levels were determined using the same NFL antibodies from UmanDiagnostics, transferred onto the Simoa™ platform using a homebrew kit (Quanterix Corp, Boston, MA, USA). The lower limit of quantification (LLoQ), determined by the blank mean signal + 10 SD, was 1.95 pg/ml. All samples measured were above LLoQ in a pilot run on plasma from 100 patients and controls, where a range from 2.2 to 403 pg/mL could be demonstrated. The analyses were performed by a board-certified laboratory technician using one batch of reagents with intra-assay coefficients of variation below 10%.

Statistical Methods

Correlation analyses were carried out with two sided Spearman's rank correlation. Differences in NFL levels between groups were tested with two sided non-parametric Kruskal-Wallis rank sum or Wilcoxon test. For comparison between different lengths of treatment paired Wilcoxon test was used. Statistical tests were carried out in Rv 3.3.2.

Results

Correlation between NFL levels in CSF and serum/plasma

We first determined NFL levels in CSF and serum in a heterogeneous group of MS patients and NINDC (Cohort 1). The mean NFL level in CSF was 341 (SD 267) and 1475 (SD 2358) pg/ml in controls and MS, respectively. In the MS cohort, the highest mean NFL levels were found in RRMS patients with a recent relapse (3840 pg/ml), whereas mean levels were lower in RRMS patients in remission (1343 pg/ml) and SP/PPMS patients (535 pg/ml). Subsequently, using the same antibodies transferred onto the Simoa™ platform, NFL levels were determined in paired serum samples. At the group level mean NFL levels were 8.2 (SD 3.58) and 17.0 (SD 16.94) pg/ml in controls and MS, respectively. The levels of NFL in paired CSF and serum samples were highly correlated (rho=0.671, $p<10^{-9}$, Figure 1A). As a subsequent step we also tested the degree of correlation between paired serum and plasma samples in a subpopulation (n=16) of patients from Cohort 1, again demonstrating a high degree of correlation (Sr=0.684, p=0.009, Figure 1B).

NFL levels in patients switching to fingolimod

Next we determined NFL levels in samples collected in context of a large nationwide post-marketing study for fingolimod (Cohort 2). The samples were selected based on the availability of a baseline sample and at least one follow up sample as well as information on prior treatment with injectable therapies (interferons, glatiramer acetate), and was limited to patients with RRMS at treatment start but were otherwise randomly chosen. Cohort 2 consisted of 243 individuals and a total of 603 samples (Table 2).

As a sensitivity analysis, given that samples had been sent by surface mail with a variable time in the post, we calculated mean NFL levels in relation to number of days between sampling and arrival, which showed that the time (range from 1 to 7 days) the sample had been in the post did not affect the mean level (rho=0.046, p=0.60) (Figure 2). Therefore, we did not correct for this in the subsequent analyses. In addition, re-testing to exclude batch effects revealed an excellent reproducibility (data not shown).

At the group level mean plasma NFL measured at baseline, 12 and 24 month after start of fingolimod therapy demonstrated a significant 34% reduction at 12 months (13.5 pg/ml, SD 7.34) compared to baseline (20.4 pg/ml, SD 17.79), where levels remained similar at 24 months (13.20 pg/ml, SD 6.19) compared to 12 months (Table 2; Figure 3). The reduction in NFL was associated with a reduced MS Severity Score (MSSS) from a

median of 2.30 at start of therapy, 2.00 at 12 months and 1.70 at 24 months. While most subjects (n=191) demonstrated lowered levels, a few subjects displayed increased NFL levels after start of fingolimod (n=49).

Sub-group analyses

Sex did not affect NFL levels at baseline, but males had a tendency for lower levels than females at both 12 and 24 months (p=0.09 and p=0.16, respectively). Age was positively correlated with NFL levels at all time points; baseline (rho=0.216 p<0.0008), 12 (rho=0.306, p< 2 10-6) and 24 months (rho=0.221 p<0.02). MSSS was positively correlated with NFL levels at baseline (rho=0.19, p<0.03), with a tendency for correlation at 12 months (rho=0.14. p=0.109 but not at 24 months (r=0.10, p=ns).

Blood lymphocyte counts were not correlated with NFL at baseline (rho=0.023, p=0.80), while there was a weak trend, however going in the opposite directions, at 12 (rho=-0.133, p=0.013) and 24 (rho=0.062, p=0.63) months. In contrast, the change in lymphocyte numbers displayed a correlation with log change in NFL between baseline and 12 months of treatment (rho=0.273 p<0.009).

Discussion

We here determined NFL levels in paired CSF and serum samples in MS patients and controls, as well as in a large cohort of MS patients switching from injectable therapies to fingolimod, where we provide preliminary evidence for the usefulness of NFL levels in blood as a biomarker to assess disease activity and therapeutic efficacy in MS. Thus, in a large group of MS patients switching from injectable MS therapies to fingolimod a highly significant drop in plasma NFL levels had occurred by 12 months and was retained at 24 months. All samples were collected in context of a nationwide post-marketing study, which means that measurements represent a large real world population with population-based sampling. However, weaknesses include incomplete information on some of the clinical variables, especially EDSS and relapses, and lack of MRI data, which limit the possibilities of correlation with clinical outcome variables.

Currently there are no validated fluid biomarkers for disease activity in MS that could function as a complement to MRI[4]. Even if inflammatory activity detectable on MRI is correlated with clinical disease measures, standard measures assessing focal active inflammation mainly in the brain white matter through

contrast-enhancing or newly appearing T2 lesions only reflect certain aspects of MS disease processes. Thus, there is a growing body of evidence from pathological studies that MS comprise also diffuse axonal injuries occurring independent of lesions, as well as grey matter pathology [19] [20]. Volumetric MRI techniques can show combined effects of focal and diffuse injuries, but likely will be insensitive for changes over shorter time periods, at least at the individual level. Since it is well established that degree of neurodegeneration is the most important determinant of long term disability, it would prove useful to identify biomarkers to assess the rate of ongoing neurodegeneration. Among such markers the evidence base for CSF NFL is the strongest, including its usefulness as a marker for therapeutic response [8] [9] [10] [11] [12] [13].

In order to have a more widespread clinical use of neurofilament in MS the assay will have to be developed for measuring levels in blood. Until recently this has not been possible, since the sensitivity of most assays have been well above of what is seen in healthy controls, which also is the treatment target for patients with MS. However, promising preliminary results have been demonstrated with the Simoa™ platform, where the analytical sensitivity is well below that of neurofilament levels seen in healthy controls. Interestingly, using this analytical technique in a recent study serum NFL levels was shown to accurately detect the onset of neurodegeneration in transgenic animal models of neurodegenerative diseases, and in the same study elevated blood NFL levels were demonstrated in the human diseases they model [21]. Increased serum or plasma NFL concentrations further reflect neuroaxonal injury in human neurodegenerative diseases such as atypical parkinsonian disorders (Hansson O et al., Neurology. 2017 Feb 8. pii: 10.1212/WNL.0000000000003680; Rojas JC et al., Ann Clin Transl Neurol. 2016 Feb 1;3(3):216-25), frontotemporal dementia (Rohrer JD et al., Neurology. 2016 Sep 27;87(13):1329-36), Alzheimer's disease (Mattsson N et al., in press JAMA Neurol) and HIV-associated neurocognitive dysfunction (Gisslén M et al., EBioMedicine. 2015 Nov 22;3:135-40), as well as in traumatic brain injury (Shahim P et al., Sci Rep. 2016 Nov 7;6:36791). Furthermore, at an Ectrims platform presentation interesting data from the Freedoms I study, a phase III study examining the effect of fingolimod compared to placebo in RRMS, was communicated by the group of Jens Kuhle. Thus, increased levels of NFL in blood were associated not only with the occurrence of gadolinium-enhancing lesions, relapses and increased EDSS, but also with brain atrophy [22]. Collectively, these findings suggest that blood NFL can be used as a biomarker to assess disease activity and therapeutic efficacy in MS. In this context our findings are important as they represent a proof-of-principle of the feasibility of testing in a routine clinical context.

This also opens interesting perspectives that comprise the possibility to re-analyse archival blood samples from clinical trials, sometimes with several years of follow up after the double blinded period, which can be used to generate important information on how well this biomarker can predict long term clinical outcomes. Still, some caution is warranted with regard to the validity of blood NFL measurements to assess inflammatory disease activity at the individual level. Thus, the dynamic range of blood NFL levels is much compressed compared to measurements in CSF and might also be affected by individual variability in blood-brain barrier function. For example, it is not known if sampling during a low grade inflammation or infection might affect the leakage of neurofilament over the blood-brain barrier and thus result in falsely elevated levels in the peripheral compartment. Therefore repeated measures over longer periods of time might be needed to more accurately assess individual NFL levels. We here also find that NFL levels on fingolimod treatment correlate with age, which replicate previous findings from CSF NFL measurements in healthy controls [23], which suggest that adjustment for age might be needed.

In conclusion, we here demonstrate a high degree of correlation between serum and CSF levels of NFL in MS patients and controls. Furthermore, we show that in patients switching from injectable MS therapies the plasma NFL levels are reduced. Additional studies are needed to demonstrate correlation between blood NFL levels and long term clinical outcomes.

Conflict of Interest Statement

Dr Piehl has received unrestricted academic research grants from Biogen, Genzyme and Novartis, and travel support and/or compensation for lectures and/or participation in advisory boards from Biogen, Merckserono, Novartis, Genzyme and Teva, which have been exclusively used for the support of research activities. Dr Blennow has served as a consultant or at advisory boards for Alzheon, Eli-Lilly, Fujirebio Europe, IBL International and Roche Diagnostics, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Venture-based platform company at the University of Gothenburg. Dr Zetterberg is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Venture-based platform company at the University of Gothenburg and has served at advisory boards of Eli Lilly, Roche Diagnostics, Lundbeck and Pharmasum Therapeutics. Dr Olsson has received compensation for lectures and /or advisory boards, or unrestricted MS research grants

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Figure legends

Figure 1. Correlation of neurofilament-light (NFL) levels in cerebrospinal fluid/serum (A) and serum/plasma (B). Paired samples of cerebrospinal fluid and serum were obtained during diagnostic procedures from patients with MS and non-inflammatory neurological disease controls and show a high degree of correlation between the two compartments. In a subset of patients also plasma samples were available, also demonstrating a high degree of correlation between plasma and serum. Correlations analyzed with two-sided Spearman's rank correlation.

Figure 2. Neurofilament-light (NFL) levels in plasma obtained from a nationwide post-marketing study of fingolimod where samples are sent by surface mail. The time delay between day of sampling and day of arrival did not noticeably affect the mean NFL level, suggesting that the assay is robust with regard to handling of samples. Correlation analyzed with two-sided Spearman's rank correlation.

Figure 3. Serum neurofilament-light (NFL) levels in a cohort of patients (n=243) switching from injectable therapies to fingolimod, with sampling at baseline and at 12 and 24 months after start of therapy with fingolimod. A: At the group level mean plasma NFL demonstrated a significant 34% reduction at 12 months compared to baseline, where levels remained similar at 24 months compared to 12 months. B: Plasma NFL levels showed a significant correlation with increasing age. Differences between groups analyzed with non-parametric Kruskal-Wallis rank sum and correlation two-sided Spearman's rank correlation.