Avidity of antineurocytoskeletal antibodies in Alzheimer's disease patients

Libuse Noskova[a], Lenka Fialova[a], Ales Bartos[b,c], Tomas Zima[a]

Aims. To optimise the ELISA method for the avidity of IgG antibodies against neurofilament heavy chain (NfH) and to determine the levels and avidity of anti-NfH antibodies in patients with Alzheimer's disease (AD) and a healthy control group.
Methods. Various dilutions of sera and concentrations of urea and sodium chloride as chaotropic reagents were tested in the process of the ELISA optimisation. The levels and avidity of anti-NfH antibodies were determined in 30 patients with Alzheimer's disease and 30 age-matched cognitively normal elderly adults.
Results. Sera dilution 1:200 and urea as a chaotrope in a concentration 6 mol/L were chosen to be the most suitable for the avidity assay of anti-NfH antibodies by ELISA. The results showed no differences in either level or avidity of IgG anti-NfH antibodies between AD patients and cognitively normal persons. The levels of anti-NfH IgG antibodies inversely correlated with their avidities.
Conclusions. We optimised the ELISA method for the determination of anti-NfH antibody avidity determination which is suitable for research of anti-NfH antibody avidity in patients with neurological diseases associated with neurocytoskeletal defects. The determination of serum anti-NfH antibody avidity in AD patients seems to have limited diagnostic significance.

Key words: Alzheimer's disease, antibodies, avidity, heavy chain of neurofilament, neurofilament

Received: December 14, 2016; Accepted: March 24, 2017; Available online: April 26, 2017
https://doi.org/10.5507/bp.2017.017

[a]Institute of Medical Biochemistry and Laboratory Diagnostics, First Faculty of Medicine, Charles University and General University Hospital in Prague, Czech Republic
[b]National Institute of Mental Health, Klecany, Czech Republic
[c]Department of Neurology, Third Faculty of Medicine, Charles University and Faculty Hospital Kralovske Vinohrady, Prague, Czech Republic
Corresponding author: Libuse Noskova, e-mail: Noskova.Libuse@seznam.cz

INTRODUCTION

Alzheimer's disease (AD) is the most frequent neurodegenerative disorder. Various alterations in the neurocytoskeleton are associated with the neurodegenerative process[1,2]. When the axon is damaged or neurodegeneration is initiated, the structures of the neuronal cytoskeleton normally localised inside in neurons are released from the neurons into the interstitial fluid, cerebrospinal fluid and bloodstream.

The cytoskeletal proteins in the extracellular space as a result of various pathological processes may interact with the cells of the immune system and induce an inflammatory or autoimmune response[3]. The levels of autoantibodies against neurocytoskeletal autoantigens in the serum might be potential biomarkers for neurodegenerative diseases involving continuous neuronal death[4,5]. Moreover, the study of autoantibodies is becoming important in association with intensive investigation and clinical trials testing the possibility of immunotherapy in AD (ref.[6]).

In addition to the changes in antibody levels, their avidity is a substantial qualitative characteristic and could also be of clinical significance[7-9]. The examination of antibody avidity in clinical practice is usually used for the differentiation of primary and chronic infection[10] or for monitoring the effectiveness of vaccination[11-13].

Current knowledge about the avidity of autoantibodies is less complex than that about antibodies against exogenous antigens. High-avidity autoantibodies may be associated with autoimmune processes in the organism[7,9,14], but decreased avidity of antibodies against the β-amyloid protein in Alzheimer's disease has also been described[15]. However, information about the avidity of neuron-specific antibodies is sporadic so far, although it is a relevant issue of antibodies, which may determine their protective or injurious potency.

Recently, we found increased levels of intrathecally synthesised antibodies against NfH and tau proteins in patients with Alzheimer's disease[16]. Serum levels of anti-NfH IgG antibodies in AD have also been studied[16,17], but we are not aware of any study evaluating the avidity of these antibodies. Therefore we decided to continue with a more detailed study of anti-NfH IgG antibodies in serum by the determination of their avidity in the patients with dementia.

The aim of this study was to design and optimise ELISA for the determination of anti-NfH IgG antibody avidity. Various methods and procedures for the measurement of antibody avidity have been designed. One of

the methods suitable for clinical use is ELISA (Enzyme-Linked Immuno-Sorbent Assay), which is modified by the addition of substances with a chaotropic effect on the immune complexes formed during the ELISA procedure[18]. These agents are necessary for the disruption of the immune complexes containing weakly bound antibodies. The lower the antibody avidity is, the more immune complexes are disrupted and the released antibodies are then washed away after incubation with the chaotropic agent.

Using our optimised ELISA, we evaluated the avidity of the anti-NfH IgG antibodies in the serum of patients with Alzheimer's disease and an appropriate control group. We aimed to better characterise the humoral immune response against neurocytoskeletal components and to consider a possible diagnostic significance of anti-NfH IgG antibody avidity.

MATERIAL AND METHODS

Participants

We examined two groups of elderly persons. Serum samples were obtained from patients at the Department of Neurology, Charles University. The AD patients were recruited from the Memory Clinic. The diagnosis was based on objective evidence of a progressive decline in cognition, functional and detailed neuropsychological assessments, hippocampal atrophy seen on brain magnetic resonance imaging (or computer tomography in case of contraindications), temporo-parietal hypoperfusion seen using single photon emission computed tomography, or increased total or phosphorylated tau proteins and/or decreased β-amyloid concentrations in cerebrospinal fluid using cut-offs established in our previous studies[19-21]. The diagnosis of dementia due to AD had been already established before blood sampling[22-24]. They had mild form of dementia according to MMSE scores (Tab. 1). Many of them have already died after long-term clinical decline. Subjects in the control group (n=30; mean age 72 years) were cognitively normal elderly adults having normal Mini-Mental State Examination (MMSE) scores[25]. They were age-matched with the AD patients.

Table 1. Sociodemografic characteristics of participant groups.

	Controls	Alzheimer's disease patients
Number of patients	30	30
Age at blood draw (years)	72 ± 5	74 ± 8
Gender	63% females	77% females
Education (years)	15 ± 3	13 ± 3
MMSE score (0-30 points)	29 ± 1	19 ± 6

Data are expressed as average ±SD
MMSE - Mini-Mental State Examination score

The basic characteristics of the two groups are shown in Table 1. The AD patients and patients in the control group did not differ in age and other parameters correlates with epidemiological findings[26].

Patients included in the study signed the informed consent. The study was approved by the Ethics Commitee of the Third Faculty of Medicine, Charles University.

Only selected samples were used for the optimisation of ELISA. The influence of pH was tested in five samples (control and AD patients' samples). A comparison of chaotropic agents was performed in 20 samples (10 controls and 10 AD patients).

Methods

ELISA for anti-NfH IgG antibody level and avidity determination in the serum

Anti-NfH antibody IgG levels were determined by ELISA originally described by Silber et.al.[27] and later modified for the purpose of our previous studies[28,29].

Two kinds of chaotropic agents – NaCl and urea – in various concentrations were tested for avidity method optimisation. Solutions at different concentrations were chosen for initial experiment concentrations based on previous works[29,30]. Solutions of NaCl were used at concentrations 0.25 mol/L, 0.5 mol/L, 1.0 mol/L, 2.0 mol/L and 3.0 mol/L and urea at 2.0 mol/L, 4.0 mol/L, 6.0 mol/L and 8.0 mol/L. Since pH values change in the chaotropic agent solutions of different concentrations, we also tested the influence of pH on the avidity determination. Therefore, the avidity experiments were performed with the chaotropic agent solution both at the actual (original) pH and at pH adjusted to the uniform value of 7.4. Serum samples were analysed in serial dilution from 1:50 to 1:400.

Procedure of ELISA for the determination of anti-NfH IgG antibody levels and their avidity

The heavy neurofilament subunit isolated from a bovine spinal cord (purity is > 98%, declared by the manufacturer, Progen, Germany) was used as the antigen. An aliquot from the stock solution of the antigen was diluted in carbonate immobilisation buffer (10 mmol/L $NaHCO_3$, 0.15 mmol/L NaN_3, pH 9.6) to the concentration of 2.5 μg/mL. One half of the wells of 96-well plate type Maxisorp (Nunc, Denmark) was coated with 100 μL of diluted antigen and the other half of the wells was coated only with immobilisation buffer. The plate was incubated overnight at 4 °C.

After the binding of the antigen, the wells were washed (wash solution 0.1 M NaCl + Tween 20) and blocked with 1% BSA (bovine serum albumin) fraction V in PBS (phosphate buffered saline), pH 7.4. Then, the serum samples in appropriate dilution (in 1% BSA fr. V in PBS) were applied in an amount of 100 μL. Serum samples were incubated in the wells for 2 hours at room temperature.

After five washings of the wells, the chaotropic agents NaCl or urea in tested concentrations or PBS alone were applied to the appropriate wells in an amount of 100 μL/well. It was followed by incubation for 10 minutes at room temperature. The wells were washed (4×).

100 µL/well of secondary antibody (conjugate) against the Fc region of human immunoglobulin IgG conjugated with peroxidase (Southern Biotech, USA, diluted 5000x in 1% BSA fr. V in PBS) was added to the wells in the next step and incubated for one hour at room temperature. After washings (4×), 100 µL of peroxidase substrate TMB (tetramethylbenzidine) with H_2O_2 (TestLine, Czech Republic) was added to the wells. The enzyme reaction was stopped by adding 2 mol/L H_2SO_4 (100 µL/well) after incubation for 15-20 min at room temperature in the dark. Absorbance (A) of the wells was measured at 450 nm against a reference wavelength of 620 nm using a Tecan Sunrise Plate Reader (Tecan, Austria) with the Magellan 6 program.

The absorbance of the blank sample (serum replaced with diluent) was subtracted from the absorbance value of each sample. For comparative purposes, the same pooled serum was used as the internal control in all analytical series of patients' samples (n=60). The intra- and inter-assay of the ELISA method did not exceed 10%.

Expression of anti-NfH IgG antibody concentration

Because no appropriate generally-used standards are available for anti-NfH IgG antibodies, a pooled serum prepared by mixing sera from patients with high levels of anti-NfH IgG antibodies (according their absorbance in the previous ELISA determination) served as a calibration standard for the determination of the arbitrary units (AU) of anti-NfH IgG antibody concentration. A calibration curve constructed from the absorbance of our standard diluted geometrically from 1:50 to 1:400 was run on every plate. Arbitrary units of concentrations were determined from the calibration curve created in the Magellan 6 program (Tecan, Austria).

Expression of the avidity value

The avidity of antibodies is expressed as an avidity index (AI), which is defined as the ratio of the absorbance or AU value for the well with a chaotropic agent to the absorbance or AU value of the well without this agent. Usually, AI is expressed in the percentage. The high-avidity antibodies are commonly classified as those with an avidity index higher than 60% and the low-avidity ones as those with an avidity index lower than 40%. The avidity index values of the antibodies from 40% to 60% are usually marked as a "grey zone", when the decision about the avidity is not clear[31].

Statistical analysis

Non-parametric statistics were used because the data were not normally distributed. This statement was verified using a graphical evaluation and Shapiro-Wilk's test. The median and interquartile range were used for patients and the control group characterisation. The correlation was tested using the Spearman correlation coefficient. The statistical evaluation of paired measurements was tested using by the Wilcoxon matched pairs test. The Mann-Whitney test was used for comparisons between groups. The level of significance was set to 0.05. All statistical calculations were done in the Statistica program (StatSoft, Czech Republic).

RESULTS

Expression of the anti-NfH IgG antibody levels and avidity

The concentrations of anti-NfH IgG antibodies can be expressed in the values of absorbance (450 nm) or in the AU obtained from a calibration curve described in the Methods section. Non-linear regression with an extrapolation factor of 2 was used for the construction of a calibration curve. An example of the calibration curve for the expression of anti-NfH IgG antibody concentration in arbitrary units is shown in Fig. 1.

Levels of anti-NfH IgG antibodies in the AU correlated with their expression in the absorbance (R=0.6; P<0.0001; n=60; Spearman correlation coefficient). Similarly, the avidity index calculated from absorbance values correlated with the calculation of the avidity index derived from the AU (R=0.976 for urea 2 mol/L, P<0.0001; R=0.959 for urea 6 mol/L, P<0.0001; Spearman correlation coefficient).

Optimisation of the ELISA method for anti-NfH IgG antibody avidity determination

To optimise the method for avidity determination we focused on (1) the optimal dilution of serum samples; (2) to test the effect of chaotropic solution pH; (3) to find the optimal concentration of chaotropic agents and further (4) to select the suitable chaotropic agent.

Serum dilution

The best dilutions of sera that corresponded with the linear part of the calibration curve appeared to be 1:200 and 1:400. However, the higher dilution 1:400 was optimal only for samples with elevated levels of antibodies. The absorbance values of sera diluted 1:400 were too low for medium or low levels of antibodies and the results

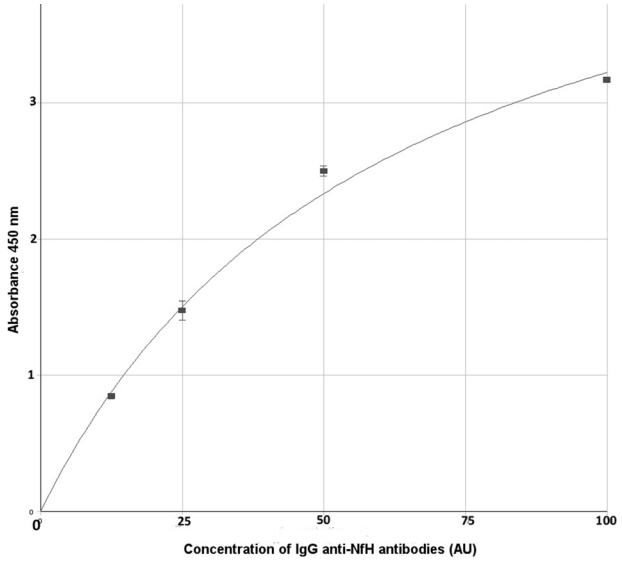


Fig. 1. Example of the calibration curve for the expression of anti-NfH IgG antibody concentration in arbitrary units.
(R value > 0.95); AU – arbitrary unit; NfH – neurofilament heavy subunit

were less precise. It was evident for both tested chaotropic agents (urea and NaCl) especially, when their higher concentration was used.

pH of the chaotropic agent solutions

Both chaotropic agent solutions - urea and NaCl - generated a pH gradient depending on the concentration of the agent. The mean change in pH values was 0.25 for NaCl solutions and 0.1 for urea solutions. The pH effect of the chaotropic agent solution for avidity determination was tested by analysing five samples (controls and AD patients, low, medium and high values of anti-NfH IgG antibodies). The avidity of each sample was determined using a chaotropic agent solution, in which the pH was adjusted to a uniform pH value (7.4) as well as with a chaotropic agent solution with original pH value arisen at preparation of the solution. The pH of the chaotropic agent solution in various concentrations did not significantly influence the determination of the anti-NfH IgG antibody avidity. Therefore, it was not necessary to modify the pH of the chaotropic agent solution in the following experiments.

Concentrations of chaotropic agents

It was crucial to find a concentration of the chaotropic agent that would clearly differentiate antibodies with high avidity from those with low avidity.

Low concentrations of NaCl (0.25 mol/L and 0.5 mol/L) showed minimal effect on the disruption of the immune complexes. Differentiation between high- or low-avidity antibodies was allowed by higher concentrations of NaCl (1 mol/L, 2 mol/L and 3 mol/L). These concentrations of NaCl were used for the further assays. After testing an additional four samples, the concentration of 1 mol/L and 3 mol/L NaCl were selected for further study because no significant differences between the 1 mol/L and 2 mol/L NaCl concentrations were seen.

Higher concentration of urea (6 mol/L and 8 mol/L) influenced the disruption of immune complexes by a similar intensity. Because of the increased risk of denaturation of immune complexes in the presence of higher concentration of urea, we chose urea concentrations of 6 mol/L for avidity determination. A significant difference between 4 mol/L and 6 mol/L urea concentrations was not found. Finally, the 2 mol/L and 6 mol/L urea concentrations were chosen for further analyses.

The result of the avidity assay depends on antibody levels[18]. The higher concentration of the chaotrope may be important for samples with high levels of antibodies, in which a lower concentration of the chaotrope could be insufficient for the disruption of a greater amount of immune complexes. Therefore, we performed the avidity determination at two different concentrations of chaotropic agents in the following avidity tests. Significant differences were seen between 1 mol/L and 3 mol/L NaCl (P=0.0001, Wilcoxon matched paired test) and 2 mol/L and 6 mol/L urea (P<0.0001, Wilcoxon matched paired test) - Fig. 2.

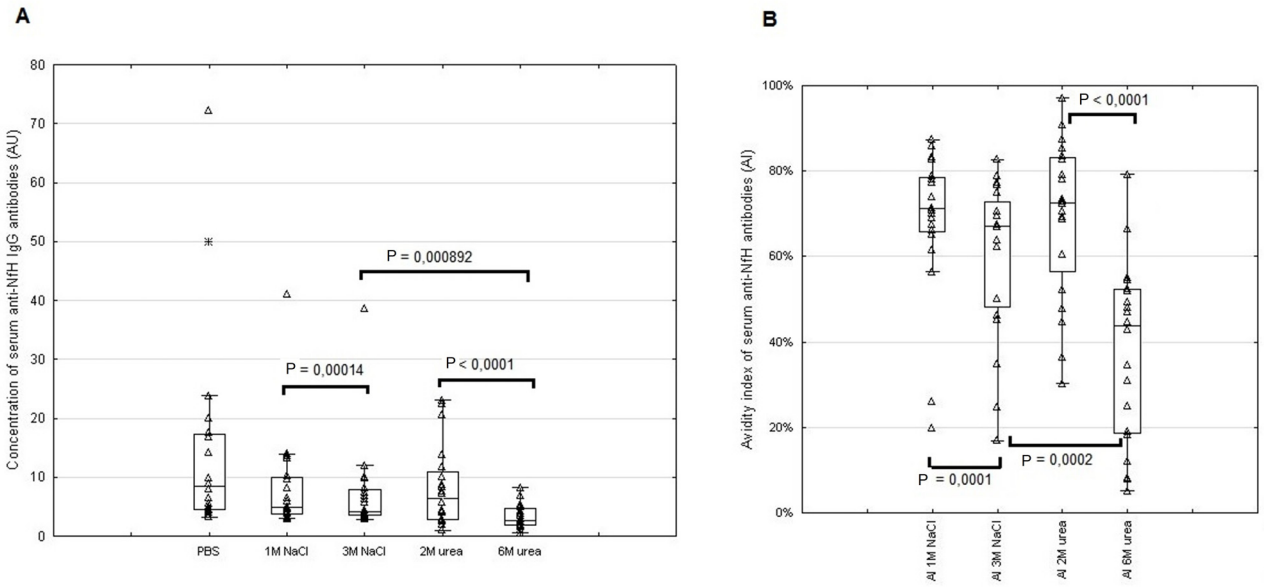


Fig. 2. Comparison of NaCl and urea as chaotropic agents in the ELISA for the avidity determination of anti-NfH IgG antibodies. The box plots include median (horizontal line —), values from the 25th to the 75th percentiles (boxes); bars (whiskers) above and below the box indicate the range of non-outliers. Non-outliers are indicated by triangles. Outliers and extremes are shown by circles and asterisks.

A. Levels of serum anti-NfH IgG antibodies analysed by ELISA method in presence of chaotropic agents (NaCl and urea) in different concentrations. The results obtained in absence of chaotropic agents (PBS) are also shown for comparison. The results are expressed in arbitrary units. A significant differences were seen between 1 mol/L and 3 mol/L of NaCl (P=0.00014), 2 mol/L and 6 mol/L urea (P<0.0001) or 3 mol/L NaCl and 6 mol/L urea (P=0.000892).

B. Avidity of serum anti-NfH IgG antibodies analysed by ELISA in the presence of chaotropic agents (NaCl and urea) in different concentrations. The results of avidity are expressed as an avidity index (%). Significant differences were seen between 1 mol/L and 3 mol/L NaCl (P=0.0001), 2 mol/L and 6 mol/L urea (P<0.0001) or 3 mol/L NaCl and 6 mol/L urea (P=0.0002).

AU – arbitrary unit; NfH – heavy subunit of neurofilaments; PBS – phosphate buffered saline; M – mol/L

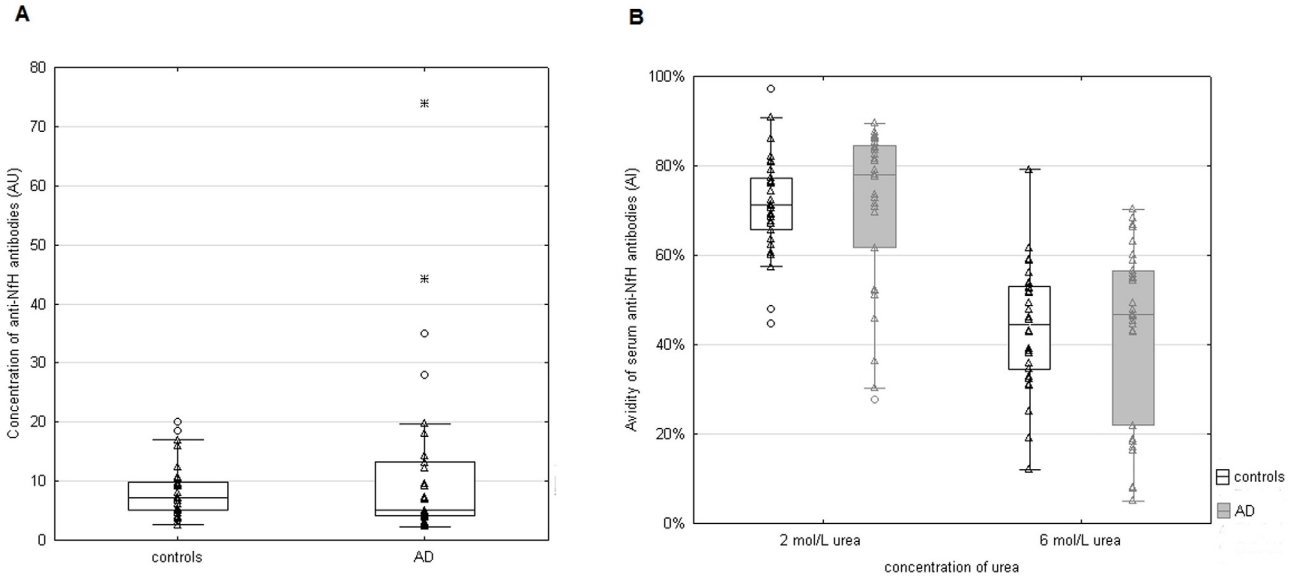


Fig. 3. Comparison of serum levels and avidities of anti-NfH antibodies IgG in the group of Alzheimer's disease patients (n=30) and in the control group (n=30).
The box plots include median (horizontal line —), values from the 25th to the 75th percentiles (boxes); bars (whiskers) above and below the box indicate the range of non-outliers. Non-outliers are indicated by triangles. Outliers and extremes are shown by circles and asterisks.
A. Levels of serum IgG anti-NfH antibodies without a significant difference between group of Alzheimer's disease patients and the control group
B. Avidity of anti-NfH autoantibodies in serum of Alzheimer's disease patients and the control group. The results of avidity are expressed as an avidity index (%). A significant difference between these two groups was not found (P>0.05).

AD – Alzheimer's disease; AI – avidity index; AU – arbitrary unit; NfH – neurofilament heavy subunit

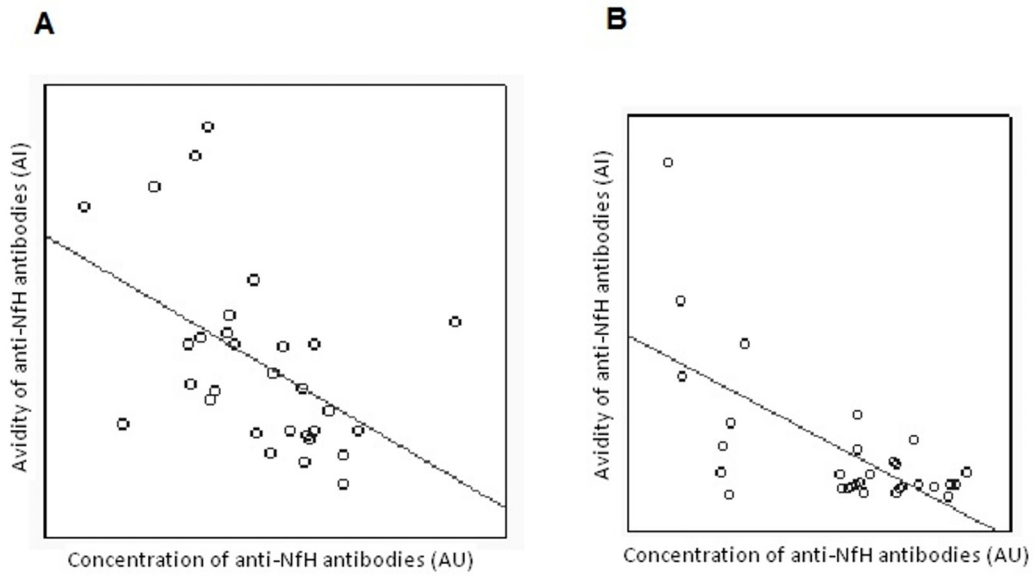


Fig. 4. Relationships between levels and avidities of anti-NfH IgG antibodies. Avidity was determined by ELISA using urea 6 mol/L as chaotropic agent.
A. The relationship between levels and avidities of anti-NfH antibodies IgG in the control group (n=30). A significant inverse correlation was found (R=-0.55, P=0.00147).
B. The relationship between levels and avidities of IgG anti-NfH antibodies in the group of the Alzheimer's disease patients (n=30). A significant inverse correlation was found R=-0.409, P=0.025).

AI – avidity index; AU – arbitrary unit; NfH – neurofilament heavy subunit

Comparison of NaCl and urea as chaotropic agents

The ability of two chaotropic agents (NaCl and urea) to distinguish between high- and low-avidity anti-NfH IgG antibodies was tested. Lower (1 mol/L for NaCl or 2 mol/L for urea) and higher (3 mol/L for NaCl or 6 mol/L for urea) concentration of these agents were selected and twenty serum samples (10 controls and 10 Alzheimer's disease patients) were analysed. The analyses were assessed in triplicates for the samples and in duplicates for calibration samples.

The influence of chaotropic agents on avidity determination was assessed according to the results both in arbitrary units and avidity indices. Different abilities of chaotropic agents to disrupt the immune complexes were observed. Levels of anti-NfH IgG antibodies (in AU) in the presence of different concentrations of chaotropic agents are shown in Fig. 2.

The values of avidity indices are similar when the 1 mol/L NaCl and 2 mol/L urea are used. A significant difference was observed between 3 mol/L NaCl and 6 mol/L urea (P=0.00016; Wilcoxon matched paired test). A reliable estimation of antibody avidity requires about 50% decrease in an antibody binding after treatment of immune complexes by chaotrophic agents in the ELISA procedure[32]. Thus, urea in the concentration of 6 mol/L resulted in the most effective disruption of the immune complexes. Urea was evaluated as a suitable chaotropic agent for the immunochemical (ELISA) determination of anti-NfH IgG antibody avidity (Fig. 2B). The optimised ELISA method using 2 mol/L and 6 mol/L urea as a chaotropic agent was used for the avidity determination in the serum samples for the clinical part of our study.

Avidity of anti-NfH IgG autoantibodies in serum of Alzheimer's disease patients

Using the optimised ELISA, the avidity of the anti-NfH IgG antibodies was studied in sera of Alzheimer's disease patients and controls.

We found no significant differences either in the levels or in the avidities of serum anti-NfH IgG antibodies between patients with Alzheimer's disease and those in the control group (Fig. 3AB). Also there was no significant correlation between anti-NfH IgG antibodies or their avidity and the relevant sociodemographic characteristics such as age of participants or sex (Spearman correlation coefficient, n.s.).

The levels of anti-NfH IgG antibodies inversely correlated with their avidities. The relationship between levels of antibodies and their avidities was expressed more closely in the control group than that of the AD group. The correlation in the control group was significant for both concentrations of urea used for avidity determination (urea 2 mol/L: R=-0.38, P=0.038; urea 6 mol/L: R=-0.55, P=0.00147, Spearman correlation coefficient). On the contrary, the relationship between levels and avidities of anti-NfH IgG antibodies was only seen in the presence of 6 mol/L urea in the AD group (urea 2 mol/L: R=-0.151, n.s.; urea 6 mol/L: R=-0.409, P=0.025; Spearman correlation coefficient) – Fig. 4AB.

DISCUSSION

Avidity represents an important feature of antibodies, which significantly influences the formation of immune complexes and affectivity of antibodies. In this study we focused on the avidity of antibodies against NfH, which belong to the cytoskeletal structure specific for nervous tissue[5]. We optimised the avidity assay for IgG antibodies against NfH by assessing a suitable dilution of sera and by selecting a chaotrope and its concentration; we then examined sera of patients with Alzheimer's disease and control individuals.

Avidity (or functional affinity) is defined as the overall forces that hold together an antigen with a corresponding antibody. It is dependent on the affinity between antigen-binding sites of the antibody and epitopes of the antigen as well as valences of the antigen and the antibody[33]. ELISA is suitable method for the determination of antibody avidity for clinical purposes. Since non-covalent interactions in the certain immune complex are characteristic for a specific antibody and appropriate antigen, it is necessary to test the optimal conditions of the ELISA avidity assay independently for individual antibodies and to select the suitable chaotropic agents and their concentration or suitable dilution of samples. Chaotropic agents such as urea, ammonium thiocyanate, guanidine hydrochloride, diethylamine or sodium chloride have already been used for the determination of antibody avidity[7,8,10,18,34,35]. We chose two chaotropic agents varying in their chemical character – urea as a nonelectrolyte acting as a mild denaturing agent and sodium chloride with an ionic nature. Both chaotropes can impair non-covalent forces such as hydrophobic interactions, hydrogen bridges and Van der Waals forces, which enable the formation of immune complexes[18]. In our experiments urea in a certain concentration disrupted immune complexes of anti-NfH IgG antibodies with NfH more effectively than tested concentrations of NaCl. The concentration of 6 mol/L urea dissociated about 45% immune complexes and met the requirement about a 50% decrease in an antibody binding after adding chaotropic agents to immune complexes in the ELISA procedure[32]. Urea is often used for avidity determination, especially for antibodies against infectious agents and it was shown to be a suitable chaotrope for the avidity assay. Therefore, we performed ELISA analyses in samples of our patients with urea solutions[13].

Research over recent years has suggested the participation of humoral autoimmune processes involved in the pathogenesis of AD (ref.[36]). Multiple autoantibodies against various autoantigens were investigated in the serum and/or CSF of AD patients[37,38]. One group of autoantibodies included those against structures specific for nervous tissue, such as neurofilaments (NfL, NfH), tau proteins, glial fibrillary acidic proteins and others[16,39-42]. It seems that some autoantibodies may act pathogenically while others could have protective effects[37]. Animal experiments suggested an association between anti-NfH antibodies and some manifestation of AD (ref.[43]). Rats immunised with the NfH of Torpedo cholinergic neurons developed specific antibodies, which accumulated

in the perikarya and neurites of neurons in the septum and hippocampus and in white matter tracts in the rats' forebrains[44]. The immunised rats showed a significant deficit in short-term memory and in a reversal of choice paradigm in a position discrimination test in contrast to long-term memory, which was not affected. Oron et. al.[45] found that cognitive deficit in rats with experimental autoimmune dementia after immunisation by the cholinergic NfH evolved more slowly than the decrease in the density of cholinergic neurons in the septum of rats and the accumulation of IgG in the same brain area.

The use of certain autoantibodies as diagnostic or prognostic markers is under investigation. Levels of these antibodies were studied in sera and/or CSF (ref.[27,28,46]). We were mainly interested in the antibodies against autoantigens originating from the neurocytoskeleton in past. We found the differences in avidities of anti-NFM and anti-tau antibodies in serum and CSF in the patients with multiple sclerosis[29]. In addition, differences in avidities of various anti-neurocytoskeletal antibodies were seen. These are not surprising findings. Totland et. al.[47] also observed various avidities of two onconeuronal antibodies Hu and Yo in patients with paraneoplastic neurological syndromes. Therefore, it is important to test the avidity of each autoantibody individually.

The pathogenic autoantibodies are mostly characterised by a higher avidity for autoantigens in the target tissue[9,48-50], but the occurrence of low-avidity autoantibodies in immune-mediated diseases have also been described[15,51]. Jianping et. al.[15] investigated the avidity of anti-Aβ antibodies in AD and found that both levels and avidities of anti-Aβ antibodies were statistically lower in AD patients than in the age-matched control group. They assumed that avidity declination would have a great influence on the clearance of Aβ by the cells of the immune system. We assumed similar changes in avidity of serum anti-NfH antibodies. Unfortunately, the comparison of anti-NfH IgG antibody levels and avidities showed no differences between AD patients and control. However, the avidity of antibodies may change with the progression of the disease. The follow-up study of individual AD patients might find a certain trend in the avidity of anti-NfH IgG antibodies in the serum.

A statistically significant inverse relationship between avidities and levels of anti-NfH antibodies in both groups was found. This might reflect that high-avidity anti-NfH IgG antibodies are bound in the immune complexes with their antigens. Conversely, the low-avidity antibodies form immune complexes less effectively and they tend to reach higher levels as free molecules.

CONCLUSION

We optimised the ELISA method in order to find anti-NfH antibody avidity determination. This method has been used for the study of anti-NfH antibody avidity in serum of patients with Alzheimer's disease, but it is also suitable for research of anti-NfH antibody avidity in patients with other neurological diseases associated with neurocytoskeletal defects. Neither levels nor avidity of IgG anti-NfH antibodies differed between AD patients and cognitively normal persons. In the future, we plan to investigate if the levels and avidity of antineurocytoskeletal antibodies will change during the follow-up study. In addition, we are going to determine separately, free anti-NfH IgG antibodies and those bound in immune complexes.

Acknowledgement: The study was supported by the following: Charles University Grant Agency GAUK 275015, Specific Academic Research Projects 260263, PROGRES Q25/LF1, PROGRES Q35 and the project of Ministry of Health, Czech Republic for conceptual development of research organization RVO-VFN64165 - General University Hospital in Prague, Czech Republic.

Author contributions: LN: study design, laboratory analysis, data analysis and interpretation, writing of the manuscript; LF: study design, data analysis and interpretation, writing of the manuscript; AB: collection and analysis of clinical data, clinical evaluation, general corrections; TZ: supervising the study.

Conflict of interest statement: The authors state that there are no conflicts of interest regarding the publication of this article.

REFERENCES

1. Eira J, Silva CS, Sousa MM, Liz MA. The cytoskeleton as a novel therapeutic target for old neurodegenerative disorders. Prog Neurobiol 2016;141:61-82.
2. Liu Q, Xie F, Alvarado-Diaz A, Smith MA, Moreira PI, Zhu X, Perry G. Neurofilamentopathy in neurodegenerative diseases. Open Neurol J 2011;5:58-62.
3. Zaffaroni M. Biological indicators of the neurodegenerative phase of multiple sclerosis. Neurol Sci 2003;24 Suppl 5:S279-82.
4. Huizinga R, Linington C, Amor S. Resistance is futile: antineuronal autoimmunity in multiple sclerosis. Trends Immunol 2008;29:54-60.
5. Petzold A. Neurofilament phosphoforms: surrogate markers for axonal injury, degeneration and loss. J Neurol Sci 2005;233:183-98.
6. Sigurdsson EM. Tau Immunotherapy. Neurodegener Dis 2016;16:34-8.
7. Cucnik S, Kveder T, Artenjak A, Ulcova Gallova Z, Swadzba J, Musial J, Iwaniec T, Stojanovich L, Alessandri C, Valesini G, Avcin T, Cohen Tervaert JW, Rozman B, Bozic B. Avidity of anti-beta2-glycoprotein I antibodies in patients with antiphospholipid syndrome. Lupus 2012;21:764-5.
8. Cucnik S, Kveder T, Ulcova-Gallova Z, Swadzba J, Musial J, Valesini G, Avcin T, Rozman B, Bozic B. The avidity of anti-beta2-glycoprotein I antibodies in patients with or without antiphospholipid syndrome: a collaborative study in the frame of the European forum on antiphospholipid antibodies. Lupus 2011;20:1166-71.
9. Cui Z, Zhao MH. Avidity of anti-glomerular basement membrane autoantibodies was associated with disease severity. Clin Immunol 2005;116:77-82.
10. Woznicová V. [Immunoglobulin G avidity in infectious diseases]. [Article in Czech]. Epidemiol Mikrobiol Imunol 2004;53:4-11.
11. Boxus M, Lockman L, Fochesato M, Lorin C, Thomas F, Giannini SL. Antibody avidity measurements in recipients of Cervarix vaccine following a two-dose schedule or a three-dose schedule. Vaccine 2014;32:3232-6.
12. Curdt I, Praast G, Sickinger E, Schultess J, Herold I, Braun HB, Bernhardt S, Maine GT, Smith DD, Hsu S, Christ HM, Pucci D, Hausmann M, Herzogenrath J. Development of fully automated determination of marker-specific immunoglobulin G (IgG) avid-

ity based on the avidity competition assay format: application for Abbott Architect cytomegalovirus and Toxo IgG Avidity assays. J Clin Microbiol 2009;47:603-13.
13. Flori P, Tardy L, Patural H, Bellete B, Varlet MN, Hafid J, Raberin H, Sung RT. Reliability of immunoglobulin G antitoxoplasma avidity test and effects of treatment on avidity indexes of infants and pregnant women. Clin Diagn Lab Immunol 2004;11:669-74.
14. Menge T, Lalive PH, von Budingen HC, Genain CP. Conformational epitopes of myelin oligodendrocyte glycoprotein are targets of potentially pathogenic antibody responses in multiple sclerosis. J Neuroinflammation 2011;8:161.
15. Jianping L, Zhibing Y, Wei Q, Zhikai C, Jie X, Jinbiao L. Low avidity and level of serum anti-Abeta antibodies in Alzheimer disease. Alzheimer Dis Assoc Disord 2006;20:127-32.
16. Bartos A, Fialova L, Svarcova J, Ripova D. Patients with Alzheimer disease have elevated intrathecal synthesis of antibodies against tau protein and heavy neurofilament. J Neuroimmunol 2012;252:100-5.
17. Soussan L, Tchernakov K, Bachar-Lavi O, Yuvan T, Wertman E, Michaelson DM. Antibodies to different isoforms of the heavy neurofilament protein (NF-H) in normal aging and Alzheimer's disease. Mol Neurobiol 1994;9:83-91.
18. Dimitrov JD, Lacroix-Desmazes S, Kaveri SV. Important parameters for evaluation of antibody avidity by immunosorbent assay. Anal Biochem 2011;418:149-51.
19. Bartoš A, Zach P, Diblíková F, Tintěra J, Řípová D, Brunovský M. Visual rating of medial temporal lobe atrophy on magnetic resonance imaging in Alzheimer's disease. Psychiatrie 2007;11:49-52.
20. Bartoš A, Čechová L, Švarcová J, Říčný J, Řípová D. Cerebrospinal fluid triplet (tau proteins and beta-amyloid) in the diagnosis of Alzheimer-Fischer disease [Likvorový triplet (tau proteiny a beta-amyloid) v diagnostice Alzheimerovy-Fischerovy nemoci]. Cesk Slov Neurol N 2012;75/108:587-94.
21. Scheltens P, Leys D, Barkhof F, Huglo D, Weinstein HC, Vermersch P, Kuiper M, Steinling M, Wolters EC, Valk J. Atrophy of medial temporal lobes on MRI in "probable" Alzheimer's disease and normal ageing: diagnostic value and neuropsychological correlates. J Neurol Neurosurg Psychiatry 1992;55:967-72.
22. Dubois B, Feldman HH, Jacova C, Dekosky ST, Barberger-Gateau P, Cummings J, Delacourte A, Galasko D, Gauthier S, Jicha G, Meguro K, O'Brien J, Pasquier F, Robert P, Rossor M, Salloway S, Stern Y, Visser PJ, Scheltens P. Research criteria for the diagnosis of Alzheimer's disease: revising the NINCDS-ADRDA criteria. Lancet Neurol 2007;6:734-46.
23. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. Neurology 1984;34:939-44.
24. McKhann GM, Knopman DS, Chertkow H, Hyman BT, Jack CR, Jr., Kawas CH, Klunk WE, Koroshetz WJ, Manly JJ, Mayeux R, Mohs RC, Morris JC, Rossor MN, Scheltens P, Carrillo MC, Thies B, Weintraub S, Phelps CH. The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement 2011;7:263-9.
25. Bartoš A, Raisová M. The Mini-Mental State Examination (MMSE) – Czech norms and cut-offs for mild dementia and mild cognitive impairment due to Alzheimer disease. Dementia and Geriatric Cognitive Disorders 2016;42:50-7.
26. Povova J, Ambroz P, Bar M, Pavukova V, Sery O, Tomaskova H, Janout V. Epidemiological of and risk factors for Alzheimer's disease: a review. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2012;156:108-14.
27. Silber E, Semra YK, Gregson NA, Sharief MK. Patients with progressive multiple sclerosis have elevated antibodies to neurofilament subunit. Neurology 2002;58:1372-81.
28. Bartos A, Fialova L, Soukupova J, Kukal J, Malbohan I, Pitha J. Antibodies against light neurofilaments in multiple sclerosis patients. Acta Neurol Scand 2007;116:100-7.
29. Fialova L, Svarcova J, Bartos A, Malbohan I. Avidity of anti-neurocytoskeletal antibodies in cerebrospinal fluid and serum. Folia Microbiol (Praha) 2012;57:415-9.
30. Cucnik S, Bozic B, Kveder T, Tomsic M, Rozman B. Avidity of anti-beta2-glycoprotein I and thrombosis or pregnancy loss in patients with antiphospholipid syndrome. Ann N Y Acad Sci 2005;1051:141-7.
31. Almanzar G, Ottensmeier B, Liese J, Prelog M. Assessment of IgG avidity against pertussis toxin and filamentous hemagglutinin via an adapted enzyme-linked immunosorbent assay (ELISA) using ammonium thiocyanate. J Immunol Methods 2013;387:36-42.
32. Pullen GR, Fitzgerald MG, Hosking CS. Antibody avidity determination by ELISA using thiocyanate elution. J Immunol Methods 1986;86:83-7.
33. Gharavi A, Reiber H. Affinity and avidity of autoantibodies. In: Peter J, Shoenfeld Y, eds. Autoantibodies. Amsterdam: Elsevier, 1996: 13-23.
34. Cucnik S, Kveder T, Krizaj I, Rozman B, Bozic B. High avidity anti-beta 2-glycoprotein I antibodies in patients with antiphospholipid syndrome. Ann Rheum Dis 2004;63:1478-82.
35. Dauner JG, Pan Y, Hildesheim A, Kemp TJ, Porras C, Pinto LA. Development and application of a GuHCl-modified ELISA to measure the avidity of anti-HPV L1 VLP antibodies in vaccinated individuals. Mol Cell Probes 2012;26:73-80.
36. D'Andrea MR. Add Alzheimer's disease to the list of autoimmune diseases. Med Hypotheses 2005;64:458-63.
37. Colasanti T, Barbati C, Rosano G, Malorni W, Ortona E. Autoantibodies in patients with Alzheimer's disease: pathogenetic role and potential use as biomarkers of disease progression. Autoimmun Rev 2010;9:807-11.
38. Papuc E, Kurys-Denis E, Krupski W, Tatara M, Rejdak K. Can Antibodies Against Glial Derived Antigens be Early Biomarkers of Hippocampal Demyelination and Memory Loss in Alzheimer's Disease? J Alzheimers Dis 2015;48:115-21.
39. Ishida K, Kaneko K, Kubota T, Itoh Y, Miyatake T, Matsushita M, Yamada M. Identification and characterization of an anti-glial fibrillary acidic protein antibody with a unique specificity in a demented patient with an autoimmune disorder. J Neurol Sci 1997;151:41-8.
40. Mecocci P, Parnetti L, Donato R, Santucci C, Santucci A, Cadini D, Foa E, Cecchetti R, Senin U. Serum autoantibodies against glial fibrillary acidic protein in brain aging and senile dementias. Brain Behav Immun 1992;6:286-92.
41. Rosenmann H, Meiner Z, Geylis V, Abramsky O, Steinitz M. Detection of circulating antibodies against tau protein in its unphosphorylated and in its neurofibrillary tangles-related phosphorylated state in Alzheimer's disease and healthy subjects. Neurosci Lett 2006;410:90-3.
42. Tanaka J, Nakamura K, Takeda M, Tada K, Suzuki H, Morita H, Okado T, Hariguchi S, Nishimura T. Enzyme-linked immunosorbent assay for human autoantibody to glial fibrillary acidic protein: higher titer of the antibody is detected in serum of patients with Alzheimer's disease. Acta Neurol Scand 1989;80:554-60.
43. d'Abramo C, Acker CM, Jimenez HT, Davies P. Tau passive immunotherapy in mutant P301L mice: antibody affinity versus specificity. PLoS One 2013;8:e62402.
44. Chapman J, Alroy G, Weiss Z, Faigon M, Feldon J, Michaelson DM. Anti-neuronal antibodies similar to those found in Alzheimer's disease induce memory dysfunction in rats. Neuroscience 1991;40:297-305.
45. Oron L, Dubovik V, Novitsky L, Eilam D, Michaelson DM. Animal model and in vitro studies of anti neurofilament antibodies mediated neurodegeneration in Alzheimer's disease. J Neural Transm Suppl 1997;49:77-84.
46. Bartos A, Fialova L, Soukupova J, Kukal J, Malbohan I, Pit'ha J. Elevated intrathecal antibodies against the medium neurofilament subunit in multiple sclerosis. J Neurol 2007;254:20-5.
47. Totland C, Aarseth J, Vedeler C. Hu and Yo antibodies have heterogeneous avidity. J Neuroimmunol 2007;185:162-7.
48. Bozic B, Cucnik S, Kveder T, Rozman B. Avidity of anti-beta-2-glycoprotein I antibodies. Autoimmun Rev 2005;4:303-8.
49. Božič B, Čučnik S, Kveder T, Rozman B. Affinity and avidity of autoantibodies. In: Shoenfeld Y, Gershwin ME, Meroni PL, eds. Autoantibodies. Amsterdam: Elsevier, 2007: 21-28.
50. Schlosser M, Koczwara K, Kenk H, Strebelow M, Rjasanowski I, Wassmuth R, Achenbach P, Ziegler AG, Bonifacio E. In insulin-autoantibody-positive children from the general population, antibody affinity identifies those at high and low risk. Diabetologia 2005;48:1830-2.
51. Cao Z, Lv J, Quan W. Low avidity and level of serum anti-Abeta antibodies in patients with cerebral amyloid angiopathy-related cerebral hemorrhage. Int J Neurosci 2010;120:760-4.

doi: 10.48095/cccsnn202211

Analytické a preanalytické aspekty stanovení lehkých řetězců neurofilament v biologických tekutinách

Analytical and pre-analytical aspects of neurofilament light chain determination in biological fluids

Souhrn

Tento přehled si klade za cíl seznámit klinické a laboratorní pracovníky s nejdůležitějšími preanalytickými a analytickými aspekty stanovení lehkých řetězců neurofilament (NfL) v biologických tekutinách. NfL představují perspektivní nespecifický biomarker poškození neuronů a axonů, k němuž dochází u celé řady neurologických onemocnění. Před zavedením vyšetřování NfL do širší klinické praxe je nutné charakterizovat preanalytické a analytické stránky stanovení, které mohou významným způsobem ovlivnit správnost výsledku analýzy. Při hodnocení koncentrací NfL je zapotřebí brát v úvahu věk pacienta a vliv může mít i body mass index. Výhodou NfL je jejich dlouhodobá stabilita při různých teplotách skladování i odolnost vůči opakovaným cyklům zmrazování a rozmrazování. Koncentrace NfL v klinických studiích se stanovují především imunoanalytickými metodami, které se liší citlivostí. Pro stanovení NfL existuje několik imunoanalytických přístupů vhodných pro spolehlivé vyšetření v mozkomíšním moku (MMM) i v séru/plazmě. Volba optimálního analytického přístupu závisí mimo jiné na koncentracích NfL v biologických tekutinách. Pro stanovení NfL v MMM lze využít metod ELISA, které vykazují dostačující citlivost pro vyšší koncentrace NfL vyskytující se v této biologické tekutině. Postupně zaváděné nové technologie charakterizované výrazně vyšší citlivostí ve srovnání s metodou ELISA umožnily spolehlivé vyšetřování NfL i v séru či plazmě. Podrobněji jsou zmiňovány principy metod postavené na technologii Simoa®, SimplePlex™ a imunomagnetické redukce.

Abstract

The aim of this review is to inform clinical and laboratory workers about the most important pre-analytical and analytical aspects of neurofilament light chain (NfL) determination in biological fluids. NfLs represent a promising nonspecific biomarker of neuronal and axonal damage that occurs in a variety of neurological diseases. Before introducing NfL determination into routine clinical practice, it is necessary to characterize the pre-analytical and analytical aspects of the assays, which can significantly affect the accuracy of the analysis results. When evaluating NfL concentrations, the patient's age should be taken into account and body mass index may also have an effect. The advantages of NfLs are their long-term storage stability at different temperatures as well as resistance to repeated freezing and thawing cycles. NfL concentrations in clinical trials are determined primarily by immunoassay methods that vary in sensitivity. There are several immunoassay technologies for the determination of NfL suitable for reliable determination in cerebrospinal fluid (CSF) and serum/plasma. The choice of the optimal analytical approach depends, among other things, on the concentration of NfL in biological fluids. ELISA methods can be used to determine NfL in CSF, which show sufficient sensitivity for higher concentrations of NfL occurring in this biological fluid. Newly introduced technologies characterized by significantly higher sensitivity in comparison with the ELISA methods enabled reliable examination of NfL also in serum/plasma. The principles of methods based on Simoa® technology, SimplePlex™, and immunomagnetic reduction are mentioned in more detail.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.
The Editorial Board declares that the manuscript met the ICMJE "uniform requirements" for biomedical papers.

L. Fialová[1], L. Nosková[1], M. Kalousová[1], T. Zima[1], T. Uher[2], A. Bartoš[3]

[1] Ústav lékařské biochemie a laboratorní diagnostiky, 1. LF UK a VFN v Praze
[2] Centrum pro demyelinizační onemocnění, Neurologická klinika 1. LF UK a VFN v Praze
[3] Neurologická klinika 3. LF UK, Praha

✉ MUDr. Lenka Fialová, CSc.
Ústav lékařské biochemie a laboratorní diagnostiky
1. LF UK a VFN v Praze
Kateřinská 32
121 08 Praha
e-mail: lfial@lf1.cuni.cz

Přijato k recenzi: 26. 10. 2021
Přijato do tisku: 20. 1. 2022

Klíčová slova

biomarker – krev – mozkomíšní mok – neurofilamenta – preanalytická fáze

Key words

biomarker – blood – cerebrospinal fluid – neurofilaments – pre-analytical phase

Úvod

Neurologům se dostává do rukou slibný biomarker, kterým jsou lehké řetězce neurofilament (NfL). Někteří jejich význam přirovnávají k troponinům v kardiologii [1].

Neurofilamenta v CNS jsou heteropolymery sestavené ze čtyř polypeptidových řetězců, k nimž patří triplet neurofilamentových řetězců (NfL, střední řetězce neurofilament a těžké řetězce neurofilament) a α-internexin. V periferním nervovém systému (PNS) je součástí neurofilament společně s tripletem neurofilamentových řetězců další protein – peripherin. Struktura neurofilamentových řetězců je podobná, ale jednotlivé polypeptidy se liší relativní molekulovou hmotností (Mr) a mírou fosforylace – lehké řetězce mají Mr 70 000, střední řetězce (NfM) 150 000 a těžké řetězce (NfH) 200 000 [2]. Schéma molekuly NfL je znázorněno na obr. 1. NfL tvoří nejhojnější podjednotku v tripletu neurofilamentových řetězců, zastoupení NfL : NfM : NfH v neurofilamentech je přibližně v tomto poměru 5 : 3 : 1 [3].

NfL představují nespecifický biomarker odrážející poškození axonů a neuronů, k němuž dochází z různých příčin, ať už se jedná např. o zánět, neurodegeneraci nebo trauma [4–8]. NfL se uvolňují do intersticiální tekutiny a z ní mohou pronikat do mozkomíšního moku (MMM) a také do krve. Při některých patologických stavech v oblasti nervové soustavy se množství NfL přestupujících do různých kompartmentů výrazně zvyšuje [9–11].

Detailnější výzkum NfL jako biomarkeru u neurologických onemocnění umožnili již v roce 1996 Rosengren et al [12], když jako první popsali metodu ELISA (enzyme-linked immunosorbent assay) jako vhodnou ke stanovení NfL. Citlivost metody ELISA však dovoluje správně stanovovat koncentrace NfL pouze v MMM, jehož odběr představuje invazivní výkon. Proto se další vlna zájmu o NfL dostavila až poté, co se začaly úspěšně vyvíjet vysoce citlivé metody na jejich stanovení v krvi [13]. Od té doby se datuje prudký nárůst nových poznatků o NfL využitelných v péči o pacienty se závažným neurologickým onemocněním. Značná pozornost je věnována přínosu stanovení NfL u nemocných s RS [1,5,14–17]. Vysoká hladina NfL predikuje aktivitu nemoci u RS [18–20], naopak nízká hladina je spojená s absencí radiologické aktivity nemoci a mohla by v budoucnosti u některých pacientů doplnit, nebo dokonce nahradit kontrolní zobrazení CNS MR [17]. Povzbudivé výsledky přinášejí i studie zaměřené na využití NfL u dalších neurologických onemocnění spojených se ztrátou nebo poškozením neuronů [6,9,21,22]. Existují studie ukazující přínos NfL při diferenciální diagnostice různých typů demencí, extrapyramidových neurodegenerativních onemocnění nebo také amyotrofické laterální sklerózy [9,10]. Měření hladin NfL může pomoci při predikci vývoje stavu po ischemické CMP, po úrazech mozku nebo po kardiopulmonální resuscitaci [20,22,23].

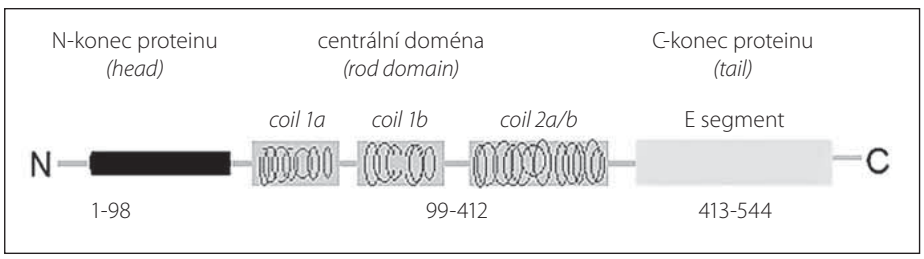
Před zavedením vyšetřování NfL do širší klinické praxe je nutné charakterizovat preanalytické a analytické stránky jejich stanovení, které mohou významným způsobem ovlivnit správnost výsledku analýzy, a je zapotřebí k nim přihlížet při interpretaci výsledků vyšetření. Tento text si klade za cíl seznámit klinické a laboratorní pracovníky s nejdůležitějšími preanalytickými a analytickými aspekty stanovení NfL v biologických tekutinách.

Koncentrace NfL v jednotlivých biologických tekutinách

Běžnými biologickými tekutinami, v nichž se NfL vyšetřují, jsou MMM, sérum a plazma (tab. 1). Nejvyšších koncentrací dosahují NfL v MMM (cNfL). V porovnání s plazmou či sérem jsou koncentrace cNfL mnohonásobně vyšší. Např. Disanto et al [5] uvedli, že medián koncentrací NfL byl 42× nižší v séru než v MMM.

Jak vyplynulo z metaanalýzy, mezi koncentracemi cNfL a koncentracemi NfL v séru (sNfL) nebo v plazmě (pNfL) existuje středně silná korelace, která je více vyjádřena u pacientů s onemocněním CNS nebo PNS než u kontrolních skupin. Těsnější vztah byl pozorován ve studiích, které použily pro stanovení NfL citlivějších imunoanalytických metod [24].

NfL byly testovány i ve slinách, které představují velice snadno dostupný biologický materiál [25]. Zatím se ale zdá, že stanovení NfL ve slinách bude mít omezený klinický význam, alespoň pro neurodegenerativní onemocnění.



Obr. 1. Schéma struktury lehkého řetězce neurofilamenta.
Fig. 1. Scheme of the neurofilament light chain structure.

Biologické faktory ovlivňující koncentrace NfL

Věk

Důležitý faktor, který ovlivňuje koncentrace cNfL i koncentrace NfL v krvi, představuje věk. Pozitivní korelaci mezi věkem a koncentrací cNfL u kontrolních skupin i u řady neurologických onemocnění publikovali v rozsáhlé metaanalýze, na které se podíleli i někteří autoři tohoto článku, Bridel et al [4]. U zdravých osob metaanalýza uvádí nárůst koncentrací cNfL na každý rok věku o 3,3 %.

Vztah mezi věkem a koncentrací NfL vyjádřený u zdravých jedinců i u pacientů je dobře dokumentován i v krvi [5,26]. Pro zdravé kontroly se udává vzestup koncentrací sNfL o 2,2 % na každý rok věku [5]. Podle některých studií není zvyšování koncentrací sNfL s věkem lineární a zrychluje se po dosažení věku 60 let, kdy navýšení může být až dvojnásobné, tj. o 4,3 % [27]. U různých neurologických onemocnění byla také prokazována významná korelace mezi NfL v krvi a věkem [5,19,28].

Pozitivní vztah mezi koncentracemi NfL a věkem je připisován věkem podmíněným změnám v CNS spojeným se ztrátou neuronů, ale přispívat může i výraznější pokles renálních funkcí, zejména u starších osob [29,30]. Proto je nezbytné souvislost mezi věkem a koncentracemi NfL zohledňovat při interpretaci jejich vyšetření.

Pohlaví a rasa

Koncentrace cNfL ve vztahu k pohlaví hodnotili v nedávné metaanalýze Bridel at al [4]. Byly vyšší o 26 % u zdravých mužů a zvýšení u mužů bylo pozorováno i u některých neurologických onemocnění. V EDTA (kyselina ethylendiamintetraoctová) plazmě a séru ale není závislost koncentrací NfL na pohlaví v řadě prací prokazována [5,26,28,29]. Pohlaví se proto při normalizaci koncentrací NfL nebere v úvahu.

Ukazuje se, že etnika mohou mít odlišné koncentrace. V MMM byly koncentrace NfL

nižší u kognitivně zdravých Afroameričanů než u osob kavkazské rasy [31]. Naopak v séru byly koncentrace NfL nižší u kavkazské rasy než u jiných ras [30].

Body mass index a objem krve

Dalším faktorem, který ovlivňuje koncentrace NfL v krvi, je index tělesné hmotnosti (body mass index; BMI) a objem krve. Manouchehrinia et al [32] tento vztah analyzovali na velkém souboru kontrol (n = 662) a prokázali významnou negativní korelaci mezi koncentrací pNfL a BMI s poklesem o 0,02 pg/ml na vzestup BMI o jednotku. Vztah mezi koncentracemi p/sNfL a BMI popsaný Manouchehriniou et al u zdravých platil také pro pacienty s RS a mentální anorexií [32–34]. Snížení koncentrace pNfL o 0,15 pg/ml bylo zaznamenáno při zvýšení objemu krve o 1 l u zdravých kontrol. Jiná je pak situace v průběhu fyziologického těhotenství, které je doprovázeno vzestupem koncentrací NfL v krvi [20].

Biologická variabilita

Důležitou vlastností analytu je jeho stabilita u jedince v čase. Intraindividuální kolísání koncentrací sNfL v průběhu dne a mezi dny je minimální (3,1 %), ale interindividuální variabilita je výrazná (35,6 %) [35].

Další biologické vlivy

Kromě výše uvedených vlivů byly popsány i další faktory s dopadem na koncentrace NfL v krvi, jako jsou kouření, systolický krevní tlak, fibrilace síní, onemocnění PNS, užívání neurotoxických léků, které většinou přispívají ke zvýšení koncentrací. Přihlížet je zapotřebí i ke stavu hematoencefalické bariéry [20,24].

Vliv teploty a doby skladování na stabilitu NfL v biologických tekutinách

NfL je stabilním analytem při skladování vzorku při nízkých i vyšších teplotách. Stabilita NfL byla prokázána jak v MMM, tak v séru/plazmě.

Mozkomíšní mok

Koncentrace cNfL ve vzorcích zpracovaných po jedné nebo 24 h po odběru se významně nelišily bez ohledu na teplotu skladování (pokojová teplota, 4 °C). Stabilita koncentrací cNfL je zachována i po dobu 21 dnů při různých teplotách v rozmezí od –20 °C do 37 °C při srovnání s teplotou –80 °C [36].

NfL v MMM jsou odolná vůči opakovanému zmrazování a rozmrazování (–80 °C a pokojová teplota). Byly testovány čtyři cykly bez významných změn v koncentraci cNfL [36].

Tab. 1. Koncentrace NfL v různých biologických tekutinách u zdravých osob analyzované pomocí souprav Simoa NF-light™ Advantage Kit Quanterix*.

Biologická tekutina	Koncentrace NfL medián pg/ml	Citace
mozkomíšní mok	317 (n = 35)	[51]
sérum	6,96 (n = 132)	[51]
plazma (EDTA)	5,87 (n = 129)	[51]
sliny	2,3** (n = 17)	[25]

*nejedná se o referenční rozmezí; **hodnota odpovídá průměru
EDTA – kyselina ethylendiamintetraoctová; n – počet; Nfl – neurofilamenta

Sérum, plazma

Koncentrace s/pNfL zůstávají stabilní 7–8 dnů, když jsou uchovávány při pokojové teplotě nebo při 4 °C [37,38]. Stabilita NfL je za situace několikadenního uchování při pokojové teplotě mírně vyšší v séru než v EDTA plazmě [38]. Stabilitu si NfL udržují i při opakovaném zmrazování a rozmrazování. Koncentraci sNfL a EDTA plazmy významně neovlivnil ani 3–5× opakovaný cyklus rozmrazení a zmrazení [37,39].

Další preanalytické vlivy na koncentraci NfL

Mozkomíšní mok

Nízká, tj. 0,05% (odpovídá 2 500 erytrocytů/µl) kontaminace vzorku MMM krví neměla při 1–2hodinovém skladování při pokojové teplotě dopad na změnu koncentrace cNfL, ale větší, 5% kontaminace (odpovídá 250 000 erytrocytů/µl) se již odrazila ve zvýšení koncentrace cNfL až o 66 %. Nižší teplota při 4 °C snižovala míru nárůstu koncentrací cNfL. Podobné ovlivnění koncentrací cNfL se projevilo i při skladování vzorků po delší dobu (4–24 h) [36].

Sérum, plazma

Koncentraci v plazmě může ovlivnit použitý typ odběrové zkumavky s rozdílnými antikoagulačními přísadami. Plazmy s různými antikoagulačními prostředky pro stanovení NfL by neměly být zaměňovány, i když hodnoty koncentrací spolu významně korelují [39]. Podle studie Ashtona et al [39] nejnižší koncentrace pNfL stanovené pomocí Simoa® (single molecule array) (Quanterix, Billerica, MA, USA), na HD-X analyzátoru poskytuje citrátová plazma, následuje plazma s EDTA a nejvyšší koncentrace jsou získávány z plazmy ze zkumavek s heparinem lithným. Koncentrace NfL v citrátové plazmě byly o 20 % nižší než v plazmě EDTA. Podle různých studií jsou koncentrace NfL v séru vyšší asi o 11 % než v EDTA plazmě, ale je mezi nimi vyjádřena signifikantní korelace nebo se neliší [26,39,40]. Při srovnání mezi sérem a plazmou s heparinem lithným nebyl pozorován rozdíl [39].

Pro stanovení s/pNfL je zapotřebí přihlédnout k hemolýze vzorku vzhledem ke skutečnosti, že erytrocyty obsahují některé cytoskeletální struktury, vč. NfL [41]. Pomocí vysoce citlivé metody Simoa® byly v lyzátech z erytrocytů prokázány nízké koncentrace NfL, které korelovaly s koncentracemi NfL v plazmě [42].

Dalším preanalytickým faktorem, který může mít dopad na přesnost stanovení koncentrace NfL v plazmě, je doba, jež uplyne mezi odběrem a centrifugací. I v tomto případě se projevuje dobrá stabilita NfL. Koncentrace NfL v EDTA plazmě, která byla získána standardním způsobem (centrifugací krve během dvou hodin po odběru) signifikantně korelovala s koncentracemi NfL v plazmě získané oddálenou centrifugací krve za různých podmínek (po 48 h stání krve při pokojové teplotě, při 37 °C), i když bylo pozorováno mírné zvýšení [43]. Dobré výsledky přineslo i testování koncentrací NfL v eluátech z krve odebrané ve formě skvrn na speciální kartičky, hodnoty koncentrací NfL získané z těchto tekutin však byly signifikantně nižší [43].

Metody pro stanovení NfL v biologických tekutinách

Koncentrace NfL v klinických studiích se stanovují především imunoanalytickými metodami, které se liší citlivostí. Proto volba opti-

málního analytického přístupu závisí mimo jiné na koncentracích NfL v biologických tekutinách.

Stanovení NfL v mozkomíšním moku

Pro stanovení cNfL lze využít metod ELISA, které vykazují dostačující citlivost pro vyšší koncentrace NfL vyskytující se v této biologické tekutině. Před dostupností imunoanalytických souprav pro měření NfL v krvi byly koncentrace NfL ve většině klinických studií analyzovány pomocí komerčně dostupné soupravy NF-light® (Neurofilament light) ELISA firmy UmanDiagnostics (Umeå, Švédsko). Tato sendvičová ELISA využívá dvou vysoce specifických monoklonálních protilátek. Jejich reaktivita je namířena proti odlišným epitopům lokalizovaným v centrální tyčovité oblasti (rod domain) molekuly NfL (obr. 1) [44,45].

Stanovení NfL v krvi (plazmě, séru)

Vyšetření v krvi zpočátku bránila nedostatečná citlivost metod ELISA pro stanovení nízkých koncentrací NfL, o čemž jsme se sami přesvědčili [14]. O něco vyšší citlivosti dosahují imunoanalytické metody založené na detekci elektrochemiluminiscenčního signálu, který využívá pro elektrochemiluminiscenční metody (ECL) společnost MESO Scale Discovery™ (Rockville, MD, USA) [37]. Nově jsou dostupné i citlivější ELISA soupravy pro stanovení NfL v séru, s nimiž ale zatím nemáme zkušenosti. Postupně zaváděné nové technologie charakterizované výrazně vyšší citlivostí umožnily spolehlivé vyšetřování NfL i v séru či plazmě, ale při vhodném ředění jimi lze samozřejmě stanovovat i koncentrace v MMM [13].

Vysoce citlivé metody jsou postavené na technologii Simoa®, SimplePlex™ (Protein-Simple, San Jose, CA, USA) a IMR (immunomagnetic reduction) [46,47]. Principy uvedených imunoanalytických technik se liší.

Vysoce citlivé imunoanalytické metody pro stanovení NfL

Simoa®

Simoa®, o níž se hovoří také jako o digitální ELISE, využívá základních principů ELISY, jejichž modifikací je dosaženo schopnosti detekovat koncentrace proteinů v jednotkách fg–pg/ml [13,48–50].

V průběhu stanovení NfL metodou Simoa® se na paramagnetických částicích, které jsou označeny specifickými anti-NfL protilátkami, vytvoří sendvičový imunokomplex. Jeho součástí je protilátka na částici, dále NfL ze vzorku, popř. standardu a druhá anti-NfL protilátka, k níž se prostřednictvím vazby biotin-streptavidin připojí enzym β-galaktosidáza. Po vytvoření sendvičového komplexu se jednotlivé paramagnetické částice s roztokem fluorogenního substrátu rozdělí do pole obsahujícího několik set tisíc mikrojamek. Poté se v každé jamce detekuje fluorescence vzniklá rozkladem substrátu prostřednictvím β-galaktosidázy přítomné v imunokomplexu. Fluorescenční signál je dále vyhodnocován pomocí softwaru, který je součástí analyzátoru [13,49,50]. Soupravy Simoa® využívají stejné protilátky, jako jsou ty v soupravách ELISA UmanDiagnostics [51].

Metoda stanovení NfL na principu Simoa® je v současnosti považována za referenční metodu pro stanovení NfL v séru či plazmě [46]. Kromě vysoké citlivosti a nízké spotřeby vzorku patří k jejím výhodám i široký rozsah měřitelných koncentrací umožňující stanovení NfL v MMM a v séru či plazmě, a to dokonce i u osob bez onemocnění CNS i PNS [51]. Další výhodou technologie Simoa® je dostupnost komerčních souprav, které lze zpracovávat na několika typech analyzátorů firmy Quanterix (Billerica, MA, USA). Pro každý z analyzátorů jsou vyvinuty speciální soupravy a k dispozici jsou i takové, v nichž lze NfL analyzovat společně s dalšími proteiny ve formě multiplexu. Koncentrace pNfL stanovené samostatně vysoce korelovaly s výsledky NfL měřených jako součást multiplexu [52]. Souprava Simoa NF-light® Advantage Kit je určena pro stanovení NfL v séru, plazmě a MMM [51]. Jako kalibrátor využívá rekombinantní lidský NfL, který nahradil v minulosti používaný kalibrátor hovězího původu. Starší výsledky získané prostřednictvím hovězího kalibrátoru lze přepočítat pomocí poměru 5 : 1 (koncentrace NfL určená pomocí hovězího kalibrátoru = 5× koncentrace určená pomocí rekombinantního lidského kalibrátoru).

SimplePlex

Další citlivou imunoanalytickou metodou vhodnou pro stanovení nízkých koncentrací NfL v krvi je technika založená na nové automatizované platformě SimplePlex™ [46]. Imunochemická reakce probíhá v kanálcích, z nichž v každém jsou zabudovány tři skleněné komůrky (nanoreaktory) umožňující provedení analýzy každého vzorku v triplikátu. Na vnitřním povrchu komůrek je navázána specifická protilátka, která zachytí antigen obsažený ve vzorku. K detekci vzniklého imunokomplexu slouží další protilátka označená fluorescenčním barvivem. Analýza je plně automatizovaná pomocí analyzátoru ELLA™, jenž měří fluorescenční signál [53]. Platforma SimplePlex™ je schopna i multiplexových analýz pro stanovení několika biomarkerů současně. Zcela nedávno byla publikována studie srovnávající koncentrace sNfL stanovené technologiemi SimplePlex™ ELLA™ a Simoa® [46]. Srovnání ukázalo silnou korelaci mimo jiné i díky tomu, že obě metody používají stejné anti-NfL protilátky. Na rozdíl od protilátek byl v obou soupravách odlišný standard, což se odrazilo v rozdílných absolutních koncentracích sNfL. Vzhledem k linearitě vztahu je možný přepočet koncentrací získaných oběma metodami pomocí korekčního faktoru.

Imunomagnetická redukce

Pro nízké koncentrace NfL byla vypracována i metoda na bázi imunomagnetické redukce (IMR), jejíž vývoj je spojen se společností MagQu Co. Ltd. (Taiwan) / MagQu LLC (USA) [47,54]. IMR je založena na měření poklesu magnetického signálu, ke kterému dochází po vytvoření imunokomplexu na povrchu magnetických nanokuliček. Tyto nanokuličky vyrobené z Fe_3O_4 mají na povrchu navázané specifické protilátky. V magnetickém poli střídavého proudu kuličky oscilují. Po navázání antigenu na kuličku prostřednictvím specifické protilátky se kuličky zvětšují a některé shlukují, což má za následek snížení rychlosti jejich oscilace. Tato změna rychlosti oscilace je úměrná množství vázaného analytu a lze ji přímo změřit. Signál IMR je analyzován velice citlivým magnetometrem. Na rozdíl od výše uváděných imunoanalytických metod je zapotřebí pouze jedné protilátky a provedení metody nevyžaduje promývací kroky.

Metoda IMR pro měření NfL nevyužívá anti-NfL protilátky UmanDiagnostics, nýbrž anti-NfL protilátky Santa Cruz Biotechnology, Inc. Standard má povahu rekombinantního lidského NfL. Předklinické testování metody pro vyšetřování NfL pomocí IMR ukázalo, že test se vyznačuje mimořádnou citlivostí a vysokou specifičností [47].

Základní údaje týkající se různých technologií pro stanovení NfL jsou shrnuty v tab. 2.

Perspektivy v analytice

Další krok v analytice NfL představuje adaptace vysoce citlivé metody pro rutinní imunoanalytickou platformu Siemens – ADVIA Centaur® a Atellica® (Siemens, Mnichov, Ně-

Tab. 2. Základní údaje týkající se souprav pro stanovení NfL v biologických tekutinách.

Platforma	Dolní limit stanovitelnosti	Standard (původ)	Protilátky	Výrobce souprav	Citace
ELISA	cca 50 pg/ml	hovězí	anti-NfL mAb 47 : 3	UmanDiagnostics AB, (Umea, Sweden)**	[51]
			anti-NfL mAb 2 : 1		
ECL	15,6 pg/ml	hovězí	anti-NfL mAb 47 : 3	MESO Scale Discovery™ (MSD, Maryland, USA)	[37]
			anti-NfL mAb 2 : 1		
ECL	3,4 pg/ml	–	–	MESO Scale Discovery™	[60]
(technologie MULTI-ARRAY)				(Rockville, Maryland, USA)	
SimplePlex™	2,7 pg/ml	hovězí	anti-NfL mAb 47 : 3	ProteinSimple (San Jose, CA, USA)	[46]
			anti-NfL mAb 2 : 1		
Siemens ADVIA CentaurÒ	1,62 pg/ml	–	–	Siemens Healthineers AG	[55]
Quanterix SimoaÒ	0,686 pg/ml	lidský rekombinantní	anti-NfL mAb 47 : 3	Quanterix (Billerica, MA USA)	[51]
(NF-lightÒ Advantage Kit HD)			anti-NfL mAb 2 : 1		
IMR	0,18 fg/ml*	lidský rekombinantní	anti-NfL mAb (Santa Cruz Biotechnology)	MagQu (New Taipei City, Taiwan/ Surprise, AZ, USA)	[47]

*limit detekce; **od konce roku 2021 je od stejného výrobce k dispozici i souprava ELISA pro stanovení NfL v séru s dolním limitem stanovitelnosti 0,8 pg/ml
ECL – electrochemiluminescence; ELISA – enzyme-linked immunosorbent assay; IMR – immunomagnetic reduction; mAb – monoklonální protilátky; Nfl – neurofilamenta

mecko) [55]. Nová imunoanalýza vykazuje vysokou korelaci s metodou Simoa® (NfL-light® Advantage Kit, Quanterix) [55] .

Jedním z trendů v biochemické diagnostice jsou multiplexové analýzy. Pro neurologické biomarkery jsou ve formě až kvadruplexu vč. NfL k dispozici od společnosti Quanterix. Nový analytický přístup volí Olink Proteomics (Uppsala, Švédsko). Využívá technologie PEA (Proximity Extension Assay) spojující princip imunochemické reakce s polymerázovou řetězovou reakcí [56]. Uvedená technologie umožňuje vytváření multiplexů pro stanovení vysokého počtu analytů, mezi nimiž jsou zařazena i NfL, které jsou součástí panelu Olink® Target 96 Neuro Exploratory.

Závěr

NfL představují perspektivní nespecifický biomarker poškození neuronů a axonů, k němuž dochází u celé řady neurologických onemocnění. Pro stanovení NfL existuje několik imunoanalytických přístupů vhodných pro spolehlivé vyšetření v MMM i v séru/plazmě. Postupně jsou studovány preanalytické a analytické vlivy, které mají dopad na výsledek stanovené koncentrace NfL. Při hodnocení koncentrací je zapotřebí brát v úvahu věk pacienta a BMI. Výhodou NfL je jejich dlouhodobá stabilita při různých teplotách i odolnost vůči opakovaným cyklům zmrazování a rozmrazování. Je zřejmé, že se postupně rozšiřuje nabídka nových technologických přístupů, které se zdají být perspektivními pro vyšetřování i velmi nízkých koncentrací NfL. I v ČR existují pracoviště, která provádějí stanovení NfL v krvi, zatím na výzkumné bázi. V této souvislosti se naléhavým problémem stává standardizace stanovení NfL, zahrnující výběr referenční analytické metody a přípravu certifikovaného referenčního materiálu. Při zavedení vyšetřování NfL do klinické praxe bude také nutné zajistit systém externí kontroly kvality, jako je tomu u jiných analytů. Bude zajímavé sledovat, jaké místo zaujmou sérové koncentrace NfL v diagnostice neurologických onemocnění vedle zobrazovacích metod mozku, vyšetření MMM a kognitivních testů [57–59].

Grantová podpora

Práce byla podpořena výzkumnými záměry RVO VFN 64165, Cooperatio Laboratory Diagnostics and Neuroscience Charles University, BBMRI-CZ LM2018125, OP VVV CZ.02.1.01/0.0/0.0/18_046/0015959, grantem FV40032, CleverAge Biota v programu Trio, AZV grantem NV-18-08-00062 a projektem SVV 260 523.

Konflikt zájmů

Autoři deklarují, že v souvislosti s předmětem práce nemají žádný konflikt zájmů.

Literatura

1. Thebault S, Booth RA, Freedman MS. Blood neurofilament light chain: the neurologist's troponin? Biomedicines 2020; 8(11): 523. doi: 10.3390/biomedicines8110523.
2. Yuan A, Rao MV, Veeranna et al. Neurofilaments and neurofilament proteins in health and disease. Cold Spring Harb Perspect Biol 2017; 9(4): a018309. doi: 10.1101/cshperspect.a018309.
3. Lobsiger CS, Cleveland DW. Neurofilaments: organization and function in neurons. In: Squire LR (eds). Encyclopedia of neuroscience. Amsterdam: Elsevier 2009: 433–436. doi: 10.1016/B978-008045046-9.00728-2.
4. Bridel C, van Wieringen WN, Zetterberg H et al. Diagnostic value of cerebrospinal fluid neurofilament light protein in neurology: a systematic review and meta-analysis. JAMA Neurol 2019; 76(9): 1035–1048. doi: 10.1001/jamaneurol.2019.1534.
5. Disanto G, Barro C, Benkert P et al. Serum neurofilament light: a biomarker of neuronal damage in multiple sclerosis. Ann Neurol 2017; 81(6): 857–870. doi: 10.1002/ana.24954.
6. Fialová L. Neurofilamenta u traumatického poškození mozku – současné znalosti. Klin Biochem Metab 2018; 26(2): 68–75.
7. Piťha J. Biomarkery roztroušené sklerózy – současné možnosti a perspektivy. Cesk Slov Neurol N 2015; 78/111(3): 269–273.
8. Kusnierova P, Zeman D, Hradilek P et al. Neurofilament levels in patients with neurological diseases: a comparison of neurofilament light and heavy chain levels. J Clin Lab Anal 2019; 33(7): e22948. doi: 10.1002/jcla.22948.
9. Khalil M, Teunissen CE, Otto M et al. Neurofilaments as biomarkers in neurological disorders. Nat Rev Neurol 2018; 14(10): 577–589. doi: 10.1038/s41582-018-0058-z.
10. Gaetani L, Blennow K, Calabresi P et al. Neurofilament light chain as a biomarker in neurological disorders. J Neurol Neurosurg Psychiatry 2019; 90(8): 870–881. doi: 10.1136/jnnp-2018-320106.
11. Andersson E, Janelidze S, Lampinen B et al. Blood and cerebrospinal fluid neurofilament light differentially detect neurodegeneration in early Alzheimer's disease.

Neurobiol Aging 2020; 95: 143–153. doi: 10.1016/j.neurobiolaging.2020.07.018.
12. Rosengren LE, Karlsson JE, Karlsson JO et al. Patients with amyotrophic lateral sclerosis and other neurodegenerative diseases have increased levels of neurofilament protein in CSF. J Neurochem 1996; 67(5): 2013–2018. doi: 10.1046/j.1471-4159.1996.67052013.x.
13. Kuhle J, Barro C, Andreasson U et al. Comparison of three analytical platforms for quantification of the neurofilament light chain in blood samples: ELISA, electrochemiluminescence immunoassay and Simoa. Clin Chem Lab Med 2016; 54(10): 1655–1661. doi: 10.1515/cclm-2015-1195.
14. Fialova L, Bartos A, Svarcova J et al. Serum and cerebrospinal fluid light neurofilaments and antibodies against them in clinically isolated syndrome and multiple sclerosis. J Neuroimmunol 2013; 262(1–2): 113–120. doi: 10.1016/j.jneuroim.2013.06.010.
15. Srpova B, Uher T, Hrnciarova T et al. Serum neurofilament light chain reflects inflammation-driven neurodegeneration and predicts delayed brain volume loss in early stage of multiple sclerosis. Mult Scler 2021; 27(1): 52–60. doi: 10.1177/1352458519901272.
16. Fialova L. Neurofilaments in multiple sclerosis. Multiple Sclerosis News 2019; 5(1): 6–12.
17. Uher T, Schaedelin S, Srpova B et al. Monitoring of radiologic disease activity by serum neurofilaments in MS. Neurol Neuroimmunol Neuroinflamm 2020; 7(4): e714. doi: 10.1212/nxi.0000000000000714.
18. Uher T, Kubala Havrdova E, Benkert P et al. Measurement of neurofilaments improves stratification of future disease activity in early multiple sclerosis. Mult Scler 2021; 27(13): 2001–2013. doi: 10.1177/13524585211047977.
19. Barro C, Benkert P, Disanto G et al. Serum neurofilament as a predictor of disease worsening and brain and spinal cord atrophy in multiple sclerosis. Brain 2018; 141(8): 2382–2391. doi: 10.1093/brain/awy154.
20. Barro C, Chitnis T, Weiner HL. Blood neurofilament light: a critical review of its application to neurologic disease. Ann Clin Transl Neurol 2020; 7(12): 2508–2523. doi: 10.1002/acn3.51234.
21. Fialova L, Bartos A, Svarcova J. Neurofilaments and tau proteins in cerebrospinal fluid and serum in dementias and neuroinflammation. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2017; 161(3): 286–295. doi: 10.5507/bp.2017.038.
22. Shahim P, Politis A, van der Merwe A et al. Neurofilament light as a biomarker in traumatic brain injury. Neurology 2020; 95(6): e610–e622. doi: 10.1212/wnl.0000000000009983.
23. Moseby-Knappe M, Mattsson N, Nielsen N et al. Serum neurofilament light chain for prognosis of outcome after cardiac arrest. JAMA Neurol 2019; 76(1): 64–71. doi: 10.1001/jamaneurol.2018.3223.
24. Alagaratnam J, von Widekind S, De Francesco D et al. Correlation between CSF and blood neurofilament light chain protein: a systematic review and meta-analysis. BMJ Neurol Open 2021; 3(1): e000143. doi: 10.1136/bmjno-2021-000143.
25. Gleerup HS, Sanna F, Hogh P et al. Saliva neurofilament light chain is not a diagnostic biomarker for neurodegeneration in a mixed memory clinic population. Front Aging Neurosci 2021; 13: 659898. doi: 10.3389/fnagi.2021.659898.
26. Hviid CVB, Knudsen CS, Parkner T. Reference interval and preanalytical properties of serum neurofilament light chain in Scandinavian adults. Scand J Clin Lab Invest 2020; 80(4): 291–295. doi: 10.1080/00365513.2020.1730434.
27. Khalil M, Pirpamer L, Hofer E et al. Serum neurofilament light levels in normal aging and their association with morphologic brain changes. Nat Commun 2020; 11(1): 812. doi: 10.1038/s41467-020-14612-6.
28. Mattsson N, Andreasson U, Zetterberg H et al. Association of plasma neurofilament light with neurodegeneration in patients with Alzheimer disease. JAMA Neurol 2017; 74(5): 557–566. doi: 10.1001/jamaneurol.2016.6117.
29. Akamine S, Marutani N, Kanayama D et al. Renal function is associated with blood neurofilament light chain level in older adults. Sci Rep 2020; 10(1): 20350. doi: 10.1038/s41598-020-76990-7.
30. Korley FK, Goldstick J, Mastali M et al. Serum NfL (Neurofilament Light Chain) levels and incident stroke in adults with diabetes mellitus. Stroke 2019; 50(7): 1669–1675. doi: 10.1161/STROKEAHA.119.024941.
31. Howell JC, Watts KD, Parker MW et al. Race modifies the relationship between cognition and Alzheimer's disease cerebrospinal fluid biomarkers. Alzheimers Res Ther 2017; 9(1): 88. doi: 10.1186/s13195-017-0315-1.
32. Manouchehrinia A, Piehl F, Hillert J et al. Confounding effect of blood volume and body mass index on blood neurofilament light chain levels. Ann Clin Transl Neurol 2020; 7(1): 139–143. doi: 10.1002/acn3.50972.
33. Wentz E, Dobrescu SR, Dinkler L et al. Thirty years after anorexia nervosa onset, serum neurofilament light chain protein concentration indicates neuronal injury. Eur Child Adolesc Psychiatry 2021; 30(12): 1907–1915. doi: 10.1007/s00787-020-01657-7.
34. Nilsson IAK, Millischer V, Karrenbauer VD et al. Plasma neurofilament light chain concentration is increased in anorexia nervosa. Transl Psychiatry 2019; 9(1): 180. doi: 10.1038/s41398-019-0518-2.
35. Hviid CVB, Madsen AT, Winther-Larsen A. Biological variation of serum neurofilament light chain. Clin Chem Lab Med 2021 [ahead of print]. doi: 10.1515/cclm-2020-1276.
36. Koel-Simmelink MJ, Vennegoor A, Killestein J et al. The impact of pre-analytical variables on the stability of neurofilament proteins in CSF, determined by a novel validated SinglePlex Luminex assay and ELISA. J Immunol Methods 2014; 402(1–2): 43–49. doi: 10.1016/j.jim.2013.11.008.
37. Gaiottino J, Norgren N, Dobson R et al. Increased neurofilament light chain blood levels in neurodegenerative neurological diseases. PLoS One 2013; 8(9): e75091. doi: 10.1371/journal.pone.0075091.
38. Altmann P, Ponleitner M, Rommer PS et al. Seven day pre-analytical stability of serum and plasma neurofilament light chain. Sci Rep 2021; 11(1): 11034. doi: 10.1038/s41598-021-90639-z.
39. Ashton NJ, Suarez-Calvet M, Karikari TK et al. Effects of pre-analytical procedures on blood biomarkers for Alzheimer's pathophysiology, glial activation, and neurodegeneration. Alzheimers Dement (Amst) 2021; 13(1): e12168. doi: 10.1002/dad2.12168.
40. O'Connell GC, Alder ML, Webel AR et al. Neuro biomarker levels measured with high-sensitivity digital ELISA differ between serum and plasma. Bioanalysis 2019; 11(22): 2087–2094. doi: 10.4155/bio-2019-0213.
41. Terasawa K, Taguchi T, Momota R et al. Intermediate filaments of endoskeleton within human erythrocytes. Blood 2007; 110(11): 1734. doi: 10.1182/blood.V110.11.1734.1734.
42. Lombardi V, Carassiti D, Giovannoni G et al. The potential of neurofilaments analysis using dry-blood and plasma spots. Sci Rep 2020; 10(1): 97. doi: 10.1038/s41598-019-54310-y.
43. Simren J, Ashton NJ, Blennow K et al. Blood neurofilament light in remote settings: alternative protocols to support sample collection in challenging pre-analytical conditions. Alzheimers Dement (Amst) 2021; 13(1): e12145. doi: 10.1002/dad2.12145.
44. Norgren N, Rosengren L, Stigbrand T. Elevated neurofilament levels in neurological diseases. Brain Res 2003; 987(1): 25–31. doi: 10.1016/s0006-8993(03)03219-0.
45. Norgren N, Karlsson JE, Rosengren L et al. Monoclonal antibodies selective for low molecular weight neurofilaments. Hybrid Hybridomics 2002; 21(1): 53–59. doi: 10.1089/15368590252917647.
46. Gauthier A, Viel S, Perret M et al. Comparison of Simoa and Ella to assess serum neurofilament-light chain in multiple sclerosis. Ann Clin Transl Neurol 2021; 8(5): 1141–1150. doi: 10.1002/acn3.51355.
47. Liu HC, Lin WC, Chiu MJ et al. Development of an assay of plasma neurofilament light chain utilizing immunomagnetic reduction tec hnology. PLoS One 2020; 15(6): e0234519. doi: 10.1371/journal.pone.0234519.
48. Fialova L, Zima T, Bartos A. Přehled imunoanalytických metod ke stanovení tripletu biomarkerů Alzheimerovy nemoci v mozkomíšním moku a v krvi. Chemické listy 2020; 114(8): 537–544.
49. Cohen L, Walt DR. Single-molecule arrays for protein and nucleic acid analysis. Annu Rev Anal Chem (Palo Alto Calif) 2017; 10(1): 345–363. doi: 10.1146/annurev-anchem-061516-045340.
50. Lue LF, Guerra A, Walker DG. Amyloid beta and tau as Alzheimer's disease blood biomarkers: promise from new technologies. Neurol Ther 2017; 6 (Suppl 1): 25–36. doi: 10.1007/s40120-017-0074-8.
51. Hendricks R, Baker D, Brumm J et al. Establishment of neurofilament light chain Simoa assay in cerebrospinal fluid and blood. Bioanalysis 2019; 11(15): 1405–1418. doi: 10.4155/bio-2019-0163.
52. Korley FK, Yue JK, Wilson DH et al. Performance evaluation of a multiplex assay for simultaneous detection of four clinically relevant traumatic brain injury biomarkers. J Neurotrauma 2018; 36(1): 182–187. doi: 10.1089/neu.2017.5623.
53. Dysinger M, Marusov G, Fraser S. Quantitative analysis of four protein biomarkers: an automated microfluidic cartridge-based method and its comparison to colorimetric ELISA. J Immunol Methods 2017; 451: 1–10. doi: 10.1016/j.jim.2017.08.009.
54. Yang CC, Yang SY, Chen HH et al. Effect of molecule-particle binding on the reduction in the mixed-frequency alternating current magnetic susceptibility of magnetic bio-reagents. J Appl Phys 2012; 112(2): 024704. doi: 10.1063/1.4739735.
55. Plavina T, Rudick RA, Calabresi PA et al. Development of a sensitive serum neurofilament light assay on Siemens routine immunoassay platforms. Multiple Sclerosis J 2019; 25 (2 Suppl): 278. doi: 10.1177/1352458519868078.
56. Lundberg M, Eriksson A, Tran B et al. Homogeneous antibody-based proximity extension assays provide sensitive and specific detection of low-abundant proteins in human blood. Nucleic Acids Res 2011; 39(15): e102. doi: 10.1093/nar/gkr424.
57. Šilhán D, Ibrahim I, Tintěra J et al. Parietální atrofie na magnetické rezonanci mozku u Alzheimerovy nemoci s pozdním začátkem. Cesk Slov Neurol N 2019; 82(1): 91–95. doi: 10.14735/amcsnn201991.
58. Bartoš A, Polanská H. Správná a chybná pojmenování obrázků pro náročnější test písemného Pojmenování obrázků a jejich vybavení (dveřní POBAV). Cesk Slov Neurol N 2021; 84/117(2): 151–163. doi: 10.48095/cccsnn202115.
59. Bartoš A, Čechová L, Švarcová J et al. Likvorový triplet (tau proteiny a beta-amyloid) v diagnostice Alzheimerovy-Fischerovy nemoci. Cesk Slov Neurol N 2012; 75/108(5): 587–594.
60. Stengelin M, Bathala P, Wohlstadter JN. Sensitive serum/plasma neurofilament light immunoassay. Alzheimers Dement 2019; 15(7): P1346–P1347. doi: 10.1016/j.jalz.2019.06.3846.

Journal of Clinical Lipidology (2020) 14, 675–684

Journal of Clinical Lipidology

Neuroprotective associations of apolipoproteins A-I and A-II with neurofilament levels in early multiple sclerosis

Mason McComb, BS[1], Maggie Krikheli, BS, Tomas Uher, MD, PhD[1], Richard W. Browne, MS, PhD, Barbora Srpova, MD, Johanna Oechtering, MD, Aleksandra Maleska Maceski, MSc, Michaela Tyblova, MD, PhD, Dejan Jakimovski, MD, PhD, Deepa P. Ramasamy, MD, Niels Bergsland, MS, PhD, Jan Krasensky, MS, Libuse Noskova, MS, Lenka Fialova, MD, PhD, Bianca Weinstock-Guttman, MD, PhD, Eva Kubala Havrdova, MD, PhD, Manuela Vaneckova, MD, PhD, Robert Zivadinov, MD, PhD, Dana Horakova, MD, PhD, Jens Kuhle, MD, PhD, Murali Ramanathan, MS, PhD*

Department of Pharmaceutical Sciences, State University of New York, Buffalo, NY, USA (Drs McComb, Krikheli, and Ramanathan); Department of Neurology and Center of Clinical Neuroscience, Charles University in Prague, First Faculty of Medicine and General University Hospital, Prague, Czech Republic (Drs Uher, Srpova, Tyblova, Havrdova, and Horakova); Department of Biotechnical and Clinical Laboratory Sciences, State University of New York, Buffalo, NY, USA (Dr Browne); Neurologic Clinic and Policlinic, Departments of Medicine, Biomedicine and Clinical Research, University Hospital Basel, University of Basel, Basel, Switzerland (Drs Oechtering, Maceski, and Kuhle); Buffalo Neuroimaging Analysis Center, Department of Neurology, State University of New York, Buffalo, NY, USA (Drs Jakimovski, Ramasamy, Bergsland, and Zivadinov); IRCCS, Fondazione Don Carlo Gnocchi, Milan, Italy (Dr Bergsland); Department of Radiology, Charles University in Prague, First Faculty of Medicine and General University Hospital in Prague, Czech Republic (Drs Krasensky and Vaneckova); Institute of Medical Biochemistry and Laboratory Diagnostics, First Faculty of Medicine, Charles University and General University Hospital, Prague, Czech Republic (Dr Noskova and Fialova); Department of Neurology, State University of New York, Buffalo, NY, USA (Drs Weinstock-Guttman and Ramanathan); and Center for Biomedical Imaging at Clinical Translational Science Institute, University at Buffalo, State University of New York, Buffalo, NY, USA (Dr Zivadinov)

Financial conflicts: See disclosure statement.

Confidentiality: Use of the information in this manuscript for commercial, non-commercial, research, or purposes other than peer review not permitted before publication without expressed written permission of the author.

Funding information: The project was supported by the Charles University Grant Agency (GAUK) 230217, Czech Ministry of Education under project Progress Q27/LF1, Czech Ministry of Health project RVOVFN64165, and AZV grants NV18-08-00062 and NV18-04-00168. SET study and assessment of serum neurofilament light chain levels were supported by Biogen. Support from grant MS190096 from the Department of Defense Multiple Sclerosis Research Program for the Office of the Congressionally Directed Medical Research Programs (CDMRP) to the Ramanathan laboratory is gratefully acknowledged.

[1] These authors contributed equally.

* Corresponding author. Department of Pharmaceutical Sciences, State University of New York, 355 Kapoor Hall, Buffalo, NY 14214-8033, USA.
E-mail address: Murali@Buffalo.Edu

Submitted April 3, 2020. Accepted for publication July 1, 2020.

https://doi.org/10.1016/j.jacl.2020.07.001

KEYWORDS:
Neurofilament light chain;
Lipid;
Cholesterol;
Apolipoproteins;
Blood-brain barrier;
Cerebrospinal fluid;
Brain lesions;
MRI;
Disease onset;
Multiple sclerosis

BACKGROUND: The role of cholesterol homeostasis in neuroaxonal injury in multiple sclerosis is not known.

OBJECTIVE: The objective of the study is to investigate the associations of cerebrospinal fluid (CSF) and serum neurofilament light chain levels (CSF-NfL and sNfL, respectively), which are biomarkers of neuroaxonal injury, with cholesterol biomarkers at the clinical onset of multiple sclerosis.

METHODS: sNfL, serum cholesterol profile (total cholesterol, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol), serum apolipoprotein (Apo) levels (ApoA-I, ApoA-II, ApoB, and ApoE), and albumin quotient were obtained for 133 patients (63% female, age: 29.9 ± 8.0 years) during the first demyelinating event. CSF-NfL was available for 103 (77%) patients.

RESULTS: CSF-NfL and sNfL were negatively associated with serum ApoA-II ($P = .005$, $P < .001$) and positively associated with albumin quotient ($P < .001$, $P < .0001$). In addition, higher CSF-NfL was associated with lower serum ApoA-I ($P = .009$) levels and higher sNfL was associated with lower high-density lipoprotein cholesterol ($P = .010$). In stepwise regression, age ($P = .045$), serum ApoA-II ($P = .022$), and albumin quotient ($P < .001$) were associated with CSF-NfL; albumin quotient ($P = .002$) and ApoA-II ($P = .001$) were associated with sNfL. Path analysis identified parallel pathways from ApoA-II ($P = .009$) and albumin quotient ($P < .001$) to the sNfL outcome that were mediated by CSF-NfL ($P < .001$). The associations of CSF-NfL with ApoA-I ($P = .014$) and ApoA-II ($P = .015$) and sNfL with ApoA-II ($P < .001$) remained significant after adjusting for number of contrast-enhancing lesions and T2 lesion volume.

CONCLUSION: Lower serum ApoA-II and ApoA-I levels are associated with greater neuroaxonal injury as measured by CSF-NfL.

Introduction

Neurofilament light chain (NfL) are cytoskeletal proteins in nervous tissue that are shed on axonal injury and can be measured in the cerebrospinal fluid (CSF-NfL) and serum (sNfL). NfL are a promising biomarker in multiple sclerosis (MS) and other neurological diseases.[1,2] sNfL are increased during MS disease activity[3] and predict future disease progression,[4] lesion activity, and brain atrophy on magnetic resonance imaging (MRI).[5] CSF-NfL and sNfL levels in early MS reflect mostly inflammation-mediated neurodegeneration with contributions from noninflammatory neurodegeneration.[6–8]

It is important to delineate the role of modifiable factors that can slow neuroaxonal injury in early MS. Cholesterol has critical roles in metabolism, inflammation, and neuroaxonal integrity, and its levels are modulated by genetic, environmental, and lifestyle factors. Cholesterol homeostasis plays a vital role in axonal repair and has been shown to be a rate limiting step in central nervous system (CNS) myelination in animal models, while also promoting repair when supplemental cholesterol is provided.[9,10] In addition, protective associations of high-density lipoprotein (HDL) cholesterol and apolipoproteins in established and early MS have been demonstrated.[11–14] The pleiotropic roles of cholesterol homeostasis in MS neurodegeneration and lesion formation motivated this study whose goal was to assess the interdependence between neurofilament levels and serum cholesterol biomarkers.

Material and methods

Study population

Clinical study design

The Observational Study of Early Interferon beta 1-a Treatment in High-Risk Subjects after Clinically Isolated Syndrome (CIS) (SET study, clin.gov # NCT01592474) was a prospective, longitudinal observational study coordinated by Charles University in Prague, Czech Republic.

The SET study design has been previously described.[15–17] The SET study protocol was approved by the Medical Ethics Committees of the General University Hospital and 1st Faculty of Medicine of Charles University, Prague, Czech Republic. The study screened 259 patients and enrolled 220 patients from 8 Czech Republic MS centers. Clinical data were available for 211 patients.

Informed consent

Written informed consent was obtained from all patients at the time of enrollment.

Inclusion criteria

Patients were enrolled within 4 months after their first clinical event suggestive of MS. Inclusion was based on the following characteristics: 18–55 years of age, presence of ≥ 2 T2-hyperintense lesions on diagnostic MRI, and presence of ≥ 2 oligoclonal bands in CSF obtained before corticosteroid treatment and Expanded Disability Status

Scale ≤ 3.5. This substudy included 133 patients with age ≥ 18 years for whom CSF-derived measures of blood brain barrier (BBB) permeability and sNfL levels were available.

Treatments

All patients were treated with 3–5 g of methylprednisolone for the first symptom. Baseline MRI was performed ≥ 30 days after steroid administration and within 4 months after disease onset. All patients were started on 30 μg, once weekly, intramuscular interferon beta 1-a (AVONEX) treatment at baseline.

CSF and blood draws

CSF and blood were obtained before treatment with corticosteroids at the study-coordinating center during the morning hours. A total of 20–25 mL of CSF was drawn from the L5-S1, L4-5, or L3-4 interspace. A 5-mL volume of blood was obtained.[12,17]

All biochemical and imaging measurements were performed on coded samples and images, and technicians were blinded to clinical and/or MRI data.

CSF and serum neurofilaments

CSF-NfL levels were measured by enzyme-linked immunosorbent assays using the NF-light enzyme-linked immunosorbent assay kit (UmanDiagnostics AB, Umea, Sweden) by the General Faculty Hospital laboratory, Prague. Interassay coefficients of variation for CSF samples were 1.5%.

sNfL levels were measured using a Single Molecule Array (SIMOA) assay at the University Hospital, Basel.[18] The mean intra-assay coefficient of variation of duplicate determinations for concentration was 6.4%. Interassay coefficients of variation for 3 native serum samples were below 10% (ie, 7.8%, 8.8%, and 5.5% for 7.0 pg/mL).[8]

Serum lipids and apolipoproteins

Serum was obtained from patients at the screening visit before the start of corticosteroid or interferon treatment for analysis of apolipoproteins and lipid profile.

Serum total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and triglycerides were measured with diagnostic reagent kits from Sekisui Diagnostics (Lexington, MA). Low-density lipoprotein cholesterol (LDL-C) was obtained from the Friedewald equation.[19] Apolipoprotein levels (ApoA-I, ApoA-II, ApoB, and ApoE) were analyzed by immunoturbidimetric methodology using diagnostic kits from Kamiya Biomedical (Thousand Oaks, CA). These assays were adapted to the ABX Pentra 400 automated chemistry analyzer (Horiba Instruments, Irvine, CA). The coefficient of variation of these assays is < 5%. For convenience, we refer to TC, HDL-C, LDL-C, ApoA-I, ApoA-II, ApoB, or ApoE as cholesterol biomarkers.

Albumin and immunoglobulins in serum and CSF

CSF total protein was measured photometrically with the pyrogallol red-molybdate reaction method on a Synchron LX 20 analyzer (Beckman Coulter). Serum and CSF albumin, immunoglobulin G (IgG), and immunoglobulin M (IgM) concentrations were measured by immunonephelometry (IMMAGE Immunohistochemistry system, Beckman Coulter). The albumin quotient, IgG quotient, and IgM quotient were defined as the ratio of the corresponding CSF to serum concentrations, respectively.[20–22]

MRI measures

MRI was performed with a standardized protocol on the same 1.5-T scanner (Gyroscan; Philips Medical Systems, Best, the Netherlands). Axial brain acquisitions included fluid-attenuated inversion recovery, three-dimensional T1-weighted images, and T1 spin-echo images before and 5 min after a single injection of 0.1 mmol/kg of gadopentetate dimeglumine. The MRI acquisition and analysis methods have been previously described.[23,24] This study also assessed the number and volume of T2 and contrast-enhancing lesions (CELs) at baseline.

Data analysis

SPSS (IBM Inc, Armonk, NY, version 19.0) statistical program was used. sNfL and CSF variables (albumin, IgG, IgM, and albumin quotients, IgG, IgM indices) and T2 lesion volume (T2-LV) were logarithm (base 10) transformed to reduce skew.

For regression analyses, CSF-NfL and sNfL were treated as dependent variables. The independent variables were as follows: age, sex, body mass index (BMI), and individual cholesterol biomarker of interest.

The associations of cholesterol biomarkers and other demographic variables associated with CSF-NfL or sNfL levels were investigated with stepwise regression with forward selection. The probability of the F-statistic for variable entry was 0.05 and that for variable removal was 0.1. Separate regressions were conducted for CSF-NfL and sNfL levels, which were treated as the target dependent variables of interest. The candidate variable list for the CSF-NfL and sNfL target dependent variables was as follows: age, sex, BMI, TC, HDL-C ApoA-I, ApoA-II, ApoB, LDL-C, albumin quotient, IgG quotient, IgM quotient, IgG index, and IgM index.

Mediator and moderator analyses were conducted for assessing the possible association pathways between ApoA-II, albumin quotient, and CSF-NfL using PROCESS (v3.4) regression analysis software for SPSS and methodology in the study by.Hayes.[25] Moderation was assessed with main effects and a product term of centered Z-scores of all biomarker product terms. All these analyses were adjusted for age, sex, and BMI. Bootstrap *P*-values were obtained using 5000 samples.

The interdependencies between ApoA-II, albumin quotient, CSF-NfL, and sNfL were assessed using the path analysis method in the IBM SPSS AMOS (IBM Inc, Armonk, NY, version 26) structural equation modeling software. Maximum likelihood estimation was used, and missing data were handled listwise.

We conducted additional regression analyses that adjusted for imaging markers of CNS inflammation. In these analyses, CSF-NfL and sNfL were dependent variables with age, sex, BMI, individual cholesterol biomarker of interest, CEL number, and T2-LV as independent variables.

The Benjamini-Hochberg method with $q = 0.05$ was used to control false discovery rate.[26] The tables and results summarize the raw, unadjusted *P*-values; the variables with $q \leq 0.05$ are highlighted with an asterisk.

Results

Demographic and clinical characteristics

The demographic, clinical, MRI at baseline, neurofilament, cholesterol biomarkers, CSF measures at screening, and longitudinal outcomes at 4 years of the study sample are summarized in Table 1.

Table 1 Demographic and clinical characteristics of the study sample

Demographic characteristics	All	Subset with CSF-NfL	Subset without CSF-NfL	*P*-value
Sample size, *n*	133	103 (77%)	30 (23%)	–
Age, y	29.9 ± 8.0	29.3 ± 7.7	31.1 ± 8.9	.38*
Sex				
male	49 (37%)	44 (43%)	5 (17%)	.010†
Female	84 (63%)	59 (57%)	25 (83%)	
EDSS at baseline, median (IQR)	1.5 (0.5)	1.5 (0.5)	2.0 (1.0)	.39‡
MRI measures at baseline				
CEL number	1.02 ± 3.3	0.72 ± 1.9	2.14 ± 6.1	.23‡
T2-LV, cm^3	5.37 ± 6.4	5.47 ± 6.5	5.03 ± 6.2	.43‡
Brain volume, cm^3	1500 ± 72	1510 ± 81	1500 ± 70	.31*
Neurofilament levels				
CSF-NfL, ng/mL	–	1.5 ± 1.6	–	–
Serum NfL, pg/mL	37.8 ± 53	33.2 ± 31	53.8 ± 95	.25*
Cholesterol biomarkers				
Total cholesterol, mg/dL	206 ± 47	207 ± 48	201 ± 45	.53*
HDL cholesterol, mg/dL	70.7 ± 18	71.0 ± 17	69.4 ± 18	.65*
LDL cholesterol, mg/dL	135 ± 36	136 ± 38	132 ± 33	.53*
Apolipoprotein A-I, mg/dL	160 ± 39	161 ± 39	156 ± 39	.59*
Apolipoprotein A-II, mg/dL	37.6 ± 8.0	37.8 ± 7.3	36.6 ± 10	.53*
Apolipoprotein B, mg/dL	75.0 ± 23	75.9 ± 23	71.7 ± 22	.36*
Apolipoprotein E, mg/dL	3.32 ± 1.0	3.31 ± 0.98	3.35 ± 1.3	.077*
CSF measures				
CSF albumin (mg/L)	0.222 ± 0.090	0.223 ± 0.092	0.219 ± 0.086	.83*
Albumin quotient (mg/g)	4.84 ± 1.9	4.86 ± 1.6	4.80 ± 1.9	.88*
IgG quotient (mg/g)	4.32 ± 2.5	4.24 ± 2.4	4.57 ± 2.7	.54*
IgG index	0.895 ± 0.40	0.880 ± 0.40	0.945 ± 0.43	.38*
IgM quotient (mg/g)	1.14 ± 1.3	1.1 ± 1.4	1.17 ± 1.1	.81*
IgM index	0.249 ± 0.34	0.249 ± 0.37	0.246 ± 0.21	.95*
Outcomes at 4 y§				
Relapsing activity	71 (53%)	52 (73%)	19 (27%)	.30†
PBVC, Mean (SD)	−2.39 ± 2.1	−2.21 ± 1.9	−3.03 ± 2.6	.067*
NEDA-4 status‖	15 (13%)	12 (80%)	3 (20%)	1.0†

CELs, contrast-enhancing lesions; CSF, cerebrospinal fluid; EDSS, Expanded Disability Status Scale; HDL, high-density lipoprotein; Ig, immunoglobulin; IQR, interquartile range; LDL, low-density lipoprotein; MRI, magnetic resonance imaging; NEDA, no evidence of disease activity; NfL, neurofilament light chain; PBVC, percent brain volume change; SD, standard deviation; T2-LV, T2 lesion volume.

Gadolinium contrast–enhancing lesions. Demographics, sNfL, cholesterol biomarkers, and CSF measures were obtained at screening.

*Independent samples *t*-test.

†Fisher's exact test.

‡Mann-Whitney *U*-test.

§Long-term clinical outcomes of the frequency of patients with relapsing activity, annualized percent brain volume change (PBVC), and no evidence of disease activity-4 (NEDA-4: absence of relapses, disability progression, active brain MRI lesions, and annualized PBVC < -0.4%) status at 4-y data are presented for showing that the included group is representative.

‖NEDA-4 was available for 120 patients.

Table 2 Associations of albumin quotient with CSF-NfL, sNfL, and cholesterol variables

Predictor variable†	CSF-NfL levels‡		sNfL levels‡	
	r_p	P	r_p	P
Total cholesterol	−0.19	.069	−0.16	.074
HDL cholesterol	−0.14	.18	−0.23	.010*
LDL cholesterol	−0.16	.10	-0.092	.30
Apolipoprotein A-I	−0.26	.009*	−0.19	.028
Apolipoprotein A-II	−0.28	.005*	−0.32	<.001*
Apolipoprotein B	−0.17	.098	−0.11	.23
Apolipoprotein E	−0.10	.32	−0.063	.48
Albumin quotient‡	0.36	<0.001*	0.34	<.001*

CSF-NfL, neurofilament light chain levels in cerebrospinal fluid; HDL, high-density lipoprotein; LDL, low-density lipoprotein; sNfL, neurofilament light chain levels in serum.

*Significant after Benjamini-Hochberg adjustment at a false discovery rate of 0.05.

†Partial correlation (r_p) and P-value from linear regression adjusted for age, sex, and body mass index are shown.

‡Log base 10 transformed.

Table 1 also compares the demographic, clinical, and neuroimaging measures in the included subset with CSF-NfL, which were generally similar to the excluded subset that did not have CSF-NfL. The only exception was the proportion of females to males in the included subset without CSF-NfL (49% female, 43% male), which was lower than that in the excluded subset that did not have CSF-NfL available (83% female, 17% male, $P = .010$, Fisher's exact test).

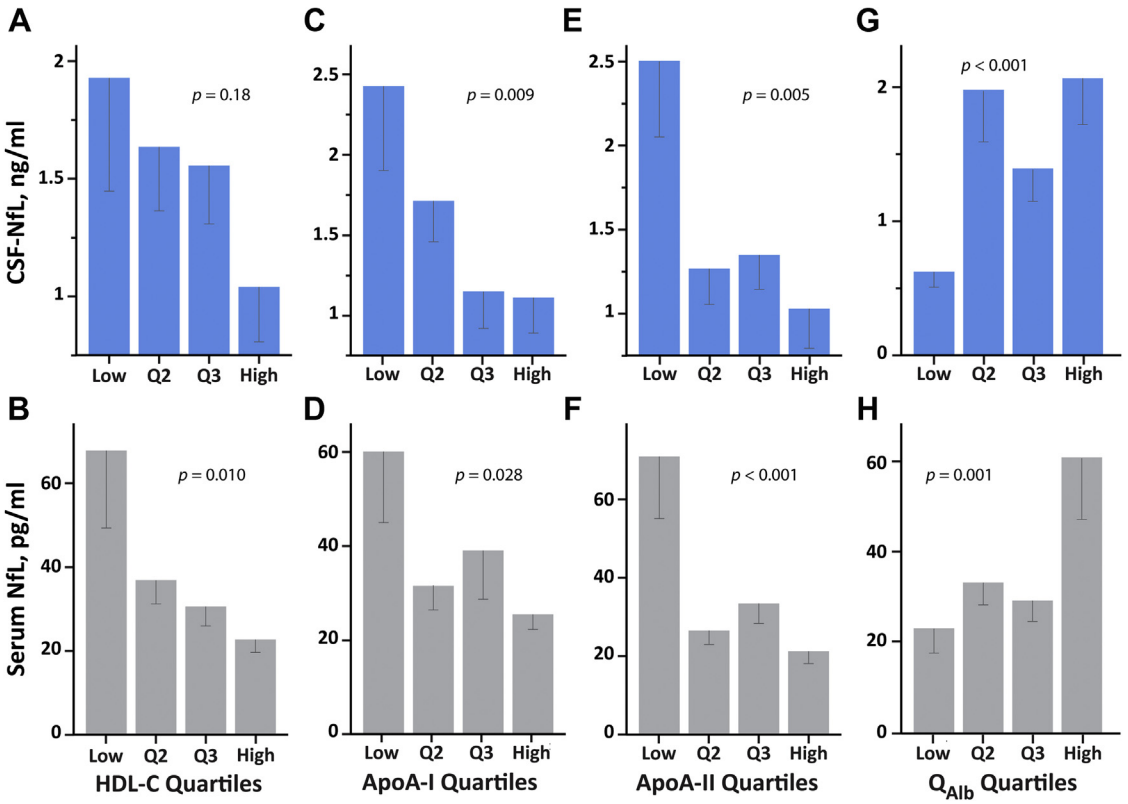


Figure 1 Associations of neurofilament light chain levels in cerebrospinal fluid (CSF-NfL in ng/mL, blue bars) are shown for the quartiles of high-density lipoprotein cholesterol (HDL-C, A), apolipoprotein A-I (ApoA-I, C), apolipoprotein A-II (ApoA-II, E), and albumin quotient (Q_{Alb}, G). The corresponding associations of serum neurofilament light chain levels (sNfL in pg/ml, gray bars) are shown for the quartiles of HDL-C (B), ApoA-I (D), ApoA-II (F), and albumin quotient (Q_{Alb}, H). The bars compare mean values of the CSF-NfL or sNfL as shown on the y-axis for quartiles of the HDL-C biomarker or albumin quotient shown on the x-axis. The error bars are standard error of the mean. The P-values from the linear regression results shown in Table 1 adjusted for age, sex, and body mass index are shown.

Associations of CSF-NfL and sNfL with HDL-C biomarkers

As summarized in Table 2 and Figure 1, CSF-NfL levels were associated with serum ApoA-I (partial correlation (r_p) = −0.26, P = .009), serum ApoA-II (r_p = −0.28, P = .005), and albumin quotient (r_p = .36, P < .001), whereas sNfL levels were associated with HDL cholesterol (r_p = −0.23, P = .010) and ApoA-II (r_p = −0.32, P < .001) and albumin quotient (r_p = 0.34, P = <.001).

Next, we conducted stepwise linear regression to identify a parsimonious model that included the contribution of the variables acting together. Stepwise regression of CSF-NfL identified age (r_p = −0.12, P = .045, Benjamini-Hochberg q-value = 0.045), albumin quotient (r_p = 0.36, P < .001, q = 0.003), and ApoA-II (r_p = −0.24, P = .022, q = 0.033) in the final model. Stepwise regression of sNfL identified albumin quotient (r_p = .28, P = .002, q = 0.002) and ApoA-II (r_p = −0.29, P = .001, q = 0.002) in the final model. The stepwise regression models for CSF-NfL and sNfL, which identified ApoA-II and albumin quotient as predictors, were confirmed using random forest regression (see Supplemental Information).

Based on these results, we evaluated mediation and moderation models for understanding the interdependencies among ApoA-II, albumin quotient, CSF-NFL, and sNfL. Figure 2A summarizes the regression coefficients for all effects in the mediation model with ApoA-II, CSF-NfL, and sNfL as predictor, mediator, and outcome variables, respectively. We recapitulated evidence for a total effect (c = −0.25, P = .01) for the association of ApoA-II with sNfL, which was concordant with the initial regression analysis. The total effect was abrogated when the mediation path involving the ApoA-II and CSF-NfL was considered (c' = −0.04, P = .53). Further support for mediation by the ApoA-II–sNfL association by CSF-NfL was provided by the indirect effect (ab = −0.21 ± 0.08, 95% confidence interval (CI): −0.35, −0.064), which was also significant based on the bootstrap CIs. These results suggest that the association of ApoA-II with sNfL is accounted for by the mediation model terms for the association of ApoA-II with CSF-NfL and the association of CSF-NfL with sNfL.

Similarly, Figure 2B summarizes the regression coefficients for all effects in the mediation model with albumin quotient, CSF-NfL, and sNfL as predictor, mediator, and outcome variables, respectively. As expected, we found the evidence for a total effect for the albumin quotient–sNfL association (c = 0.28, P = .005). The total effect was abrogated (c' = 0.002, P = .98) when the mediation path involving the albumin quotient and CSF-NfL (ab = 0.28 ± 0.08, 95% CI: 0.13, 0.42) was considered.

In the mediation analyses, Figure 2A shows the total and indirect effects of ApoA-II are negatively associated with the sNfL outcome variable. Figure 2B shows the total and

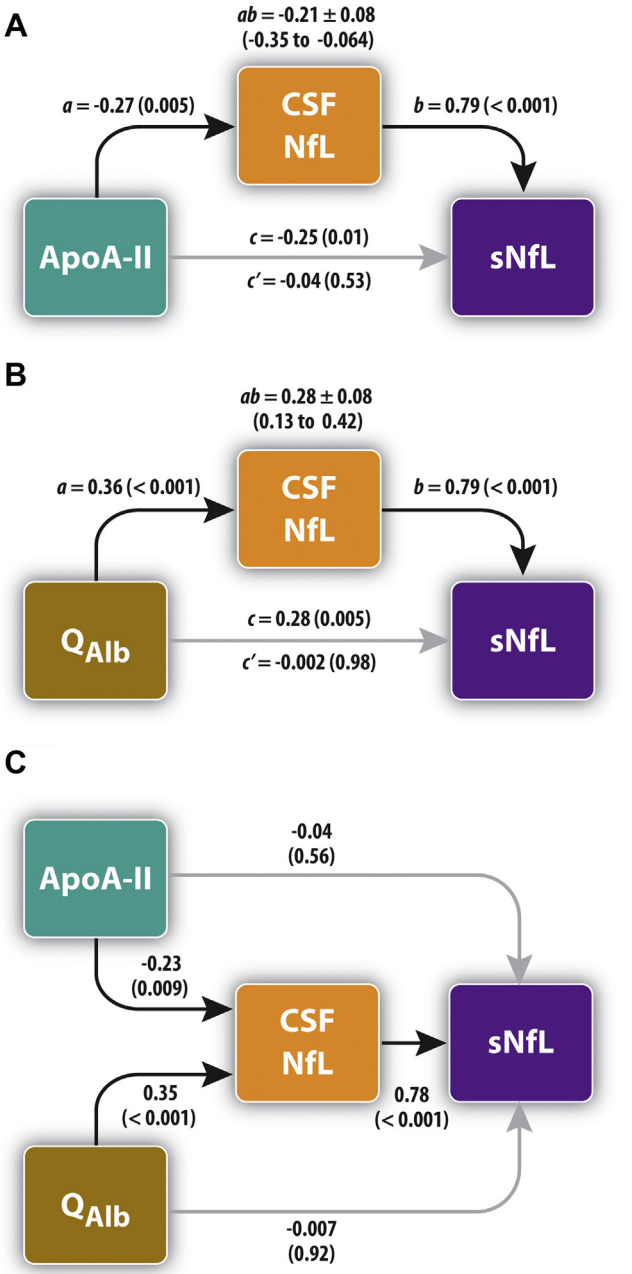


Figure 2 Results from mediation analysis for ApoA-II (A) and albumin quotient (B). The values of the standardized regression coefficients from mediation analysis are shown against the arrows; a represents the standardized coefficient for the effect of ApoA-II in (A) and albumin quotient in (B); b represents the standardized coefficient for the effect of CSF-NfL on sNfL in (A and 2); c represents the standardized coefficient for the total effect in the absence of mediation between ApoA-II and sNfL in (A) and mediation between albumin quotient and sNfL in (B); *c'* represents the standardized coefficient for the direct effect between ApoA-II and sNfL in the presence of mediation in (A) and between albumin quotient and sNfL in (B). The *P*-values for *a*, *b*, *c*, and *c'* are shown in parenthesis. For *a b*, the coefficient ± standard error and 95% confidence intervals from 5000 bootstrap samples are shown. (C) summarizes the results of path analysis using the SPSS AMOS structural equation modeling software tool. The standardized regression coefficients are shown against the arrows with the corresponding *P*-values in parenthesis. The black lines indicate the path that includes the mediator, whereas the gray lines indicate the direct path.

indirect effects of albumin quotient are positively associated with the sNfL outcome variable.

We assessed the combined effects of ApoA-II and albumin quotient on sNfL via CSF-NfL mediation using the path analysis model in Figure 2C that incorporated both variables simultaneously with CSF-NfL mediation. Figure 2C summarizes standardized coefficients of each direct and indirect pathway. The pathways from ApoA-II to CSF-NfL (standardized coefficient = −0.23, P = .009), albumin quotient to CSF-NfL (standardized coefficient = 0.35, $P < .001$), and CSF-NfL to sNfL (standardized coefficient = 0.78, $P < .001$) remained significant, whereas the direct pathways from ApoA-II to sNfL (standardized coefficient = −0.035, P = .56) and albumin quotient to sNfL (standardized coefficient = −0.02, P = .915) were not significant, recapitulating the results from the individual mediation models. These results suggest parallel but oppositely acting associations for ApoA-II and albumin quotient with sNfL that are mediated via the CSF-NfL associations.

We also examined the corresponding moderation models that included main effects and centered interaction terms for ApoA-II with CSF-NfL, albumin quotients with CSF-NfL, or both ApoA-II and albumin quotients with CSF-NfL. The interaction terms were not significant in any of the models (data not shown).

Associations of CSF-NfL and sNfL with HDL-C biomarkers persist after adjusting for lesions

sNfL are known to be associated with CELs, which represent focal areas of blood-brain barrier breakdown, and with T2-LV, which are related to disease burden in MS.[6] The goal of this analysis was to determine if ApoA-II explained variability in CSF-NfL and sNfL that was not accounted for by lesional injury.[6] In regression analyses adjusted for age, sex, and BMI, we investigated whether the associations of CSF-NfL and sNfL with ApoA-II remained significant on including number of CELs and T2-LV.

Table 3 shows that CSF-NfL remained associated with ApoA-I (r_p = −0.25, P = .014) and ApoA-II (r_p = −0.25, P = .015), whereas sNfL remained associated with ApoA-II (r_p = −0.28, P = .001). Figure 3 graphically summarizes CSF-NfL and sNfL levels in the groups with and without CELs for the lowest and higher quartiles of ApoA-II levels. These results are concordant with the possibility that ApoA-II has protective associations with neurodegeneration occurring at CNS sites outside of brain MRI-evident lesions that could also contribute to CSF-NfL in early MS.

Discussion

The associations of cholesterol biomarkers, BBB permeability, and neuroaxonal injury were investigated in a cohort of 133 patients with MS during the first demyelinating event. Neuroaxonal injury was assessed by measuring CSF-NfL and sNfL, and BBB integrity was assessed using the albumin quotient. Using the path analysis approach, we found evidence that ApoA-II has negative (protective) associations with CSF-NfL, which is a mediator for sNfL levels.

Our group has previously found that HDL-C and ApoA-I had negative (protective) associations with BBB injury,[12] and this finding provided the initial motivation for investigating the associations of HDL-C and ApoA-I with NfL. Although neuroprotective associations of ApoA-I and ApoA-II with CSF-NfL were identified, we did not obtain evidence for associations with HDL-C. This suggests that the ApoA apolipoproteins rather than the HDL lipoprotein particle containing HDL-C are important for conferring the potential neuroprotective associations with CSF-NfL. ApoA-II, which comprises approximately 20% of HDL proteins, is the second most abundant apolipoprotein in the HDL lipoprotein after ApoA-I.[27] Although the critical roles of ApoA-I in mediating HDL recognition by cells and other lipoproteins and its protective associations with cardiovascular disease risk are well established, the physiological functions of ApoA-II are poorly understood.[27] The association of ApoA-II with CSF-NfL and sNFL remained significant after adjusting for both CEL number and T2-LV. Lesion burden represents only the tip of the

Table 3 Associations of CSF-NfL and sNfL with cholesterol variables after adjusting for the number of CELs and T2-lesion volume

	CSF-NfL levels‡		sNfL levels‡	
Predictor variable†	r_p	P	r_p	P
HDL cholesterol	−0.086	.40	−0.17	.055
Apolipoprotein A-I	−0.25	.014*	−0.18	.045
Apolipoprotein A-II	−0.25	.015*	−0.28	.001*

CSF-NfL, neurofilament light chain levels in cerebrospinal fluid; HDL, high-density lipoprotein; sNfL, neurofilament light chain levels in serum.
*Significant after Benjamini-Hochberg adjustment at a false discovery rate of 0.05.
†Partial correlation (r_p) and P-value from linear regression adjusted for age, sex, and body mass index, number of CELs, and log-transformed T2-lesion volume are shown.
‡Log base 10 transformed.

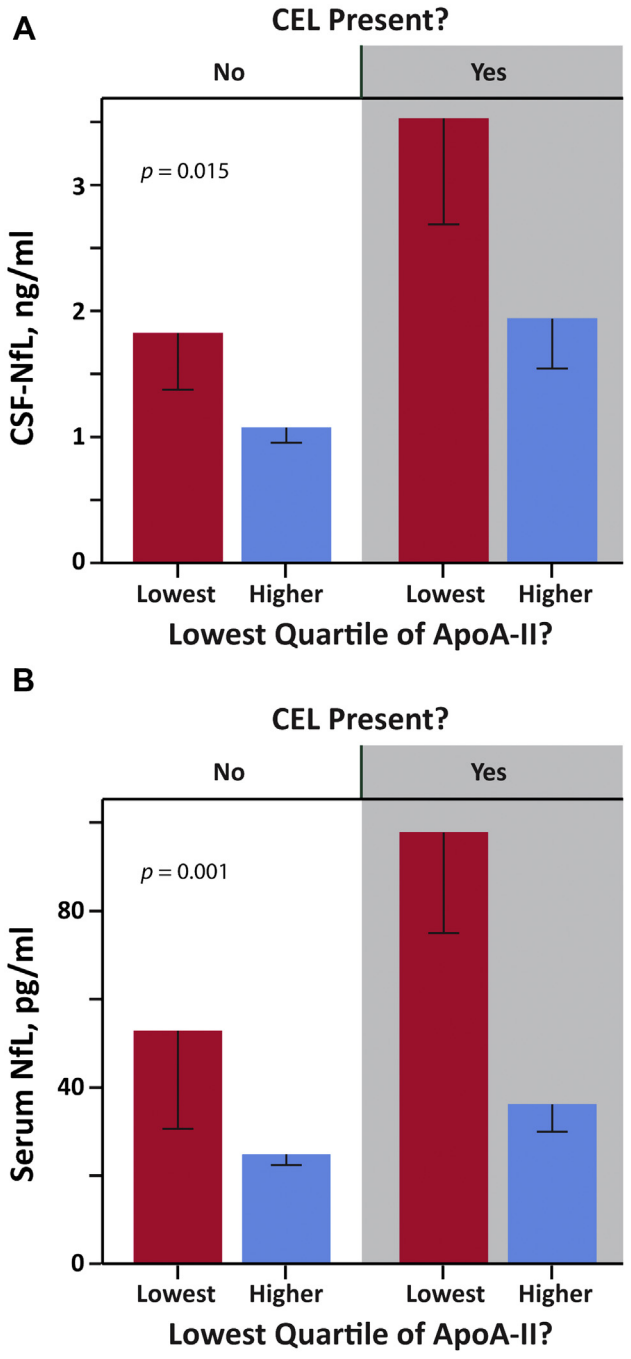


Figure 3 Dependence of CSF-NfL (A) and sNfL (B) in the groups with (gray region) and without (clear region) gadolinium contrast–enhancing lesions present. The red bars represent mean values for the group with the lowest quartile of ApoA-II levels, and the blue bars represent the mean value for the higher quartiles of ApoA-II. The error bars are standard errors of the mean. The *P*-values for ApoA-II from Table 3 are also shown.

iceberg of MS pathology, and neuroinflammatory changes are often found in normal-appearing white and gray matter of the brain and spinal cord.[5,28] This might explain, why association between neurofilament levels and ApoA-II remained even after adjustment for focal lesion pathology. On the other hand, ApoA-II may also be negatively associated with neuroaxonal injury at CNS sites outside of MRI-evident brain lesions, which is another pathophysiological mechanism responsible for brain pathology in MS. However, the mechanisms that could mediate this apparent neuroprotection are not known and we caution against inferring a causal role from our results.

Our results raise intriguing questions regarding the site of ApoA-II action and the MS pathophysiological processes that are the primary target of its mechanism of action. It is not clear, whether or not ApoA-II acts primarily in the CNS to inhibit neurodegeneration and neuroinflammation or whether there are also contributions from protective anti-inflammatory and metabolic processes in the vascular space. Although ApoA-I and ApoA-II proteins are present in the CSF, ApoA-I or ApoA-II mRNA expression is not found in CNS tissue.[29] ApoA-I and ApoA-II proteins found in CSF likely enter the CNS from the periphery via the choroid plexus.[29] This may potentially explain why serum ApoA-II levels are associated with lower CSF-NfL. Although it is thought that HDL particles do not cross the BBB, there is evidence that a subset of small diameter ApoA-I- and ApoA-II-containing particles enter the CSF.[30–32] These small diameter particles are cholesterol poor, which may explain the absence of evidence for HDL-C associations with CSF-NfL. HDL-C is a poor surrogate for HDL-C particle numbers and functionality. Impaired HDL functionality has been reported in patients with MS,[33,34] but the significance of these findings in patients with early MS after the first demyelinating event is not clear.

A limitation of our work is that we did not have CSF apolipoprotein levels: analysis of a more comprehensive panel of CSF apolipoproteins including ApoAI/ApoAII and ApoJ vs ApoE could help differentiate blood- vs CNS-derived HDL, which could help clarify the association between CSF ApoE level and neuroaxonal injury biomarkers. CSF ApoA-I levels (0.37 ± 0.08 mg/dL) are more than 400-fold lower than in serum, and ApoA-II levels are lower than ApoA-I levels in CSF.[29,31,35] ApoA-I and ApoA-II correlate between serum and CSF, whereas ApoE does not.[36] ApoE is the most abundant apolipoprotein in CSF. The CSF ApoE levels reported by Koch et al.[36] had a median of 0.73 (interquartile range: 0.059–0.097) mg/dL, whereas Wahrle et al.[37] reported a mean of 0.91 ± standard deviation 0.27 mg/dL. For comparison, our serum ApoE levels (mean 3.32 ± 1.0 mg/dL) were nearly 4-fold greater than the reported CSF ApoE. As CSF apolipoproteins have unknown diagnostic value, there are no commercially available ApoE assays with the required sensitivity. We did not have adequate CSF samples and validated assays with the required sensitivity to conduct CSF apolipoprotein analysis.

Mediation analysis and structural equation modeling are rigorous methodologies for assessing potentially causal pathways to surmise a directed statistical relationship between an exposure variable and an outcome that involves interdependence with other variables.[25,38] An indirect

pathway occurs when the exposure variable exhibits a statistically significant effect on the outcome through a mediator variable. In this study, we performed 2 independent mediation analyses using ApoA-II and albumin quotient as the exposure variables, CSF-NfL as the mediator, and sNfL as the outcome. These models provided support for an indirect effect of ApoA-II or albumin quotient on sNfL through CSF-NfL mediation. These pathways were combined into a single path analysis model in the structural equation modeling framework, wherein ApoA-II and albumin quotient provide parallel yet opposing indirect effects on sNfL through CSF-NfL mediation. The indirect effect of ApoA-II was negatively associated with increased CSF-NfL levels, whereas albumin quotient was positively associated with increased CSF-NfL levels.

Many MS treatments, including interferon beta, corticosteroids, dimethyl fumarate, and fingolimod, are known to modulate HDL levels.[39,40] However, it should be noted that our lipid profiles were obtained at the screening visit before the administration of corticosteroids for the demyelinating event or initiation of the interferon beta-1a treatment. Age, sex, and BMI, which are known to affect HDL levels, were adjusted for in the regression analyses. None of the patients were on statin therapies.

Our results demonstrating that ApoA levels have protective associations against neuroaxonal injury provide support for a role for apolipoproteins and cholesterol homeostasis in MS neurodegeneration. However, further investigation of the underlying mechanisms is warranted in next steps.

Acknowledgments

The authors thank the patients who participated in this study.

Authors' contributions: MM and TU contributed to data acquisition and analysis and manuscript drafting. MK contributed to data analysis, manuscript drafting. BS, JO, AMM, and MT contributed to data acquisition. DJ, DPR, NB, MV, RZ, and JK contributed to MRI data analysis. LN and LF contributed to biochemical data analysis. BW-G contributed to manuscript review and data interpretation. EKH contributed to clinical study design. DH contributed to clinical study design, data acquisition and interpretation. JK contributed to data acquisition and manuscript preparation. MR contributed to study concept and design, data analysis, and manuscript preparation.

Financial disclosure

M.M., M.K., R.W.B., A.M., D.J., D.P.R., N.B., L.N., and L.F. have nothing to disclose.

T.U. received financial support for conference travel and honoraria from Biogen Idec, Novartis, Roche, Genzyme, and Merck Serono, as well as support for research activities from Biogen Idec and Sanofi (GZ-2017-11718).

B.S. received compensation for traveling and conference fees from Novartis, Sanofi, Genzyme, and Biogen.

J.O. received travel grants from Biogen-Idec, Novartis, and Bayer and served on an advisory board by Roche.

M.T. received compensation for travel and honoraria from Biogen Idec, Sanofi, Teva, and Merck Serono.

J.K. received financial support for research activities from Biogen Idec.

B.W-G. received honoraria for serving in advisory boards and educational programs from Teva Pharmaceuticals, Biogen Idec, Novartis, Acorda, EMD Serono, Pfizer, Novartis, Genzyme, and Sanofi. She also received support for research activities from the National Institutes of Health, National Multiple Sclerosis Society, National Science Foundation, Department of Defense, EMD Serono, Biogen Idec, Teva Neuroscience, Cyberonics, Novartis, Acorda, and the Jog for the Jake Foundation.

E.K.H. received speaker honoraria and consultant fees from Biogen Idec, Merck Serono, Novartis, Genzyme, Teva, Actelion, and Receptos, as well as support for research activities from Biogen Idec and Merck Serono.

M.V. received speaker honoraria and consultant fees from Biogen Idec, Novartis, Merck Serono, Genzyme, Roche, and Teva, as well as support for research activities from Biogen Idec and supported by Czech Ministry of Health, grant RVO-VFN 64165.

R.Z. received personal compensation from EMD Serono, Genzyme-Sanofi, Celgene, and Novartis for speaking and consultant fees. He received financial support for research activities from Genzyme-Sanofi, Novartis, Celgene, Mapi Pharma, Protembo, V-VAWE Medical. R.Z. serves on the editorial board of Journal of Alzheimer's Disease, BMC Medicine, BMC Neurology, Vein and Lymphatics and Clinical CNS Drugs.

D.H. received compensation for travel, speaker honoraria, and consultant fees from Biogen Idec, Novartis, Merck Serono, Bayer Schering, and Teva, as well as support for research activities from Biogen Idec.

J.K. received speaker fees, research support, travel support, and/or served on advisory boards by ECTRIMS, Swiss MS Society, Swiss National Research Foundation, (320030_160221), University of Basel, Bayer, Biogen, Celgene, Genzyme, Merck, Novartis, Protagen AG, Roche, Teva.

M.R. received research funding from the National Institutes of Health and Department of Defense. Funding for the Ramanathan laboratory from MS190096 from Department of Defense Congressionally Directed Medical Research Programs, USAMRDC, Multiple Sclerosis Research Program. He has also received research funding from Otsuka Pharmaceutical Research and Development. These are unrelated to the research presented in this report.

References

1. Khalil M, Teunissen CE, Otto M, et al. Neurofilaments as biomarkers in neurological disorders. *Nat Rev Neurol*. 2018;14:577–589.
2. Dujmovic I. Cerebrospinal fluid and blood biomarkers of neuroaxonal damage in multiple sclerosis. *Mult Scler Int*. 2011;2011:767083.
3. Disanto G, Adiutori R, Dobson R, et al. Serum neurofilament light chain levels are increased in patients with a clinically isolated syndrome. *J Neurol Neurosurg Psychiatry*. 2016;87:126–129.
4. Matute-Blanch C, Villar LM, Alvarez-Cermeno JC, et al. Neurofilament light chain and oligoclonal bands are prognostic biomarkers in radiologically isolated syndrome. *Brain*. 2018;141:1085–1093.
5. Bo L, Geurts JJ, Mork SJ, van der Valk P. Grey matter pathology in multiple sclerosis. *Acta Neurol Scand Suppl*. 2006;183:48–50.
6. Varhaug KN, Torkildsen O, Myhr KM, Vedeler CA. Neurofilament light chain as a biomarker in multiple sclerosis. *Front Neurol*. 2019;10:338.
7. Uher T, McComb M, Galkin S, et al. Neurofilament levels are associated with blood-brain barrier integrity, lymphocyte extravasation, and risk factors following the first demyelinating event in multiple sclerosis. *Mult Scler*. 20201352458520912379.
8. Srpova B, Uher T, Hrnciarova T, et al. Serum neurofilament light chain reflects inflammation-driven neurodegeneration and predicts delayed brain volume loss in early stage of multiple sclerosis. *Mult Scler*. 20201352458519901272.
9. Berghoff SA, Gerndt N, Winchenbach J, et al. Dietary cholesterol promotes repair of demyelinated lesions in the adult brain. *Nat Commun*. 2017;8:14241.
10. Saher G, Brugger B, Lappe-Siefke C, et al. High cholesterol level is essential for myelin membrane growth. *Nat Neurosci*. 2005;8:468–475.
11. Browne RW, Weinstock-Guttman B, Horakova D, et al. Apolipoproteins are associated with new MRI lesions and deep grey matter atrophy in clinically isolated syndromes. *J Neurol Neurosurg Psychiatry*. 2014;85:859–864.
12. Fellows K, Uher T, Browne RW, et al. Protective associations of HDL with blood-brain barrier injury in multiple sclerosis patients. *J Lipid Res*. 2015;56:2010–2018.
13. Murali N, Browne RW, Fellows Maxwell K, et al. Cholesterol and neurodegeneration: longitudinal changes in serum cholesterol biomarkers are associated with new lesions and gray matter atrophy in multiple sclerosis over 5 years of follow-up. *Eur J Neurol*. 2019;27:188-e4.
14. Zhornitsky S, McKay KA, Metz LM, Teunissen CE, Rangachari M. Cholesterol and markers of cholesterol turnover in multiple sclerosis: relationship with disease outcomes 2016;5:53–65.
15. Zivadinov R, Havrdova E, Bergsland N, et al. Thalamic atrophy is associated with development of clinically definite multiple sclerosis. *Radiology*. 2013;268:831–841.
16. Kalincik T, Vaneckova M, Tyblova M, et al. Volumetric MRI markers and predictors of disease activity in early multiple sclerosis: a longitudinal cohort study. *PLoS One*. 2012;7:e50101.
17. Uher T, Horakova D, Tyblova M, et al. Increased albumin quotient (QAlb) in patients after first clinical event suggestive of multiple sclerosis is associated with development of brain atrophy and greater disability 48 months later. *Mult Scler*. 2016;22:770–781.
18. Disanto G, Barro C, Benkert P, et al. Serum neurofilament light: a biomarker of neuronal damage in multiple sclerosis. *Ann Neurol*. 2017;81:857–870.
19. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem*. 1972;18:499–502.
20. Freedman MS, Thompson EJ, Deisenhammer F, et al. Recommended standard of cerebrospinal fluid analysis in the diagnosis of multiple sclerosis: a consensus statement. *Arch Neurol*. 2005;62:865–870.
21. Wada H. Blood-brain barrier permeability of the demented elderly as studied by cerebrospinal fluid-serum albumin ratio. *Intern Med*. 1998;37:509–513.
22. Reiber H, Teut M, Pohl D, Rostasy KM, Hanefeld F. Paediatric and adult multiple sclerosis: age-related differences and time course of the neuroimmunological response in cerebrospinal fluid. *Mult Scler*. 2009;15:1466–1480.
23. Uher T, Horakova D, Bergsland N, et al. MRI correlates of disability progression in patients with CIS over 48 months. *Neuroimage Clin*. 2014;6:312–319.
24. Uher T, Schaedelin S, Srpova B, et al. Monitoring of radiologic disease activity by serum neurofilaments in MS. *Neurol Neuroimmunol Neuroinflamm*. 2020;7:e714.
25. Hayes AF. Introduction to Mediation, Moderation, and Conditional Process Analysis: a Regression-Based Approach. 2nd ed. New York: Guilford Press; 2018.
26. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J Roy Stat Soc B*. 1995;57:289–300.
27. Remaley AT. Apolipoprotein A-II: still second fiddle in high-density lipoprotein metabolism? *Arterioscler Thromb Vasc Biol*. 2013;33:166–167.
28. Mallucci G, Peruzzotti-Jametti L, Bernstock JD, Pluchino S. The role of immune cells, glia and neurons in white and gray matter pathology in multiple sclerosis. *Prog Neurobiol*. 2015;127-128:1–22.
29. Elliott DA, Weickert CS, Garner B. Apolipoproteins in the brain: implications for neurological and psychiatric disorders. *Clin Lipidol*. 2010;51:555–573.
30. de Vries HE, Breedveld B, Kuiper J, de Boer AG, Van Berkel TJ, Breimer DD. High-density lipoprotein and cerebral endothelial cells in vitro: interactions and transport. *Biochem Pharmacol*. 1995;50:271–273.
31. Koch S, Donarski N, Goetze K, et al. Characterization of four lipoprotein classes in human cerebrospinal fluid. *J Lipid Res*. 2001;42:1143–1151.
32. Ladu MJ, Reardon C, Van Eldik L, et al. Lipoproteins in the central nervous system. *Ann N Y Acad Sci*. 2000;903:167–175.
33. Jorissen W, Wouters E, Bogie JF, et al. Relapsing-remitting multiple sclerosis patients display an altered lipoprotein profile with dysfunctional HDL. *Sci Rep*. 2017;7:43410.
34. Mukhopadhyay S, Fellows K, Browne RW, et al. Interdependence of oxysterols with cholesterol profiles in multiple sclerosis. *Mult Scler*. 2017;23:792–801.
35. Borghini I, Barja F, Pometta D, James RW. Characterization of subpopulations of lipoprotein particles isolated from human cerebrospinal fluid. *Biochim Biophys Acta*. 1995;1255:192–200.
36. Koch M, Furtado JD, Falk K, Leypoldt F, Mukamal KJ, Jensen MK. Apolipoproteins and their subspecies in human cerebrospinal fluid and plasma. *Alzheimers Dement (Amst)*. 2017;6:182–187.
37. Wahrle SE, Shah AR, Fagan AM, et al. Apolipoprotein E levels in cerebrospinal fluid and the effects of ABCA1 polymorphisms. *Mol Neurodegener*. 2007;2:7.
38. Lange T, Rasmussen M, Thygesen LC. Assessing natural direct and indirect effects through multiple pathways. *Am J Epidemiol*. 2014;179:513–518.
39. Uher T, Fellows K, Horakova D, et al. Serum lipid profile changes predict neurodegeneration in interferon-beta1a-treated multiple sclerosis patients. *J Lipid Res*. 2017;58:403–411.
40. Blumenfeld Kan S, Staun-Ram E, Golan D, Miller A. HDL-cholesterol elevation associated with fingolimod and dimethyl fumarate therapies in multiple sclerosis. *Mult Scler J Exp Transl Clin*. 2019;52055217319882720.

Appendix

Supplemental material

Random forest regression methods

The stepwise regression results were confirmed using random forest regression (RFR), an ensemble learning-based method for identifying associations between predictor variables and dependent variables. The R package *randomForestSRC*[1] was used with mean squared error for the split rule and the hyperparameters set to default values of 500 trees (*ntrees*), 5 random variables tried at each split (*mtry*), and an average of 5 observations in each terminal node in the forest (*nodesize*). Missing values were imputed.

The general framework of stepwise regression was maintained, wherein the dependent variables were either serum neurofilament (sNfL) or cerebrospinal fluid neurofilament (CSF-NfL) levels; the predictor variables included total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), apolipoprotein A-I (ApoA-I), ApoA-II, ApoB, ApoE, albumin quotient, immunoglobulin G (IgG) quotient, immunoglobulin M (IgM quotient), IgG index, IgM index, age, body mass index, and sex. Variable importance (VIMP)[2] was used to identify the highest contributing associations among the predictor variables, where larger values indicate greater importance.

Random forest regression results

Supplementary Table 1A shows the predictor variables ordered by RFR VIMP for the dependent variables sNfL and CSF-NfL. The most important predictor variables associated with sNfL were ApoA-II (VIMP = .00699) and albumin quotient (0.00649), which are concordant with the results from stepwise regression (Supplemental Table 1B). However, age, which was identified in the stepwise regression model for sNfL, was the fifth most important predictor variable from RFR VIMP. Albumin quotient (0.0243) and ApoA-II (0.00850) were identified as the most important variables associated with CSF-NfL, similar to the stepwise regression results. The qualitative similarity of the results from stepwise regression and RFR VIMP confirmed the usage of ApoA-II and albumin quotient in subsequent mediation and moderation analyses.

Supplemental Table 1A Random forest regression variable importance (VIMP) values for the different cholesterol biomarkers for CSF-NfL and sNfL levels

Predictors*	sNfL VIMP	Predictors*	CSF-NfLVIMP
Apolipoprotein A-II	0.00699	Albumin quotient	0.0243
Albumin quotient	0.00649	Apolipoprotein A-II	0.00850
IgG quotient	0.00174	Apolipoprotein E	0.00691
LDL cholesterol	0.00122	IgG quotient	0.00662
Total cholesterol	0.00117	Age	0.00494
Age	7.19×10^{-4}	Total cholesterol	0.00393
IgM quotient	6.39×10^{-4}	Apolipoprotein A-I	0.00350
HDL cholesterol	3.99×10^{-4}	IgG index	0.00311
IgM index	7.67×10^{-5}	Apolipoprotein B	0.00297
IgG index	3.20×10^{-5}	LDL cholesterol	0.00256
BMI	-6.23×10^{-4}	IgM quotient	0.00114
Sex	-8.39×10^{-4}	BMI	0.00110
Apolipoprotein B	−0.00116	HDL cholesterol	6.29×10^{-4}
Apolipoprotein A-I	−0.00328	Sex	2.04×10^{-4}
Apolipoprotein E	−0.00330	IgM index	-9.15×10^{-5}

CSF-NfL, neurofilament light chain levels in cerebrospinal fluid; HDL, high-density lipoprotein; LDL, low-density lipoprotein; sNfL, neurofilament light chain levels in serum; BMI, body mass index; IgG, immunoglobulin G; IgM, immunoglobulin M; r_p, partial correlation value.

*Log base 10 transformed values of sNfL, CSF-NfL, albumin quotient, IgG quotient, IgM quotient, IgG index, and IgM index were used.

Supplemental Table 1B Stepwise regression results for CSF-NfL and sNfL levels. The partial correlation (r_p) and *P*-values are shown

Predictors*	sNfL r_p (*P*-value)	Predictors*	CSF-NfL r_p (*P*-value)
Apolipoprotein A-II	0.360 (.00043)	Albumin quotient	−0.294 (.0010)
Albumin quotient	−0.238 (.022)	Apolipoprotein A-II	0.279 (.0019)
Age	−0.209 (.045)		

CSF-NfL, neurofilament light chain levels in cerebrospinal fluid; HDL, high-density lipoprotein; LDL, low-density lipoprotein; sNfL, neurofilament light chain levels in serum; BMI, body mass index; IgG, immunoglobulin G; IgM, immunoglobulin M; r_p, partial correlation value.

*Log base 10 transformed values of sNfL, CSF-NfL, albumin quotient, IgG quotient, IgM quotient, IgG index, and IgM index were used.

Supplemental references

1. Ishwaran H, Kogalur U. Fast Unified Random Forests for Survival, Regression, and Classification (RF-SRC). R package version 293. 2020.
2. Ishwaran H. Variable importance in binary regression trees and forests. *Electron J Stat*. 2007;1:519–537.

Multiple Sclerosis and Related Disorders 46 (2020) 102534

Contents lists available at ScienceDirect

Multiple Sclerosis and Related Disorders

journal homepage: www.elsevier.com/locate/msard

The weak association between neurofilament levels at multiple sclerosis onset and cognitive performance after 9 years

Lucie Friedova[a], Jiri Motyl[a], Barbora Srpova[a], Johanna Oechtering[b], Christian Barro[b], Karolina Vodehnalova[a], Michaela Andelova[a], Libuse Noskova[c], Lenka Fialová[c], Eva Kubala Havrdova[a], Dana Horakova[a], Ralph HB Benedict[d], Jens Kuhle[b], Tomas Uher[a,*]

[a] Department of Neurology and Center of Clinical Neuroscience, First Faculty of Medicine, Charles University and General University Hospital, Katerinska 30, 120 00 Prague, Czech Republic
[b] Departments of Medicine, Clinical Research, Biomedicine and Biomedical Engineering, Neurologic Clinic and Policlinic, University Hospital and University of Basel, Switzerland
[c] Institute of Medical Biochemistry and Laboratory Diagnostics, First Faculty of Medicine, Charles University and General University Hospital, Prague, Czech Republic
[d] Department of Neurology, University at Buffalo, Buffalo, NY United States

ARTICLE INFO

Keywords:
Multiple sclerosis
Neurofilament light chain
Cognition
Information processing
Longitudinal studies
Predictors

ABSTRACT

Background: Neurofilament light chain level in serum (sNfL) and cerebrospinal fluid (CSF-NfL) is a promising biomarker of disease activity in multiple sclerosis (MS). However, predictive value of neurofilaments for development of cognitive decline over long-term follow-up has not been extensively studied.
Objective: To investigate the relationship between early neurofilament levels and cognitive performance after 9-years.
Methods: We included 58 MS patients from the SET study. sNfL levels were measured at screening, at 1 and 2 years. CSF-NfL were measured in 36 patients at screening. Cognitive performance was assessed by the Brief International Cognitive Assessment for Multiple Sclerosis and the Paced Auditory Serial Addition Test-3 s at baseline, at 1, 2 and 9 years. Association between neurofilament levels and cognition was analyzed using Spearman´s correlation, logistic regression and mixed models.
Results: We did not observe associations among early sNfL levels and cross-sectional or longitudinal cognitive measures, except of a trend for association between higher sNfL levels at screening and lower California Verbal Learning Test-II (CVLT-II) scores at year 1 (rho = -0.31, unadjusted $p = 0.028$). Higher sNfL level was not associated with increased risk of cognitive decline, except of a trend for greater risk of CVLT-II decrease in patients with higher sNfL levels at 1 year (OR = 15.8; 95% CI = 1.7–147.0; unadjusted $p = 0.015$). Similar trends were observed for CSF-NfL.
Conclusion: We found only weak association between sNfL levels at disease onset and evolution of cognitive performance over long-term follow-up.

1. Introduction

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system characterized by a wide range of physical and cognitive symptoms, which are known to be closely related to quality of life and frequently lead to premature deterioration of employment status (Benedict et al., 2017; Sumowski et al., 2018; Uher et al., 2018).

Neurofilament light chain (NfL) is a promising biomarker of disease activity in MS (Comabella and Montalban, 2014; J. Kuhle et al., 2016). Although number of studies showed predictive value of early cerebrospinal fluid NfL (CSF-NfL) or serum NfL (sNfL) levels for prognostication of future MRI or clinical disease activity (J. Kuhle et al., 2016; Barro et al., 2018; Disanto et al., 2017), there are only few studies investigating relationship between NfL levels and cognition, and the reported findings are contradictory (Gaetani et al., 2019; Kalatha et al., 2019; Quintana et al., 2018; Modvig et al., 2015; Chitnis et al., 2018; Jakimovski et al., 2019).

For example, in a recent cross-sectional study of 28 patients, CSF-NfL levels were significantly higher in cognitively impaired patients (Gaetani et al., 2019). Another cross-sectional study of 39 patients found an association between CSF-NfL levels and cognition only in seven patients with progressive MS (Kalatha et al., 2019), while another

* Corresponding author.
E-mail address: tomas.uher@vfn.cz (T. Uher).

https://doi.org/10.1016/j.msard.2020.102534
Received 4 May 2020; Received in revised form 22 July 2020; Accepted 25 September 2020

cross-sectional small sample study performed on 51 patients showed a moderately strong association already in the early stages of MS (Quintana et al., 2018).

More negative results were obtained in a few longitudinal small sample size studies where either only a weak correlation between sNfL levels and cognitive performance over 5 years (Jakimovski et al., 2019), a trend towards an association between CSF-NfL levels and cognition over almost 14 years (Modvig et al., 2015) or no association between sNfL levels and cognitive decline over 9 years was found (Chitnis et al., 2018). In the context of the previous inconclusive research, the main aim of this study was to investigate the predictive value of sNfL levels in newly diagnosed MS patients for development of cognitive decline after long-term follow-up. To the best of our knowledge, this is the first longitudinal study which investigated homogenous sample of MS patients at disease onset, employed repeated cognitive testing from baseline and included cognitive screening battery assessing more cognitive domains. Early identification of patients at higher risk of cognitive decline is clinically relevant, because it might help to identify patients profiting from more intensive cognitive monitoring or from early initiation of high efficacy disease-modifying treatment (Comi et al., 2017; Gudesblatt et al., 2018).

2. Methods

2.1. Study population

The SET (Study of Early Interferon beta-1a Treatment) study was an investigator-initiated, observational, prospective multicenter clinical study in the Czech Republic (Kalincik et al., 2012; Uher et al., 2014). Patients were enrolled between October 2005 and July 2009. Inclusion criteria included: age between 18 and 55 years, enrollment within 4 months from the first demyelinating event, Expanded Disability Status Scale (EDSS) score at baseline of 3.5 or less, at least two T2-hyperintense lesions on diagnostic MR images (before high dose corticosteroid treatment) and ≥ 2 oligoclonal bands (OCB) exclusively in the CSF. SET study patients were originally diagnosed with clinically isolated syndrome according to the McDonald 2005 criteria (Polman et al., 2005). Patients' diagnosis was reclassified to relapsing-remitting multiple sclerosis based on the 2017 McDonald criteria (Thompson et al., 2018). Together, 43 (74%) of patients developed at least one relapse over 9 years and had clinically confirmed MS. Screening visit was early after disease onset prior to corticosteroid treatment (all patients were treated with 3–5 g methylprednisolone). Baseline brain MRI was acquired at least 30 days after steroid treatments and prior disease modifying therapy (DMT) initiation. All patients started intramuscular IFNb-1a once a week (30 mg; Biogen-Idec, Cambridge, MA, USA) (Kalincik et al., 2012; Zivadinov et al., 2013; Uher et al., 2015). Patients were monitored for 48 months with evaluation of EDSS at baseline (DMT initiation) and then every 3 months. Clinical visits occurred every 3 months for 24 months and long-term follow-up in routine clinical practice followed. Clinical assessments were performed using the EDSS.

Of the 220 SET study patients, 58 consecutive individuals underwent cognitive assessment at all required timepoints (baseline, year 1, year 9) and had available analysis of sNfL levels at 1 year.

The study was approved by the Medical Ethics Committees of the General University in Prague and the First Faculty of Medicine, Charles University in Prague and by local ethics committees in the participating centers. Written informed consent was obtained from each patient.

2.2. CSF and blood sampling, and sNfL measurement

CSF-NfL and sNfL samples were collected at screening and stored at −80 °C. Sampling procedures were performed according to the standard protocol (Teunissen et al., 2009).

CSF-NfL concentrations were measured by enzyme-linked immunosorbent assays (ELISA) using the NF-light (neurofilament light) ELISA kit (UmanDiagnostics AB, Umea, Sweden) at the General Faculty Hospital in Prague. This kit provided measuring range 100 – 10 000 pg/mL with the limit of detection 32 pg/mL. The procedure was performed according to the instructions from manufacturer. Recommended temperatures, time of incubation and agitation was in agreement with the instructions. All measurements were run on the Tecan Sunrise ELISA reader with the Magellan software (*Tecan Group Ltd., Switzerland*). The calibration curves from supplied bovine NfL standard was created in Magellan software, the 4-parameter Marquardt transformation was set according to the manufacturer´s instruction. The samples were analyzed in duplicate. Internal control sample was used, inter- and intra-assay coefficient of variation did not exceed 10%. All CSF samples assembled from screening visit were analyzed by accredited laboratory. sNfL concentration was measured using a sensitive immunoassay on the Simoa platform at the University Hospital Basel as described previously (Barro et al., 2018; Disanto et al., 2017; J. Kuhle et al., 2016). Inter-assay coefficients of variation (CV) for 3 native serum samples were below 10% (i.e. 7.8%, 8.8% and 5.5% for 7.0., 18.8 and 81.3 pg/ml, respectively). The mean intra-assay CV of duplicate determinations for concentration was 6.4%. One patient´s samples showed an sNfL value below 1.3 pg/ml (i.e. the lower limit of quantification). This patient was excluded from the analysis. sNfL levels were assembled from screening and at 1 and 2 years after DMT initiation as described previously (Srpova et al., 2020). All patients had available serum samples at year 1. In addition, there were also available sNfL data at other timepoints: at screening ($n = 49$), at baseline ($n = 22$), at 1 month ($n = 57$), and at 24 months ($n = 53$). Due to the limited number of sNfL samples from the baseline, we analyzed only sNfL samples from screening, year 1, and year 2.

Measurements of CSF-NfL and sNfL were performed on coded samples. None of the laboratory personnel had access to clinical data and remained blinded to treatment allocation and diagnosis.

Patients were divided into two groups based on the age-adjusted pathological sNfL levels at screening and year 1, which were defined using 90th percentile cut-off. Age-adjusted sNfL percentiles were derived from a normative dataset of healthy controls (Disanto et al., 2017) (Supplementary Table 1).

2.3. Neuropsychological assessment

Patients were tested using the Czech-validated version of the Brief International Cognitive Assessment for Multiple Sclerosis (BICAMS) (Benedict et al., 2012; Dusankova et al., 2012) and the Rao adaptation of the Paced Auditory Serial Addition Test – 3 s (PASAT-3) (Rao, 1990).

The PASAT-3 assesses auditory information processing speed and working memory (Rao, 1990). The BICAMS battery consists of the Symbol Digit Modalities Test (SDMT) (Smith, 1982), California Verbal Learning Test - second edition (CVLT-II) (Delis et al., 2000) and the Brief Visuospatial Memory Test - Revised (BVMT-R) (Benedict, 1997). The SDMT assesses cognitive processing speed and visual scanning (Smith, 1982). The CVLT-II assess the verbal learning and memory (Delis et al., 2000), and the BVMT-R evaluates visuospatial memory (Benedict, 1997). For both the CVLT-II and the BVMT-R only the initial learning trials were administered (Benedict et al., 2012; Dusankova et al., 2012). For the assessment of depressive symptoms, the Beck Depression Inventory-Second Edition (BDI-II) was used.

Cognitive assessment was administered during the clinical visits at baseline, at 1 (mean [M] 1.01, 95% confidence interval [CI] 0.99–1.02) year, at 2 (M 2.00, 95% CI 1.99–2.01) years and at 9 (M 8.87, 95% CI 8.72–9.02) years. All patients were relapse-free for at least 30 days prior to testing. Raw scores of the cognitive tests were used in the statistical models and presented results. The outcome of this study was a worsening of cognitive performance over 9 years, which was defined as a presence of decrease (categorical variable) of cognitive test raw score between baseline and follow-up timepoint. In addition, to create unified composite measure of cognitive performance, we have

calculated the composite z-score from all cognitive tests and compared the composite z-score at baseline and after 9 years.

2.4. MRI measures

MRI was performed with a standardized protocol on the same 1.5-T scanner (Gyroscan; Philips Medical Systems, Best, the Netherlands). Axial brain acquisitions included fluid attenuated inversion recovery, three-dimensional (3D) T1-weighted images, and T1 spin-echo images before and 5 min after a single injection of 0.1 mmol/kg of gadopentetate dimeglumine. Semi-automated image analysis of the T2 lesion volume (T2LV) and brain parenchymal fraction (BPF) was performed with the ScanView software as previously described (Uher et al., 2017).

2.5. Statistical analysis

All statistical analyses were carried out with IBM SPSS Statistics software 22.0 (IBM Corp. Armonk, NY).

The demographic, clinical, neuropsychological, MRI and biochemical characteristics of the patients were compared using the Mann–Whitney rank-sum test and Chi-Square test. The associations between sNfL levels at individual timepoints, MRI and cognitive measures were evaluated using the non-parametric Spearman correlation test. We analysed association with an average sNfL levels within first 2 years (with or without screening levels). Patients were divided into two groups using the 90th percentile cut-off (Disanto et al., 2017) of their sNfL levels at screening and at year 1. Alternatively, patients were also divided into two groups based on the presence of cognitive decline in each cognitive test over 9 years of the study. Cognitive decline was defined as a categorical variable of an absence or presence of decrease in each neuropsychological test raw score between baseline and follow-up timepoints, or defined as a decrease of cognitive composite z-score over follow-up. Logistic and linear regression analysis was used to test the relationships between cognitive decline or performance and pathological sNfL levels defined using the 90th percentile cut-off. Logistic and linear regression was also used to investigate relationship between early MRI and cognitive outcomes after 9 years. All regression models were adjusted for age, sex, cognitive performance at baseline or at year 1, and treatment over follow-up, as classified in two categories: 1) low efficacy DMTs (glatiramer-acetate, dimethyl fumarate, interferons and teriflunomide), 2) high or moderately high efficacy DMTs (fingolimod, natalizumab and rituximab). Because of multicollinearity between age and disease duration, our models were only adjusted for age. In linear regression, log-transformed values of average sNfL values within first 2 years of follow-up (with or without screening levels) were used as continuous independent variable.

Finally, we performed adjusted linear mixed model analysis to consider all available cognitive measures over follow-up in one model. In mixed models log-transformed values of sNfL were used as independent continuous variable. The equation of the mixed model was used as follows:

Change in cognitive performance in each timepoint ~ log-*transformed sNfL level in certain timepoint + Sex + Age + Education + DMT category at certain timepoint + Time from baseline + (1|Subject), where (1|Subject) is a random intercept for each subject*

The Benjamini-Hochberg (BH) procedure with $q = 0.05$ was used to control false discovery rate. Relationships that lost significance after BH procedure are described as trends.

3. Results

3.1. Baseline and follow-up demographic, clinical, MRI and sNfL concentration characteristics

Table 1 shows demographic, clinical, neuropsychological and biochemical characteristics of the patients ($n = 58$) at each timepoint, separated by the age-related pathological sNfL levels at year 1 using 90th percentile cut-off. T2LV (1.4 ± 2.3 ml vs. 1.9 ± 3.1 ml; $p = 0.23$) and BPF (86.8 ± 1.5 vs. 86.9 ± 1.7; $p = 0.92$) in 58 SET study patients included in the study did not differ from MRI measures of the 162 SET study patients who were not included in this study.

Except of an association between high sNfL levels at screening and higher T2LV ($p = 0.016$) at baseline, we did not find any associations between baseline MRI measures and sNfL levels during the first 2 years of follow-up. sNfL concentration levels at individual timepoints are shown in Supplementary Table 2.

At screening ($n = 49$), 28 (57%) patients had sNfL level above the 90th percentile. The prevalence of cognitive decline over 9 years was not greater in patients with higher sNfL levels (M: 46.31 ± 32.22 pg/ml) at screening compared with those with lower sNfL levels (M: 10.65 ± 4.36 pg/ml). At year 1 ($n = 58$), 24 (41%) patients had sNfL level above the 90th percentile. The mean sNfL concentration in a group above the 90th percentile was 28.35 ± 12.74 pg/ml and 10.20 ± 4.53 pg/ml in those with lower sNfL levels at year 1.There were no differences in clinical and demographical measures between patients with high or low sNfL levels at 1 year, except of higher age in patients with low sNfL levels ($p = 0.013$). A decline in raw scores of cognitive subtests between year 1 and year 9 was present in 16 (28%) patients in the SDMT, 12 (21%) patients in the CVLT-II, 12 (21%) patients in the BVMT-R, and 27 (47%) of patients in the PASAT-3 (Fig. 1). High sNfL levels at 1 year were not associated with more frequent occurrence of cognitive decline over follow-up, except of sNfL levels at year 1 and decline in the CVLT-II over follow-up ($p = 0.008$) (Table 2).

At 9 year follow-up, the median EDSS was 2 and the following percentage of patients reached ≥2 points in individual functional systems scores of EDSS: 43% in pyramidal, 10% in cerebellar, 7% in brainstem, 36% in sensory, 3% in bowel and bladder, 3% in visual and 2% in mental functional system.

3.2. The correlation between neuropsychological performance and sNfL levels

We did not observe associations among early sNfL levels (at screening or at year 1) and cross-sectional or longitudinal cognitive measures (at year 1, 2 a 9), except of an association between higher sNfL levels at screening ($n = 49$) and lower CVLT-II scores at year 1 (rho = −0.31, $p = 0.028$; $q = 0.112$). We did not observe association between average sNfL levels within first 2 years of follow-up (with or without screening levels) and cross-sectional or longitudinal cognitive measures (at year 1, 2 a 9).

3.3. Risk of cognitive decline over 9 years according to early sNfL levels

sNfL levels above 90th percentile at year 1 were associated with a greater risk of CVLT-II decrease over 9 years compared with patients with lower sNfL levels [odds ratio (OR) 15.8; 95% CI 1.7–147.0; $p = 0.015$; $q = 0.060$ after BH procedure). We did not observe other relationships between sNfL levels at year 1 and higher risk of cognitive decline in other neuropsychological tests over 9 years (Table 3). Also, we did not find an association between higher sNfL levels at year 1 and higher risk of the overall memory decline (decrease in CVLT-II or BVMT-R), information processing speed decline (decrease in SDMT or PASAT-3) or abnormal BICAMS battery outcome (decrease in at least one subtest)(Supplementary Table 3).

There was also a tendency towards an association between high sNfL level at 2 years and PASAT-3 decline at year 9 (OR = 3.9; 95% CI: 0.8–19.0; $p = 0.091$; $q = 0.198$). We did not observe any other associations between sNfL levels at 2 years and cognitive measures.

We did not observe relationships among average sNfL levels within first 2 years and higher risk of decline in each individual neuropsychological test over 9 years. There was also no association in

Table 1
Demographic, clinical, neuropsychological and serum neurofilament light chain concentration characteristics of the patients.

Characteristic	Total sample (n = 58)	sNfL level at year 1		
	sNfL level > 90th percentile (n = 24)	sNfL level < 90th percentile (n = 34)		p value
At baseline				
Sex, female (%)	40 (68.97%)	16 (66.67%)	24 (70.59%)	0.751*
Education (years)‡	13.00 (3.00)	13.00 (4.00)	13.50 (3.00)	0.598
Clinical				
Age at disease onset (years)	27.78 ± 8.85	24.46 ± 7.34	30.12 ± 9.17	0.013
Time to baseline (days)	81.53 ± 19.32	83.42 ± 19.72	80.21 ± 19.22	0.487
EDSS‡	1.50 (0.50)	1.50 (0.50)	1.50 (0.60)	0.650
Proportion of low efficacy DMTs (%)	58 (100%)	24 (100%)	34 (100%)	*
At year 1				
Age (years)	29.318 ± 8.80	26.07 ± 7.38	31.618 ± 9.11	0.015
Proportion of low efficacy DMTs (%)	58 (100%)	24 (100%)	34 (100%)	*
Neuropsychological				
SDMT	63.29 ± 8.63	64.21 ± 8.29	62.65 ± 8.92	0.687
CVLT-II TL	56.66 ± 7.80	57.00 ± 7.87	56.41 ± 7.85	0.647
BVMT-R TL	27.69 ± 4.95	27.38 ± 4.73	27.91 ± 5.17	0.516
PASAT-3	53.84 ± 8.57	54.96 ± 8.03	53.09 ± 8.96	0.594
BDI	5.49 ± 6.00	5.25 ± 6.28	5.64 ± 5.92	0.985
	sNfL level			
	sNfL (pg/ml)	17.72 ± 12.60	28.38 ± 12.73	10.20 ± 4.53
At 9 years				
Age (years)	37.24 ± 8.70	34.07 ± 7.43	39.48 ± 8.93	0.016
EDSS‡	2.00 (1.3)	1.5 (0.90)	2.5 (1.5)	0.020
Proportion of low efficacy DMTs (%)**	44 (75.86%)	18 (75.00%)	26 (76.47%)	0.568*
Neuropsychological				
SDMT	64.79 ± 8.26	64.33 ± 8.60	65.12 ± 8.13	0.658
CVLT-II TL	62.38 ± 9.01	61.75 ± 9.77	62.82 ± 8.55	0.906
BVMT-R TL	30.69 ± 3.20	30.63 ± 3.12	30.74 ± 3.30	0.806
PASAT-3	53.95 ± 6.88	55.04 ± 6.55	53.21 ± 7.09	0.338
BDI	7.21 ± 8.18	8.20 ± 8.50	6.61 ± 8.06	0.247
Absolute changes between year 1 and 9 years				
Neuropsychological				
SDMT	1.50 ± 5.13	0.13 ± 5.94	2.47 ± 4.31	0.156
CVLT-II TL	5.72 ± 7.27	4.75 ± 8.17	6.41 ± 6.61	0.434
BVMT-R TL	3.00 ± 4.65	3.25 ± 5.04	2.82 ± 4.41	0.516
PASAT-3	0.11 ± 5.14	0.09 ± 5.31	0.12 ± 5.10	0.250
BDI	1.72 ± 5.64	2.95 ± 3.98	0.97 ± 6.38	0.160

Note. Unless otherwise indicated, mean ± standard deviation (SD) are reported. ‡ median (inter-quartile range). Differences between the pathological sNfL levels group and normal sNfL levels group were tested using the Chi-Square Test (categorical)* and Mann–Whitney rank-sum test (other).
sNfL: serum neurofilament light chain, EDSS: Expanded Disability Status Scale, SDMT: Symbol Digit Modalities Test, DMTs: disease modifying therapies, CVLT-II: California Verbal Learning Test - Second Edition, TL: total learning, BVMT-R: Brief Visuospatial Memory Test-Revised, TL: total learning, PASAT-3: Paced Auditory Serial Addition Test- 3 s, BDI: Beck Depression Inventory.
** Proportion of low efficacy DMTs (glatiramer-acetate, dimethyl fumarate, interferons and teriflunomide) to high or moderately high efficacy DMTs (fingolimod, natalizumab and rituximab).

linear regression models using sNfL levels as a continuous variable.

Higher sNfL levels were not associated with increased risk of cognitive decline, defined as a composite measure (an average z-score of all cognitive tests).

In mixed model analysis, we found only a trend for association between sNfL level at 12 months and evolution of BVMT-R scores over follow-up (p = 0.04).

3.4. The association between neuropsychological performance and csf-nfl levels

The mean concentration of CSF-NfL in 36 patients with available CSF samples was 1501,81 ± 1102.73 pg/ml. We observed a weak negative association between CVLT-II at year 1 and CSF-NfL levels at screening (rho = −0.34; p = 0.042; q = 0.120). There was only a trend for association among higher CSF-NfL levels at screening and lower SDMT scores at baseline and year 1 (rho = −0.30 to −0.32; p = 0.060–0.074; q = 0.132–0,148). We did not observe any relationship between CSF-NfL levels at screening and higher risk of cognitive decline over 9 years.

3.5. The association between neuropsychological performance and mri measures

T2LV at baseline, at year 1 and 2 (n = 58), was not associated with any cognitive tests at year 9 (Supplementary Table 4). We did not observe a relationship between early T2LV and higher risk of cognitive decline over 9 years.

BPF at baseline, at year 1 and 2 (n = 58), was not associated with any cognitive tests at year 9. There was only a cross-sectional association between BPF and BVMT-R at baseline (rho = 0.25; p = 0.044). We also observed a tendency towards an association between lower BPF at baseline and BVMT-R decline at year 9 (OR 2.03; 95% 0.9–4.6; p = 0.089). No other associations between early MRI measures and risk of cognitive decline over 9 years were found.

4. Discussion

In this study, we investigated the predictive value of sNfL levels at disease onset for identification of MS patients with cognitive decline over 9-years follow-up.

Our small sample size longitudinal study found only a trend for weak association between sNfL levels at 1 year and verbal memory decline over 9 years. However, this association was not observed for

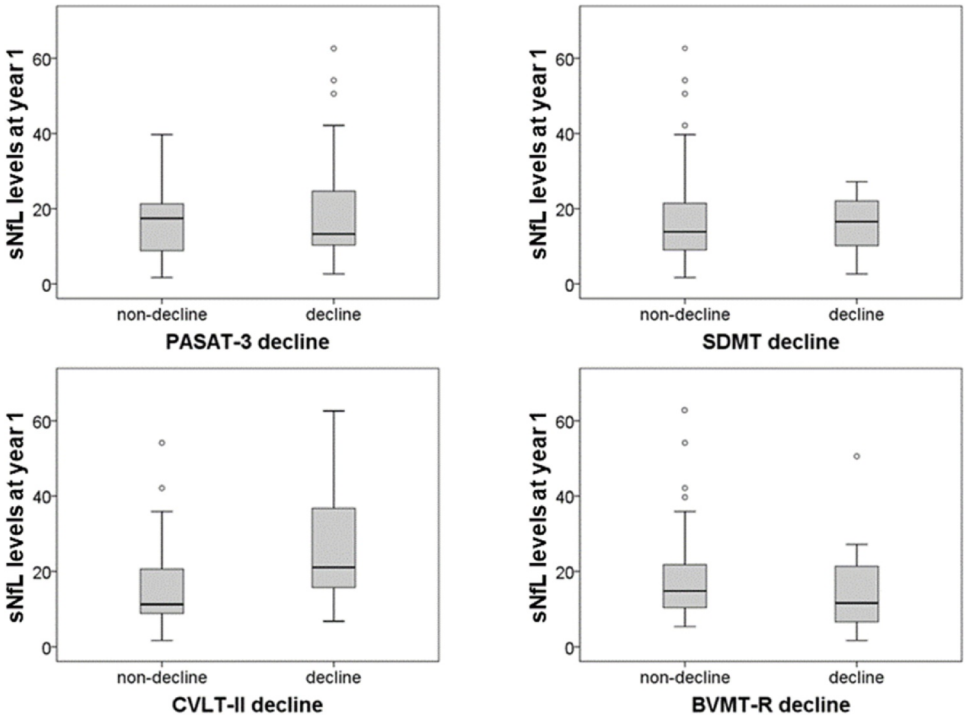


Fig. 1. Box plots corresponding to sNfL levels at year 1 (pg/ml) stratified by absolute decline in cognitive tests over the 9 years (Captions: PASAT-3: Paced Auditory Serial Addition Test- 3 s; SDMT: Symbol Digit Modalities Test; CVLT-II: California Verbal Learning Test - second edition; BVMT-R: Brief Visuospatial Memory Test – Revised; sNfL: serum neurofilament light chain).

sNfL levels measured at screening or at 2 years, for average sNfL levels during the first 2 years, neither in longitudinal mixed model analysis.

Taken together, our findings are consistent with a previous sNfL longitudinal study which unexpectedly also did not show a clear association between sNfL levels and cognitive decline over time (Chitnis et al., 2018). In our study, all patients had CSF restricted oligoclonal bands. This might select patients with slightly increased disease activity (Arrambide et al., 2018). On the other hand, an average sNfL level at disease onset in our cohort was lower (19.1 pg/ml) compared with some recent studies in CIS patients (the median sNfL level = 22.0 - 28.39 pg/ml) (Dalla Costa et al., 2019; Siller et al., 2018). To the best of our knowledge, there is only one study conducted on 20 CIS patients at disease onset with available sNfL and long-term cognitive data (Jakimovski et al., 2019). Compared with this study, an average sNfL levels were comparable (median 19.9 vs. 19.1 pg/ml) and similarly no associations between cognitive performance over 5 years and sNfL were observed in this study (Jakimovski et al., 2019). Disability scores at baseline, as assessed by EDSS were also comparable between both studies (1.5 vs 1.5 points). We hypothesize that in future studies

Table 3
Risk of absolute decline in cognitive tests raw scores between year 1 and year 9 in association with increased serum neurofilament light chain levels (>90th percentile) at year 1.

Neuropsychological Test	OR (95% CI)	*p* value	*q* value[1]
SDMT	1.88 (0.49–7.21)	0.360	0.480
CVLT-II TL	15.80 (1.70–147.01)	0.015	0.060
BVMT-R TL	1.46 (0.27–8.01)	0.661	0.661
PASAT-3	0.40 (0.11–1.43)	0.158	0.316

Note: OR: Odds Ratio, 95% CI: 95% Confidence Interval, SDMT: Symbol Digit Modalities Test, CVLT-II: California Verbal Learning Test – Second Edition, BVMT-R: Brief Visuospatial Memory Test-Revised, TL: total learning, PASAT-3: Paced Auditory Serial Addition Test- 3 s.
OR was estimated using logistic regression model adjusted for age, sex, treatment over follow-up and cognitive performance at 1 year.
[1] adjusted *p* value after The Benjamini-Hochberg procedure with $q = 0.05$ was used to control false discovery rate.

Table 2
The overall prevalence of cognitive decline over 9 years in association with increased serum neurofilament light chain levels (>90th percentile) at screening (A) and 1 year (B).

A)	sNfL level > 90th percentile at screening ($n = 28$)		sNfL level < 90th percentile at screening ($n = 21$)		*p* value	*q* value[1]
	Cognitive decline	No cognitive decline	Cognitive decline	No cognitive decline		
SDMT	3 (11%)	25 (89%)	2 (10%)	19 (90%)	0.892	0.892
CVLT-II TL	10 (36%)	18 (64%)	9 (43%)	12 (57%)	0.612	0.828
BVMT-R TL	7 (25%)	21 (75%)	4 (19%)	17 (81%)	0.621	0.828
PASAT-3*	3 (11%)	25 (89%)	6 (30%)	14 (70%)	0.091	0.364
B)	sNfL level > 90th percentile at year 1 ($n = 24$)		sNfL level < 90th percentile at year 1 ($n = 34$)			
	Cognitive decline	No cognitive decline	Cognitive decline	No cognitive decline		
SDMT	8 (33%)	16 (67%)	8 (24%)	26 (76%)	0.411	0.548
CVLT-II TL	9 (37%)	15 (63%)	3 (9%)	31 (91%)	0.008	0.032
BVMT-R TL	5 (21%)	19 (79%)	7 (21%)	27 (79%)	0.982	0.982
PASAT-3*	9 (39%)	14 (61%)	18 (53%)	16 (47%)	0.306	0.548

Note: absolute numbers and percentages (%) are reported. sNfL: serum neurofilament light chain, SDMT: Symbol Digit Modalities Test, CVLT-II: California Verbal Learning Test – Second Edition, BVMT-R: Brief Visuospatial Memory Test-Revised, TL: total learning, PASAT-3: Paced Auditory Serial Addition Test- 3 s.
[1] adjusted *p* value after The Benjamini-Hochberg procedure with $q = 0.05$ was used to control false discovery rate.
* missing data for one patient in PASAT-3.

including patients with higher disease activity or greater disease burden could be expected stronger association between cognitive and other paraclinical measures, such as sNfL.

The presence of high sNfL levels at early disease stages without accompanying future cognitive decline may be explained by several factors. Firstly, the cognitive-pathologic dissociation with an absence of relationship between cognitive and paraclinical markers is well known from the imagining studies (Uher et al., 2018; Sumowski and Leavitt, 2013). This phenomenon is obvious especially in the early stages of MS and may be attributed to brain and cognitive reserves preventing measurable cognitive decline in most patients during the early disease stages. Moreover, the study patients had very low levels of disability even after 9 years of follow-up (median EDSS = 2.0). We suggest that, as in imaging studies (Uher et al., 2018), the association between paraclinical markers such as sNfL and disease activity measures including cognitive outcomes might be substantially stronger in more advanced disease stages. Moreover, an effect of disease-modifying treatment might play a role in preventing of disease progression in patients with high sNfL levels before treatment initiation. Dynamic character of neurofilaments levels with only transient increase of neurofilament levels following neuro-axonal injury may represent another factor explaining our results (Lycke et al., 1998). Finally, our study had a small sample size which together with a small proportion of patients with evident cognitive deterioration over follow-up resulted in decreased statistical power to prove statistically significant associations between sNfL and longitudinal cognitive measures. These arguments are also supported by the absence of association between early MRI measures and cognition after 9 years or early sNfL levels. Hence, future studies investigating a predictive role of sNfL levels in early MS should include larger sample size.

Our study has also some other limitations. The cognitive assessment included only information processing speed and memory examination. However, information processing speed and memory are known to be the most commonly impaired cognitive domains in MS (Benedict et al., 2012; Chiaravalloti and DeLuca, 2008), therefore implementation of more comprehensive neuropsychological batteries, such as the Minimal Assessment of Cognitive Function in MS (MACFIMS) would probably lead to very similar results. Despite that, future studies would benefit from more complex cognitive assessment.

In conclusion, although we found some trends for association between high sNfL levels at disease onset and cognitive decline over subsequent long-term follow-up, our results do not provide convincing support for this association. Whether associations between high sNfL levels and verbal memory decline will be observed also in other studies or represent rather random finding remains to be elucidated in future research.

Role of funding source

This study was funded by the Charles University Grant Agency (GAUK) 1154218, Czech Ministry of Education project Progres Q27/LF1, Czech Ministry of Health project RVO-VFN64165, and AZV grant NV18-08-00062. The SET study was supported by Biogen. Statistical analysis was also supported by Sanofi research grant (GZ-2017-11718).

Declaration of Competing Interest

Lucie Friedova received compensation for traveling, conference fees and speaker honoraria from Biogen and Roche.

Jiri Motyl received compensation for traveling, conference fees and speaker honoraria from Sanofi Genzyme, Biogen, Novartis and Merck.

Barbora Srpova received compensation for traveling and conference fees from Novartis, Sanofi Genzyme, Biogen Idec, Roche and Merck as well as support for research activities from Biogen. Johanna Oechtering received travel grants from Bayer, Novartis, and Biogen Idec.

Christian Barro received conference travel grant from Teva and Novartis.

Karolina Vodehnalova received compensation for traveling, conference fees and consulting fees from Merck, Sanofi Genzyme, Biogen Idec, Novartis.

Michaela Andelova received financial support for conference travel from Novartis, Genzyme, Merck Serono, Biogen Idec and Roche.

Libuse Noskova and Lenka Fialova report no disclosures.

Eva Kubala Havrdova received speaker honoraria and consultant fees from Biogen Idec, Merck Serono, Novartis, Genzyme and Teva, as well as support for research activities from Biogen Idec and Merck Serono.

Dana Horakova received compensation for travel, speaker honoraria, and consultant fees from Biogen Idec, Novartis, Merck, Bayer, Sanofi Genzyme, Roche and Teva, as well as support for research activities from Biogen Idec.

Ralph HB Benedict received personal compensation from Neurocog Trials, Genentech, Roche, Takeda, Abbvie, Novartis, Sanofi, and EMD Serono for speaking and consultant fees.

Jens Kuhle received speaker fees, research support, travel support, and/or served on advisory boards by ECTRIMS, Swiss MS Society, Swiss National Research Foundation, (320,030_160,221), University of Basel, Bayer, Biogen, Genzyme, Merck, Novartis, Protagen AG, Roche, Teva.

Tomas Uher received financial support for conference travel from Biogen Idec, Novartis, Sanofi, Roche and Merck Serono and speaker honoraria from Biogen Idec, Novartis and Roche as well as support for research activities from Biogen Idec and Sanofi.

We certify that all co-authors fulfill the ICMJE authorship definition and have approved the submitted manuscript. The submission is not under review at any other publication.

CRediT authorship contribution statement

Lucie Friedova: Writing - original draft, Formal analysis. **Jiri Motyl:** Investigation, Project administration. **Barbora Srpova:** Funding acquisition, Resources. **Johanna Oechtering:** Data curation, Validation. **Christian Barro:** Data curation, Validation. **Karolina Vodehnalova:** Investigation, Writing - review & editing. **Michaela Andelova:** Investigation, Writing - review & editing. **Libuse Noskova:** Investigation, Formal analysis. **Lenka Fialová:** Investigation, Formal analysis. **Eva Kubala Havrdova:** Writing - review & editing, Supervision. **Dana Horakova:** Funding acquisition, Writing - review & editing, Supervision. **Ralph HB Benedict:** Supervision, Writing - review & editing. **Jens Kuhle:** Supervision. **Tomas Uher:** Conceptualization, Methodology, Writing - review & editing.

Acknowledgments

The project was supported by Czech Ministry of Education project PROGRES-Q27/LF1, the Charles University Grant Agency (GAUK) 230217, Czech Ministry of Health project RVO-VFN64165 and grant NV18–04–00168. Part of the project was funded through a Biogen Sponsored Research Agreement.

Lucie Friedova received compensation for traveling, conference fees and speaker honoraria from Biogen and Roche. Jiri Motyl received compensation for traveling, conference fees and speaker honoraria from Sanofi Genzyme, Biogen, Novartis and Merck. Barbora Srpova received compensation for traveling and conference fees from Novartis, Sanofi Genzyme, Biogen Idec, Roche and Merck as well as support for research activities from Biogen. Johanna Oechtering received travel grants from Bayer, Novartis, and Biogen Idec. Christian Barro received conference travel grant from Teva and Novartis. Karolina Vodehnalova received compensation for traveling, conference fees and consulting fees from Merck, Sanofi Genzyme, Biogen Idec, Novartis. Michaela Andelova received financial support for conference travel from Novartis, Genzyme, Merck Serono, Biogen Idec and Roche. Libuse Noskova and Lenka Fialova report no disclosures. Eva Kubala Havrdova received speaker

honoraria and consultant fees from Biogen Idec, Merck Serono, Novartis, Genzyme and Teva, as well as support for research activities from Biogen Idec and Merck Serono. Dana Horakova received compensation for travel, speaker honoraria, and consultant fees from Biogen Idec, Novartis, Merck, Bayer, Sanofi Genzyme, Roche and Teva, as well as support for research activities from Biogen Idec. Ralph HB Benedict received personal compensation from Neurocog Trials, Genentech, Roche, Takeda, Abbvie, Novartis, Sanofi, and EMD Serono for speaking and consultant fees. Jens Kuhle received speaker fees, research support, travel support, and/or served on advisory boards by ECTRIMS, Swiss MS Society, Swiss National Research Foundation, (320030_160221), University of Basel, Bayer, Biogen, Genzyme, Merck, Novartis, Protagen AG, Roche, Teva. Tomas Uher received financial support for conference travel from Biogen Idec, Novartis, Sanofi, Roche and Merck Serono and speaker honoraria from Biogen Idec, Novartis and Roche as well as support for research activities from Biogen Idec and Sanofi.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.msard.2020.102534.

References

Benedict, R.H.B., DeLuca, J., Enzinger, C., Geurts, J.J.G., Krupp, L.B., Rao, S.M., 2017. Neuropsychology of multiple sclerosis: looking back and moving forward. J. Int. Neuropsychol. Soc. https://doi.org/10.1017/S1355617717000959.

J.F. Sumowski, R. Benedict, C. Enzinger, M. Filippi, J.J. Geurts, P. Hamalainen, H. Hulst, M. Inglese, V.M. Leavitt, M.A. Rocca, E.M. Rosti-otajarvi, S. Rao, State of the field and priorities for the future, (2018). doi:10.1212/WNL.0000000000004977.

Uher, T., Krasensky, J., Sobisek, L., Blahova Dusankova, J., Seidl, Z., Kubala Havrdova, E., Sormani, M.P., Horakova, D., Kalincik, T., Vaneckova, M., 2018. Cognitive clinico-radiological paradox in early stages of multiple sclerosis. Ann. Clin. Transl. Neurol. 5, 81–91. https://doi.org/10.1002/acn3.512.

Comabella, M., Montalban, X., 2014. Body fluid biomarkers in multiple sclerosis. Lancet Neurol 13, 113–126. https://doi.org/10.1016/S1474-4422(13)70233-3.

Kuhle, J., Barro, C., Disanto, G., Mathias, A., Soneson, C., Bonnier, G., Yaldizli, Ö., Regeniter, A., Derfuss, T., Canales, M., Schluep, M., R. Du Pasquier, Krueger, G., Granziera, C., 2016a. Serum neurofilament light chain in early relapsing remitting MS is increased and correlates with CSF levels and with MRI measures of disease severity. Mult. Scler. 22, 1550–1559. https://doi.org/10.1177/1352458515623365.

Barro, C., Benkert, P., Disanto, G., Tsagkas, C., Amann, M., Naegelin, Y., Leppert, D., Gobbi, C., Granziera, C., Yaldizli, O., Michalak, Z., Wuerfel, J., Kappos, L., Parmar, K., Kuhle, J., 2018. Serum neurofilament as a predictor of disease worsening and brain and spinal cord atrophy in multiple sclerosis. Brain 141, 2382–2391. https://doi.org/10.1093/brain/awy154.

Disanto, G., Barro, C., Benkert, P., Naegelin, Y., Schädelin, S., Giardiello, A., Zecca, C., Blennow, K., Zetterberg, H., Leppert, D., Kappos, L., Gobbi, C., Kuhle, J., 2017. Serum Neurofilament light: a biomarker of neuronal damage in multiple sclerosis. Ann. Neurol. 81, 857–870. https://doi.org/10.1002/ana.24954.

Gaetani, L., Salvadori, N., Lisetti, V., Eusebi, P., Mancini, A., Gentili, L., Borrelli, A., Portaccio, E., Sarchielli, P., Blennow, K., Zetterberg, H., Parnetti, L., Calabresi, P., Filippo, M.Di, 2019. Cerebrospinal fluid neurofilament light chain tracks cognitive impairment in multiple sclerosis. J. Neurol. https://doi.org/10.1007/s00415-019-09398-7.

Kalatha, T., Arnaoutoglou, M., Koukoulidis, T., Hatzifilippou, E., Bouras, E., Baloyannis, S., Koutsouraki, E., 2019. Does cognitive dysfunction correlate with neurofilament light polypeptide levels in the CSF of patients with multiple sclerosis? J. Int. Med. Res., 030006051984055. https://doi.org/10.1177/0300060519840550.

Quintana, E., Coll, C., Salavedra-Pont, J., Muñoz-San Martín, M., Robles-Cedeño, R., Tomàs-Roig, J., Buxó, M., Matute-Blanch, C., Villar, L.M., Montalban, X., Comabella, M., Perkal, H., Gich, J., Ramió-Torrentà, L., 2018. Cognitive impairment in early stages of multiple sclerosis is associated with high cerebrospinal fluid levels of chitinase 3-like 1 and neurofilament light chain. Eur. J. Neurol. 25, 1189–1191. https://doi.org/10.1111/ene.13687.

Modvig, S., Degn, M., Roed, H., Sørensen, T.L., Larsson, H.B.W., Langkilde, A.R., Frederiksen, J.L., Sellebjerg, F., 2015. Cerebrospinal fluid levels of chitinase 3-like 1 and neurofilament light chain predict multiple sclerosis development and disability after optic neuritis. Mult. Scler. 21, 1761–1770. https://doi.org/10.1177/1352458515574148.

Chitnis, T., Gonzalez, C., Healy, B.C., Saxena, S., Rosso, M., Barro, C., Michalak, Z., Paul, A., Kivisakk, P., Diaz-Cruz, C., Sattarnezhad, N., Pierre, I.V., Glanz, B.I., Tomic, D., Kropshofer, H., Häring, D., Leppert, D., Kappos, L., Bakshi, R., Weiner, H.L., Kuhle, J., 2018. Neurofilament light chain serum levels correlate with 10-year MRI outcomes in multiple sclerosis. Ann. Clin. Transl. Neurol. 5, 1478–1491. https://doi.org/10.1002/acn3.638.

D. Jakimovski, R. Zivadinov, M. Ramanthan, J. Hagemeier, B. Weinstock-guttman, D. Tomic, H. Kropshofer, T.A. Fuchs, C. Barro, D. Leppert, Ö. Yaldizli, J. Kuhle, R.H.B. Benedict, Serum neurofilament light chain level associations with clinical and cognitive performance in multiple sclerosis : a longitudinal retrospective 5-year study, (2019). doi:10.1177/1352458519881428.

Comi, G., Patti, F., Rocca, M.A., Mattioli, F.C., Amato, M.P., Gallo, P., Centonze, D., Pozzilli, C., Saccà, F., Bergh, F.T., Bartezaghi, M., Turrini, R., Filippi, M., 2017. Efficacy of fingolimod and interferon beta-1b on cognitive, MRI, and clinical outcomes in relapsing–remitting multiple sclerosis: an 18-month, open-label, rater-blinded, randomised, multicentre study (the GOLDEN study). J. Neurol. 264, 2436–2449. https://doi.org/10.1007/s00415-017-8642-5.

Gudesblatt, M., Wissemann, K., Zarif, M., Bumstead, B., Fafard, L., Wilken, J., Blitz, K., Buhse, M., Santra, S., Hotermans, C., Lee, L., 2018. Improvement in cognitive function as measured by neurotrax in patients with relapsing multiple sclerosis treated with natalizumab: a 2-year retrospective analysis. CNS Drugs 32, 1173–1181. https://doi.org/10.1007/s40263-018-0553-1.

Kalincik, T., Vaneckova, M., Tyblova, M., Krasensky, J., Seidl, Z., Havrdova, E., Horakova, D., 2012. Volumetric MRI markers and predictors of disease activity in early multiple sclerosis: a longitudinal cohort study. PLoS ONE 7, e50101. https://doi.org/10.1371/journal.pone.0050101.

Uher, T., Blahova-Dusankova, J., Horakova, D., Bergsland, N., Tyblova, M., Benedict, R.H.B., Kalincik, T., Ramasamy, D.P., Seidl, Z., Hagermeier, J., Vaneckova, M., Krasensky, J., Havrdova, E., Zivadinov, R., 2014. Longitudinal MRI and neuropsychological assessment of patients with clinically isolated syndrome. J. Neurol. 261, 1735–1744. https://doi.org/10.1007/s00415-014-7413-9.

Polman, C.H., Reingold, S.C., Edan, G., Filippi, M., Hartung, H.-P., Kappos, L., Lublin, F.D., Metz, L.M., McFarland, H.F., O'Connor, P.W., Sandberg-Wollheim, M., Thompson, A.J., Weinshenker, B.G., Wolinsky, J.S., 2005. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria. Ann. Neurol. 58, 840–846. https://doi.org/10.1002/ana.20703.

Thompson, A.J., Banwell, B.L., Barkhof, F., Carroll, W.M., Coetzee, T., Comi, G., Correale, J., Fazekas, F., Filippi, M., Freedman, M.S., Fujihara, K., Galetta, S.L., Hartung, H.P., Kappos, L., Lublin, F.D., Marrie, R.A., Miller, A.E., Miller, D.H., Montalban, X., Mowry, E.M., Sorensen, P.S., Tintoré, M., Traboulsee, A.L., Trojano, M., Uitdehaag, B.M.J., Vukusic, S., Waubant, E., Weinshenker, B.G., Reingold, S.C., Cohen, J.A., 2018. Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. Lancet Neurol 17, 162–173. https://doi.org/10.1016/S1474-4422(17)30470-2.

Zivadinov, R., Havrdová, E., Bergsland, N., Tyblova, M., Hagemeier, J., Seidl, Z., Dwyer, M.G., Vaneckova, M., Krasensky, J., Carl, E., Kalincik, T., Horáková, D., 2013. Thalamic atrophy is associated with development of clinically. Radiology 268, 831–841. https://doi.org/10.1148/radiol.13122424/-/DC1.

Uher, T., Horakova, D., Kalincik, T., Bergsland, N., Tyblova, M., Ramasamy, D.P., Seidl, Z., Vaneckova, M., Krasensky, J., Havrdova, E., Zivadinov, R., 2015. Early magnetic resonance imaging predictors of clinical progression after 48 months in clinically isolated syndrome patients treated with intramuscular interferon β-1a. Eur. J. Neurol. 22, 1113–1123. https://doi.org/10.1111/ene.12716.

C.E. Teunissen, A. Petzold, J.L. Bennett, F.S. Berven, L. Brundin, M. Comabella, D. Franciotta, J.L. Frederiksen, J.O. Fleming, R. Furlan, R.Q. Hintzen, S.G. Hughes, M.H. Johnson, D.E. Krasulova, J. Kuhle, M.C. Magnone, C. Rajda, K. Rejdak, H.K. Schmidt, V. Van Pesch, E. Waubant, C. Wolf, G. Giovannoni, B. Hemmer, H. Tumani, F. Deisenhammer, A consensus protocol for the standardization of cerebrospinal fluid collection and biobanking Supplemental data at www.neurology.org VIEWS & REVIEWS, (2009). www.neurology.org.

Kuhle, J., Barro, C., Andreasson, U., Derfuss, T., Lindberg, R., Sandelius, A., Liman, V., Norgren, N., Blennow, K., Zetterberg, H., 2016b. Comparison of three analytical platforms for quantification of the neurofilament light chain in blood samples: ELISA, electrochemiluminescence immunoassay and Simoa. Clin. Chem. Lab. Med. 54, 1655–1661. https://doi.org/10.1515/cclm-2015-1195.

Srpova, B., Uher, T., Hrnciarova, T., Barro, C., Andelova, M., Michalak, Z., Vaneckova, M., Krasensky, J., Noskova, L., Havrdova, E.K., Kuhle, J., Horakova, D., 2020. Serum neurofilament light chain reflects inflammation-driven neurodegeneration and predicts delayed brain volume loss in early stage of multiple sclerosis. Mult. Scler. J. 1–9. https://doi.org/10.1177/1352458519901272.

Benedict, R.H.B., Amato, M.P., Boringa, J., Brochet, B., Foley, F., Fredrikson, S., Hamalainen, P., Hartung, H., Krupp, L., Penner, I., Reder, A.T., Langdon, D., 2012. Brief International Cognitive Assessment for MS (BICAMS): international standards for validation. BMC Neurol 12, 1. https://doi.org/10.1186/1471-2377-12-55.

Dusankova, J.B., Kalincik, T., Havrdova, E., Benedict, R.H.B., 2012. Cross cultural validation of the minimal assessment of cognitive function in multiple sclerosis (MACFIMS) and the brief international cognitive assessment for multiple sclerosis (BICAMS). Clin. Neuropsychol. 26, 1186–1200. https://doi.org/10.1080/13854046.2012.725101.

Rao, S.M., 1990. A Manual For the Brief Repeatable Battery of Neuropsychological Tests in Multiple Sclerosis. National Multiple Sclerosis Society, New York.

Smith, A., 1982. Symbol Digit Modalities Test (SDMT): Manual. Western Psychological Services, Los Angeles, CA.

Delis, D.C., Kramer, J.H., Kaplan, E., Ober, B.A., 2000. California Verbal Learning Test – second edition. Adult version. Manual, Psychological Corporation, San Antonio, TX.

Benedict, R.H., 1997. Brief Visuospatial Memory Test - Revised: Professional Manual.

Uher, T., Krasensky, J., Vaneckova, M., Sobisek, L., Seidl, Z., Havrdova, E., Bergsland, N., Dwyer, M.G., Horakova, D., Zivadinov, R., 2017. A novel semiautomated pipeline to measure brain atrophy and lesion burden in multiple sclerosis: a long term comparative study. J Neuroimaging. https://doi.org/10.1111/jon.12445.

Arrambide, G., Tintore, M., Espejo, C., Auger, C., Castillo, M., Río, J., Castilló, J., Vidal-Jordana, A., Galán, I., Nos, C., Mitjana, R., Mulero, P., de Barros, A., Rodríguez-Acevedo, B., Midaglia, L., Sastre-Garriga, J., Rovira, A., Comabella, M., Montalban, X., 2018. The value of oligoclonal bands in the multiple sclerosis diagnostic criteria.

Brain 141, 1075–1084. https://doi.org/10.1093/brain/awy006.
Dalla Costa, G., Martinelli, V., Sangalli, F., Moiola, L., Colombo, B., Radaelli, M., Leocani, L., Furlan, R., Comi, G., 2019. Prognostic value of serum neurofilaments in patients with clinically isolated syndromes. Neurology 92https://doi.org/10.1212/WNL.0000000000006902. e733 LP-e741.
Siller, N., Kuhle, J., Muthuraman, M., Barro, C., Uphaus, T., Groppa, S., Kappos, L., Zipp, F., Bittner, S., 2018. Serum neurofilament light chain is a biomarker of acute and chronic neuronal damage in early multiple sclerosis. Mult. Scler. J. 25, 678–686. https://doi.org/10.1177/1352458518765666.
Sumowski, J.F., Leavitt, V.M., 2013. Cognitive reserve in multiple sclerosis. Mult. Scler. J. https://doi.org/10.1177/1352458513498834.
Lycke, J.N., Karlsson, J.E., Andersen, O., Rosengren, L.E., 1998. Neurofilament protein in cerebrospinal fluid: a potential marker of activity in multiple sclerosis. J. Neurol. Neurosurg. Psychiatry. 64, 402–404 http://www.ncbi.nlm.nih.gov/pubmed/9527161%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2170011.
Chiaravalloti, N.D., DeLuca, J., 2008. Cognitive impairment in multiple sclerosis. Lancet Neurol 7, 1139–1151. https://doi.org/10.1016/S1474-4422(08)70259-X.

P.09-155-Tue
Antineurocytoskeletal antibodies and their immune complexes in patients with neurodegenerative diseases

L. Nosková[1], L. Fialová[1], A. Bartoš[2]
[1]*Institute of Medical Biochemistry and Laboratory Diagnostics, 1st Faculty of Medicine, Charles University, Prague 2, Czech Republic,* [2]*Department of Cognitive Disorders, National Institute of Mental Health, Klecany, Czech Republic*

Neurocytoskeletal proteins, such as neurofilaments (Nf), may be released from neurons during a neurodegenerative process and induce the synthesis of specific autoantibodies. These antibodies could form immune complexes with corresponding antigens. The autoantibodies are present both in a free form and bound in immune complexes. An analysis of free antibodies alone cannot give the information on their total production. The aim of our study was to introduce and optimise the ELISA (Enzyme-Linked Immuno-Sorbent Assay) method for the determination of specific immune complexes of IgG antibodies against the heavy subunit of neurofilament (NfH) with the corresponding neurofilament subunit. Levels of anti-NfH antibodies and immune complexes determined by our in-house ELISA method were expressed in the same arbitrary units of concentration using the commercial calibrator for immunoglobulins. We evaluated this method on the pilot groups of patients in serum and cerebrospinal fluid (CSF) samples. Simultaneous determination of free antibodies against the heavy subunit of neurofilament and corresponding immune complexes have been performed in patients with mild cognitive impairment (MCI), Alzheimer's disease (AD) and age-matched control subjects. Levels of free antibodies and antibodies bound in immune complexes were significantly lower in patients with MCI than in the patients with AD and control subjects. Significant differences between levels of free antibodies and antibodies bound in the immune complexes were observed especially in CSF. Our ELISA method is suitable for analysis of both serum and CSF. The parallel analysis of free anti-Nf antibodies and their immune complexes could be evaluated together and provide more complex information about autoantibody response against neurocytoskeletal proteins in neurodegenerative diseases. The study is supported by PROGRES Q25/LF1 and RVO-VFN64165.

FEBS Open Bio **8** (Suppl. S1) (2018) 107–496 DOI: 10.1002/2211-5463.12453

P094 Protilátky proti neurofilamentům u pacientů s neurodegenerativním onemocněním

Nosková L[1], Fialová L[1], Bartoš A[2,3]

[1] *Ústav lékařské biochemie a laboratorní diagnostiky, 1. LF UK a VFN v Praze*

[2] *Oddělení kognitivních poruch, Národní ústav duševního zdraví, Klecany*

[3] *Neurologická klinika 3. LF UK a FN Královské Vinohrady, Praha*

Úvod: V průběhu neurodegenerativního procesu dochází k uvolnění neurocytoskeletálních proteinů (typicky neurofilamenta) do oběhu. Uvolněné proteiny mohou indukovat syntézu specifických autoprotilátek, které by mohly být dalšími potenciálními biomarkery pro neurodegenerativní onemocnění. Autoprotilátky jsou v oběhu přítomny jako volné molekuly a také ve formě imunokomplexů s odpovídajícím antigenem. Vyšetření pouze volných protilátek tedy neposkytuje komplexní informaci o autoprotilátkách u neurodegenerativních onemocnění.

Cíl: Stanovit hladiny volných autoprotilátek i odpovídajících imunokomplexů s neurofilamenty v mozkomíšním moku (MM) a séru pacientů s neurodegenerativním onemocněním pomocí modifikované a optimalizované metody ELISA a zhodnotit možný klinický přínos tohoto vyšetření.

Metodika: Námi optimalizovaná metoda ELISA byla použita pro stanovení autoprotilátek proti těžkým řetězcům neurofilament (NfH) a imunokomplexů těchto protilátek s odpovídajícím antigenem. Pro možnost porovnání hladin volných i vázaných anti-NfH protilátek v biologických tekutinách byla využita jednotná kalibrace. Stanovení bylo provedeno párově v séru i MM u souboru 72 pacientů rozdělených na věkově si odpovídající skupinu kontrolních pacientů, pacientů s mírnou kognitivní poruchou (MKP) a Alzheimerovou chorobou (ACH).

Výsledky: Hladiny anti-NfH IgG volných protilátek i imunokomplexů v MM a séru byly signifikantně nižší u pacientů s MKP oproti kontrolní skupině i pacientům s ACH. V MM byly rozdíly více signifikantní. Byla prokázána vysoká korelace jak volných autoprotilátek, tak imunokomplexů mezi sérem a MM ($p < 0{,}001$). Významná byla i korelace volných autoprotilátek a odpovídajících imunokomplexů ($p < 0{,}05$), ale lišila se v rámci jednotlivým skupin.

Závěr: Optimalizovaná metoda ELISA je i díky jednotné kalibraci vhodná pro stanovení volných autoprotilátek i jejich imunokomplexů s daným antigenem. U pacientů s MKP je signifikantně snížená hladina anti-NfH IgG autoprotilátek jak volných, tak i ve formě imunokomplexů. Stanovení jak volných, tak vázaných autoprotilátek proti neurocytoskeletálním proteinům může vhodně doplnit informace o autoprotilátkové odpovědi u neurodegenerativních onemocnění.

Studie byla podpořena PROGRES Q25/LF1, PROGRES Q35, SVV 260373 a RVO-VFN64165.