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Published in:
Journal of Neurology, Neurosurgery and Psychiatry

DOI:
10.1136/jnnp-2019-321321

Publication date:
2019

Document version
Final published version

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Citation for published version (APA):

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Download date: 10. Jan. 2020
Dimethyl fumarate decreases neurofilament light chain in CSF and blood of treatment naïve relapsing MS patients

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ABSTRACT
Objectives In a prospective phase IV trial of the first-line oral treatment dimethyl fumarate (DMF), we examined dynamics of neurofilament light (NFL) chain in serum, plasma and cerebrospinal fluid (CSF) samples collected over 12 months from relapsing-remitting multiple sclerosis (RRMS) patients. NFL changes were related to disease activity.

Methods We examined NFL levels by single-molecule array in 88 CSF, 348 plasma and 131 sera from treatment-naive RRMS patients (n=52), healthy controls (n=23) and a placebo group matched by age, sex and NFL (n=52). Plasma/sera were collected at baseline, and 1, 3, 6 and 12 months after DMF. CSF samples were collected at baseline and 12 months after DMF.

Results NFL concentration in CSF, plasma and serum correlated highly (p<0.0001 for all), but plasma levels were only 76.9% of paired serum concentration. After 12 months of DMF treatment, NFL concentration decreased by 73%, 69% and 55% in the CSF, serum and plasma (p<0.0001, respectively). Significant reduction in blood was observed after 6 and 12 months treatment compared with baseline (p<0.01 and p<0.0001, respectively) and to placebo (p<0.0001). Patients with NFL above the 807.5 pg/mL cut-off in CSF had 5.0-times relative risk of disease activity (p<0.001).

Conclusions This study provides Class II evidence that first-line DMF reduces NFL in both blood and CSF after 6 months and normalises CSF levels in 73% of patients. High NFL concentration in CSF after a year reflected disease activity. NFL levels were higher in serum than in plasma, which should be considered when NFL is used as a biomarker.

INTRODUCTION
Levels of the cytoskeleton protein neurofilament light (NFL) are increased in the cerebrospinal fluid (CSF) and blood in diseases associated with axonal damage.1 If measured by sensitive methods, blood and CSF concentrations of NFL correlate.2-4 Studies have shown that in the blood and CSF of patients with multiple sclerosis (MS), NFL concentration is elevated at the time of diagnosis compared with healthy controls (HC) and is associated with disease severity and prognosis.5 NFL levels are also increased during relapse and when new lesions are detected by MRI.6,7 Treatment with highly effective disease modifying therapies (DMTs), such as natalizumab, fingolimod, cladribine, daclizumab and alemtuzumab resulted in significant reduction in NFL in the CSF and blood.8-15 Switch from injectables to fingolimod reduced NFL concentration in the plasma, and switch to rituximab decreased NFL levels in the CSF.14,16 In patients treated with natalizumab, such reduction in NFL in the CSF correlated with brain atrophy changes.16 Therefore, NFL has been suggested as a supplementary biomarker in the management of MS.17

Prospective studies that investigate changes of NFL in the blood and CSF during treatment with first-line DMTs are lacking. In this prospective open label phase IV study, we investigated dynamic changes of NFL concentration in parallel samples of serum, plasma and CSF taken from newly diagnosed treatment-naive relapsing-remitting multiple sclerosis (RRMS) patients after treatment with dimethyl fumarate (DMF). We also compared NFL levels in MS patients before and after DMF treatment to healthy controls and placebo patients from the randomised phase III trial: Pegylated interferon β−1a for relapsing-remitting multiple sclerosis (ADVANCE).18

METHODS
Study design TRENEND (Tecfidera in Relapsing-Remitting Multiple Sclerosis: Endothelial Dysfunction) was a prospective open label phase IV trial which enrolled newly diagnosed MS patients with RRMS from March 2014 till August 2016 (EudraCT 2014-000254-11) (see online supplementary figure 1). TRENEND was a study designed to identify potential biomarkers in DMF-treated MS patients. The primary and secondary objectives were the change in the levels of biomarkers from baseline by month 12 and 24 in subjects with RRMS receiving DMF. Analysing NFL was one of the biomarker endpoints, and part of the original protocol.

We included untreated (naïve) newly diagnosed MS patients with RRMS according to the McDonald 2010 criteria (table 1).19 All patients had oligoclonal bands (OCBs) in the CSF (n=52). The reason for including only patients with positive OCBs was to ensure that participants had active disease since absence of OCBs has been described a marker of low disease activity.20 and support the
inclusion of patients with MS even after a single clinical event. These patients were matched to new patients diagnosed with MS, treated with placebo in the ADVANCE trial (phase III trial of beta interferon) and ADVANCE (Pegylated interferon beta-1a for relapsing-remitting multiple sclerosis) (n=48)\(^\text{18}\); mean±SD is shown. P values were used to define their specificity and sensitivity.

We used ordinary one-way analysis of variance and Holm-Sidak’s multiple comparisons test in normal distributed data. In the absence of Gaussian distribution, non-parametric analysis was performed with Kruskal-Wallis test and Dunn’s multiple comparisons.

To analyse relative risk between high (above cut-off) and low (below cut-off) NFL levels in CSF and blood, we used Fisher’s exact test. Statistical analysis and data management were performed using GraphPad Prism 7.

RESULTS
NFL levels correlate in body fluids and are the lowest in plasma
To examine NFL in body fluids obtained from MS and HC, we first compared NFL levels in the CSF to paired plasma and serum samples (n=88); plasma levels were also compared with the paired serum (n=129) (figure 1). We observed the strongest correlation between plasma and serum levels (p<0.0001; Spearman r: 0.96). NFL levels highly correlated with both plasma (p<0.0001; Spearman r: 0.79) and serum NFL levels (p<0.0001; Spearman r: 0.72). We also determined the ratio between CSF, serum and plasma NFL concentrations. NFL levels in plasma were on average 0.45% of NFL in the CSF, serum NFL levels were on average 0.52% of CSF levels and plasma NFL levels were on average 76.9% of NFL levels in the serum.

NFL levels are higher in serum, plasma and CSF of newly-diagnosed MS patients compared to HC
Next, we compared NFL concentration in serum, plasma and CSF obtained from treatment-naïve, newly diagnosed MS patients to HC. NFL levels in the CSF were higher in MS patients (2368±1947 pg/mL) compared with HC (417±191 pg/mL) (p<0.001) (figure 2A). Plasma NFL levels were also higher in plasma (16.4±14.4 pg/mL) and in sera (25.0±43.9 pg/mL) of the untreated MS patients (baseline) compared with healthy controls (7.3±3.0) (p<0.01 and p<0.0001, respectively) (figure 2B).
Multiple sclerosis

Figure 1  Correlation between NFL levels in CSF, plasma and serum. Linear regression analysis of respective body fluids, depicting Spearman r value and levels of significance. CSF, cerebrospinal fluid; NFL, neurofilament light. \( n_{\text{CSF}} = 88, n_{\text{plasma}} = 129, n_{\text{serum}} = 129 \).

Cut-off concentrations discriminate between HC and MS patients

In order to identify cut-offs that discriminate NFL levels in HC from MS patients, we performed ROC analysis (figure 3). ROC analysis showed an area under curve 0.933 (95% CI 0.865 to 1.002, \( p < 0.0001 \)) in the CSF (\( n = 53 \)), 0.757 (95% CI 0.645 to 0.868, \( p < 0.001 \)) in the plasma (\( n = 70 \)) and 0.778 (95% CI 0.669 to 0.888, \( p < 0.001 \)) in the serum (\( n = 68 \)). To discriminate between MS patients and healthy controls, a cut-off concentration of 807.5 pg/mL in the CSF showed 100% specificity and 80% sensitivity; a cut-off concentration of 13.0 pg/mL in the plasma showed 100% specificity and 47% sensitivity and a cut-off concentration of 15.6 pg/mL in the serum showed 100% specificity and 43.2% sensitivity. Using other cut-off values did not improve overall sensitivity and specificity (data not shown).

By comparing individual CSF samples with increased NFL (ie, above cut-off) to paired plasma and serum, we found that NFL was below cut-off, that is, in the normal range in 43.3% of sera and 35.5% of plasma samples.

Continuous decline of NFL levels after treatment with DMF

We next examined the change of NFL levels after DMF treatment in serum, plasma and CSF samples. After 1 year treatment, NFL levels were reduced by 1340 pg/mL in the CSF (\( p < 0.0001 \)), by 17.3 pg/mL in the serum (\( p < 0.0001 \)) and by 9.3 pg/mL in the plasma (\( p < 0.01 \)). These changes corresponded to a reduction of 73%, 69% and 57% in NFL levels in the CSF, serum and plasma, respectively. All sera samples, 96% of plasma samples, and 72% of CSF samples showed levels similar to healthy controls, that is, below cut-off after 1 year treatment (figures 2C, D and 4).

In the placebo-treated group, plasma NFL levels remained unchanged, when samples collected at baseline (17.5 ± 14.0 pg/mL) were compared with samples obtained after 1 year placebo treatment (16.6 ± 14.0 pg/mL) (\( p > 0.99 \)) (figure 4A).

We also examined the dynamics and temporal profile of NFL reduction in the blood. After DMF treatment, plasma levels remained unchanged from baseline (16.4 ± 14.4 pg/mL) to 1 month (15.8 ± 11.0 pg/mL, \( p > 0.99 \)) and 3 months after treatment (12.0 ± 7.8 pg/mL, \( p = 0.78 \)). Significant reduction in NFL

Figure 2  NFL in the CSF and plasma of MS patients at baseline, after DMF treatment and in controls. NFL concentrations in MS patients at baseline compared with healthy controls (A, B) and after 12 months treatment with DMF (C, D). Cut-offs determined by receiver operating characteristic analysis (see figure 3) are shown with dotted line. Significance in difference between groups is shown: ****: \( p < 0.01 \), ****: \( p < 0.0001 \). BL, baseline; CSF, cerebrospinal fluid; DMF, dimethylfumarate; HC, healthy control; MS, multiple sclerosis; NFL, neurofilament light. \( n_{\text{CSF BL}} = 30, n_{\text{plasma BL}} = 47, n_{\text{CSF Healthy}} = 23, n_{\text{plasma Healthy}} = 22, n_{\text{CSF 12}} = 35, n_{\text{plasma 12}} = 47 \).
plasma concentration was observed after 6 (9.1±5.7 pg/mL, p<0.01) and 12 months (7.4±3.1 pg/mL, p<0.0001) treatment with DMF. NFL concentration in the plasma of MS patients became similar to that of HC after 6 and 12 months treatment (figure 4A). NFL concentration in the serum showed similar dynamics: concentration reached the cut-off after 3 months treatment similar to plasma, became similar to HS and showed a significant reduction from baseline (p<0.0001) after 1 year of DMF (figure 4B). After 1 year, NFL levels showed a pattern of decline and below cut-off in 16 out of 22 CSF samples (73%) (figure 5A).

High NFL levels after one year treatment with DMF are associated with disease activity

We also investigated, if disease activity during the first and second year after DMF was associated with change in NFL levels during the first year of treatment. Clinical data before and after DMF are presented in online supplementary table 1.

First we examined, if disease activity, that is, relapses and/or new or enlarging T2-weighted lesions on MRI during DMF treatment was associated with increased NFL levels after 1 year of treatment. NFL levels were below cut-off in the CSF in 72.8% of 22 MS patients (figure 5A), and below the cut-off in the plasma in 96.2% of 52 MS patients after 1 year treatment, respectively. Patients with NFL in the CSF above cut-off (807.5 pg/mL) after 1 year of treatment had a relative risk of 5.0 of relapse and/or new T2-weighted lesions on MRI (p<0.001) during the first year of treatment (figure 5B). Patients with disease activity and NFL levels above cut-off in CSF at 12 months had disease activity between months 4 to 12 and patients with disease activity between month 0 to 4 were below cut-off in CSF after 12 months treatment with DMF (figure 5C). Disease activity during the second year of DMF was not in association with NFL levels below or above with cut-off in CSF (p=0.66). Associations of disease activity with NFL levels in the blood could not be found in the first and second year of DMF treatment. There was no significant difference between the subgroups with and without disease activity at any time point (see online supplementary figure 2).

DISCUSSION

We determined NFL levels in the CSF, serum and plasma during and at the end of 1 year treatment with DMF in a prospective phase IV trial that enrolled newly diagnosed, treatment-naive relapsing MS patients (see online supplementary figure 1). Results were compared with a healthy control group and a
placebo control group from a phase III trial on pegylated interferon β–1a (ADVANCE). Although a randomised placebo group in our TREMEND trial would have been more optimal, such phase IV trials cannot be approved in Denmark for first-line DMTs. The placebo-treated group from the ADVANCE trial was matched to the DMT-treated patients in TREMEND based on age, sex and NFL levels, and EDSS at baseline was also similar between the two trials. We also examined the dynamics of NFL in the serum and plasma during 1 year of DMF treatment in MS patients, and compared the sensitivity of blood and CSF NFL for detection of disease activity by using paired samples.

This study showed that NFL values in the CSF were approximately 200 times higher than in the plasma and serum, and thus support results seen in other MS cohorts.4 7 15 25 We found the highest correlation between plasma and serum, with plasma demonstrating 23% lower NFL values than serum (figure 1). Samples were collected at the same time following international guidelines; therefore, a systematic bias is unlikely. Difference between serum and plasma are known for other biomarkers, since clotting factor and tube material may affect the composition of the two body fluids.26 The differences between NFL levels in plasma and serum were significant and should be considered when biomarker outcomes in peripheral blood are planned in future studies, for example, when designing trials with either plasma or serum NFL as outcomes.

We also identified NFL cutoffs with ROC analysis to discriminate between patients with newly diagnosed treatment naïve RRMS and healthy controls (figures 2 and 3). The identified blood cutoffs were similar to previous data,27 28 but some other studies demonstrated a wide range of mean NFL from 9 to 36 pg/mL in blood.7 25 28 29 Those studies however did not define cutoff based on ROC analysis, and the enrolled patient populations were heterogeneous regarding treatment and clinical disease course. We found a CSF cutoff of 807.5 pg/mL, which is similar to results published within the last 5 years; these studies detected cutoffs above and below 1000 pg/mL with mean values from 463 to 2500 pg/mL.1 11 27 28 30 31 (figure 3).

Observing differences between newly diagnosed untreated MS patients and HC, we next examined the impact of 1 year DMF treatment on NFL concentration in the serum/plasma/CSF compared with a placebo group. In addition, we investigated the dynamics of NFL changes and the temporal profile of normalisation in the plasma after DMF. We found a continuous decrease of NFL in the blood after starting DMF that became significant after 6 months treatment and continued declining up to 12 months (figure 4). Such decrease in the blood was also supported by a significant similar reduction of NFL in the CSF after 12 months (figure 2). The trend of reduction was observed after 3 months (figure 4), the time point when clinical effects became significant in both the DEFINE (The Determination of the Efficacy and Safety of Oral Fumarate in Relapsing–Remitting MS study) and CONFIRM (The members of the Comparator and Oral Fumarate in Relapsing Remitting Multiple Sclerosis) trials.2 3 3 3

The observed dynamics of decline are related to the effect of treatment and not to the natural course, as we did not see any decline in patients treated with placebo for 1 year. The relative larger reduction in serum NFL compared with plasma NFL could suggest that serum is more useful in detecting changes, but this should be addressed by specifically designed studies.

Our data indicates that DMF used as a first-line treatment has similar potential in reducing NFL levels in the blood and CSF compared with highly effective DMTs even though this cohort demonstrated mild disease burden prior to initiation of treatment. A few studies have already shown that natalizumab,8 9 22 24 34 fingolimod,10 11 15 28 cladribine15 29 dactuzumab13 and alemtuzumab15 are able to reduce NFL levels in both the CSF and/or blood.

Our data would suggest that NFL may be used as biomarker to monitor treatment responses during the first treatment of MS. Therefore, we also examined, if higher NFL levels after 1 year of DMF treatment was associated with higher disease activity.5 8 9 15 28 We were able to directly compare blood and CSF NFL levels in detecting clinical activity by utilising paired serum/plasma-CSF samples. By using the calculated NFL cutoff in CSF (807.5 pg/mL), we could retrospectively identify patients who had experienced disease activity during the first year of treatment (figure 5). However, NFL levels in paired plasma and serum were unable to identify the same patients. In addition NFL levels in CSF at 12 months did not predict disease activity in month 12 to 24. Further trials designed for examination of dynamics and clinical significant difference of NFL are needed.
This discrepancy between blood and CSF measurement probably reflects the lower NFL concentration in the systemic compartment compared with the CSF, possibly because CSF is closer to the damaged tissue. We found that despite the increased NFL concentration in the CSF, 13.6% and 22.1% of the respective paired serum/plasma samples showed normal values. Another study also showed higher sensitivity of CSF NFL for detecting disease activity compared with serum.27 This lower sensitivity of NFL measurement in the peripheral compartment may limit the value of evaluating clinical activity, although blood is a more accessible body fluid than CSF and can be repeatedly analysed. Since serial lumbar puncture is not possible in an ordinary clinical setting, modifications in the assay that increases sensitivity in the plasma are needed. Alternatively, regular monitoring with blood samples at predefined intervals, for example 2 to 3 months should be necessary in order to avoid misinterpreting normal plasma values at time points, when CSF still shows mild elevation suggesting active disease. Further studies are necessary to understand the required frequency of such laboratory tests.

Our study is not without limitations. MRI lesions with gado-linium enhancement have not been included in the study, and we considered disease activity based on clinical relapses and new/enlarging T2 lesions. The placebo group was not part of the TREMEND trial for ethical reasons, and was added based on matching age, sex and baseline NFL levels. We did not find association between NFL levels and EDSS or age in univariate analysis (data not shown), but this is related to the trial design, and the cohort with narrow age and EDSS range in newly diagnosed, treatment-naive patients.

In conclusion, we demonstrate that DMF as first-line treatment reduces NFL levels in both the blood and CSF, and such reduction becomes significant after 6 months. NFL levels in CSF above the calculated cut-off reflected disease activity within the first year but did not predict disease activity in the second year. NFL levels in plasma are lower than in paired serum samples, and this should be considered during future trial design. Additional studies are needed to implement NFL as a clinical monitoring tool in patients with MS receiving DMF and determine the minimum frequency of blood NFL to detect subclinical disease activity.

Contributors  TS contributed with: concept and design, acquisition, analysis and interpretation of data, drafting of the manuscript, revision of the manuscript, critical revision of the manuscript for important intellectual content, statistical analysis. HHN contributed with: analysis, acquisition, interpretation of data and revision of manuscript. NP contributed with: analysis, interpretation of data and revision of manuscript. TP contributed with: analysis, interpretation of data and revision of manuscript. JPM contributed with: analysis, interpretation of data and revision of manuscript. MML contributed with: acquisition of data and manuscript. MLE contributed with: acquisition of data and manuscript. MHR contributed with: concept and design, acquisition, analysis, interpretation, manuscript and revision of manuscript. ZI contributed with concept and design, writing, the protocol of the trial, obtaining the necessary research grants, analysis and interpretation of data, drafting of the manuscript, revision of the manuscript, critical revision of the manuscript for important intellectual content, study supervisor.

Funding  This trial was funded by research grants from Biogen (TREMEND to ZI and TS), University of Southern Denmark (14/24200 to TS and ZI), Odense University Hospital (5798002573633 to TS and ZI) and The Danish Multiple Sclerosis Society (R367-A25341 to ZI).

Competing interests  TS has served on scientific advisory boards, received support for congress participation, received speaker honoraria and received research support from Biogen and Novartis. NP is employee of and holds stock/stock options in Biogen. TP is employee of and holds stock/stock options in Biogen. JPM is employee of and holds stock/options in Biogen. HHN has served on scientific advisory boards, received support for congress participation, received speaker honoraria and received research support from Biogen, Merck-Serono, Lundbeck and Novartis. ZI has served on scientific advisory boards, served as a consultant, received support for congress participation, received speaker honoraria and received research support from Biogen, Merck-Serono, Sanofi-Genzyme, Lundbeck and Novartis.

Patient consent for publication  Obtained.

Ethics approval  This study was approved by the regional Committee of Health Research Ethics (S-20140015HLH(csf)), the Danish Health and Medicines Authority (2014013769) and the European Clinical Trials Database (2014-000254-11).

Provenance and peer review  Not commissioned; externally peer reviewed.

Data availability statement  Data are available upon reasonable request.

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