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Comparison of plasma and CSF Neurofilament-light in an MS trial

Running title: CSF and plasma NFL in an MS clinical trial

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Conflicts of Interest:

Kaj Blennow is one of the founders of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg, and has served as a consultant or at advisory boards for Alzheon, BioArctic, Biogen, Eli Lilly, Fujirebio Europe, IBL International, Merck, Novartis, Pfizer, and Roche Diagnostics.

Henrik Zetterberg is one of the founders of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg, has served at advisory boards of Eli Lilly and Roche Diagnostics and has received travel support from TEVA.

Pierre de Flon, Katarina Laurell, Peter Sundström, Lars Söderström, Martin Gunnarsson and Anders Svenningsson declare no conflicts of interest.

Abstract

Objective: The main objective of this study was to evaluate the axonal component neurofilament light protein (NFL) in plasma and cerebrospinal fluid (CSF) as an outcome measure in a clinical trial on disease-modifying treatments in multiple sclerosis.

Materials & Methods: Seventy-five patients with clinically stable relapsing-remitting multiple sclerosis (RRMS) participating in the clinical trial "Switch-To RItuXimab in MS" (STRIX-MS) were switched to rituximab from first-line injectable therapy and then followed for two years. Thirty patients from the extension trial (STRIX-MS extension), accepting repeated lumbar punctures, were followed for an additional three years. Plasma and CSF samples were collected yearly during the follow-up. NFL concentration in plasma was measured by an in-house NF-light assay on the Simoa platform with a Homebrew kit. NFL concentration in CSF was measured by sandwich ELISA.

Results: The mean levels of NFL, in both CSF and plasma, were low. The reduction of CSF-NFL was 25% during the first year of follow-up (from a mean of 471 (SD 393) to 354 (SD 174) pg/mL; p=0.006) and was statistically significant. The corresponding reduction in plasma-NFL was 18% (from 9.73 (SD 7.04) to 7.94 (SD 3.10) pg/mL; p=0.055) and did not reach statistical significance.

Conclusion: This study indicates that NFL in plasma is less sensitive as an endpoint in group comparisons than NFL in CSF. Given that plasma NFL is far easier to access, it is a promising and awaited method but further studies are needed to optimise the use in clinical trials.

Key words: Multiple sclerosis, rituximab, treatment, clinical trial, neurofilament light, cerebrospinal fluid, plasma

Introduction

Neurofilaments, structural components of the axonal cytoskeleton, are proteins composed of three chains, light (NFL, 68 kDa), medium (NFM, 150 kDa) and heavy (NFH, 190-210 kDa)[1,2]. NFL is released into the extracellular space upon acute axonal damage and was already in the late 1990s suggested as a potential biomarker for inflammatory activity in multiple sclerosis (MS)[3]. The levels of NFL in cerebrospinal fluid (CSF) correlates with clinical and radiological disease activity[4-6] and has shown to be a useful marker for treatment response in MS[7]. However, the use of CSF-NFL, especially for longitudinal evaluations, is limited by the inconvenience of the lumbar puncture (LP) needed to obtain samples.

The development of highly sensitive analytical techniqes has enabled analysis of NFL in peripheral blood[8]. Comparison of analyses in serum and plasma show high correlation admitting interchangebility between the two[9,10]. Several studies have confirmed a high correlation between serum or plasma-NFL (s/p-NFL) and CSF-NFL[11-13]. The concentration of NFL in plasma is shown to be increased in patients with a clinically isolated syndrome (CIS)[14] and to correlate with the inflammatory activity in RRMS [12,13,15]. Decrease of s/p-NFL levels in response to disease modifying drugs has also been described[10,15]. Despite this growing knowledge, it is still a remaining challenge to establish the appropriate use of s/p-NFL as a tool in clinical trials as well as in clinical routine[16].

We have, in an earlier paper, described a reduction in CSF-NFL after a therapy switch from first-line injectables to rituximab in RRMS in the trial "Switch-To-RItuXimab in MS (the STRIX-trial)[17]. The objective of this study was to to evaluate the use of p-NFL as an end-point measure in a clinical MS-trial, here represented by the STRIX trial.

Method

Study design

The STRIX-MS-trial was an open label, multicenter phase II trial performed in collaboration between the neurological departments in Umeå, Östersund and Örebro, Sweden. This trial investigated the effect on disase activity measured via MRI and CSF-NFL upon switch from injectable MS therapies to rituximab in a group of clinically stable RRMS patients. The study design, methods and main results are described in more detail in an earlier publication [17]. The extension trial (STRIX-MS extension), is a three-year extension trial open for patients that completed the STRIX-MS trial. In the extension trial, treatment with rituximab was re-introduced according to either a low-dose protocol (500 mg IV every six months for one year) for patients >50 year of age and without any signs of inflammatory activity during the STRIX-trial, or a high dose protocol (1000 mg IV every six months for one year followed by 1000 mg IV every 12 months for the remaining two years of the study) for patients not fulfilling the criteria for the low-dose protocol. During the extension trial, the MRI has been evaluated unblinded as a part of the clinical and safety follow-up. The final results of the extension trial are not yet compiled. For details on the study design of both trials, see Fig. 1. During the STRIX-extension trial, LPs were optional.

Study participants

The study population in this study was recruited from the 75 patients that performed the therapy switch in the STRIX-MS trial and from the study population that had completed the STRIX-MS extension trial at the Umeå and Östersund sites with an LP performed at least once during the extension trial follow-up (n=30). Demographic data are presented in Table 1. During the first year of the STRIX trial, one patient was treated with natalizumab as rescue therapy due to a clinical relapse (isolated optic neuritis). Data from this patient were excluded

from the longitudinal analyses in agreement with the original protocol, i.e. evaluating the treatment effect of rituximab. Three patients where re-treated with rituximab during the second year of the STRIX trial due to clinical and/or radiological signs of inflammation. One of these patients also received an extra dose of rituximab 1000 mg IV at month 42. For one patient, the treatment was discontinued at month 48 due to adverse effects. Data from these patients were included in the statistical analyses. One patient was excluded because being re-diagnosed as CADASIL after the closure of the STRIX trial. One patient with a ventriculo-peritoneal shunt displayed obviously aberrant values of NFL in the CSF deemed not related to the MS diease and was therefore excluded. Among the thirty patients included from the extension trial there was one who experienced a clinical relapse (isolated optic neuritis) and one patient with one new T2 lesion. For details regarding the number of samples and drop-outs at each time point, see Fig 2.

CSF and blood sampling

Samples of CSF and blood were obtained before treatment switch and then yearly during the follow-up in the STRIX- and STRIX-extension trial. CSF was collected in 10 ml polypropylene tubes (Sarstedt) and centrifuged at 400G for 10 minutes. The supernatant was pipetted off and dispensed in 9 fractions of 1 ml in 1.5 ml polypropylene tubes (Sarstedt). Blood was drawn in EDTA tubes (10 ml) and centrifuged at 1300G for 15 minutes. The plasma was pipetted off and dispensed in aliquots of 2ml. All samples were stored at -80°C.

NFL analysis

The concentration of NFL in plasma was determined by an NF-light assay adapted for the Simoa platform with a Homebrew kit (Quanterix Corp, Boston, MA, USA) at the Clinical Neurochemistry Laboratory at the Sahlgrenska University Hospital, Sweden, as previously

described in detail[18]. The concentration of NFL in CSF collected during the STRIX-trial (month 0, 12 and 24 was determined by a sandwich ELISA method (NF-light ELISA kit; UmanDiagnostics AB, Umea, Sweden) at the Umeå Center of Molecular Medicine, Sweden, according to the instructions of the manufacturer. The concentrations in CSF from month 36-60 (the extension trial) were analysed by an in house sandwich ELISA method, as previously decribed in detail[19], at the Clinical Neurochemistry Laboratory at the Sahlgrenska University Hospital, Sweden. The correlation between the two methods used for CSF was tested by re-testing of samples analysed in Umeå.

Statistics

All statistical calculations were performed in the SPSS v24 software. Correlation analyses were performed with the Spearman rank test. Differences between groups were tested for statistical significance by paired student t-test. The level of significance was set to $p<0.05$.

Ethics and regulatory statements

The STRIX- and the STRIX-extension trials were approved by the Ethics Committee in Umeå 2010-315-31M and 2013-301-31) and the analysis of p-NFL was approved in a supplementary application (2017-37-32M) as it was not a part of the initial study protocol. The studies are registered in the European Union (EU) Clinical Trials Register with EudraCT number 2010-023012-38 and 2013-002378-26. Written informed consent was obtained from each patient.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author after appropriate ethical review board approval.

Results

Correlations between CSF- and p-NFL

The levels of NFL in plasma and CSF at various time points are presented in Fig 2. The overall correlation between NFL in plasma and CSF in this study was ρ=0.445 (p<0.01), Fig 3. The correlation between the two methods used for analysis of CSF-NFL was 0.881 (p<0.01)

Comparison between p-NFL and CSF-NFL as study outcome measures

Mean levels of CSF-NFL at month 12 compared with month 0 was a primary outcome measures in the STRIX trial. At this time point, the mean level of CSF-NFL was reduced at a statistically significant level (p=0.006). The corresponding reduction in plasma did not reach the level of statistical significance (p=0.055). The relative difference in CSF and plasma was 25% and 18%, respectively. At month 24, the difference compared with month 0 reached the level of statistical significance in plasma but not in CSF (p=0.046 versus 0.083).

After re-treatment in the STRIX-MS extension trial, CSF-NFL at month 36 was reduced with 36% (p=0.032) compared with month 0 while the corresponding value for plasma was 17% (p=0.088). The remaining differences in the extension trial between month 0 and month 48 and month 60, respectively, were not statistically significant in either CSF or plasma. These data are summarised in Fig 2.

The study did not admit any further statistical analysis of relevance on the individual patient level.

Discussion

In this study population with clinically stable RRMS, a reduction of NFL in plasma was seen at 12 months after treatment shift to rituximab but the statistical significance, demonstrated for the corresponding values in CSF, could not been reproduced. Equally, the reduction in CSF-NFL observed in the extension trial after re-treatment with rituximab at month 36 was not paralleled by a significant reduction of NFL in plasma. This indicates a lower sensitivity for p-NFL to detect differences on a group level than CSF-NFL.

The correlation between NFL in plasma and CSF in this study appeared less strong compared to MS populations in earlier studies[11,12,15], but similar to what is described for healthy controls[15]. One explanation to this could be the overall low values in both CSF and plasma in this study, which are at similar levels as described for healthy controls. The ratio between plasma and CSF in our study was of the same magnitude as in earlier findings.[13] An underestimation of the p-value by the use of Spearmans rank correlation test could be argued due to to the inevitable intra-patient correlation since repetead measures for each patient are used. The potential error is estimated to be small and acceptable in relation to the final conclusions of the study. Given the low values and the low magnitude of changes over time, the interpretation needs to be related to the rather low statistical power of the study as a possible contributor to the discordant pattern over time in CSF and plasma.

The generally low concentrations of NFL in plasma as well as in CSF in this study were expected with inclusion criteria selecting a study population with clinically stable RRMS. Only a few patients displayed CSF-NFL values above the age adjusted reference value[20]. In plasma, the median values were below the levels reported for healthy controls in earlier

studies at all time points [13,15]. Despite the low values while on treatment with injectable therapy, the mean CSF-NFL was reduced by 25% one year after therapy switch. It has been demonstrated that the magnitude of the relative changes of NFL differs in CSF compared with peripheral blood. A rise in CSF-NFL with 10 % was associated with a 5.9 % increase in s/p-NFL[13]. This may further support the interpretation of s/p-NFL as less sensitive for changes of low magnitude at low concentrations. Since the study populations in MS trials are generally less inflammatory active in the era of modern treatment[21], the sensitivity for small changes in damage-related markers is of relevance. This may, as is implied by this study, be an important aspect when calculating the power for future clinical trials with s/p-NFL as an outcome measure.

In clinical practice, the relevance of small changes at low levels of NFL might be disputed. A recent publication, using serum NFL as a tool for individual follow-up, demonstrated that there was a moderate increased odds ratio of 1.5 of the occurrence of a Gd positive lesion if an increase of 10 pg/mL in s-NFL was detected[22]. Our study did not have the prerequisites to explore such aspects further.

The use of data from the STRIX-trial is associated with limitations regarding the conclusions that can be drawn. Although it provides the opportunity to explore s/p-NFL as an outcome measure in a trial that was adequately powered for detecting differences by CSF-NFL, the design including patients with clinically stable disease limits the possibilities to evaluate the dynamics of NFL in relation to inflammatory activity. The overall low values also precludes the possibility to evaluate correlations between NFL in plasma and CSF over time on the level of individual patients.

In summary, our results suggest that s/p–NFL is less sensitive as an outcome measure in clinical trials than CSF-NFL but shows similar dynamics of change in relation to a immunomodulatory treatment intevention. Given that s/p-NFL is far easier to access, it is a promising and awaited method. We do agree with earlier statements[16] that further studies are needed to optimise the use in both clinical routine as well as in clinical trials.

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Table 1. Demographic data on the STRIX-MS and STRIX-MS extension trials

	STRIX (n=75)	STRIX-extension (n=30)†
Gender Female, n(%)	52 (69)	19 (63)
Age at inclusion, mean (SD)	41 (8.1)	39 (6.9)
EDSS, median (range)	1.5 (0-5)	1.75 (0-3.5)
MRI activity‡ during run-in of the STRIX trial, n (%)	17 (23)	7 (23)

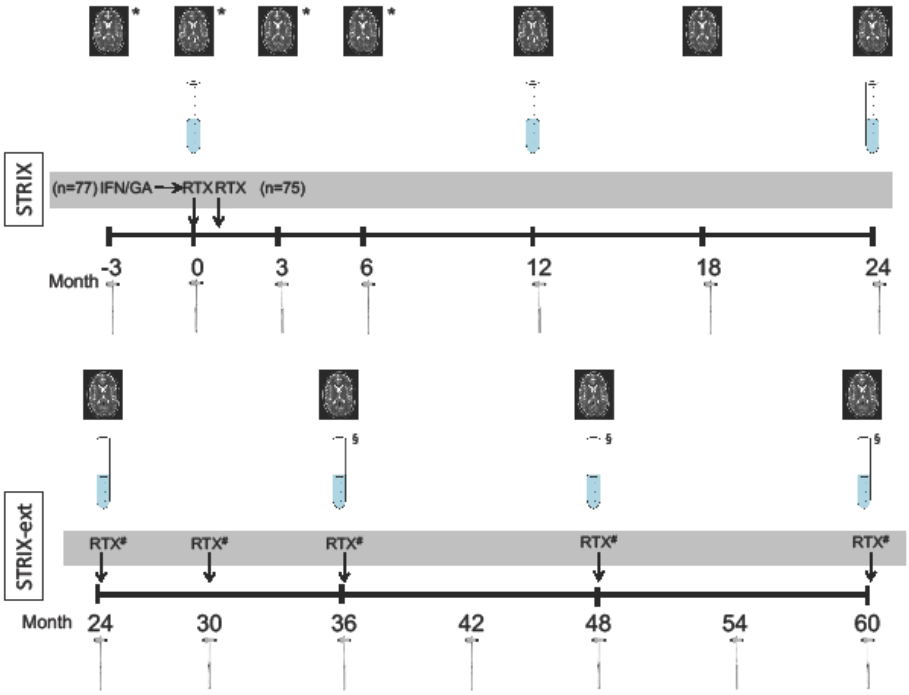
SD- standard deviation, † One patient treated according to the low-dose protocol, twenty-nine patients treated according to the high-dose protocol. ‡ MRI activity defined as Gd-enhancing lesiond on either of the two run-in scans or new T2-lesions on the second run-in scan.

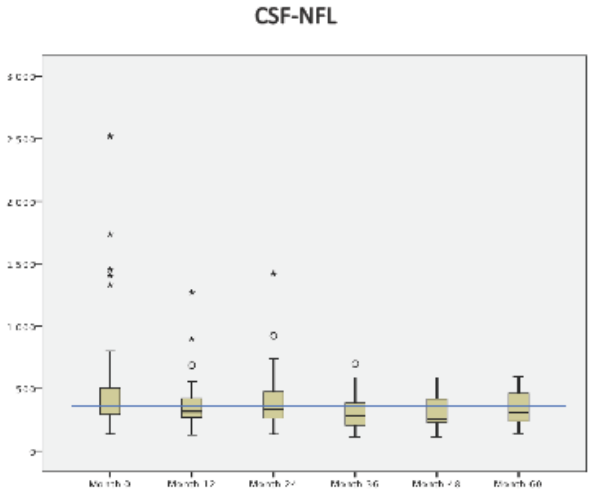
Figures legends

Figure 1. Study design of the STRIX-MS and the STRIX-MS extension trials. Month 24 was the last visit in the STRIX-trial and at the same time the first visit in the extension trial. RTX= rituximab 1000 mg IV. RTX[#]= rituximab 500 or 1000 mg IV according to the protocols of the STRIX-MS extension trial. MRI pictures indicate the time of radiological evaluation, the asterisks denote investigations performed with double-dose contrast. The sample tubes indicate timing of CSF and blood samples. [§] indicates optional LP according to the STRIX-MS extension protocol. The reflex-hammers indicate the timing of clinical evaluation.

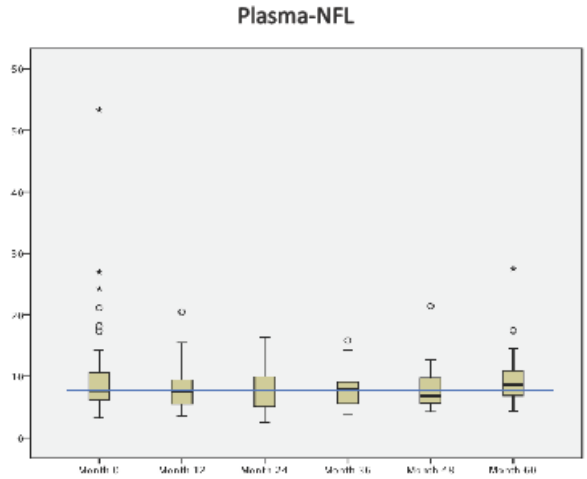
Figure 2. Concentrations of NFL (pg/mL) in CSF (A) and plasma (B). The boxes represent the IQR with the line within the box marking the median and the whiskers marking the levels for upper and lower extreme. o= outliers, *=extreme outliers. The blue horizontal line represents the median at month 0 for visual clarity. SD=standard deviation. No CSF was obtained (procedure failure or patient declined LP) from 2 patients at month 0, from three patients at month 12 and from ten patients month 24. Of the 30 patients included from the STRIX-MS extension trial, one declined LP at month 36, four at month 48 and seven at month 60.

Figure 3. Correlation between NFL in CSF and plasma $\rho=0.445$ ($p<0.01$), with 281 datapairs included in the analysis. All values in pg/mL.





CSF	Month 0	Month 12	Month 24	Month 36	Month 48	Month 60
Available samples	71	69	62	29	26	23
Mean (SD)	471 (393)	354 (174)	382 (202)	303 (140)	310 (130)	346 (136)
Median (min-max)	367 (132-2518)	315 (127-1270)	334 (130-1417)	281 (109-697)	253 (109-590)	308 (140-600)
p-value versus month 0	-	**p= 0.006**	p= 0.083	**p= 0.032**	p= 0.129	p= 0.487



Plasma	Month 0	Month 12	Month 24	Month 36	Month 48	Month 60
Available samples	71	68	62	29	26	23
Mean (SD)	9.73 (7.04)	7.94 (3.36)	7.99 (3.36)	8.04 (3.12)	7.87 (3.67)	9.69 (5.01)
Median (min-max)	7.60 (3.20-53.3)	7.44 (3.48-20.4)	7.62 (2.50-16.32)	7.85 (3.81-15.8)	6.80 (4.17-21.4)	8.62 (4.24-27.4)
p-value versus month 0	-	p= 0.055	**p= 0.046**	p= 0.088	p= 0.052	p= 0.296