Claus Vinter Bødker Hviid, Anne Tranberg Madsen and Anne Winther-Larsen*

Biological variation of serum neurofilament light chain

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Abstract

Objectives: The neurofilament light chain (NfL) has emerged as a versatile biomarker for CNS-diseases and is approaching clinical use. The observed changes in NfL levels are frequently of limited magnitude and in order to make clinical decisions based on NfL measurements, it is essential that biological variation is not confused with clinically relevant changes. The present study was designed to evaluate the biological variation of serum NfL.
Methods: Apparently healthy individuals (n=33) were submitted to blood draws for three days in a row. On the second day, blood draws were performed every third hour for 12 h. NfL was quantified in serum using the Simoa™ HD-1 platform. The within-subject variation (CV_I) and between-subject variation (CV_G) were calculated using linear mixed-effects models.
Results: The overall median value of NfL was 6.3 pg/mL (range 2.1–19.1). The CV_I was 3.1% and the CV_G was 35.6%. An increase in two serial measurements had to exceed 24.3% to be classified as significant at the 95% confidence level. Serum NfL levels remained stable during the day (p=0.40), whereas a minute variation (6.0–6.6 pg/mL) was observed from day-to-day (p=0.02).
Conclusions: Serum NfL is subject to tight homeostatic regulation with none or neglectable semidiurnal and day-to-day variation, but considerable between-subject variation exists. This emphasizes serum NfL as a well-suited biomarker for disease monitoring, but warrants caution when interpreting NfL levels in relation to reference intervals in a diagnosis setting. Furthermore, NfL's tight regulation requires that the analytical variation is kept at a minimum.

Keywords: biological variation; biomarker; monitoring; neurofilament proteins.

***Corresponding author: Anne Winther-Larsen**, MD, PhD, Department of Clinical Biochemistry, Aarhus University Hospital, Palle Juul-Jensens Boulevard 99, 8200 Aarhus, Denmark, Phone: +0045 7844 0000, E-mail: anlarsen@rm.dk
Claus Vinter Bødker Hviid, Department of Clinical Biochemistry, Aarhus University Hospital, Aarhus, Denmark; and Department of Clinical Biochemistry, Horsens Regional Hospital, Horsens, Denmark
Anne Tranberg Madsen, Department of Microbiology and Immunology, Albert Einstein College of Medicine, New York, NY, USA.
https://orcid.org/0000-0001-9146-5943

Introduction

Neurofilaments are cytoskeletal proteins that have emerged as versatile and sensitive biomarkers of neuron injury and are approaching clinical use as biomarkers [1–4]. These intermediate filament structures consist of a neurofilament-heavy, -medium, and -light chain (NfL), which are obligate intracellular and particularly abundant in high caliper myelinated axons [5]. The proteins are released to the extracellular environment upon neuron injury in quantities proportional to the amount of injured nervous tissue [6, 7]. Studies have documented solid biomarker capabilities of NfL measurement in diverse types of neurological conditions ranging from degenerative to traumatic disease [2–4, 8–11]. Furthermore, it appears that NfL may serve as a highly sensitive measure of neuron affection that enable biochemical detection of even mild traumatic brain injury [12, 13].

The implementation of NfL as a biomarker for routine clinical use has been facilitated by the development of ultra-sensitive assays, which allow detection of NfL in the peripheral circulation, as well as the consistent finding that plasma NfL levels closely reflect levels in the cerebrospinal fluid [2, 4, 10, 14–16]. As such, NfL represents a promising, versatile, and highly sensitive biochemical biomarker of neuron decay that can be measured directly in the blood. Compared with CSF sampling, this offers reduced invasiveness and increased feasibility, in addition to increased patient comfort with potential use for repeated monitoring in research as well as patient treatment.

While the evidence that support clinical use of NfL for monitoring, prediction, and prognostication is rapidly evolving, essential aspects regarding the biological behavior of the biomarker still remain to be clarified [1]. We recently established a reference interval for serum NfL and observed a wide reference interval among healthy individuals, which leads to speculations that NfL levels could be subject to considerable biological variation [17].

Understanding the magnitude of the between- and within-subject variation is essential in order to determine whether a NfL level change actually represents a clinically relevant change and not exclusively a natural dynamic owing to the biological variation. This is particularly true since studies have shown only small to moderate changes in NfL levels, especially for patient groups with more limited disease burden [13, 18].

The current study aimed to evaluate the magnitude of the biological variation of NfL in healthy individuals and, based on this, calculate the reference change value (RCV). This will help to elucidate how to interpret NfL analyses and assist the translation into clinical practice.

Materials and methods

Subjects

Apparently healthy individuals were recruited from June 2018 until October 2018 at Aarhus University Hospital, Denmark. The study population, inclusion, and exclusion criteria have previously been described in detail [19]. In short, the subjects were eligible for enrollment if they were non-smokers, non-pregnant, had no chronic infection or inflammation, and did not receive any medication. The study was conducted according to the Helsinki Declaration and approved by the Central Denmark Region Committees on Biomedical Research Ethics (1-10-72-452-17). Subjects provided written informed consent before inclusion.

Study design

Subjects were subjected to blood draws at 9 AM for three days in a row. On the second day, blood samples were also drawn every third hour for 12 h (12, 3, 6, and 9 PM). Sampling and processing of all blood samples were performed by the same four technicians. During the study period, subjects were not allowed to drink alcohol or engage in high-impact exercise defined as exercise harder than walking or low intensity bicycling. Furthermore, subjects had to refrain from physical activity and food an hour prior to each blood draw. The study was designed according to the checklists for biological variation studies [20, 21].

Blood samples

Ten ml serum were collected into serum tubes (BD vacutainer®) at each time point. After 30 min of incubation, the samples were centrifuged for 10 min at 1,800*g* at room temperature (22–24 °C). Subsequently, serum was isolated and frozen at −80 °C until further analysis.

Laboratory analysis

The quantification of NfL level has previously been described [11, 17]. In short, the NF-light® assay was established on the ultra-sensitive Simoa™ HD-1 platform (Quanterix©, Lexington, MA, USA) [14]. According to the manufacturer, the limit of detection and limit of quantitation for NfL are 0.038 pg/mL and 0.174 pg/mL, respectively. The calibrator range is 0–500 pg/mL with linearity from 4 to 128 times dilution. The intermediate precision of our assay on the levels 4.4 and 18.1 pg/mL are 12.9 and 11.2% [17]. Positive controls in two levels (3.63–5.71 and 125–187 pg/mL) are supplied by the manufacturer and were analyzed in duplicate in each run. To minimize analytical variance, all samples from each individual were batch analyzed, and all analyses were performed by a single analyst. Each sample was analyzed in duplicates.

Statistical analysis

The steady state of the subjects was evaluated using a linear regression of the median value for each blood drawing vs. the blood drawing number. Outlier analyses were performed on three levels; analytical, within-subject, and between-subject. Cochran's *C* test was used for determining analytical and within-subject outliers, and the Dixon-Reed criterion was used for between-subject outliers [22, 23]. Assessment for variance homogeneities of within-subject variability was performed by Bartlett's test on the ln transformed results. The analytical variation (CV_A) was estimated from duplicate results of every sample according to Fraser et al. [22]. The within-subject variation (CV_I) and between-subject variation (CV_G) were calculated using linear mixed effects models with day, sample, and age as fixed effects and subject as a random effect. Age was included in the model as a fixed effect as an approximately 3% increase in serum NfL level per year has been observed previously [17]. Adjusting for the age-dependent NfL increases provided almost identical results. The 95% confidence intervals (CIs) for the biological variation estimates were calculated as described by Røraas et al. [24]. Normality of the residuals was confirmed visually and using Shapiro–Wilk test. Data followed a ln-normal distribution and was thus transformed accordingly. Data is presented as median and range. Pair-wise comparisons of day-to-day and semidiurnal median values were adjusted for multiple comparisons with the Bonferroni correction. Index of individuality (II), and number of samples that are required to estimate a subject's homeostatic set point (n) within ±15% with 95% confidence were calculated using the following equations: $II = \sqrt{(CV_I^2 + CV_A^2)}/CV_G$; $n = (z \cdot \sqrt{(CV_I^2 + CV_A^2)}/D)^2$, where z is the z-score and D is the desired percentage closeness to the homeostatic set point according to ref. [22]. For ln-transformed data, RCV was calculated as $RCV = \exp(\pm z \cdot \sqrt{2} \cdot \sigma) - 1$, where $\sigma = \sqrt{\ln(CV_{I+A}^2 + 1)}$ [25]. $p<0.05$ was considered statistically significant. All statistical calculations were performed in STATA 14 (StataCorp).

Results

Description of the healthy subjects

A total of 184 blood samples were collected from 33 apparently healthy individuals during the study. Of these, 23 were women and the median age of all individuals was 39 years (range 22–66). Fourteen participants were subjected to blood draws on all seven time points, 14 participants were subjected to all but the 6 and 9 PM blood draws,

and one participant had all samples drawn except for the 3, 6, and 9 PM blood draws. Lastly, four participants only participated in the three 9 AM blood draws. No outliers were identified on the any of the three levels. All individuals were in steady state.

Components of variation

Median values, ranges, and variance components are presented in Table 1. The overall median value of NfL was 6.3 pg/mL (range 2.1–19.1). Figure 1 shows the individual median and range of NfL levels for all subjects. The CV_I was very low at 3.1% (95% CI 1.2–5.0) and much lower than the CV_G, which was 35.6% (95% CI 25.5–45.8). Consequently, the II was only 0.22. The CV_A was 7.2% (95% CI 6.6–7.9) and higher than the CV_I. The RCV for an increase in serum NfL was 24.3% at a 95% level of significance. One sample was found to be sufficient to provide an estimate of the homeostatic setting point within ±15% with 95% confidence. No difference in CV_A, CV_I, and CV_G were found between sexes (data not shown). If the variation components were calculated excluding age from the mixed model, CV_I (3.1% [95% CI 1.2–5.0]) and CV_G (46.2% [95% CI 35.4–57.0]) remained unchanged.

Day-to-day and semidiurnal variance

Day-to-day variance components were obtained for the 9 AM measurements on each of the three days. Overall, a systematic day-to-day difference was observed in serum NfL level (p=0.02). However, NfL levels on days 1, 2, and 3 were median 6.0 pg/mL (range 2.1–17.1), 6.2 pg/mL (range 2.3–16.7) and 6.6 pg/mL (range 2.6–17.5), respectively, and a significantly difference was found only between days 2 and 3 (p=0.03; Figure 2). Semidiurnal variance components were obtained for all the measurements on day 2 (9 AM, 12 PM, 3 PM, 6 PM, 9 PM). There was no significant difference in NfL levels during the day (p=0.40; Figure 3). The day-to-day and semidiurnal variance components were comparable to the total and are shown in Table 2.

Table 1: Components of biological variation.

	NfL
Number of subjects	33
Number of samples[a]	184
Median, pg/mL	6.3
Range, pg/mL	2.1–19.1
CV_I, %	3.1 (1.2–5.0)
CV_G, %	35.6 (25.5–45.8)
CV_A, %	7.2 (6.6–7.9)
II[b]	0.22
RCV[c], %	
Increasing	24.3
Decreasing	19.5
Samples required, n[d]	1

Values in parentheses are the 95% confidence intervals. [a]Number of samples analyzed after exclusion of outliers; [b]II, Index of individuality; [c]RCV, reference change value at 95% significance; [d]n, required to estimate homeostatic set point within 15% with 95% confidence. CV_I, within-subject coefficient of variation; CV_G, between-subject coefficient of variation; CV_A, analytical coefficient of variation; NfL, neurofilament light chain.

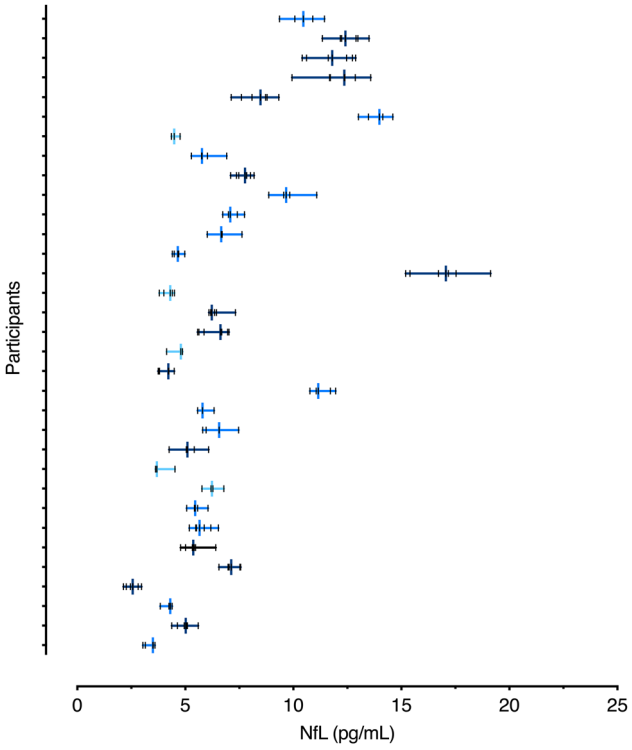

Figure 1: Neurofilament light chain (NfL) levels in apparently healthy subjects. Subjects are color-coded according to the number of samples they contributed with: dark blue, seven samples; blue, five samples; light blue, three samples and the blue vertical line illustrates the median value. The vertical black lines indicate each NfL level (mean value of duplicates) measured in the patient. The subjects are ordered according to age with the youngest subject at the bottom and the oldest subject at the top. The horizontal line illustrates the range from minimum to maximum.

Discussion

In this study, we investigated the biological variation of NfL in apparently healthy subjects. We found a minute within-subject variation resulting in a low reference change value and a neglectable day-to-day variation without semidiurnal

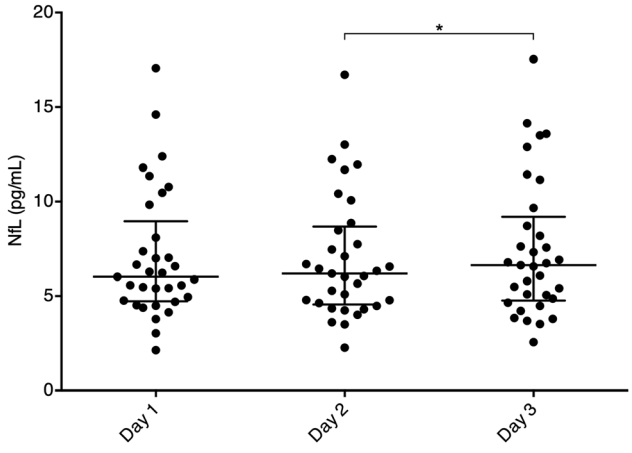

Figure 2: Day-to-day neurofilament light chain (NfL) levels. Individual results are shown with mean (longest horizontal line) and *interquartile range* (the smallest horizontal lines). Significant differences from a pairwise comparison are marked with *p<0.05.

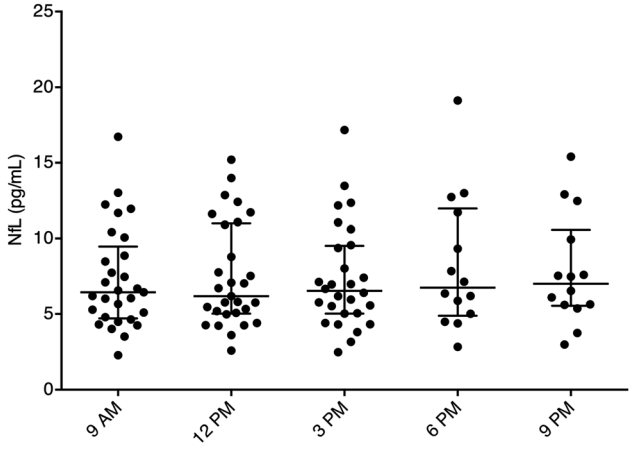

Figure 3: Within-day neurofilament light chain (NfL) levels on day 2. Individual results are shown with mean (longest horizontal line) and *interquartile range* (the smallest horizontal lines).

changes. In contrast to the tight within-subject variation, a considerable between-subject variation was observed. To our knowledge, this is the first study to evaluate the biological variation of NfL and it provides important clues to the interpretation of studies on NfL.

The within-subject variation is quantified by the CV_I which is defined as the fluctuation of a biomarker around a homeostatic set point in steady state conditions [22]. In our study, the CV_I for serum NfL was 3.1% only, which is comparable to the variation reported for hematologic parameters such as hemoglobin or hematocrit and much lower than the variation observed in parameters affectable by physiologic stress such as cardiac troponins [26, 27]. This clearly demonstrates that NfL levels are under tight homeostatic control, which is comprehendible

Table 2: Day-to-day and semidiurnal components of biological variation.

	NfL
Day-to-day	
Number of subjects	33
Day 1, pg/mL	6.0
Day 2 pg/mL	6.2
Day 3 pg/mL	6.6
CV_I, %	2.0 (–0.08–4.0)
CV_G, %	36.4 (26.1–46.6)
Semidiurnal	
Number of subjects	29
12 PM, pg/mL	6.2
03 PM, pg/mL	6.5
06 PM, pg/mL	6.7
09 PM, pg/mL	7.0
CV_I, %	3.8 (1.4–6.2)
CV_G, %	35.4 (25.0–45.9)

Day-to-day and semidiurnal cfDNA levels are presented as medians. Values in parentheses are the 95% confidence intervals. CV_I, within-subject coefficient of variation; CV_G, between-subject coefficient of variation.

for a cytoskeletal neuron-restricted protein. This low level of within-subject variation was further reflected in a modest RCV, which reached only 24.3% for an increase. The RCV express that the difference between two serial NfL measurements in one individual must vary more than 24.3% to represent a significant change that exceeds the natural variation. In comparison, the RCV is 30.2% for neuron-specific enolase and 29.3 % for S100-β protein [28]. This clearly suggests NfL to be an ideal biomarker for disease monitoring. While the exact mechanisms that lead to NfL liberation after neuron decay remain unknown, the increases observed are often profoundly above the threshold and there are strong indications that NfL levels reflect the extent of the neuron injury [6, 10, 12, 13, 18, 29]. As such, NfL represents a strong and versatile candidate to monitor neurological disease, but also calls for caution if the disease of interest is associated with more restricted neuron affection. A large number of studies are available on longitudinal monitoring of serum NfL and its relation to clinical disease development. In studies on neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease, and amyotrophic lateral sclerosis, increased NfL levels are associated with disease activity and progression [2, 6, 30, 31]. Likewise, changes in serum NfL follow the disease course in mild traumatic brain injury [12, 13]. However, these average and group level changes are frequently of limited magnitude or subject to considerable variation between individuals [2, 12, 31]. So, while valuable insight into disease pathophysiology stem

from such investigations, caution is warranted when translating this into clinical use of NfL as a biomarker, since a considerable proportion of patients could fail to reach the 24.3% increase in serum NfL despite relevant disease progression. Hence, the information gain from the RCV is very helpful for interpreting serial measurements and to assist in the translation of NfL into clinical practice. Moreover, it highlights that careful selection of which diseases to monitor with NfL measurements is required.

While serum NfL was not subject to semidiurnal variations, it was surprising to observe a systematic, though minute, day-to-day variation. However, the post-hoc test revealed a significant difference only between days two and three with an absolute level of only 0.4 pg/mL. No obvious biological phenomenon associated with repeated blood sampling would explain this observation, and we retrospectively cross-checked and excluded errors in all steps of sampling, collection, storage, and biochemical analyses. Whether the observed variation represents a statistical type-I error or a discrete biological phenomenon remains to be clarified. Yet, the magnitude of the observed day-to-day variation was so limited that it will hardly be of any relevance for the clinical application. Overall, our observations indicate that when samples are collected for analyzing NfL levels that have to be longitudinal compared, the exact day and time point on the day for the blood drawn is unimportant. This clearly eases the use of NfL as a biomarker for monitoring of diseases.

In contrast to the tightly regulated within-subject variation, we observed a marked variation between individuals manifested in a CV_G of 35.6 %, and thus an II reaching only 0.22. This emphasizes that NfL shows great individuality and reflects that the use of conventional reference intervals can be of less value in diagnosis and screening settings as the between-subject variability may cause individual values in healthy and diseased populations to overlap [32]. Thus, there is a risk of erroneously interpreting a NfL value included within the reference interval as normal, even though it is far from the homeostatic set point in an individual. Hence, comparison of serial results from a single individual seems to be of increased value for appropriate clinical interpretation of the patient's NfL level in diagnosis and screening settings [33].

The fact that serum NfL have a very low CV_I introduces high demands for the analytical performance of the NfL analysis. The desirable analytical performance for CV_A is below or equal to half the CV_I [22]. As such, the CV_A was higher than desired in this study. Yet, few analytical methods would fulfill these requirements and therefore careful precautions need to be taken in order to minimize variation as much as possible. We sought to minimize pre-analytical variation through a standardized collection procedure performed by four technicians only. The analytical variation was minimized by batch analysis of the samples in duplicate and by use of a standardized protocol performed by one laboratory technician only. The analysis was performed by the commercial Simoa Nf-light assay utilizing the Simoa HD-1 analyzer which includes an integrated automated pipettor. This is the current state-of-the-art set up for NfL analysis most frequently used, and the analytical performance of the method in our laboratory with the procedures described are at least on the level achieved by others [1, 34].

Despite the strengths of our study, there are also limitations to consider. Samples were stored at −80 °C for up to 30 months before analysis. Yet, NfL is a highly stable protein and this is unlikely to have affected the biological variation as all samples from the same individual was analyzed in the same run and frozen for the same time period. Furthermore, the duration of the study period was only three days, which potentially could underestimate the within-subject variation as it has been shown for some hematological parameters such as reticulocytes and platelets that serial sampling over a few days results in a smaller CV_I than if samples are taken over weeks or months [35]. However, such a difference could not be observed for several other hematological parameters, creatinine, and glucose [35–37]. Lastly, we only included healthy subjects and no diseased subjects in the study. Thus, we are unable to conclude if comparable biological variation exits in diseased subjects. However, it has been demonstrated for a high number of analytes that the biological variation is similar in healthy and diseased subjects [37, 38].

Conclusions

Serum NfL is a tightly controlled biomarker that displays little within-subject variation with none or neglectable semidiurnal and day-to-day variation. This tight within-subject regulation is contrasted by a considerable between-subject variation. Altogether, this suggests serum NfL to be a well-suited biomarker for disease monitoring; yet, warrants caution when interpreting NfL levels in relation to reference intervals. Furthermore, high analytical performance is required to analyze serum NfL owing to its low within-subject variation. This study provides essential knowledge on the variation of serum NfL which is groundwork for the interpretation and implementation of NfL as a reliable and unambiguous clinical biomarker.

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Competing interests: Authors state no conflicts of interest.

Informed consent: Informed consent was obtained from all individuals included in this study.

Ethical approval: Research involving human subjects complied with all relevant national regulations, institutional policies and is in accordance with the tenets of the Helsinki Declaration (as revised in 2013), and has been approved by the Central Denmark Region Committees on Biomedical Research Ethics (1-10-72-452-17).

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