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ORIGINAL COMMUNICATION

Serum phosphorylated neurofilament heavy-chain levels reflect phenotypic heterogeneity and are an independent predictor of survival in motor neuron disease

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Abstract

To investigate the prognostic role and the major determinants of serum phosphorylated neurofilament heavy -chain (pNfH) concentration across a large cohort of motor neuron disease (MND) phenotypes. Enzyme-linked immunosorbent assay (ELISA) was used to measure serum pNfH concentration in 219 MND patients consecutively enrolled in our tertiary MND clinic. A multifactorial analysis was carried out to investigate the major clinical determinants of serum pNfH. Kaplan–Meier survival curves and Cox regression analysis were performed to explore the prognostic value of serum pNfH. Serum pNfH levels were not homogenous among MND phenotypes; higher concentrations in pyramidal, bulbar, and classic phenotypes were observed. *C9orf72*-MND exhibited higher pNfH concentrations compared to non-*C9orf72* MND. Multiple linear regression analysis revealed mean MEP/cMAP and disease progression rate as the two major predictors of serum pNfH levels ($R^2 = 0.188$; $p \leq 0.001$). Kaplan–Meier curves showed a significant difference of survival among MND subgroups when divided into quartiles based on pNfH concentrations, log-rank $X^2 = 53.0$, $p \leq 0.0001$. Our study evidenced that higher serum pNfH concentration is a negative independent prognostic factor for survival. In Cox multivariate model, pNfH concentration showed the highest hazard ratio compared to the other factors influencing survival included in the analysis. pNfH differs among the MND phenotypes and is an independent prognostic factor for survival. This study provides supporting evidence of the role of pNfH as useful prognostic biomarker for MND patients. Neurofilament measurements should be considered in the future prognostic models and in clinical trials for biomarker-based stratification, and to evaluate treatment response.

Keywords Amyotrophic lateral sclerosis · ALS · FTD · C9orf72 · Biomarkers

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Introduction

Motor neuron disease (MND) includes a heterogeneous group of relentless neurodegenerative disorders defined and characterized by the degeneration of motor neurons. Amyotrophic lateral sclerosis (ALS) is the most common and severe form of MND, affecting upper (UMN) and lower motor neurons (LMN) leading to respiratory failure and death within 3–5 years after symptoms onset [1, 2]. Despite this stereotypical definition, MND clinical spectrum includes extremely heterogeneous phenotypes characterized by a varying involvement of UMN and LMN, site of onset, cognitive and genetic characteristics, resulting in a different rate of progression and prognosis [3–6].

While extensive studies have been performed to better characterize clinical, cognitive, genetic and neuroimaging biomarkers of progression, and many potential biochemical markers in both cerebrospinal fluid (CSF) and serum have been proposed, no diagnostic and prognostic biomarkers are currently available in clinical practice [7–13]. Phosphorylated neurofilament heavy chain (pNfH) and the neurofilament light chain (NfL) are nowadays considered as the most promising candidate biomarkers for MND [14]. Both pNfH and NfL have been extensively studied across the neurological disorders and are considered to be reliable markers of acute and chronic neuronal injury [15]. The diagnostic role of neurofilaments has already been extensively explored. Previous studies provided evidence of a better diagnostic performance and higher sensitivity and specificity of pNfH when tested in the CSF compared to serum; however, a strong correlation between serum and CSF concentration has been reported [9, 16, 17]. Furthermore, pNfH correlated with disease severity and survival parameters but no studies have so far investigated serum pNfH as an independent predictor of survival [10, 17–20]. On the contrary, NfLs were demonstrated as independent predictors of survival in both CSF and serum [18, 21, 22]. Currently no biochemical prognostic biomarkers for assessing neuronal damage and disease progression across MND phenotypes are been explored. There is, however, an urgent need for a biochemical prognostic biomarker for MND to facilitate the estimation of the progression rate, survival, and patient's stratification [8, 23]. Definition of subtypes of MND is required to predict disease course and to correlate the phenotypes to the serum pNfH as a disease marker.

We performed a monocentric study in a large MND cohort of patients aimed at exploring serum pNfH across the different phenotypes, ascertaining the major determinants of the serum pNfH levels and assessing the prognostic role of the serum pNfH in MND patients.

Materials and methods

Patient's selection and clinical data collection

Two hundred and nineteen consecutive MND patients, referred to the ALS center of the San Raffaele Scientific Institute, Milan, were consecutively enrolled between December 2014 and January 2019 in accordance with the Awaji and revised cEl-Escorial criteria [24, 25]. Serum blood samples for pNfH assay were collected at the first evaluation at our center. At collection time, demographics and clinical history were recorded and neurological assessment was performed as follows: Medical Research Council (MRC) scale of 0 to 5 (12 muscles for each side; score 0–120 points) [26]; MRC progression rate (ΔMRC) calculated as [(120 − MRC score)/disease duration at the serum sample]; ALS Functional Rating Scale-revised (ALSFRS-R) was obtained and disease progression rate (ΔALSFRS-R) was calculated as (48 − ALSFRS-R)/disease duration); UMN burden was assessed with the UMN score, calculated by totaling the number of pathological UMN signs at examination (score 0–16) [26]. Patients were staged in agreement with King's clinical staging system and categorized, at the time of diagnosis, into eight different MND phenotypes, according to the previously published classification criteria and fulfilling the period of clinical observation required for a reliable phenotyping [3, 27].

Patients who underwent respiratory function assessment for clinical purposes were assessed with a standard spirometry recording forced vital capacity (FVC) expressed as a percentage of predicted volume and with arterial blood gas (ABG) analysis recording partial oxygen (PaO_2) and carbon dioxide ($PaCO_2$) pressures. Height and weight were measured, and body mass index (BMI) calculated. A subset of patients underwent metabolic assessment to determine predicted and measured resting energy expenditure by indirect calorimetry and metabolic index (MI) calculated as predicted/measured resting energy expenditure.

Neuropsychological screening was performed with Edinburgh Cognitive and Behavioural ALS Screen (ECAS) [28]. A further neuropsychological evaluation as recommended by the Diagnostic Criteria for the Behavioural Variant of Frontotemporal Dementia and the ALS-FTD Consensus Criteria was performed [29, 30]. MND patients were grouped in agreement with the ALS-FTD Consensus Criteria into five categories: frontotemporal dementia (FTD) (ALS-FTD); behavioral impairment (ALS-bi); cognitive impairment (ALS-ci); combined cognitive and behavioral impairment (ALS-cbi), which includes patients who fulfill criteria for both ALS-ci and ALS-bi; normal neuropsychological evaluation (ALS-motor). Only patients who underwent neuropsychological

testing after publication of the ALS-FTD Consensus Criteria were considered [5].

Motor nerve conduction studies were routinely performed to determine bilateral median and common peroneal compound muscle action potential (cMAP) amplitude (peak to peak), and the mean cMAP at the four limbs was then calculated. Routine trans-cranial magnetic stimulation (TMS) was performed to measure motor-evoked potential (MEP) amplitude at the four limbs. MEP/cMAP amplitude ratio was calculated for each limb, expressed as a proportion of the cMAP elicited after peripheral nerve stimulation in the same target muscle (MEP/cMAP ratio) [31, 32]. Subsequently, mean MEP/cMAP at the four limbs was calculated. Only patients who underwent neurophysiological testing in a period between ± 2 months from serum sample collection were considered and data were retrospectively retrieved from medical records.

Survival defined as time from serum sample to survival event defined as death/tracheostomy, and time to King's stage 4 defined as time from symptoms onset to significant feeding or respiratory failure were calculated. Additionally, survival time from symptoms onset to death/tracheostomy was calculated. Patients were followed up by periodical phone calls; survival status was last updated in October 2019. The Ethics Committee of the San Raffaele Scientific Institute of Milan approved the current study and all the participants gave written informed consent.

Sample collection and pNfH assay

Serum samples were processed within 1 h of blood collection and were stored at − 80 °C prior until analysis. pNfH serum levels were measured in duplicate blinded to disease status by commercial ELISA using human phosphorylated Neurofilament H antibody (Biovendor, RD191138300R, Brno, Czech Republic). Kits were used according to the manufacturers' instructions. Serum concentrations below the analytical sensitivity were nominated 23.5 pg/ml. The mean intra-assay coefficient of variation was 6.8%. The median serum pNfH concentration in an age- and gender-matched control cohort of 27 healthy subjects (11 women and 16 men; median age at venipuncture: 60.2 years, IQR 53.5–64.8) was 28.5 pg/ml (IQR 23.5–81.2). DNA was available from all patients in the study and patients were screened for hexanucleotide expansion in *C9orf72* gene, to investigate the correlation of serum pNfH in the presence of the hexanucleotide expansion as previously reported in the CSF [33]. The *C9orf72* gene analysis was performed as previously described [34].

Statistical methods

Normality data distribution was assessed with the Shapiro–Wilk test. Continuous variables are reported with median and interquartile range (IQR) while categorical variables with number and relative frequencies. Mann–Whitney *U* test was used to evaluate differences between two groups. Kruskal–Wallis test and Dunn post hoc comparison were performed to verify differences among groups at a significance level of 5%. To investigate correlation between serum pNfH and clinical variables Pearson correlation analyses was carried out for continuous variables and Spearman correlation for ordinal variables. Subsequently, hierarchical multiple univariate linear regression analysis (stepwise procedure) was performed to underline which variables best explained the variance of serum pNfH. Kaplan–Meier (KM) univariate analysis was carried out to determine the effect of serum pNfH on survival from serum sample, time to King stage 4 and survival from symptom onset. log rank tests (Mantel–Cox) were used to test for differences among groups and, when more than two ordinal strata were used, the linear trend for factor level test was performed. Subsequently, multivariable analysis with Cox proportional hazards model (enter method) was performed to estimate the proportional hazard ratios of pNfH on survival and on time to King stage 4. Cox regressions were adjusted for known factors that negatively influence survival in ALS patients [14]. Serum pNfH concentration was set up as quartile groups as follows: first quartile (23.5–40.1 pg/ml); second quartile (40.2–174.3 pg/ml); third quartile (174.4–363.6 pg/ml); fourth quartile (> 363.6 pg/ml). All statistical tests were performed using SPSS 22.0 software (Technologies, Inc., Chicago, IL, USA). p value was set at $p < 0.05$.

Results

A total of two hundred and nineteen (87 women and 132 men) MND patients were enrolled in the current study. Demographics and clinical characteristics of the MND patients are summarized in Table 1. In our cohort, the median serum pNfH concentration was 174.3 pg/ml (IQR 40.1–363.3 pg/ml). Serum pNfH concentration was significantly different among MND phenotypes: higher levels were detected in pyramidal, bulbar, and classic phenotypes while flail arm, pure lower motor neuron (PLMN), and pure upper motor neuron (PUMN) phenotypes showed the lowest concentrations as shown in Fig. 1a and Table 2. Genetic analysis identified the *C9orf72* hexanucleotide repeat expansion in eighteen (8.2%) patients of our cohort. *C9orf72* MND patients exhibited significantly higher pNfH concentrations (median 403.3 pg/ml, IQR 203.3–563.5 pg/ml) compared to non-*C9orf72* MND patients (median 157.4 pg/ml, IQR

Table 1 Demographics and clinical characteristics of MND patients

Gender, M/F	132/87 (60.3%/39.7%)
Age at venipuncture (years)	64.0 (57.0–71.0)
Diagnostic delay (months)	9.0 (6.0–15.0)
ALSFRS-R (points)	37.0 (32.0–42.0)
ΔALSFRS-R (points/month)	0.7 (0.4–1.2)
MRC score (points)	100.0 (84.0–111.0)
ΔMRC (points/month)	1.3 (0.6–2.5)
UMNs (points)	8.0 (3.0–11.0)
ECAS ALS SPECIFIC (points)	75.0 (56.0–85.0)
Total ECAS score (points)	99.0 (79.0–112.0)
FVC (%)	83.5 (58.3–99.0)
PO2 (mmHg)	78.4 (71.8–88.3)
PCo2 (mmHg)	41.4 (38.3–47.0)
Basal metabolic rate (%)	92.0 (84.7–102.3)
BMI (ratio)	24.1 (22.0–26.1)
Mean MEP/cMAP	0.2 (0.1–0.3)
Mean cMAP four limbs	6.0 (3.2–8.2)
C9orf72 expansion (no/yes)	201/18 (91.8%/8.2%)
Disease duration at venipuncture (months)	14.0 (9.0–24.0)
Serum pNfH (pg/ml)	174.2 (40.1–363.6)

Values shown are *n*, percentage (%) or median (interquartile range)

M male, *F* female, *ΔALSFRS-R* ALS Functional Rating Scale Progression Rate, *ΔMRC* Medical Research Council Scale Progression Rate, *ECAS* Edinburgh Cognitive and Behavioural ALS Screen, *UMNs* Upper Motor Neuron score, *FVC* forced vital capacity, *BMI* body mass index, *MEP/cMAP* motor evoked potential/compound muscle action potential, *C9orf72* chromosome 9 open reading frame 72, *pNfH* phosphorylated neurofilament heavy chain

33.3–334.6 pg/ml) (Fig. 1b). MND patients staged in King's 3 and 4 showed significantly higher pNfH concentrations compared to patients staged in King's 1 and 2 (Fig. 1c and Supplementary Table 1). One hundred and eighteen patients underwent a complete neuropsychological assessment and were categorized according to Strong criteria as shown in Table 2. No statistical differences were detected when MND patients were grouped by cognitive/behavioral phenotypes, age at symptoms onset and gender (Fig. 1d and Supplementary Fig. 1a, b).

In univariate analysis, pNfH concentration showed a moderate correlation with disease progression rate (0.317; $p \leq 0.001$) and an inverse moderate correlation with mean MEP/cMAP at the four limbs ($r = -0.342$; $p \leq 0.001$). Moreover, pNfH concentration was weakly correlated with several MND clinical characteristics as UMNs, King's stage system, diagnostic delay, and disease duration at the serum sample (Supplementary Tables 2–5; Supplementary Fig. 1c, d).

In a subset of patients in which all the data were available ($n = 129$), a hierarchical multiple regression was carried out including variables that were significant at the univariate analysis. The full model of mean MEP/cMAP at the four limbs, disease progression rate, King's stage system, disease duration at venipuncture and diagnostic delay was statistically significant, $R^2 = 0.188$; $p \leq 0.001$. The significant variables included in the model were the mean MEP/cMAP at the four limbs and the disease progression rate. MEP/cMAP at the four limbs was the major determinant of serum pNfH concentration (Supplementary Table 6).

KM survival (defined as time from serum sample to death/tracheostomy) curves showed a significant difference of cumulative survivals between MND subgroups when divided by pNfH concentrations log-rank (Mantel–Cox) $X^2 = 53.0$, $p \leq 0.0001$, with higher serum pNfH concentrations related to shorter survival (Fig. 2). The median survival was fourth quartile 9.0 months (95% CI 7.0–11.0 months), third quartile 23.0 months (95% CI 18.6–27.4 months), second quartile 28.0 months (95% CI 13.4–42.6) and first quartile 33.0 months (95% CI 16.1–49.9 months). The negative effect of pNfH concentration was confirmed when stratifying Kaplan–Meier curves by age at onset and disease duration at serum sample (data not shown). Additionally, Kaplan–Meier curves showed a significant stratification when time to King's stage 4 was considered as event log-rank (Mantel–Cox) $X^2 = 68.1$, $p \leq 0.0001$ and similarly when survival was defined as time from symptoms onset to death/tracheostomy log-rank (Mantel–Cox) $X^2 = 64.3$, $p \leq 0.0001$ (Fig. 2, Supplementary Fig. 2 and Supplementary Tables 7–12).

Multivariate Cox regression model confirmed that serum pNfH concentration is independently associated with a reduced survival (defined as time from serum sample to death/tracheostomy) in MND. Patients with serum pNfH concentration higher than 363.6 pg/ml (fourth quartile) showed an increased proportional hazard ratio (HR) of 3.67 (95% CI 1.96–6.90) when compared with the first quartile group (Table 3). This result was confirmed by defining survival as time from symptoms onset to death/tracheostomy with an HR 3.86 (95% CI 2.05–7.28) (Supplementary table 13) and considering the variables as continuous (Supplementary tables 14 and 15). Similarly, Cox multivariate analysis confirmed that serum pNfH concentration is an independent negative prognostic factor for time to King's stage 4, HR 3.55 (95% CI 1.97–6.37) (Table 3). All the variables included in the different models, HR and 95% CI are reported in Table 3 and in Supplementary Tables 13–15.

Discussion

Serum and CSF neurofilaments are promising diagnostic and prognostic biomarkers in MND. In our large cohort of MND patients, well characterized by several clinical parameters, we found that serum pNfH concentration was not homogenous among the MND phenotypes. Moreover, we demonstrated serum pNfH to be an independent predictive factor of survival in MND patients.

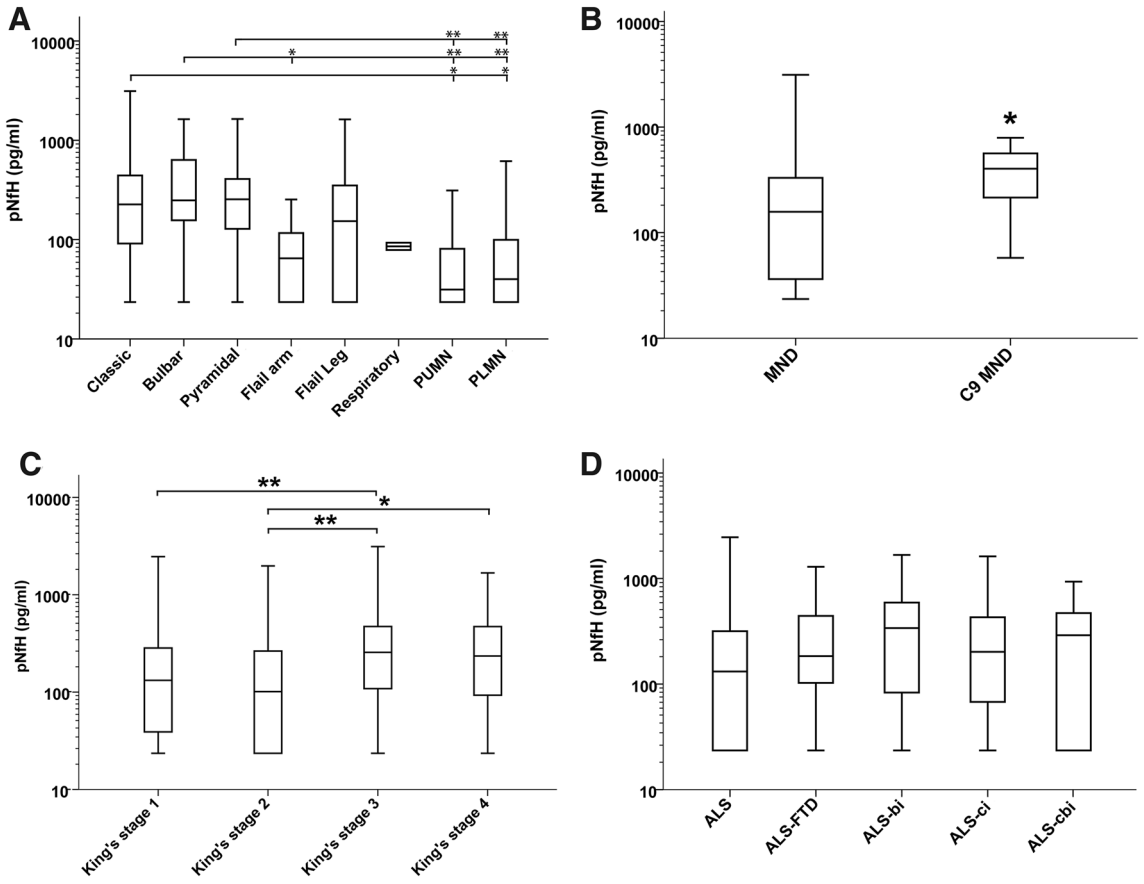

Fig. 1 Serum pNfH in MND patients. Boxplots showing pNfH concentrations among **a** MND phenotypes (overall groups comparison p value ≤ 0.0001) grouped according to Chiò criteria [3]. **b** MND and C9MND patients, **c** MND patients staged according to King's stage system (overall groups comparison p value $= 0.001$) [27], **d** MND patients classified in agreement with Strong criteria (overall groups comparison p value $= 0.345$) [30]. *p value < 0.05; **p value < 0.01. The median concentrations, 25% and 75% percentile and range values are given. pNfH levels are plotted on a 10-logarithmic scale

Indeed, pyramidal, bulbar, and classic phenotypes exhibited higher pNfH levels compared to MND patients with a selective LMN or UMN involvement. The positive correlation between pNfH and the progression rate suggests that a faster degeneration of the motor system is one of the determinants of serum pNfH concentration, explaining the lower levels detected in slow progressive phenotypes as PUMN, PLMN, and FA. We found an inverse correlation between pNfH and MEP/cMAP suggesting that an elevated UMN burden might influence the serum pNfH concentration. Although the explanation is probably more complex, our results suggest that a rapid ongoing degeneration process of the UMN/corticospinal tract might be related to an increase of the serum pNfH concentration. However, MEP/cMAP does not selectively estimate UMN impairment and it might be influenced by both central and peripheral motor conductions; therefore, experimental neurophysiological methods such as short-interval intracortical inhibition (SICI) or advanced MRI investigations are needed to confirm this correlation. Consistent with this hypothesis, previous studies reported that serum NfL levels were higher in ALS patients with a widespread UMN involvement and correlated with MRI measures of corticospinal tract degeneration, while NfL were lower in primary lateral sclerosis (PLS) patients [18, 35]. Notably, we observed significantly higher serum pNfH levels in *C9orf72* MND patients compared to non-*C9orf72* MND, in line with a previous study, which detected higher CSF pNfH levels in *C9orf72* MND carriers [33]. *C9orf72* MND has a faster disease progression rate and shorter survival [6], reflecting a widespread CNS neurodegeneration and more severe brain atrophy even involving extra motor areas compared to non- *C9orf72* MND [34]. We investigated serum pNfH levels among the cognitive phenotypes, classified according to the Strong criteria [30]. Although MND patients with a concurrence of cognitive dysfunction and/or FTD showed higher median pNfH levels compared to MND patients with normal cognition and behavior, this difference did not reach a statistical significance, suggesting that extra-motor areas involvement may not be a major

Table 2 The pNfH concentration in different MND motor and cognitive phenotypes

	Case number	pNfH (pg/ml)
Motor phenotype		
Classic	82/219 (37.4%)	226.2 (89.6–449.5)
Bulbar	31/219 (14.2%)	248.2 (153.0–651.6)
Pyramidal	31/219 (14.2%)	254.3 (108.4–407.6)
Flail arm	10/219 (4.6%)	70.6 (23.5–120.4)
Flail leg	30/219 (13.7%)	153.4 (23.5–351.1)
Respiratory	2/219 (0.9%)	85.8 (–)
PLMN	23/219 (10.5%)	40.0 (23.5–112.2)
PUMN	10/219 (4.6%)	32.7 (23.5–127.6)
Cognitive phenotype		
ALS motor	52/118 (44.1%)	131.6 (23.5–318.7)
ALS-FTD	21/118 (17.8%)	184.1 (90.4–523.7)
ALS-bi	11/118 (9.3%)	339.2 (74.2–688.0)
ALS-ci	24/118 (20.3%)	202.3 (66.9–435.3)
ALS-cbi	10/118 (8.5%)	292.3 (23.5–586.8)

Median values and interquartile range (IQR) are given

pNfH phosphorylated heavy chain, *PLMN* pure lower motor neuron, *PUMN* pure upper motor neuron, *ALS-FTD* frontotemporal dementia (FTD), *ALS-bi* behavioral impairment, *ALS-ci* cognitive impairment, *ALS-cbi* combined cognitive and behavioral impairment

determinant of the serum pNfH concentration in MND patients. However, further studies are needed to confirm our result. In support of our data, a previous study has shown higher CSF pNfH levels in ALS-FTD compared with FTD patients without evidence of motor system involvement, suggesting that the motor system involvement is the major determinant of pNfH concentrations [33].

While serum and CSF pNfH concentrations were previously correlated with survival parameters in univariate analysis, our study showed that serum pNfH concentration is a negative independent prognostic factor for survival through an accurate multivariate analysis [19]. Indeed, patients with higher pNfH concentration showed a significantly shorter median survival. Serum pNfH concentration showed the highest hazard ratio compared to all the other factors influencing survival included in the Cox multivariate model. Additionally, we showed that serum pNfH concentration was also significant as independent negative factor to predict the time to reach King's stage 4, i.e., the time to reach feeding or respiratory failure, which requires specific clinical interventions. Patients in King's stages 3 and 4 showed higher serum pNfH concentration compared to patients in King's stages 1 and 2. These findings suggest that pNfH levels in serum indicate the spreading and the global rate of CNS involvement and may offer a quick and simple measure to assess and predict neuronal damage in MND. Our results extend the previous findings that both neurofilament subunits and other wet biomarkers should be thoroughly assessed to be included in future prognostic models [18, 22, 36].

A partial limitation to the current study is that our results concerning serum pNfH were not replicable in the CSF due to the lack of serum and CSF paired matched samples in our cohort. Although previous works showed higher sensitivity and specificity of pNfH in the CSF when tested for a diagnostic purpose, a strong correlation between serum and CSF pNfH has also been demonstrated

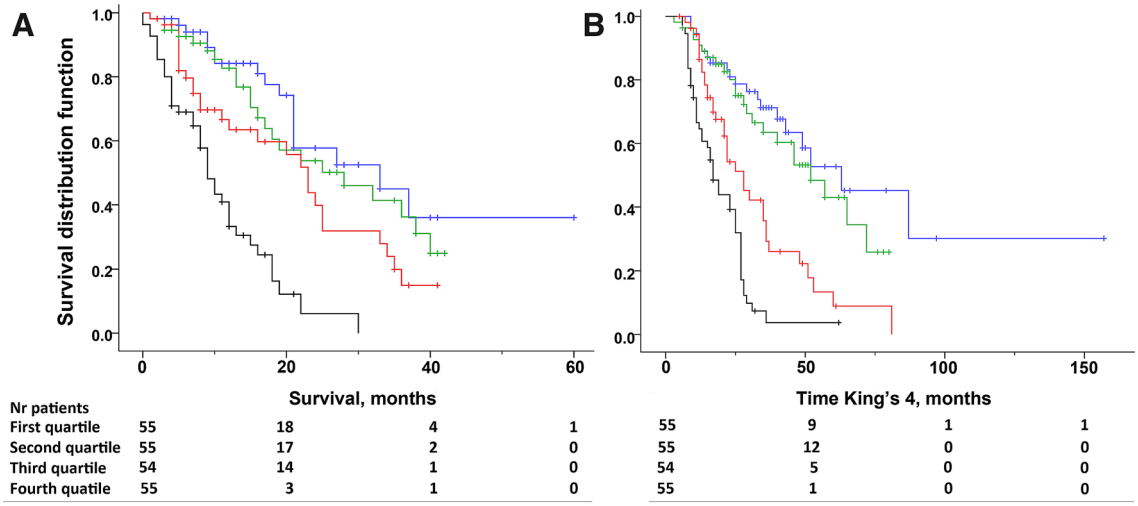

Fig. 2 Survival curves in MND patients **a** log-rank (Mantel–Cox) $X^2 = 53.0$, $p \leq 0.0001$ (event defined as time from serum sample to death/tracheostomy) **b** Kaplan–Meier time to King's stage 4 curves (event defined as time from symptoms onset to significant feeding or respiratory failure), log-rank (Mantel–Cox) $X^2 = 68.1$, $p \leq 0.0001$. MND patients were grouped according to quartile values; first quartile (blue line), second quartile (green line), third quartile (red line), and fourth quartile (black line)

Table 3 Cox proportional hazards regression multivariate analysis on survival

Factor	Survival (from serum sample to death or tracheostomy)		Time to King's stage 4	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Serum pNfH concentration (pg/ml)		**< 0.001**		**< 0.001**
23.5–40.1	1		1	
40.08–174.3	1.27 (0.66–2.43)	0.480	1.41 (0.75–2.63)	0.288
174.4–363.6	1.55 (0.83–2.89)	0.167	2.45 (1.37–4.39)	**0.003**
> 363.6	3.67 (1.96–6.90)	**< 0.001**	3.55 (1.97–6.37)	**< 0.001**
Disease duration at serum sample (months)				
≤ 14	1		1.74 (1.09–2.77)	**0.021**
> 14	1.45 (0.88–2.42)	0.148	1	
Diagnostic delay (months)				
≤ 9	1.04 (0.66–1.64)	0.874	1.23 (0.82–1.84)	0.321
> 9	1		1	
Progression rate (points/month)				
≤ 0.74	1		1	
> 0.74	2.80 (1.74–4.50)	**< 0.001**	4.32 (2.74–6.82)	**< 0.001**
Dementia				
No	1		1	
Yes	1.61 (0.92–2.82)	0.095	1.18 (0.70–1.99)	0.542
C9orf72 expansion				
No	1		1	
Yes	1.30 (0.62–2.69)	0.488	1.16 (0.59–2.27)	0.662
Age at venipuncture (years)				
31–64	1		1	
> 64	1.70 (1.13–2.57)	**0.011**	1.62 (1.11–2.36)	**0.013**
MND phenotype		0.058		**0.006**
PUMN/PLMN/FA	1		1	
CL/PY/FL	2.43 (1.16–5.09)	**0.019**	2.15 (1.10–4.20)	**0.025**
B/R	2.51 (1.07–5.91)	**0.035**	3.61 (1.63–8.00)	**0.002**

Variables included in the model: pNfH, phosphorylated neurofilament heavy chain subdivided into quartiles; disease duration at serum sample, diagnostic delay, age at onset, and progression rate divided according to the respective median values; presence of dementia no/yes; *C9orf72*, chromosome 9 open reading frame 72 hexanucleotide repeat expansion no/yes; MND phenotype, subdivided into three different groups: long survival group composed of pure upper motor neuron (PUMN), pure lower motor neuron (PLMN) and flail arm (FA); intermediate survival group composed of classic (CL), pyramidal (PY) and flail leg (FL); short survival group composed of bulbar (B) and respiratory (R)

HR hazard ratio, *CI* confidence interval

in MND patients [16, 19, 37, 38]. However, we aimed to assess the prognostic rather than the diagnostic role of serum pNfH. Furthermore, a prognostic blood-based biomarker would be preferable to a CSF biomarker to avoid an invasive practice such as the lumbar puncture. Nevertheless, we acknowledge that further independent confirmatory investigations are required to confirm our findings and to better define prognostic reference values. Furthermore, longitudinal studies will be essential to determine whether the pNfH levels increase, decrease or stay stable over time. Unfortunately, we are currently unable to raise additional experiments in the laboratory due to the lockdown related to the COVID-19 in Italy. Future studies will be essential to assess the variance of measurement of pNfH over the disease course.

In conclusion, our study shows that pNfH differs among MND phenotypes and is an independent prognostic factor for survival. This study provides evidence that supports the role of serum pNfH as useful prognostic biomarker for MND patients. Neurofilament measurements should be considered in future prognostic models and in clinical trials for biomarker-based stratification, and to evaluate treatment response.

Author contributions YMF and TD performed the experiments; YMF, TD, PC, FA, MF, AQ, and NR contributed to analysis and interpretation of the data; YMF, PS, MC, AB, LL, UD, RF, and NR recruited the patients, performed the clinical evaluation, acquired clinical data, and recruited patients; TD, LP, and PC performed the genetic analysis; YMF, NR, and AQ drafted the manuscript; YMF, FA, MF, NR, and AQ conceptualized and designed the study; FA, MF, NR, and AQ obtained the funding; NR, AQ, GC, and FM supervised the study. All authors had full access to the data in the study, critically revised, and approved the final version of the manuscript.

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Compliance with ethical standards

Conflicts of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Patient consent Informed consent was obtained from the patients who participated in the study.

Ethics approval The study was approved by the appropriate ethics committee and has, therefore, been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

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