Serum neurofilament light chain levels in healthy individuals: A proposal of cut-off values for use in multiple sclerosis clinical practice

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proposal of cut-off values for use in multiple sclerosis clinical practice

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ABSTRACT

Background

Serum Neurofilament Light (sNFL) is the most promising marker for patient's monitoring in Multiple Sclerosis (MS). However, operating reference values for use in clinical practice are still lacking. Here we defined sNFL reference cut-off values in a cohort of healthy controls (HC) and assessed their performance in Multiple Sclerosis (MS) patients, as well as the intra-individual sNFL variability.

Methods

We measured sNFL by single molecule array (Simoa) assay in 79 HC assessing their correlation with age. Changes of sNFL levels were evaluated during a short-term follow-up (median 67 days between consecutive samples) in a subgroup of 27 participants. sNFL were tested in 23 untreated MS patients, at both diagnostic time and start of therapy (median 80 days after), considering disease activity.

Results

Findings confirmed a correlation between sNFL levels and age in HC, thus cut-off values specific for age decades were calculated. sNFL did not vary significantly with time during short-term follow-up (median CV 13%).

sNFL levels in MS patients were higher and demonstrated a higher variability between diagnostic time and treatment start (median CV 39%). According to cut-off values, "pathologic" sNFL levels were found in 57% of MS patients at diagnostic time, and in 30% of samples at treatment start. In particular, "pathologic" sNFL levels were found in 80% of samples (16/20) obtained during a phase of disease activity, while a total of 85% of samples (22/26) associated with inactive disease showed sNFL in the normal range.

Conclusion

This study demonstrates an overall intra-individual stability of sNFL values in the short-term in HC and suggests age-dependent reference cut-off values that could be beneficial for sNFL implementation in clinical practice.

Keywords

Multiple Sclerosis, Neurofilament light chain, NFL, serum, reference values, biomarker, variability

1. INTRODUCTION

Multiple sclerosis (MS) is a chronic autoimmune disease resulting in demyelination and neurodegeneration (Dobson and Giovannoni, 2019; Filippi et al., 2018). An effective biological biomarker could be beneficial for a personalized medicine approach. To date, neurofilament-light chain (NFL) represents the most promising biomarker for MS disease activity, progression and prognosis (Varhaug et al., 2019). These neuronal cytoskeletal filaments (Gaetani et al., 2019; Khalil et al., 2018; Preziosa et al., 2020) are constantly released, and in increased amount with aging and axonal damage (Lambertsen et al., 2020).

In particular, recent studies quantified NFL in accessible fluids, such as plasma and serum (p/sNFL), with single molecule array (Simoa™) assay, which enables the detection of proteins up to femtomolar concentrations, increasing quality and quantity of biomarkers that could be measured (Kuhle et al., 2016).

sNFL levels correlate with age in healthy (HC) and MS individuals, with higher concentrations in the latter (Disanto et al., 2017; Khalil et al., 2020; Preziosa et al., 2020). Moreover, patients with progressive disease or experiencing relapses demonstrate higher sNFL (Akgün et al., 2019; Gaetani et al., 2019; Novakova et al., 2017; Preziosa et al., 2020; Varhaug et al., 2018) relative to stable patients. Finally, sNFL correlate with disease-modifying therapies (DMTs) efficacy (Kuhle et al., 2019; Novakova et al., 2017; Preziosa et al., 2020).

Still, sNFL implementation in clinical practice is to be addressed, mainly because the definition of cut-off values for everyday patients' monitoring is lacking.

The aim of the present study was to evaluate sNFL levels in HC and identify reference cut-off values applicable to patients' samples. Moreover, we assessed intra-individual variability after a short-term follow-up, which is fundamental for clinical biomarkers usually dosed with an interval time of a few months. We also explored the correlation between sNFL, Blood Volume (BV) and Body Mass Index (BMI), which have been associated to sNFL levels (Manouchehrinia et al., 2020). Finally, we tested the applicability of the defined cut-off values on a cohort of naïve MS patients.

2. METHODS

2.1. Participants and samples

2.1.1. HC

Serum samples from 79 HC were selected from CRESM Biobank, a structured biobank for neurological and autoimmune diseases of the regional reference center for MS (CRESM), in Piedmont (Italy). Inclusion criteria were age between 18 and 70 years, and absence of family history and individual's own neurological or autoimmune disease. For 36 HC, a paired plasma sample was also tested to evaluate the correlation between serum and plasma NFL levels. A total of 27 participants agreed to be followed-up, with 79 follow-up samples (median of 3 samples per individual, range 2-4 samples per individual; median follow-up time 67 days, range 59-134 days). Only the first sample per each participant was used to define the cut-off values.

2.1.2. MS patients

A cohort of 23 MS patients was enrolled at diagnostic evaluation between April 2019 and December 2019 and samples were prospectively collected during follow-up. Inclusion criteria were a diagnosis of Relapsing Remitting MS (RRMS) according to revised Mc Donald criteria, age between 18 and 70 years and availability of serum samples collected at the diagnostic lumbar puncture (LP) and immediately before treatment initiation (T0). The median time between LP and T0 samples was 80 days (range 49-175 days). Clinical and MRI activity associated with each sample were

blindly evaluated for sNFL levels: in particular, the presence of disease activity was defined as the occurrence of a clinical relapse and/or a new GD+ MRI lesion within 60 days before or after blood sampling.

The study was approved by the Ethical committee of San Luigi Gonzaga University Hospital (approvals number 7262/2019 and 18390/2019). All participants provided written informed consent.

2.2. NFL measurements

Blood samples for both HC and individuals with MS were collected in serum or K2-EDTA tubes (BD Vacutainer, Becton, Dickinson and Company) and processed within two hours from collection according to CRESM Biobank standard procedures and international guidelines (Teunissen et al., 2009); blood samples were centrifuged at 3000xg 10 min, and serum/plasma supernatant stored at -80°C in coded aliquots until analysis to avoid repeated freeze-thaw cycles.

NFL levels were measured by single molecule array (Simoa™) on SR-X instrument (Lambert et al., 2018) using NF-light assays (Quanterix). In each assay session, samples were run together with a titration curve and two internal controls provided in the kit. Reproducibility (intra-assay variability) and repeatability (inter-assay variability) of the assay was evaluated on three native serum samples tested in duplicate in five different assay runs on independent days. The mean coefficients of variation (CV) of duplicates (intra-assay precision) for NfL were 6.7% (6.5 pg/ml, sample 1), 6.7% (30.0 pg/ml,

sample 2) and 3.0% (166.6 pg/ml, sample 3), averaging at 5.5%. Inter-assay CVs were 11.4% (sample 1), 7.0% (sample 2) and 12.3% (sample 3), averaging at 10.2%.

BV was calculated separately for males and females based on weight and height using the Nadler's formula(Nadler et al., 1962), calculated as follows: BV (males) = $0.3669h^3$141 + 0.03219w + 0.6041; BV (females) = $0.3561h^3$ + 0.03308w + 0.1833 (where BV = blood volume in liters; h = height in meters; w = weight in kilograms).

2.3. Statistical analysis

Statistical analysis was performed using R version 4.0.2 and Graph Pad Prism 5. p value < 0.05 was considered statistically significant. Normality of distribution was evaluated by Shapiro-Wilk test and when appropriate sNFL levels were log-transformed. Paired t-test was used to compare plasma and sNFL levels. Univariate and multivariate linear regression models were used to evaluate the relation between plasma and sNFL levels, and between log sNFL and age, sex, BV and BMI. Regression coefficients of models with log sNfL as the dependent variable were back- transformed to the original scale and therefore reflect multiplicative effects. Multivariate mixed effects models were used to evaluate the effect of time on repeated measures of log sNFL, adjusting for age. The coefficient of variation (CV) between consecutive follow-up samples was also calculated. To test differences in sNFL levels between groups, the Kruskal-Wallis test with Dunn Post-hoc test, the Mann Whitney-U-test and the Wilcoxon signed rank sum test were performed, as appropriate. Fisher's exact test was used to compare categorical variables.

3. RESULTS

3.1. Technical performance

A total of 213 samples were run in 29 different assay sessions: in each session a titration curve was run, showing a median R^2 of 0.995 (range 0.964-0.999). Two internal controls provided in the kit were also run in each session falling in the expected concentration range in all runs.

sNFL were analysed in duplicate in 189 out of 213 samples and concentrations were reported as mean of duplicates: the average CV between duplicates was 6% (range 0-27%).

3.2. Correlation between serum and plasma NFL levels in paired samples from HC

NFL levels measured in both serum and EDTA plasma samples (n=36 paired samples from HC) showed a strong correlation (Pearson correlation coefficient r=0.869, R^2=0.756, p<0.0001 (Fig 1). However, mean serum NFL levels (5.18 pg/ml, SD 1.94 pg/ml) were significantly higher than those measured in paired plasma samples (4.31, SD 1.89 pg/ml; paired t-test p<0.0001).

All the following analysis of the present study were performed on serum samples.

Figure 1

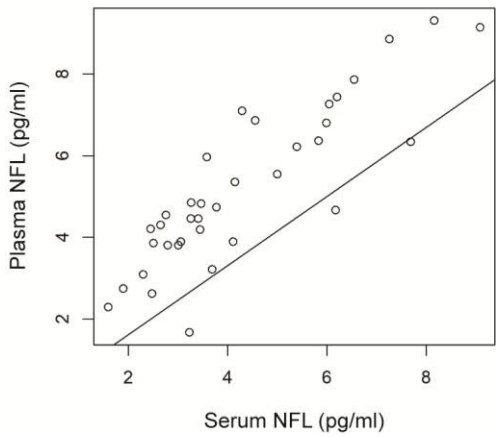

Figure 1. Correlation between serum and plasma NFL levels

Serum and plasma NFL levels evaluated on paired samples from healthy participants (n=36) show a strong correlation (Pearson correlation coefficient r=0.869, R^2=0.756, p<0.0001).

3.3. sNFL values in HC: correlation with age, and definition of decade related cut-off values

The HC cohort consisted of 79 individuals (62 females, 78%). Their median age was 35 years (range 20-68). Median sNFL level was 5.2 pg/ml (range 1.7-18.4). No significant difference in sNFL levels was observed between genders. A correlation between sNFL (log-transformed) and age was found in our cohort (Pearson correlation r=0.689, p<0.0001; R^2=0.475). Log sNFL increased by 0.025 pg/ml per year of age (95% C.I.

0.019 – 0.031, p=0.01), corresponding to a yearly mean increase of 2.5% in sNFL level (Fig 2, panel A and B). In particular, the mean increase of sNFL was 2.3% per year (R^2=0.25, p<0.0001) for participants under 50 years of age, while for those over 50 years of age it increased to 4.3% (R^2=0.31, p=0.049).

Given the relationship with age, sNFL levels were grouped according to the different age decades before defining clinically applicable cut-off values (Fig 2, panel C).

sNFL levels in the second (median 3.9 pg/ml, range 1.7-9.4) and the third decade (median 4.7 pg/ml, range 2.3-7.4) were comparable, with no significant difference between these two groups (Kruskal Wallis with Dunn post hoc test, p=0.274). Compared to the second decade, sNFL significantly increased in the fourth decade (median 6.2 pg/ml, range 4.3-10.3), in the fifth decade (median 8.9 pg/ml, range 5.4-12.5), and finally in the sixth decade (median 9.1 pg/ml, range 7.1-18.4) (Kruskal Wallis with Dunn post hoc test, p=0.003, p=0.001 and p<0.0001, respectively). Notably, sNFL values in the sixth decade showed the greatest variability.

Different cut-off values specific for each decade were calculated as the sum of the respective sNFL mean to three times the standard deviation (SD) (Table 1). The operating cut-off ranges were defined as follows: 10.0 pg/ml for individuals <40 years old (considering that sNFL levels don't differ between 2nd and 3rd decades), 11.0 pg/ml for individuals in the 4th decade, and 16 pg/ml for individuals in the 5th decade. A cut-

off value for the 6th decade was not defined, because of the high variability observed in sNFL levels in this group.

Figure 2

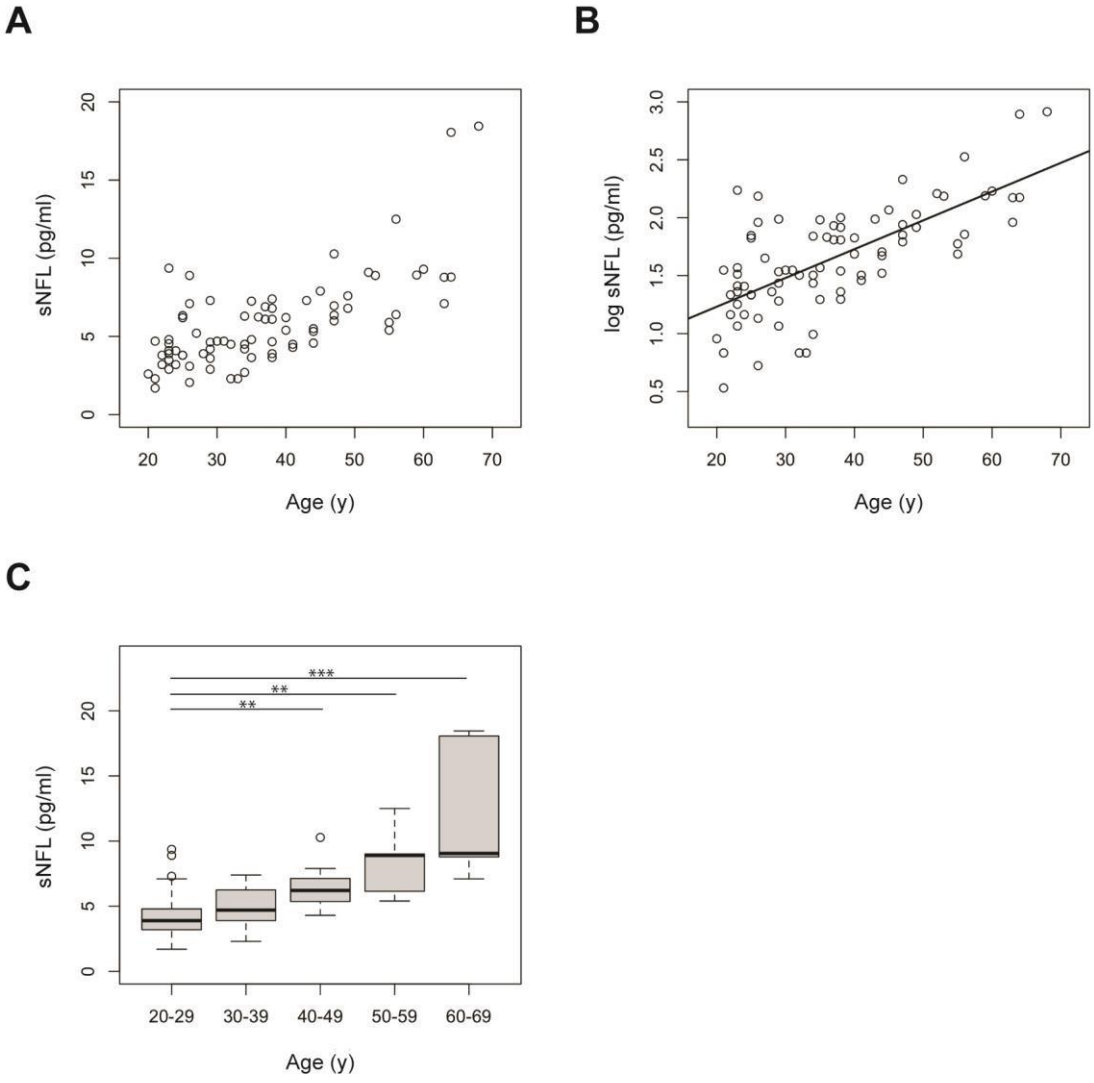

Figure 2. Association between sNFL and age

The association between sNFL levels and age in 79 healthy individuals is shown both on untransformed (A) and log-transformed sNFL values (B) (Pearson correlation r=0.689, $p<0.0001$; $R^2=0.475$). In panel C, sNFL levels are shows stratified according to age-decade: sNFL levels did not show significant differences between the second (median 3.9 pg/ml, range 1.7-9.4) and the third decade (median 4.7 pg/ml, range 2.3-7.4) (Kruskal Wallis with Dunn post hoc test, p=0.274). sNFL significantly increased in the fourth decade (median 6.2 pg/ml, range 4.3-10.3), in the fifth decade (median 8.9 pg/ml, range 5.4-12.5), and finally in the sixth decade (median 9.1 pg/ml, range 7.1-18.4) compared to the second decade (Kruskal Wallis with Dunn post hoc test, p=0.003, p=0.001 and p<0.0001, respectively).

Table 1. Cut-off reference values specific for age-decades

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Age (years)	**N**	**Mean sNFL** (pg/ml)	**SD** (pg/ml)	**Median sNFL** (pg/ml)	**Range** (pg/ml)	**Cut-off =mean+ 3 SD** (pg/ml)
20-29	30	4.4	1.9	3.9	1.7-9.4	**10.0**
30-39	21	4.9	1.6	4.7	2.3-7.4	**9.6**
40-49	15	6.3	1.6	6.2	4.3-10.3	**11.0**
50-59	7	8.2	2.5	8.9	5.4-12.5	**15.7**
60-69	6	11.7	5.1	9.1	7.1-18.4	**27.0**

2,2,5

3.4. sNFL values in HC and their relation with BMI and BV

The correlation between sNFL levels and two biometrical parameters, BMI and BV, was assessed in HC. Whereas log sNFL levels and BMI were not significantly associated after adjusting for sex and sampling age (beta coefficient=-0.01, p=0.23) (not shown), an association with BV adjusted for sex and age was observed. Log sNFL decreased by 0.22 pg/ml per liter of BV increase (95% C.I. -0.39 -0.05, p=0.01) (Fig 3, panels A and B).

Figure 3

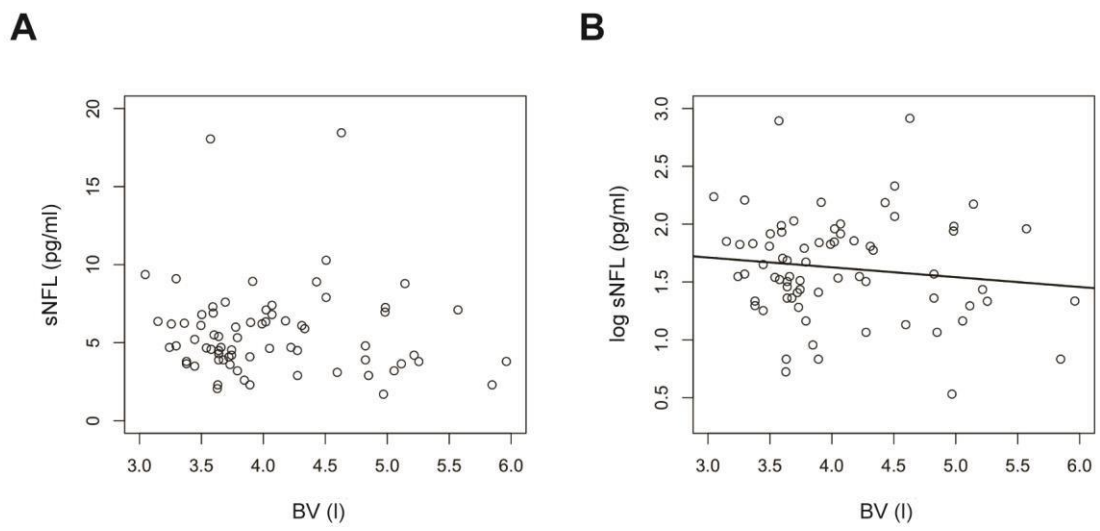

Figure 3. Association between sNFL and blood volume

The association between sNFL and blood volume evaluated in healthy individuals (n=72) is shown both on untransformed (A) and log-transformed sNFL values (B). A correlation is observed, after adjusting for sex and sampling age.

3.5. Intra-individual variability of sNFL levels in short term follow-up

For 27 HC a total of 79 sNFL measures were performed during follow-up period (Fig 4). In particular, a second blood sample was collected from 27 individuals (median follow-up period 67 days, time range 59-106 days), a third sample from 17 individuals (median follow-up period 134 days, time range 131-171 days) and a fourth sample from 8 individuals (median follow-up period 238 days, time range 238-238 days).

sNFL levels did not evidently vary with time (LMM adjusted for age, p=0.83). Median CV between pairs of consecutive samples (median 67 days, range 59-134 days) for each participant was 13% (range 1-64%).

The defined cut-off values were thus firstly tested on the follow-up samples from the HC (n=79 available samples from 27 participants). We observed sNFL levels below the proposed cut-off values for all the follow-up samples but one at the T2 (1.3%): the latter however normalized in the following available sampling (Fig 4).

Figure 4

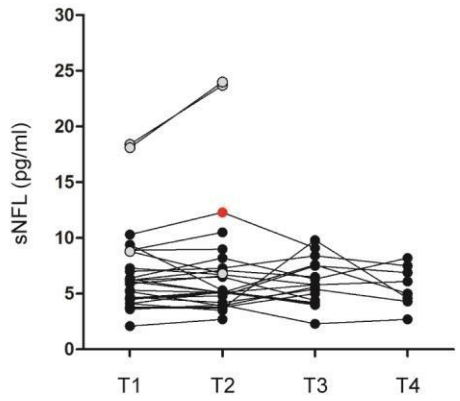

Figure 4. Intra-individual variability

Intra-individual variability was assessed on 79 follow-up samples available from 27 healthy participants: a second blood sample (T2) was collected from 27 individuals (median follow-up 67 days, time range 59-106 days), a third sample (T3) from 17 individuals (median follow-up 134 days, time range 131-171 days) and a fourth sample (T4) from 8 individuals (median follow-up 238, time range 238-238 days). Median time between consecutive samples was 67 days (range 59-134 days). Operating reference values previously calculated were applied on these follow-up samples *(black circles* indicate sNFL in the normal range, *red circles* show high sNFL levels, as *grey circles* correspond to samples from people older than 60 for which a reference value was not available): all follow-up samples showed sNFL levels in normal range, except for one (1.3%).

3.6. sNFL levels in naive MS patients at two-time points and applicability of cut-off values

In addition, sNFL levels and cut-off values identified in HC were tested on a small cohort of naive RRMS patients, consisting of 23 individuals (14 females, 61%) with a median age of 37 years (range 18-59 years). sNFL levels were evaluated on samples collected at the lumbar puncture (LP) and immediately before starting a specific DMT (T0) (Fig 5). The median time between LP and T0 sampling was 80 days (range 49-175 days). sNFL levels in naïve MS patients, either at LP (median 13.9 pg/ml, range 3.6-45.7 pg/ml) or T0 (median 7.7 pg/ml, range 3.4-77.9) were higher than HC (Mann Whitney-U-test, $p<0.0001$ and $p=0.0003$, respectively) (Fig 5, panel A). Although median sNFL value was higher at LP compared with T0 sampling, Wilcoxon signed rank sum test showed no significant difference ($p=0.16$). Median CV between paired LP and T0 samples was 39% (range 0.2-98%) (Fig 5, panel B).

sNFL cut-off values obtained from HC were therefore applied to MS patients to discriminate pathologically high from normal sNFL levels.

At the LP 13/23 patients (57%) showed pathologically high sNFL levels, while at T0 sNFL were above the cut-off in 7 of 23 patients (30%). sNFL levels were normalized in 8/13 (61%) patients between LP and treatment initiation. On the other hand, among patients with normal values at LP, 2 (20%) showed pathological levels at T0.

To investigate how the defined age-related cut-off values relate to clinical outcomes, sNFL status was correlated with the presence of disease activity, defined as the occurrence of clinical and/or radiological activity within 60 days before or after the day of blood sampling. Overall, in naïve MS patients, sNFL were measured in 46 samples, of which 20 were obtained during disease activity and 26 during a phase of disease stability: 16 out of 20 samples (80%) obtained during a phase of disease activity demonstrated sNFL levels above their age-related cut-off, as well as 4 out of 26 samples (15%) obtained during inactive status (Fisher's exact-test $p<0.0001$).

Figure 5

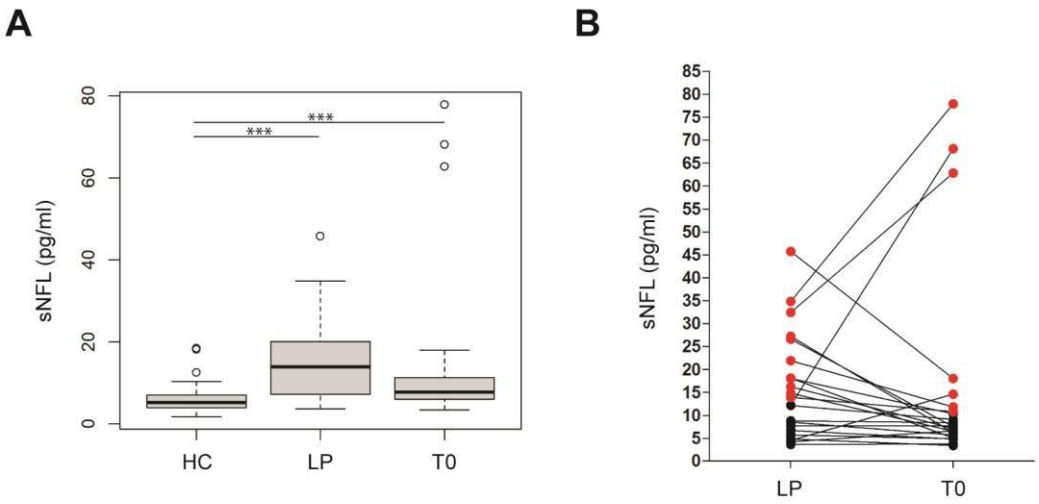

Figure 5. sNFL in naive MS patients

(A) sNFL levels are shown in naïve MS patients at diagnostic lumbar puncture (LP, median 13.9 pg/ml, range 3.6-45.7 pg/ml), before treatment start (T0, median 7.7 pg/ml, range 3.4-77.9) and in healthy participants, showing higher levels in MS patients at both time points (Mann Whitney-U-test, $p<0.0001$ and $p=0.0003$, respectively). (B) sNFL levels in paired LP and T0 samples were compared showing a median CV% equal to 39% (range 0.2-98%). According to the age-decade cut-off values previously defined, 13/23 patients (57%) at LP time point and 7/23 patients (30%) at T0 time point showed high sNFL levels (*red circles*).

4. DISCUSSION

The clinical utility of sNFL is increasingly evident, but widely accepted reference values have not been implemented yet.

The present study addresses the unmet need of defining widely accepted normative values required to interpret sNFL levels in patients with neurological diseases. Further, we described the normal fluctuations of sNFL level in a short-term period. These represent two crucial issues to consider to ensure sNFL implementation in clinical practice.

In the determination of sNFL clinically applicable cut-off values, several parameters need to be considered, as they can influence sNFL, potentially confounding their interpretation and correlation with clinical outcomes. First of all age (Khalil et al., 2020), but recently also BMI and BV were suggested to correlate with blood NFL levels (Manouchehrinia et al., 2020).

Here we describe our strategy to establish cut-off values able to distinguish normal and pathologic sNFL levels in patients, starting from the HC's information.

Regarding the age correlation, we showed a sNFL increase of 2.5% (range 2.3-4.3%) per year (Fig 2). This is in line with the previous findings describing a 2.2% increase per year in a group of 254 HC (Disanto et al., 2017), a 2.9% increase in 342 HC (Hviid et al., 2020) and a 0.9%-2.7% increase in 335 HC depending on age ranges, with a maximum slope of 4.3% in individuals >60 years-old (Khalil et al., 2020). The same

relation has been described in CSF and plasma samples as well (Khalil et al., 2020). Rising and more variable sNFL levels in individuals >60 years indicate an acceleration of neuronal injury at higher age, which may be driven by subclinical comorbid pathologies.

Upon considering age dependency, we were able to define decade-related cut-off values. Since the first two decades had similar values, they were merged into a common operating value of 10.0 pg/ml for patients <40 years old. Cut-off value for those between 40 and 49 years old was 11.0 pg/ml and 16.0 pg/ml was adopted for patients 50-59 years old. Patients >60 years were excluded from cut-off determination not only for the low number of samples and high SD, but also because of the lack of radiological proof of brain health.

Several heterogeneous studies assessed sNFL levels in healthy participants as control groups for different neurological diseases (Hviid et al., 2020; Hyun et al., 2020; Kuhle et al., 2019; Mariotto et al., 2019; Varhaug et al., 2019; Watanabe et al., 2019; Khalil et al., 2020; Jakimovski et al., 2020), but only some of these defined reference values, showing discrepancies (Hviid et al., 2020; Hyun et al., 2020; Novakova et al., 2017; Disanto et al., 2017; Verde et al., 2019).

They have been ascribed to the use of two different protocol assays for blood NFL detection, both based on Simoa technology (Barro et al., 2020): early studies using homebrew assays (Disanto et al., 2017) showed higher sNFL values (almost double)

compared to those described in studies using the actual available commercial kit (NF-light advantage kit) [(Hviid et al., 2020)(Delcoigne et al., 2020)].

In particular, two studies performed on large cohorts of healthy participants defined age-dependent reference values: in the earliest study, performed with homebrew assay (Disanto et al., 2017), 99th percentiles of sNFL distribution across different age groups were calculated, showing values ranging from 37.2 pg/ml in 30 years old participants to 87 pg/ml in 70 years old. In the more recent study (Hviid et al., 2020), performed with NF-light advantage kit, the estimated 97.5% percentiles for serum NfL by ten year intervals showed values ranging from 7.4 pg/ml for 20 years/old to 54.9 pg/ml in 90 years old individuals.

Our work, performed with the same commercial assay, showed similar results to the latter study, suggesting the efficacy of this approach and the consistency of obtained reference values, despite the different method used to define cut-off ranges.

NF-light advantage kit currently represents the assay mainly used by researchers. Multicentre assay validation studies are crucial to demonstrate reproducibility of measurements across different sites and instruments. Preliminary results of a multisite analytical validation of the NF-light assay showed optimal performance of Simoa technology using a standardized protocol and common materials (Kuhle et al., 2018).

Secondly, we assessed the variability of sNFL levels in a short-term follow-up in 27 HC. Until now, only long-term studies were carried out (Khalil et al., 2020). However, short-

time variability is fundamental to consider when analyzing a biomarker that will probably be serially measured along follow-up in clinical practice. In the present study, we assessed the variability of sNFL levels in both HC and naive MS patients', between diagnostic lumbar puncture time point and the beginning of DMT. We found an overall stability of sNFL values in HC (CV=13% between samples), in contrast to MS patients (CV=39% between LP ant T0 time points) (fig 4 and 5), with a similar follow-up time.

In order to verify their performance, we firstly applied the defined cut-off values on healthy follow-up samples (n=79 samples from 27 participants): exclusively one sample (1.3%) was above the cut-off, but subsequently normalized in the follow-up. Subsequently, we tested the cut-offs on MS patients' samples at both LP and T0, showing a higher prevalence of pathological sNFL at the time of diagnosis (56% of patients) compared with T0 time point (30% of patients).

A number of studies described an increase in sNFL levels associated with radiological and/or clinical disease activity. (Akgün et al., 2019; Gaetani et al., 2019; Preziosa et al., 2020; Varhaug et al., 2019) Therefore, to investigate whether obtained cut-off values could be clinically beneficial, we retrospectively performed a preliminary clinical evaluation of patients enrolled. In particular, we correlated sNFL status, based on our age-related cut-offs, with the presence of disease activity. According to the previous findings, we defined disease activity as the occurrence of clinical relapses and/or new

GD+ MRI lesions within 60 days before or after sampling (Disanto et al., 2017; Akgün et al., 2019; Novakova et al., 2017; Varhaug et al., 2018).

Despite the limited cohort of naïve MS patients involved, results obtained represents a further indication about the consistency of defined cut-off values: in line with the literature, our findings showed a higher prevalence of pathologic sNFL levels among patients with disease activity (80%) compared to those in the remission phase (15%).

This latter result could reflect the sNFL potential to unveil inflammatory and neurodegenerative subclinical phenomena, as well as its prognostic value (Gaetani et al., 2019; Giovannoni, 2018; Khalil et al., 2018; Thebault et al., 2020; Varhaug et al., 2019). However, the possible need of cut-off optimization cannot be ruled out.

To further address factors that could influence the proposed reference values, we used HC samples to investigate the poorly discussed relationship between sNFL levels, BV and BMI. Our results proved an inverse correlation with BV, but not with BMI, once adjusted for sex and age. However, we did not consider this association in the cut-off definition, namely because this represents a preliminary observation that needs to be confirmed in independent and larger cohorts. These findings are still of importance and suggest that BV in particular should be taken into account when analyzing the correlation between blood biomarkers and clinical outcomes (Manouchehrinia et al., 2020).

We also focused on the correlation between serum and plasma samples, observing a strong correlation, in line with previous literature (Hviid et al., 2020; Sejbaek et al., 2019) . Instead, the difference between values of these sample types is still controversial (Hviid et al., 2020; O'Connell et al., 2019; Sejbaek et al., 2019). Here, NFL levels from plasma EDTA samples demonstrated lower relative to the paired serum samples. The observed difference seems contained relative to the expected increase in NFL levels in pathological conditions, however this result possibly suggests that serum and plasma samples should not be used interchangeably in clinical practice and should be compared with caution.

The limitation of the study is undoubtedly the number of healthy participants. Indeed, future perspectives of the research group include further enrolment of HC, especially in the 5^{th} and 6^{th} age decade, validation of the identified cut-off values in new cohorts of HC and patients, further investigation of the influence of BV as well as of comorbidities on sNFL levels, and possibly the implementation of these factors in reference values.

In the ongoing process of NFL implementation in clinical practice, our work provides a concrete instrument to enable the clinical use of sNFL, represented not only by the definition of specific cut-off values, but also by the demonstration of intra-individual stability in normal condition, and by data obtained in naïve MS patients.

In conclusion, the present study suggests cut-off values obtained from a cohort of HC to interpret sNFL levels in patients. The obtained decade-related values could be beneficial

for the introduction of sNFL dosing in clinical practice, to guarantee a more personalized-medicine and a less invasive approach. The short-term intra-individual stability of the biomarker values observed in our study ensures the feasibility of serial assessment during patients' follow-up.

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