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ORIGINAL ARTICLE

Age-adjusted neurofilament light chain cutoffs for diagnosing neurodegenerative dementia: a two-threshold machine learning approach

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Abstract Clinical implementation of neurofilament light chain (NfL), a biomarker of neurodegeneration, remains challenging due to absence of reliable cutoffs and influence of confounding factors, particularly age. We aimed to develop an age-adjusted, two-threshold classification framework to support clinical interpretation of NfL in the neurodegenerative dementia diagnosis. We retrospectively enrolled subjects with cognitive/behavioral disturbances and cognitively unimpaired controls (CTR). Participants underwent a baseline diagnostic workup, including at least a neuropsychological assessment, blood test, CSF and serum NfL measurement, and MRI, and were followed up for 2 years. At follow-up, they were diagnosed with either a neurodegenerative dementia [Alzheimer's Disease (AD), Frontotemporal Dementia (FTD), or Lewy Body Dementia (LBD)] or a non-neurodegenerative condition [Other Etiology (OE)]. AD, FTD, and LBD were grouped as Neurodegenerative (NDG), while OE and CTR were grouped as Non-Neurodegenerative (non-NDG). Bayesian regression models assessed the effects of age, disease duration, renal function, and gender on NfL. A weighted support vector machine (SVM) with leave-one-out cross-validation defined age-adjusted cutoffs using a two-threshold strategy

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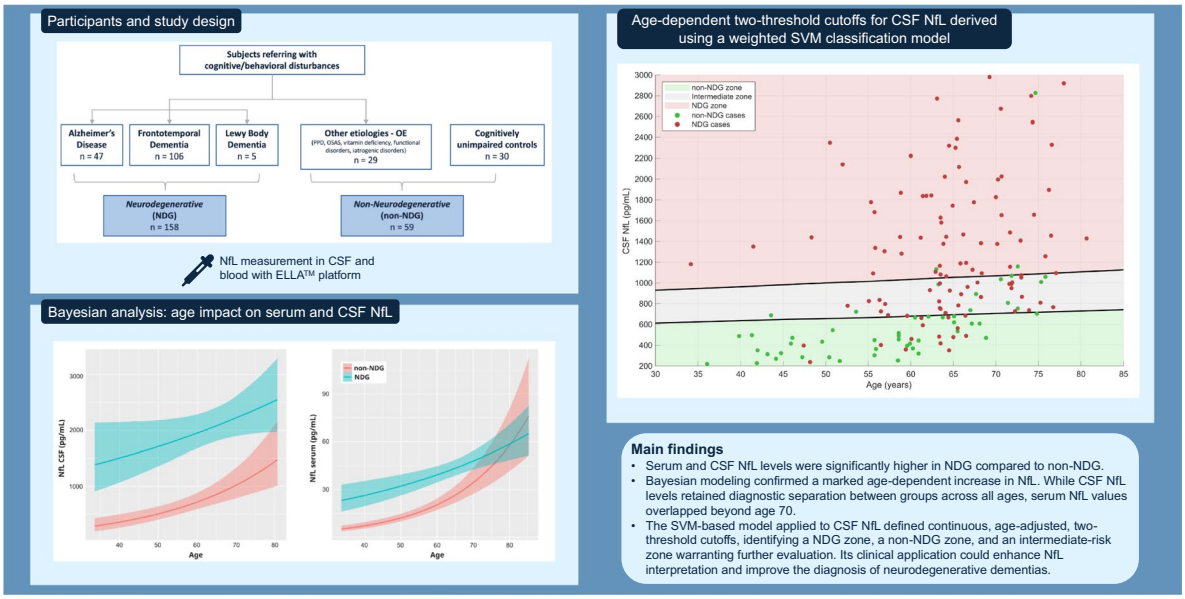
constraining sensitivity ≥ 85% and limiting the intermediate zone. We included 217 subjects (158 NDG, 59 non-NDG). Serum and CSF NfL levels were significantly higher in NDG. Age strongly increased NfL levels, particularly in serum where diagnostic separation declined beyond age 70. The SVM-based model applied to CSF NfL defined continuous, age-adjusted, two-threshold cutoffs, identifying low-risk, high-risk, and intermediate zones. The proposed framework for CSF NfL provides a clinically oriented decision-support tool to stratify the likelihood of underlying neurodegeneration and guide diagnostic workup in cognitive neurology settings, pending external validation.

Graphical abstract



Keywords Neurofilament light chain · Neurodegenerative dementia · Cutoffs · Diagnosis

Introduction

Neurofilaments are neuron-specific type IV intermediate filaments of the cytoskeleton that play a crucial role in axonal growth and structural integrity. They are composed of different subunits, with neurofilament light chain (NfL) being the most abundant and soluble. When axonal damage occurs, increased levels of NfL are released into the CSF and subsequently drained into the bloodstream [1]. Over the past decades, many advanced analytical technologies have been developed to enable a reliable quantification of NfL levels not only in CSF but also in blood, where concentrations are 50–100 times lower. Notably, a strong correlation between NfL levels in the two biofluids has been demonstrated [2, 3]. Different analytical assays yield comparable results and we have proposed conversion formulas to shift from one to the other [4].

Numerous studies have shown that NfL levels are significantly increased across a broad spectrum of neurological disorders, establishing NfL as a non-specific biomarker of neurodegeneration and axonal damage [5, 6]. In particular, NfL has garnered considerable interest in the field of neurodegenerative dementias, including Alzheimer's disease (AD), frontotemporal dementia (FTD), and Lewy body dementia (LBD). Its measurement in this context has proven valuable not only for diagnosis but also for

monitoring disease progression, assessing treatment response, and predicting phenoconversion in genetic dementias [1, 5].

Despite its promise, the clinical implementation of NfL measurement remains limited, especially in the context of dementia, primarily due to the absence of reliable and universally applicable reference ranges to be used for clinical interpretation.

Several confounding factors have so far challenged the identification of cutoff values based solely on absolute NfL concentrations. Among them, age affects NfL levels the most. Studies report annual percentage increases in NfL concentrations of at least 2.2%, with faster rises in older populations [7]. This age-related increase has been attributed to greater neuronal loss and a higher prevalence of co-pathologies associated with aging [6–8]. Other physiological factors, including renal function, blood sugar levels, gender, and body mass index (BMI), have also been implicated in variability of NfL levels, though findings are inconsistent, and their effect size appears smaller than that of age [2, 5, 9–11]. With this study, we aimed to develop age-adjusted NfL reference values to support the identification of neurodegenerative conditions in the setting of dementia clinics and to inform a more clinically meaningful use of NfL measurement.

Methods

Participant selection and classification

For our study purpose, we retrospectively included consecutive individuals who had presented to the Cognitive Neurology Clinic at Modena University Hospital, Northern Italy, due to cognitive and/or behavioral disturbances from 2009 to 2023. The clinic serves a population of about 700,000 inhabitants of the province of Modena with a focus on young onset, mild symptoms, or challenging diagnosis [12]. All participants were Caucasian and native Italian speakers. Inclusion criteria required participants to have completed a comprehensive baseline diagnostic workup, including neuropsychological assessment, blood test, and a brain MRI scan. When clinically indicated, this also included a lumbar puncture for measurement of CSF AD biomarkers (i.e., β-amyloid$_{1–42}$ and β-amyloid$_{1–40}$ ratio), a (18F)-flutemetamol PET (amyloid-PET), and/or a (18F)-fluorodeoxyglucose PET to support the clinical diagnosis with available biomarkers. Moreover, participants had to have subsequently undergone clinical follow-up for at least 2 years. We excluded individuals younger than 30 years at the time of baseline evaluation, as well as subjects with head trauma, focal brain injuries, diagnosis of Parkinson's disease, and known disease of the central and peripheral nervous system. We also excluded individuals for whom the diagnosis was still uncertain after the diagnostic workup and the 2-year follow-up. All diagnoses were established through a multidisciplinary consensus process integrating clinical assessment and follow-up, neuropsychological evaluation, neuroimaging, and laboratory testing and were retrospectively reviewed for accuracy and confirmed before inclusion in the study. Importantly, diagnostic attribution was never based on a single test or biomarker in isolation.

Subjects who had eventually received a clinical and biomarker-based diagnosis of AD [13], FTD, including both behavioral and aphasic variants [14, 15], and LBD [16] were included in the *Neurodegenerative* group (NDG). Subjects whose diagnostic workup (including at least neuropsychological assessment, blood testing, and brain MRI) had not led to a diagnosis of a neurodegenerative condition and who had not clinically progressed or worsened for at least 2 years of clinical follow-up were stratified in the Other Etiology group (OE). They included subjects with a Primary Psychiatric Disorder (PPD), cognitive disturbances due to Obstructive Sleep Apnea Syndrome (OSAS) or vitamin deficiency, functional disorders, and iatrogenic disorders. Cognitively unimpaired individuals with available blood and CSF samples were also included as a Control group (CTR) and aggregated with OE in the *Non-Neurodegenerative* group (non-NDG) (Fig. 1).

Demographic and clinical data, including symptom onset, were collected for all participants, as well as blood creatinine values as a renal function indicator.

The study was conducted in accordance with the Declaration of Helsinki. Prior to sample collection, all participants had provided written informed consent for the storage of CSF and blood samples in the Modena Neurobiobank (ethics committee approval no. 26/2017) and for their use for research purposes (ethics committee approval no. 372/2021).

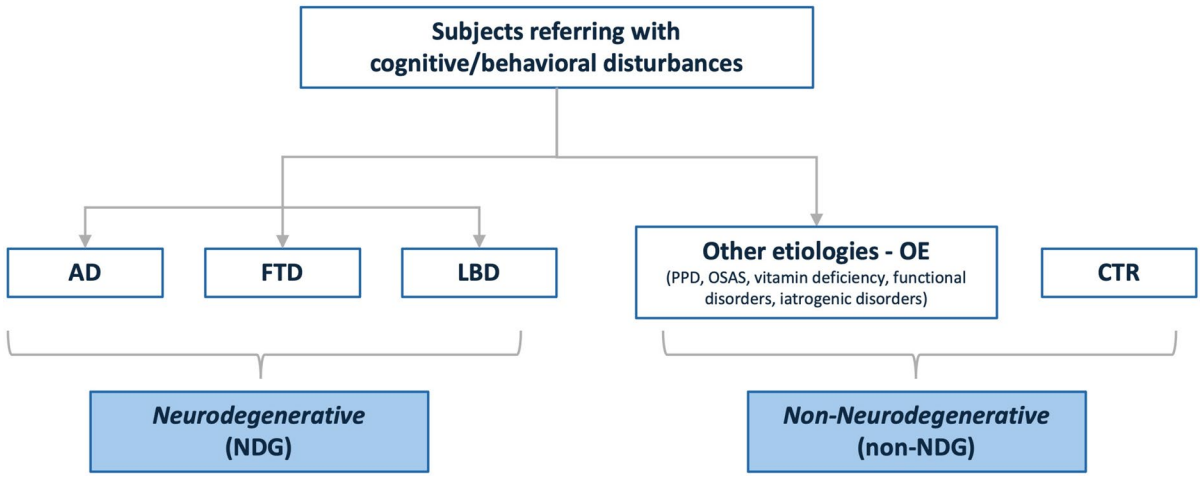


Fig. 1 Participant classification. Subjects referring for cognitive and/or behavioral disturbances were first classified according to clinical and biomarker-based diagnosis. Neurodegenerative dementias (i.e., AD, FTD, and LBD) were then classified in the Neurodegenerative group (NDG). Subjects with other non-neurodegenerative etiologies and cognitively unimpaired controls were classified in the Non-Neurodegenerative group (non-NDG).

AD Alzheimer's Disease, *FTD* Frontotemporal Dementia, *LBD* Lewy Body Dementia, *OE* Other Etiologies, *PPD* Primary Psychiatric Disorder, *OSAS* Obstructive Sleep Apnea Syndrome, *CTR* control

Laboratory testing

NfL levels were measured in serum and CSF samples. Analyses were conducted at the Neuroimmunology Laboratory of Modena University Hospital using Ella™ (Ella Simple Plex assay technology, Bio-Techne, ProteinSimple) [17]. Ella™ performs immunoassays within a microfluidic cartridge, analyzing up to 72 samples in triplicate inside glass nanoreactors with a fluorescent substrate. Samples were processed within 30 min of collection, through centrifugation at $2500 \times g$ for 10 min at controlled room temperature. The supernatant was then aliquoted into sterile polypropylene vials and stored at − 80 °C until analysis. Prior to testing, samples were thawed and centrifuged at $2500 \times g$ for 5 min at 4 °C, following manufacturer's protocols. NfL quantification was performed using the Human NF-L Simple Plex cartridge-based assay (ProteinSimple, San Jose, CA, USA) on the Ella™ device. Serum and CSF 1:2 dilutions were manually performed in accordance with the manufacturer's recommended procedures. Analytical performance characteristics of the assay were provided by the manufacturer (https://www.bio-techne.com/pdf-download-arena-document/product-insert/d10-1202-001/24).The lower limit of detection (LOD) was 1.09 pg/mL, and the lower limit of quantitation (LLOQ) was 2.70 pg/mL; the upper limit of quantitation (ULOQ) was 10,290 pg/mL. Assay precision was validated across multiple runs: intra-assay coefficients of variation (CVs) ranged from 4.7 to 4.8%, and inter-assay CVs ranged from 7.7 to 10.4%. All values were obtained from the official assay specification sheet. The curve shown in Supplementary Figure 1 represents the fitted output of the sample measurements, illustrating the distribution of observed NfL concentrations within the validated dynamic range of the assay.

Data analysis

Median concentrations and interquartile ranges (IQR) of demographic and neurobiological variables were calculated both for NDG and non-NDG groups. Given the known non-normal distribution of NfL values, non-parametric tests were applied. Group differences between NDG and non-NDG participants were assessed using the Wilcoxon-Mann-Whitney test. Comparisons across the five diagnostic subgroups (AD, FTD, LBD, OE, and CTR) were evaluated with the Kruskal-Wallis test, followed by pairwise

Wilcoxon tests with Holm correction when the global test was significant ($\alpha = 0.05$). Serum and CSF NfL levels were log-transformed to achieve normal distributions [18]. The Shapiro-Wilk test ($\alpha = 0.05$) was employed to assess the normality of these transformed distributions.

Comparison of NfL levels across groups using Bayesian statistics

We examined the effects of age, disease duration (i.e., the interval between symptom onset and NfL measurement), creatinine levels, gender, and diagnosis on log-transformed NfL levels using a Bayesian regression model. To investigate whether the relationship between diagnosis and NfL levels was moderated by other clinical variables, we included interaction terms between diagnosis and age, diagnosis and disease duration, and diagnosis and creatinine. Separate analyses were conducted for serum and CSF NfL levels. The model was implemented in R using the *brm()* function of the *brms* package [19], using the default prior specification of *brms*, and employing four Markov chain Monte Carlo, each consisting of 2000 burn-in iterations followed by 4000 sampling iterations.

Identification of an age-dependent cutoff using support vector machine

A machine learning algorithm was employed to establish a threshold differentiating NDG and non-NDG groups based on age and log-transformed NfL levels. Based on the lack of significant association between NfL and the other clinical variable tested with the regression model presented in the previous paragraph (see the "Results" section), we only included age in the machine learning algorithm. A support vector machine (SVM) classification model with a linear kernel and soft-margin regularization was developed using the MATLAB function *fitcsvm()*. SVM classifiers trained on imbalanced datasets may yield suboptimal performance, typically favoring the majority class while underperforming on the minority class [20]. To address this issue, we implemented a weighted SVM approach. More precisely, we used inverse-frequency class weights, assigning each NDG case a weight equal to 1/(number of NDG) and each non-NDG case a weight equal to 1/(number of non-NDG), so that both classes contributed approximately equally to the optimization problem.

To improve overall test accuracy and reduce the occurrence of false-positive results, we adopted a two-threshold classification strategy, in line with what has been proposed for other dementia biomarkers [21]. This approach defines an upper threshold, above which individuals are classified as definitely NDG, and a lower threshold, below which individuals are classified as definitely non-NDG. Participants with assay values falling between these two thresholds were classified as intermediate risk and recommended for additional, clinically guided evaluations aimed at resolving diagnostic uncertainty. Optimal thresholds were determined to ensure a sensitivity of at least 85%, while maximizing specificity and limiting the proportion of intermediate results to less than 25% [22]. In the absence of external validation, model performance was evaluated using leave-one-out cross-validation (LOO-CV). For each participant, a weighted SVM model was trained on the remaining participants. Then, we applied the two-threshold rule defined by that model to classify the left-out patient. This yielded an out-of-sample predicted label for each individual. Sensitivity, specificity, accuracy, and the proportion of intermediate classifications were computed from these LOO-CV predictions.

To quantify the uncertainty of our results, we examined the variability of the lower and upper thresholds across the age spectrum by performing a non-parametric bootstrap with 1000 samples. For each bootstrap sample, we re-fitted the weighted SVM and re-calculated the lower and upper CSF NfL thresholds to derive the 90% bootstrap confidence intervals (5th–95th percentile).

Results

Sample characterization

A total of 217 individuals were recruited. Among them, 158 participants were classified as NDG (i.e., having a neurodegenerative dementia), including 47 with AD [32 amnesic AD, 11 logopenic variant primary progressive aphasia (lvPPA), 3 behavioral variant AD (bvAD), and 1 posterior cortical atrophy (PCA)], 106 with FTD [78 behavioral variant FTD (bvFTD), 15 semantic variant PPA (svPPA), and 13

non-fluent variant PPA (nfvPPA)], and 5 with LBD. The remaining 59 participants were categorized as non-NDG, comprising 29 OE (18 PPD, 3 OSAS, 2 vitamin deficiency, 4 functional disorders, and 2 iatrogenic disorders) and 30 CTR.

The median concentrations of CSF and serum NfL in the NDG group were significantly higher compared to the non-NDG group [CSF 1432 pg/mL (IQR 899–2502) vs 491 pg/mL (IQR 351–703), $p < 0.001$; serum 34.6 pg/mL (IQR 23.5–53.5) vs 15.0 pg/mL (IQR 9.0–22.2), $p < 0.001$]. As expected, the median concentrations of both CSF and serum NfL did not differ significantly between CTR and OE subgroups among the non-NDG individuals [CSF 507 pg/mL (IQR 400–717) vs 471 pg/mL (IQR 337–682), $p = 0.70$; serum 16.0 pg/mL (IQR 11.6–21.6) vs 14.3 pg/mL (IQR 6.6–24.7), $p = 0.53$]. Conversely, AD individuals showed higher NfL levels both in CSF and serum compared to OE [CSF 1364 pg/mL (IQR 888–1822) vs 507 pg/mL (IQR 400–717), $p < 0.001$; serum 27.0 pg/mL (IQR 22.8–41.2) vs 16.0 pg/mL (IQR 11.6–21.6), $p = 0.02$] and CTR [CSF 1364 pg/mL (IQR 888–1822) vs 471 pg/mL (IQR 337–682), $p < 0.001$; serum 27.0 pg/mL (IQR 22.8–41.2) vs 14.3 pg/mL (IQR 6.6–24.7), $p < 0.001$] as well as FTD compared to OE [CSF 1486 pg/mL (IQR 926–3399) vs 507 pg/mL (IQR 400–717), $p < 0.001$; serum 41.5 pg/mL (IQR 24.0–58.9) vs 16.0 pg/mL (IQR 11.6–21.6), $p < 0.001$] and CTR [CSF 1486 pg/mL (IQR 926–3399) vs 471 pg/mL (IQR 337–682), $p < 0.001$; serum 41.5 pg/mL (IQR 24.0–58.9) vs 14.3 pg/mL (IQR 6.6–24.7), $p < 0.001$]. Although FTD subjects exhibited higher NfL levels than those with AD in both biofluids, the differences were not statistically significant [CSF 1486 pg/mL (IQR 926–3399) vs 1364 pg/mL (IQR 888–1822), $p = 0.50$; serum 41.5 pg/mL (IQR 24.0–58.9) vs 27.0 pg/mL (IQR 22.8–41.2), $p = 0.31$]. No significant differences were observed for the LBD group compared to the other diagnostic categories. Detailed demographic and neurobiological characteristics of the study population are summarized in Table 1, while the distribution of CSF and serum NfL levels across diagnostic groups is illustrated in Fig. 2. A descriptive stratification of the OE group by clinical subtypes is provided in Supplementary Figure 2.

Comparison of NfL levels across groups

Given the objective of the present study and the non-specific nature of NfL, subsequent analyses focused on distinguishing between the NDG and non-NDG groups. Figure 3 and Table 2 present the results of Bayesian regression models assessing the effects of age and diagnosis on NfL levels.

After excluding subjects with creatinine or CSF/serum NfL missing values from the dataset, the CSF model was trained on 105 NDG and 48 non-NDG participants, while the serum model included 113 NDG and 46 non-NDG participants (see also Supplementary Table 1 for a characterization of the excluded data). In both biofluids, NfL levels were consistently higher in NDG individuals compared to non-NDG

Table 1 Descriptive statistics of demographic and neurobiological variables

		n (males)	Age	Disease duration	Creatinine (mg/dL)	CSF NfL (pg/mL)	Serum NfL (pg/mL)
NDG		158 (80)	65.1 (61.2–71.6)	2.8 (1.3–4.3)	0.80 (0.72–0.91)	1432 (899–2502)[a]	34.6 (23.5–53.5)[a]
	AD	47 (18)	63.5 (58.0–66.7)	3.0 (1.1–4.5)	0.80 (0.72–0.90)	1364 (888–1822)[b,c]	27.0 (22.8–41.2)[b,c]
	FTD	106 (59)	66.0 (63.1–72.8)	2.8 (1.4–4.3)	0.80 (0.71–0.93)	1486 (926–3399)[d,e]	41.5 (24.0–58.9)[d,e]
	LBD	5 (3)	64.4 (61.3–67.8)	1.2 (1.0–1.8)	0.83 (0.78–0.87)	1004 (833–1206)	23.0 (22.0–25.0)
Non-NDG		59 (22)	58.6 (46.7–65.3)	0.0 (0.0–1.8)	0.74 (0.68–0.84)	491 (351–703)[a]	15.0 (9.0–22.2)[a]
	OE	29 (14)	59.9 (53.6–65.1)	2.4 (1.0–3.9)	0.76 (0.68–0.86)	507 (400–717)[b,d]	16.0 (11.6–21.6)[b,d]
	CTR	30 (8)	55.8 (44.3–66.7)	0.0 (0.0–0.0)	0.73 (0.68–0.81)	471 (337–682)[c,e]	14.3 (6.6–24.7)[c,e]

Data are reported as median score and interquartile range (IQR). Age and disease duration are expressed in years. Group comparisons were performed between NDG and non-NDG using the Wilcoxon-Mann-Whitney test and across the five diagnostic subgroups using the Kruskal-Wallis test, followed by pairwise Wilcoxon tests with Holm correction. Significance was set at $p < 0.05$. a: NDG vs non-NDG; b: AD vs OE; c: AD vs CTR; d: FTD vs OE; e: FTD vs CTR

NDG Neurodegenerative group, *non-NDG* Non-Neurodegenerative group, *AD* Alzheimer's Disease, *FTD* Frontotemporal Dementia, *LBD* Lewy's Body Dementia, *OE* Other Etiologies, *CTR* Control group

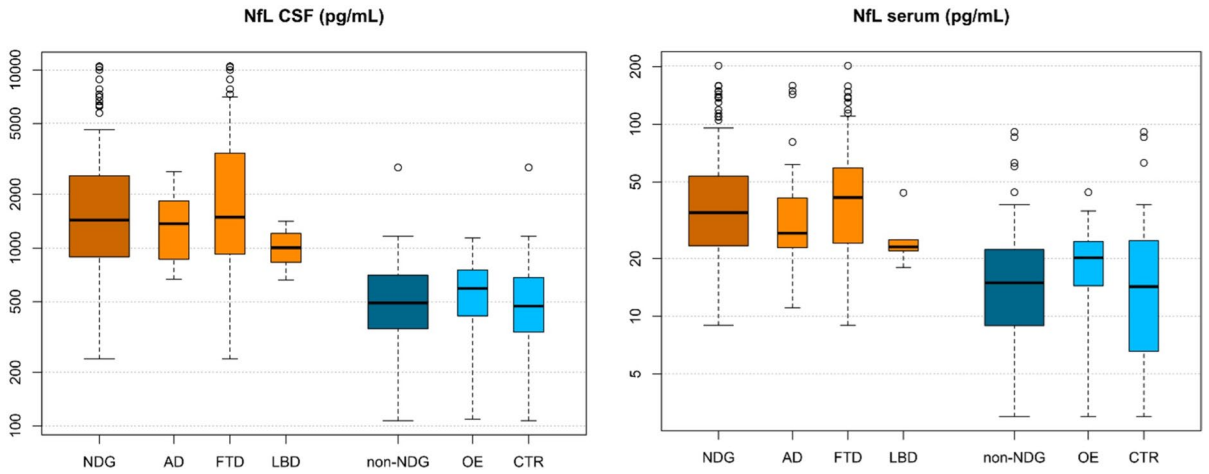


Fig. 2 Distribution of NfL values across each class and its respective subclasses. The rectangular box indicates the interquartile range (IQR), with the horizontal line within the box representing the median. Outliers are depicted as individual dots. Values are displayed on a logarithmic scale

participants. Additionally, NfL levels exhibited a strong dependence on age, indicating that it is not possible to define an age-independent cutoff between the two groups. Conversely, in both models we found no clear evidence of an association between NfL levels and disease duration, creatinine, or gender, as the posterior distributions of these variables were centered near zero and exhibited wide credible intervals (Fig. 3 and Table 2).

As illustrated in Fig. 3 and Table 2, our Bayesian regression models confirmed a robust effect of age on NfL levels in both CSF and serum. However, in serum this aging effect was more pronounced for the non-NDG group [posterior effect size 0.04, 95% credible interval (CI) 0.03−0.06] compared to the NDG group (posterior effect size 0.02, 95% CI 0.01−0.03). Consequently, the predicted serum trajectories for NDG and non-NDG converge and their posterior distributions overlap beyond approximately 70 years of age, despite the presence of a significant main effect of diagnosis. This overlap prevents us from defining clinically useful age-dependent cutoffs for serum NfL in our current dataset. In contrast, CSF NfL trajectories remained well separated across the age range. Therefore, to avoid overinterpreting potentially unstable estimates, we restricted the subsequent two-threshold SVM modeling to CSF NfL.

To assess the impact of the heterogeneity of the internal structure of the non-NDG group, a stratified Bayesian analysis separating OE and CTR was conducted; this additional analysis, reported in Supplementary Figure 3, showed no meaningful changes in the NfL trajectories between OE and CTR groups, supporting the robustness of the reported age-related patterns.

Identification of an age-dependent cutoff

To enhance overall test accuracy and reduce the incidence of false positive results compared to a single-threshold approach, we applied a two-threshold classification framework to NfL, implemented using a leave-one-out cross-validated SVM model. The model was applied to 134 NDG and 53 non-NDG participants (all participants with CSF NfL available, independently of creatinine data; see Supplementary Table 1 for more details). The model achieved a specificity of 92.8% and a sensitivity of 85.6%, while restricting the proportion of participants classified in the intermediate zone to 22.5%. Table 3 and Fig. 4 illustrate the variation of the two thresholds across the age spectrum, together with the 90% confidence interval assessed via bootstrap analysis.

To assess the robustness of the age-adjusted CSF NfL thresholds to the age imbalance between NDG and non-NDG groups, we performed an additional sensitivity analysis restricted to the middle age range (45–75 years), which is presented in the

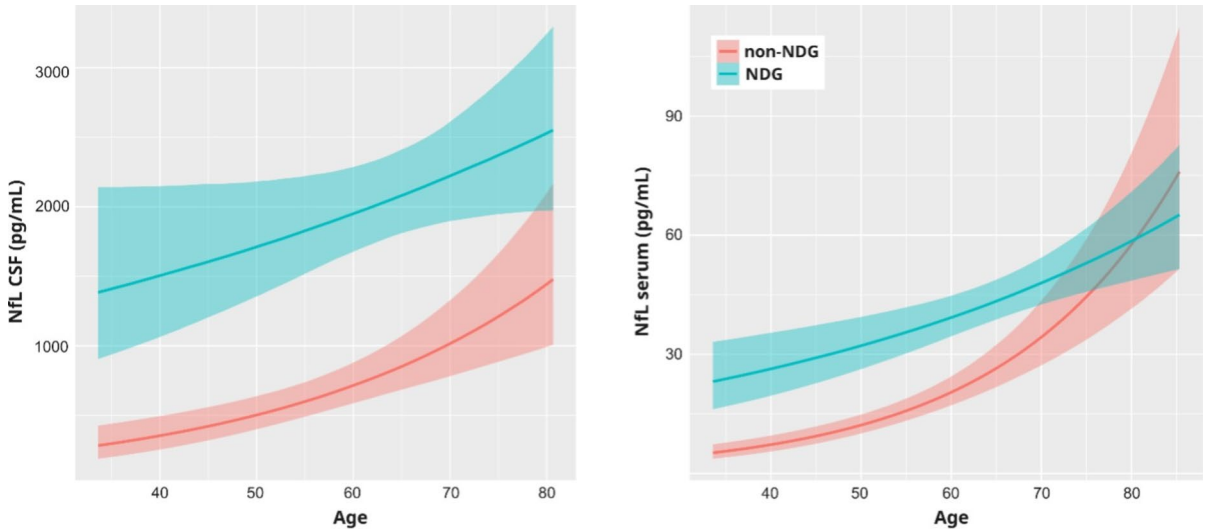


Fig. 3 Estimated mean values of NfL levels. The graphs illustrate the estimated NfL concentrations in CSF (left) and serum (right) as a function of age and diagnosis. Solid lines indicate the expected mean levels, while the shaded regions represent the 90% credible intervals

Supplementary Materials. This analysis yielded diagnostic performance metrics highly comparable to those obtained in the full cohort, supporting that the model's performance was not driven by age-related sampling imbalance.

Finally, as the proposed model was derived exclusively from CSF NfL measurements obtained with the Ella™ platform, we explored its potential applicability across analytical platforms by deriving Lumipulse-equivalent CSF NfL thresholds using a published comparison model between Ella™ and Lumipulse [4]. This analysis, which is reported in Supplementary Materials and Supplementary Table 2, is intended as an illustrative cross-assay harmonization in the presence of known inter-platform systematic differences and it should not be considered a substitute for a dedicated multiplatform validation.

Discussion

In this study, we compared NfL levels measured in CSF and serum between individuals with and without neurodegenerative conditions underlying their cognitive or behavioral symptoms, with the goal of establishing an age-adjusted reference framework aimed at supporting the identification of an underlying neurodegenerative process in clinical settings.

As expected, NfL levels were significantly elevated in neurodegenerative dementias compared to both cognitively unimpaired controls and individuals presenting with cognitive or behavioral disturbances but without evidence of an underlying neurodegenerative disease. This finding reinforces the role of NfL as a non-specific biomarker of neurodegeneration, supporting its potential use as a first-line screening and decision-support tool to identify individuals at risk for an underlying neurodegenerative dementia, particularly when other disease-specific biomarkers are lacking or insufficient (e.g., in the differential diagnosis between FTD and psychiatric disorders) [5, 23]. It is important to clarify that the non-NDG group included both cognitively unimpaired controls and individuals who were initially referred for cognitive or behavioral disturbances, but in whom a neurodegenerative disease was subsequently excluded based on diagnostic evaluation, biomarker assessment, and clinical follow-up. In both subgroups, other neurological conditions known to be associated with elevated NfL levels (e.g., multiple sclerosis, stroke, neuropathies, and traumatic brain injury) were excluded based on medical history. Moreover, we showed that both CSF and serum NfL levels did not differ significantly between CTR and OE groups. Within the OE group, NfL levels appeared broadly distributed across clinical subtypes, although, given the small sample sizes

Table 2 Posterior summaries of the Bayesian regression model

	CSF (pg/mL)				Serum (pg/mL)			
(A) Model parameters	e.s. (95% CI)	R-hat	ESS	p_{tail}	e.s. (95% CI)	R-hat	ESS	p_{tail}
Effect of diagnosis	1.79 (0.02–3.60)	1.001	8008	0.952	1.80 (0.37–3.21)	1.000	9146	0.981
Effect of age	0.03 (0.02–0.05)	1.001	10,311	0.999	0.03 (0.02–0.04)	1.000	10,612	0.999
Effect of age on NDG	0.02 (0.01–0.03)	1.001	9511	0.962	0.02 (0.01–0.03)	1.000	10,206	0.996
Effect of disease duration	0.01 (−0.04–0.05)	1.001	10,757	0.614	0.02 (−0.02–0.06)	1.000	10,901	0.790
Effect of disease duration on NDG	0.02 (−0.03–0.06)	1.001	10,664	0.727	0.02 (−0.02–0.06)	1.001	10,430	0.827
Effect of creatinine	−0.18 (−0.99–0.62)	1.000	9786	0.350	0.14 (−0.47–0.75)	1.000	10,317	0.649
Effect of creatinine on NDG	−0.13 (−1.05–0.79)	1.001	9610	0.406	0.20 (−0.38–0.77)	1.000	10,438	0.711
Effect of female gender	−0.05 (−0.31–0.23)	1.000	14,445	0.367	0.11 (−0.12–0.33)	1.000	15,159	0.825
(B) Derived quantities	e.s. (95% CI)			p_{tail}	e.s. (95% CI)			p_{tail}
Effect of age on non-NDG	0.02 (0.01–0.03)			0.999	0.04 (0.03–0.06)			0.999
Effect of disease duration on non-NDG	0.00 (−0.08–0.08)			0.493	0.02 (−0.05–0.09)			0.664
Effect of creatinine on non-NDG	−0.24 (−1.45–0.98)			0.377	0.09 (−0.91–1.11)			0.554

Panel A reports population-level regression coefficients, including posterior effect sizes (e.s.) and 95% credible intervals (CI), convergence diagnostics (R-hat and bulk effective sample size, ESS) and the posterior tail probability (p_{tail}, which ranges from 0 to 1, with values closer to 1 indicating stronger posterior evidence that the estimated effect is present). Panel B presents clinically relevant derived effects, defined as linear combinations of regression coefficients. Convergence diagnostics are reported only for the underlying model coefficients (Panel A), as the derived effects (Panel B) inherit their convergence properties. In regression models, the dependent variable was NfL (CSF or serum), while the predictors included age, diagnosis, disease duration, creatinine, and gender, as well as the interactions between diagnosis and age, diagnosis and disease duration, and diagnosis and creatinine. These interaction terms were included to account for potential differential effects of diagnosis on age, disease duration, and creatinine. Posterior predictive checks for the Bayesian regression models are reported in Supplementary Figure 4

of individual OE subcategories, this analysis was not powered to detect subgroup-specific differences. Consistently, in the Bayesian analysis stratifying the non-NDG group into OE and CTR, we did not observe meaningful differences in the overall age-related trajectories. Based on these observations, we chose to aggregate CTR and OE within the non-NDG group for the purposes of this study. Despite the inherent heterogeneity of this population, we believe that this approach might more accurately reflect the clinical scenario, in which NfL measurement is typically applied to subjects presenting with some neurological complaints rather than to strictly healthy individuals.

When evaluating potential confounding factors, we found that gender, disease duration, and renal function had a negligible impact on NfL levels across diagnostic groups. In contrast, age emerged as a critical factor, as NfL concentrations increased significantly with advancing age in both groups and biofluids. This finding highlights the limitations of fixed, age-independent thresholds, which risk misclassification by failing to account for physiological age-related increases in NfL levels.

Considering the effect of age, our model demonstrated that CSF NfL levels remained significantly different between individuals with and without an underlying neurodegenerative condition across the entire age range. Conversely, serum NfL levels exhibited a substantial overlap between groups beyond the age of 70, mainly due to a steeper age-related increase in serum NfL among non-NDG individuals. This pattern represents an empirical observation of the present dataset. However, given the age imbalance between NDG and non-NDG groups in our cohort, with fewer older non-NDG participants, part of the observed convergence may reflect extrapolation beyond the age range adequately represented in the non-NDG group. Nevertheless, this observation is consistent with previous reports suggesting a stronger age effect, non-linear increase, and greater inter-individual variability of serum NfL among older individuals [6–8]. Several physiological and pathological mechanisms have been proposed to explain this phenomenon, including reduced CSF turnover with aging and a higher prevalence of co-pathologies or clinically silent neurologic disorders

Table 3 Age-dependent cutoffs for CSF NfL derived using a weighted SVM classification model

Age	Lower cutoff (pg/mL)	Upper cutoff (pg/mL)
40	635 (464–844)	952 (685–1319)
45	646 (510–799)	969 (753–1252)
50	657 (558–759)	987 (823–1200)
55	669 (598–738)	1005 (884–1163)
60	681 (599–761)	1023 (912–1168)
65	694 (574–800)	1041 (895–1229)
70	706 (545–858)	1060 (860–1320)
75	719 (515–933)	1079 (817–1421)
80	732 (487–1021)	1098 (771–1556)

Age-specific lower and upper CSF NfL cutoffs are reported for selected age categories. Values are expressed in pg/mL, with 90% bootstrap confidence intervals shown in parentheses. Participants with CSF NfL levels below the lower cutoff are classified as non-NDG, whereas those with values above the upper cutoff are classified as NDG; values falling between the two cutoffs define an intermediate zone of diagnostic uncertainty. The discrete age-specific cutoffs reported in the table were derived from continuous age-dependent decision boundaries estimated by the weighted SVM model. Specifically, if y represents CSF NfL (pg/mL) and x age (years), the lower and upper decision boundaries are defined by the equations $y=\exp(0.003584x+6.3093)$ and $y=\exp(0.003557x+6.7166)$, respectively. The tabulated values represent the corresponding cutoffs at the indicated ages and are provided to facilitate clinical use. See also Supplementary Figure 5 for a graphical representation of the threshold stability across age ranges

(e.g., polyneuropathy, cerebrovascular brain burden) that may contribute to increased neuronal loss and elevated NfL levels in older adults [2, 5, 7]. For these reasons, in the present study we restricted the application of the age-dependent cutoff model exclusively to CSF NfL values, as only CSF data in our cohort allowed the identification of stable and clinically meaningful age-dependent decision boundaries. Replication of our approach in larger cohorts will be necessary to determine whether the observed lack of significant differences in serum NfL levels among older individuals with and without a neurodegenerative condition reflects a true biological phenomenon or is partly driven by limited statistical power and sampling imbalance. Should the overlap be confirmed, the informative value of serum NfL for distinguishing neurodegenerative conditions in advanced age may be reduced, suggesting that reliable biomarker-based assessment in older patients may preferentially require CSF NfL, with implications for its use in primary care and first-line clinical settings.

Previous approaches have defined normal NfL values by stratifying age into discrete bins of varying widths [10, 24–27]. However, these methods represent a coarse approximation of age effects and may lack precision, as they fail to capture the continuous nature of NfL variation with aging. In these models, the upper limit of normal remains identical for all individuals within a given bin, leading to greater approximation errors as bin width increases. Additionally, when sample sizes within bins are small, the precision is even lower [5]. In contrast, the SVM-based method applied here allowed us to derive a continuous age-adjusted decision boundary directly from our case-control dataset. Although we did not have so far external validation of this approach in multicenter cohorts, its stability was supported by leave-one-out cross-validation and bootstrap resampling. Additionally, the method we used is relatively robust to extreme observations (e.g., very high CSF NfL values which are clearly pathological), as such observations are unlikely to act as support vectors and therefore are not expected to materially influence the estimated decision boundaries.

A further advantage of our approach is the application of a two-threshold classification strategy to NfL assessment. This method defines three diagnostic zones based on two cutoffs: a lenient threshold and a more conservative one. It introduces an intermediate range to account for cases with ambiguous biomarker values, thereby reducing both false positives and false negatives and improving overall diagnostic confidence. In contrast, single threshold approaches must prioritize either high sensitivity—at the cost of increased false positives, particularly in older individuals—or high specificity, which increases the risk of false negatives and may lead to missed early diagnoses. From a clinical point of view, the two-threshold model allows balancing diagnostic accuracy while ensuring that cases with borderline values are appropriately flagged for additional, clinically guided evaluation rather than being prematurely assigned to a diagnostic category. These may include longitudinal clinical, neuropsychological, and radiological follow-up, as well as the use of second-level biomarkers depending on the clinical context. This approach better aligns with real-world clinical decision-making where biomarker interpretation often follows a probabilistic rather than a strictly binary process. The use of intermediate ranges with

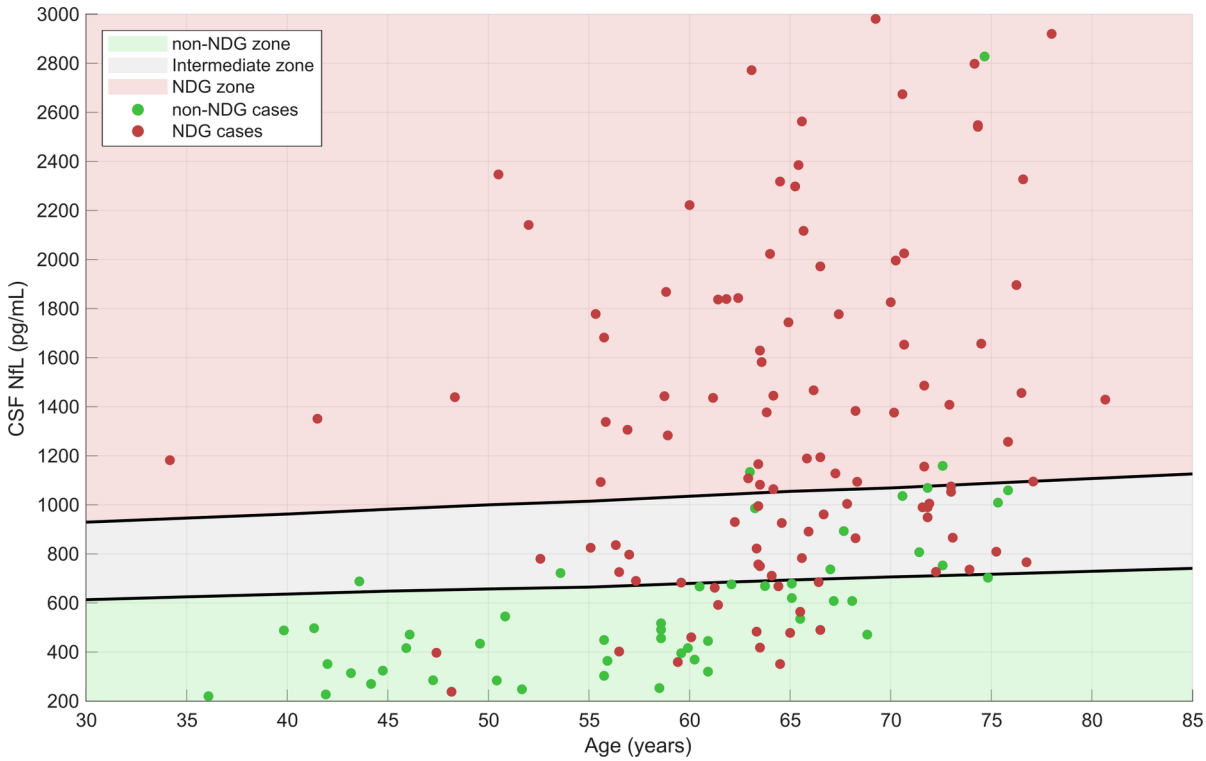


Fig. 4 Age-dependent two-threshold cutoffs for CSF NfL derived using a weighted SVM classification model. Participants falling within the green region are classified as non-NDG, while those in the red region are classified as NDG. The gray intermediate region indicates an area of uncertainty. Colored dots represent participants from the study cohort: red dots correspond to NDG participants, and green dots represent non-NDG participants. For visual clarity, the *y*-axis scale is capped at 3000 pg/mL; however, several participants exhibited CSF NfL levels exceeding this value, all of whom belonged to the NDG group

two cutoffs has already been successfully applied to other medical conditions such as prehypertension and prediabetes and has proved to be a useful construct in medicine [28]. Moreover, a two-cutoff approach has also been applied to other dementia biomarkers, including blood-based AD biomarkers and amyloid-PET, demonstrating its effectiveness [21, 22]. The novelty of our work lies in extending this established two-threshold framework to NfL measurement, a biomarker for which age dependency and the absence of validated cutoffs have so far limited its clinical translation. This approach is also highly adaptable: if the primary clinical question is to detect the earliest evidence of a neurodegenerative disease, then the lenient cutoff may be preferred. Conversely, if high diagnostic certainty is required, the conservative threshold may be more appropriate [28]. Importantly, this framework is not intended to provide a definitive diagnosis but rather to stratify individuals according to the likelihood of an underlying neurodegenerative condition, while explicitly identifying an intermediate range in which additional biomarkers, imaging, or longitudinal follow-up are required. Additional multicenter and multiplatform validations are still needed before the tool is ready for routine implementation. Furthermore, the present study was not designed to assess the rate at which individuals in the intermediate zone are subsequently reclassified following further evaluation, and this represents an important direction for future research.

Our study also reported that the various neurodegenerative dementias exhibit small (but not significant) differences in NfL levels, consistent with previous findings showing greater NfL concentration in individuals with FTD compared to both AD and LBD [1, 5, 29, 30]. However, the aim of our study was not to differentiate between specific neurodegenerative syndromes but rather to develop a tool capable of

determining whether observed cognitive or behavioral symptoms reflect an underlying neurodegenerative process—a common and urgent challenge in clinical practice. For this reason, we grouped together different neurodegenerative conditions (i.e., AD, FTD, and LBD) and the model was specifically designed to distinguish between NDG and non-NDG groups and to establish a unified cutoff framework applicable across diverse forms of dementias. This contrasts with previous approaches that have focused on individual diseases [24] or distinguished dementia subtypes based solely on NfL levels [31]. We chose this broader approach because of the biological properties of NfL, which is notably a non-specific biomarker of neuroaxonal damage [32]. As such, its primary clinical utility should lie in identifying the presence of an underlying neurodegenerative disorder, rather than in differentiating among specific etiologies. More precise disease classification can only be achieved by integrating NfL with additional disease-specific biomarkers, for example, targeting the amyloid or tau pathology in AD [33]. Accordingly, a future direction of our work could be to integrate such complementary biomarkers into the model to allow not only the identification of neurodegeneration but also etiological definition.

Our study has limitations: first, the non-NDG group was smaller and younger than the NDG group, reflecting real-world clinical practice, as lumbar puncture is preferentially proposed to individuals for whom the suspicion of a neurodegenerative disease is already high. To mitigate the risk of classification bias favoring the larger group, we implemented a weighted classification approach, which assigns greater importance to the minority class and improves classification balance. Also, within the NDG group, the distribution of individual diagnoses was not perfectly balanced and reflected the typical case mix observed at the Modena Cognitive Neurology Clinic, with a predominance of bvFTD and amnestic AD and smaller numbers of atypical presentations (lvPPA, svPPA, nfvPPA, PCA, and bvAD) and LBD. While this allowed inclusion of a broad spectrum of neurodegenerative phenotypes, the limited sample sizes warrant caution when extrapolating findings to these subgroups. In addition, the large number of FTD cases, which are known to exhibit higher NfL levels compared to AD and LBD, may have shifted the estimated decision boundaries toward higher values, potentially reducing sensitivity for detecting neurodegeneration in clinical settings where AD or LBD predominate. The magnitude of this potential bias cannot be directly quantified in the present dataset. Larger and more balanced cohorts will be necessary to generalize the performance of the proposed framework to different clinical scenarios. Furthermore, the cohort was drawn from a single Italian tertiary center and included only individuals of Caucasian ancestry, limiting the direct generalizability of the proposed cutoffs to other populations, healthcare settings, or ancestral backgrounds. Although internal validation combining cross-validation and bootstrap resampling suggests good robustness of the model within our cohort, these approaches do not substitute for external validation. Therefore, the clinical applicability of the proposed thresholds should be interpreted with caution until confirmed in independent, multicenter cohorts and across different analytical platforms.

Second, the retrospective design and long inclusion period may introduce heterogeneity related to evolving diagnostic criteria and clinical practice. However, all diagnoses were established through multidisciplinary discussion integrating multimodal data and were reviewed and confirmed prior to inclusion, mitigating the impact of temporal changes. NfL measurements were performed retrospectively using a single analytical platform and were not used in the diagnostic process to avoid circular reasoning. The exclusion of individuals with uncertain diagnoses after follow-up may introduce survivorship bias, but this strategy was deliberately adopted to maximize diagnostic certainty and minimize misclassification of reference labels. Third, although key confounding factors such as gender, renal function, and disease duration were included, data on other physiological (e.g., BMI) and pathological (e.g., cardiovascular comorbidities, diabetes) variables were unavailable. While these factors have been variably associated with NfL levels, their effects may be largely mediated by age given their higher prevalence in older individuals. Nonetheless, future studies should assess whether incorporating these variables could further improve model accuracy.

Finally, NfL quantification was performed exclusively using the Ella™ platform, which is widely used and offers a practical and accessible solution for measuring both CSF and serum NfL. While this approach ensured analytical consistency and avoided

inter-assay variability over time, it limits direct generalizability to other analytical platforms. To provide an illustrative example of cross-assay applicability, here we explored the transferability of our framework to the Lumipulse platform, using a published comparison model between the two assays (see Supplementary Materials). However, this harmonization analysis is intended as a proof of principle, and future studies are still needed for a dedicated multiplatform validation.

Conclusions

In conclusion, our study demonstrates the feasibility and advantages of an age-adaptive classification model for CSF NfL interpretation. By integrating continuous, age-adjusted thresholds and a machine learning classification framework, we propose a clinically oriented framework for the interpretation of CSF NfL, aimed at guiding the clinician's decision-making and assisting the identification of individuals at higher or lower likelihood of an underlying neurodegenerative dementia in the setting of a cognitive neurology clinic. This interpretative structure supports tailored diagnostic workup, optimized use of additional investigations, and individualized follow-up strategies based on the estimated probability of an underlying neurodegenerative condition. Importantly, our data also suggest some limitations of serum NfL measurement in older individuals, due to the substantial overlap observed between individuals with and without neurodegeneration as age increases. Through our preliminary and hypothesis-generating results, we have discussed possible explanations and the potential implication of these findings on NfL application to different clinical settings. Future research should prioritize external validation in independent, multicenter cohorts and across different analytical platforms before clinical adoption can be considered. Moreover, the applicability to blood-based NfL measurements should be explored in larger cohorts with the goal of optimizing the utility of this biomarker across different clinical contexts and age groups.

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Author contribution CG conceived the clinical and diagnostic framework of the study and was responsible for data curation. RM developed and implemented the statistical and machine learning models. Both contributed to data interpretation, manuscript drafting, and figure preparation. GZ and MT supervised the study and provided critical revision of the manuscript. RB and TU performed NfL measurements and contributed to data curation. CC, GV, SC, NI, DB, SS, and AC contributed to data curation and manuscript revision and provided intellectual input.

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Data Availability Anonymized data will be made available upon request and permission granted by our local ethics committees.

Declarations

Ethics approval and consent to participate The study was conducted in accordance with the Declaration of Helsinki. Prior to sample collection, all participants had provided written informed consent for the storage of CSF and blood samples in the Modena Neurobiobank (ethics committee approval no. 26/2017) and for their use for research purposes (ethics committee approval no. 372/2021).

Competing interests The authors declare no competing interests.

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