ANNALS of Clinical and Translational Neurology
Open Access
AMERICAN NEUROLOGICAL ASSOCIATION

BRIEF COMMUNICATION

Evaluation of peripherin in biofluids of patients with motor neuron diseases

Daniele Sabbatini[1,*], Flavia Raggi[1,*], Susanna Ruggero[2], Mara Seguso[1], Jessica Mandrioli[3,4], Annachiara Cagnin[1], Chiara Briani[1], Elisabetta Toffanin[2], Matteo Gizzi[1], Andrea Fortuna[1], Luca Bello[1], Elena Pegoraro[1], Giulia Musso[5] & Gianni Sorarù[1]

[1]Department of Neurosciences, University of Padova, Padova, Italy
[2]Department of Neurosciences, General Hospital of Padua, Padova, Italy
[3]Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Modena 41125, Italy
[4]Department of Neurosciences, Azienda Ospedaliera Universitaria Modena, Modena 41126, Italy
[5]Department of Laboratory Medicine, University of Padova, Padova, Italy

Correspondence
Gianni Sorarù, Department of Neurosciences, University of Padova, Via Nicolò Giustiniani, 5, 35128 Padova (PD), Italy. Tel: +390498213600; Fax: +390495751770; E-mail: gianni.soraru@unipd.it

Received: 24 February 2021; Revised: 6 May 2021; Accepted: 17 June 2021

Annals of Clinical and Translational Neurology **2021; 8(8): 1750–1754**

doi: 10.1002/acn3.51419

*These authors equally contributed to this work.

Abstract

Peripherin (PRPH), a type III intermediate filament, assembles with neurofilaments in neurons of the peripheral nervous system, including lower motor neurons (LMN). To evaluate the role of PRPH in LMN degeneration, we assessed PRPH and neurofilament light chain (NfL) in cerebrospinal fluid (CSF) and serum of 91 patients with motor neuron diseases (MND) and 69 controls. Overall, we found PRPH to be more concentrated in serum than in CSF. Serum PRPH resulted significantly increased in MND patients but it was unrelated to CSF-NfL or survival in the amyotrophic lateral sclerosis (ALS) subset. PRPH might represent a marker of LMN involvement.

Introduction

Neurofilament (NF) levels in cerebrospinal fluid (CSF) and peripheral blood of patients with neurological and neurodegenerative disorders are recognized as promising biomarkers of neuronal injury and disease activity.[1] The increase of both neurofilament light (NfL) and heavy chain (NfH) appear to be particularly significant in patients with amyotrophic lateral sclerosis (ALS), where NFs levels strongly correlate with disease prognosis and contribute to discriminating between ALS and disease controls.[2–5]

Peripherin (PRPH) is a type III intermediate filament, 58 kD protein, which assembles with NFs mostly in neurons of the peripheral nervous system (PNS) and also in neurons of the central nervous system (CNS) with projections to peripheral structures, such as lower motor neurons (LMNs).[6] PRPH function is incompletely understood though a role in neurite growth and stability has been suggested.[7,8] PRPH seems to be involved in motor neuron vulnerability in motor neuron diseases (MND).[9] Overexpression of wild-type PRPH in mice provokes defective axonal transport of NF proteins,[10] leading to late onset degeneration of motor axons.[11] PRPH, on the other hand, is upregulated in damaged LMNs after nerve injury.[12] Together with NFs, PRPH accumulates in the majority of axonal inclusion bodies, called spheroids, in motor neurons of ALS patients.[13] Lastly, PRPH gene mutations have been reported to disrupt NF assembly in sporadic ALS.[14,15] Unlike NFs, PRPH levels have never been assessed in healthy or disease human biofluids.

In this study, we exploratively evaluated PRPH concentrations in cerebrospinal fluid (CSF) and serum from patients with MNDs and assessed possible correlation with motor neuron degeneration.

Materials and Methods

Study cohort

This is a retrospective, longitudinal study on a cohort of 160 patients referring to the Department of Neurosciences

of the University of Padua, (Italy), from January 2016 to May 2020, who underwent lumbar puncture and blood collection for diagnostic purposes. CSF and serum data from 91 patients with MNDs (63 probable or definite ALS according to the El Escorial criteria[16]; 20 Spinal Muscular Atrophy, SMA, type 3, and 8 Spinal and Bulbar Muscular Atrophy, SBMA) were compared to those from 26 patients with dementia, 14 with peripheral neuropathy (PN) and 29 healthy subjects (healthy controls, HC) who underwent lumbar puncture because of suspected but later unconfirmed neurological disease. Demographic and clinical characteristics of the patients were obtained from medical records or directly from treating physicians and patients, as well as controls (Table 1).

Of ALS patients we further recorded: disease extension assessed with the ALS Functional Rating Scale–Revised (ALSFRS-R); progression rate (ΔFS) calculated as follows: ΔFS = 48 − ALSFRS-R score at diagnosis/months from onset to diagnosis[17]; and overall survival calculated from onset to death (or tracheostomy or last follow-up) (months).

The study was approved by the local Ethics Committee. All participants provided written informed consent to the study.

Assays

CSF and serum samples were centrifuged, aliquoted and stored at −80°C. CSF-NfL levels were measured using an enzyme-linked immunoassay (ELISA) (NF-light ELISA kit, UmanDiagnostic) as previously described.[3]. CSF-PRPH and serum PRPH (S-PRPH) levels were determined using a competitive binding enzyme-linked immunoassay (Human Peripherin ELISA kit, Abbexa). Each plate contained calibrators (0.312–20 ng/mL). For each kit, samples were distributed on the plate undiluted and measured in duplicate. The inter-assay and intra-assay coefficients of variance were all below 10%. According to manufacturer's instructions, analytical sensitivity was set <0.156 ng/mL. Manufacturer declared a Sample recovery range after spiking of 91–105% and a linearity range of 90–116% in dilutions up to 1:8 both in serum; spike recovery test on CSF and serum samples and dilution linearity test on serum samples were conducted with protocols adapted from Andreasson et al. with modifications.[18]

Table 1. Characteristics of the study groups.

Group	N°	Male/ female	Age at LP and blood sample (median)
ALS	63	36/27	65
SMA	20	12/8	30.5
SBMA	8	8/0	56.5
Dementia (18 FTD, 4 AD, 4 NPH)	26	17/9	62.5
PN (7 CIDP, 2 diabetic polyneuropathies, 1 HDMN, 4 MMN)	14	13/1	65
HC	29	7/22	50

LP, lumbar puncture; FTD, frontotemporal dementia; AD, Alzheimer's disease; NPH, normal pressure hydrocephalus; IQR, interquartile range; SMA, spinal muscular atrophy; SBMA, spinal and bulbar muscular atrophy; PN, peripheral neuropathy; CIDP, chronic inflammatory demyelinating polyneuropathy; HDMN, hereditary distal motor neuropathy; MMN, multifocal motor neuropathy; HC, healthy controls.

Statistics

To verify any deviation from the normal distribution of the considered variables, the Shapiro–Wilk test was applied. A natural logarithmic transformation has been applied to NfL concentrations (log[NfL]) as previously described.[3] Kruskal–Wallis and pairwise Wilcoxon rank sum tests were used to compare NfL and PRPH levels among disease/HC groups. ANOVA model, Pearson's product-moment correlation coefficient and Spearman's rho correlation coefficient were assessed to verify a possible correlation between S-PRPH and CSF-NfL levels, separately, with age at lumbar puncture or blood sample, and sex (for each patient group) and progression rate (ΔFS) (only for ALS group). It has been evaluated also the possible correlation between S-PRPH and CSF-NfL levels for each patients group.

In ALS group, survival was analyzed with death (or tracheostomy) as event while, being a predominantly living cohort study, 41 patients who were alive by the last follow-up were censored. ALS patients were divided into different groups corresponding to S-PRPH and CSF-NfL concentration quartiles. These groups were used to graphically represent survival data in relation to PRPH and NfL levels.

A Cox proportional hazards regression model was devised in the ALS group for S-PRPH and CSF-NfL levels, separately, with sex, ΔFS, and age at onset as covariates. Hazard ratios (HRs) were calculated for each quantitative covariate and each level of categorical covariates. Reference levels were assigned HRs of 1.0. Statistical analyses were performed in R (R Foundation, version 4.0.2), with statistical significance set at $p < 0.05$ for all tests (Holm correction applied). Survival[19] and survminer[20] packages were used for Kaplan–Meier analysis. To graphically represent data tidyverse[21] and ggpubr[22] packages were used.

Results

We analyzed CSF and serum of all the patients except for those with SBMA, who had only serum available for

testing. All CSF samples had PRPH measurements below the detection limit of the calibration curve of the assay. Average recovery range was: 80%–92% in CSF with serum spiked, 148%–183% in CSF with calibrator spiked and 147%–177% in serum with calibrator spiked; average linearity range was 99%–103% in serum (data not shown). A Kruskal–Wallis rank sum Test showed a significant difference in S-PRPH (Chi-squared = 68.623, df = 5, $p < 0.001$), and CSF-NfL (Chi-squared = 98.51, df = 4, $p < 0.001$) levels among the study groups. S-PRPH levels were significantly higher in all MND patients [ALS (median, 5.376 ng/mL; interquartile range [IQR], 3.569–7.422 ng/mL); SMA (median, 5.653 ng/mL; IQR, 2.982–7.654 ng/mL); SBMA (median, 10.224 ng/mL; IQR, 8.896–14.584)] compared to healthy controls (median, 3.168 ng/mL; IQR 2.422 to −4.726 ng/mL), dementia (median, 1.793 ng/mL; IQR, 1.287–2.226 ng/mL), and PN (median, 2.21 ng/mL; IQR 1.313–4.293 ng/mL). Among MND patients, SBMA group showed the highest values of S-PRPH (Fig. 1; Table 2).

Gender and age did not influence S-PRPH levels, considering the phenotypic groups either separately or together.

We confirmed NfLs values to be significantly higher in ALS group (median, 4357.8 pg/mL; IQR, 1768.3–6429.1 pg/mL) compared to healthy controls (median, 376.6 pg/mL; IQR, 240.7–557.1 pg/mL), SMA (median, 168.0 pg/mL; IQR, 141.0–203.5 pg/mL), and PN (median, 1016.7 pg/mL; IQR, 775.9–1294.4 pg/mL), with no significant difference between ALS and dementia groups (median, 2923.4 pg/mL; IQR, 1469.3–6688.4 pg/mL) (Fig. 2; Table 3). A possible correlation between S-PRPH and CSF-NfL levels was not found in any patient group.

In ALS group, Cox proportional hazards regression analysis showed that PRPH levels and overall survival did not correlate, even after adjusting for the concurrent

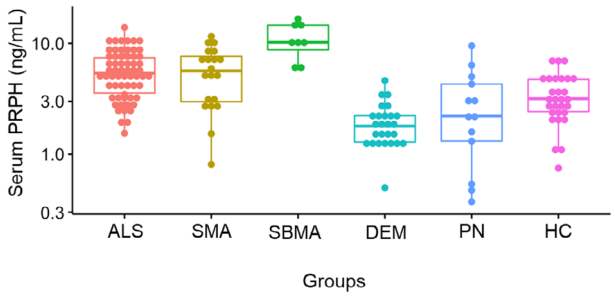

Figure 1. [PRPH] in serum (ng/mL) in the six main study groups. The central location, scatter, and dispersion of the observations are shown. In red amyotrophic lateral sclerosis (ALS); in yellow spinal muscular atrophy (SMA); in green spinal and bulbar muscular atrophy (SBMA); in light blue dementia (Dem); in blue peripheral neuropathy (PN); in pink healthy controls (HC). For a better interpretation of the graph, a standard log transformation was applied.

Table 2. Pairwise comparisons of PRPH concentrations in serum using Wilcoxon rank sum test; *p*-value adjustment method: Holm.

Group	ALS	SMA	SBMA	DEM	PN
SMA	0.98	–	–	–	–
SBMA	0.006	0.02	–	–	–
DEM	<0.001	<0.001	<0.001	-	–
PN	0.007	0.04	<0.001	<0.001	–
HC	<0.001	0.02	<0.001	0.001	0.7

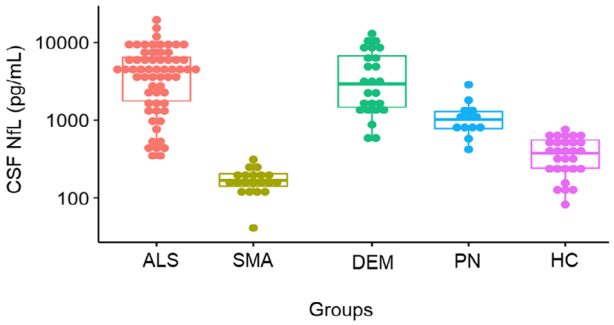

Figure 2. [NfL] in CSF (pg/mL) in the ALS, SMA, DEM, NP, and CTRLS study groups. The central location, scatter, and dispersion of the observations are shown. In red amyotrophic lateral sclerosis (ALS); in yellow spinal muscular atrophy (SMA); in green dementia (Dem); in light blue peripheral neuropathy (PN); in pink healthy controls (HC). For a better interpretation of the graph a standard log transformation was applied.

Table 3. Pairwise comparisons of NfL concentrations in CSF using Wilcoxon rank sum test; *p*-value adjustment method: Holm.

Group	ALS	SMA	DEM	PN
SMA	<0.001	–	–	–
DEM	0.55	<0.001	–	–
PN	<0.001	<0.001	<0.001	–
HC	<0.001	<0.001	<0.001	<0.001

effect of sex, ΔFS, and age at onset (Fig. S1A and B; Table S1). S-PRPH levels did not correlate with ΔFS score. Conversely, a significant inverse correlation between log[NfL] levels and overall survival was found (HR, 17.75; 95% CI, 3.70–85.2; $p < 0.001$), adjusting for the concurrent effect of sex, ΔFS, and age at onset (Fig. S2A and B; Table S2). Moreover, log[NfL] levels and ΔFS score were found to be strongly correlated (r (58) = 0.48, $p < 0.001$), as previously described.[3]

Discussion

In this report, we describe the first attempt to quantify PRPH in biofluids of MND patients and, more generally,

of human subjects. A preliminary analysis indicates that PRPH can be measured in the serum, whereas the protein levels in CSF fall beneath the analytical sensitivity of the ELISA assay. Additional spike and recovery test reported comparable results in CSF and serum, thus pointing a matrix effect of CSF in the assay might be excluded. These findings suggest that PRPH is more concentrated in serum than in CSF, which might reflect the predominant localization of PRPH in the neurite cytoskeleton of PNS neurons.[23]

In our cohort, S-PRPH was found to be increased in all MND patients compared to controls, consistently with the LMN involvement occurring in these patients. Among MND patients, S-PRPH levels were higher in the SBMA group, data that can be ascribed to the concurrent sensory axonal neuropathy observed in the disease.[24] S-PRPH resulted low in patients with dementia, thus indirectly further supporting the peripheral release of the protein.

The results of our study confirm that CSF-NfL values are elevated in patients with ALS and dementia. Low NfL levels were instead found in SMA CSF. The latter finding, along with the previous reports of normal NfLs in serum of SMA and SBMA patients,[25,26] suggests that S-PRPH may be more sensitive than NfLs to reveal LMN injury.

We failed to observe any correlation between S-PRPH and CSF-NfL concentrations in all patient groups. Moreover, no correlation was found between S-PRPH levels and disease severity or survival in ALS patients. This was not an unexpected finding, since PRPH is not only released by damaged neurites, but it is also upregulated due to the neural injury.[12] As a consequence, S-PRPH levels might not exactly reflect the number of damaged neurons and thus the degree of motor disability.

In conclusion, high serum levels of PRPH might be a general marker of LMN axon disorders. Further studies including a larger number of patients and more advanced techniques of PRPH quantification (i.e., single-molecule array, SiMoA, technology) are certainly needed to validate our results.

Acknowledgments

The authors are grateful to ASLA Onlus.

Conflicts of Interest

All other authors declare no conflict of interest.

Author Contributions

G.S., F.R., and D.S. conceived the project and designed experiments. S.R., M.S., and E.T. carried out the experiments. G.S., F.R., S.R., M.S., and E.T. supervised the data acquisition. F.R. analyzed the experimental data. DS carried out the statistical analysis. G.S., F.R., D.S, J.M., A.C., C.B., M.G., A.F., L.B., and E.P. visualized and interpreted the data. G.S., E.P., J.M., A.C., and C.B. recruited the patients. G.S, D.S., and F.R. wrote the manuscript.

Funding Information

The authors are grateful to ASLA Onlus.

References

1. Gordon BA. Neurofilaments in disease: what do we know? Curr Opin Neurobiol 2020;61:105–115.
2. Zucchi E, Bonetto V, Sorarù G, et al. Neurofilaments in motor neuron disorders: towards promising diagnostic and prognostic biomarkers. Mol Neurodegener 2020;15:58.
3. Gaiani A, Martinelli I, Bello L, et al. Diagnostic and prognostic biomarkers in amyotrophic lateral sclerosis. JAMA Neurol 2017;74:525.
4. Steinacker P, Feneberg E, Weishaupt J, et al. Neurofilaments in the diagnosis of motoneuron diseases: a prospective study on 455 patients. J Neurol Neurosurg Psychiatry 2016;87:12–20.
5. Lu C-H, Macdonald-Wallis C, Gray E, et al. Neurofilament light chain: a prognostic biomarker in amyotrophic lateral sclerosis. Neurology 2015;84:2247–2257.
6. Zhao J, Liem RKH. α-Internexin and peripherin. Expression, assembly, functions, and roles in disease. Methods Enzymol 2016;568:477–507.
7. Larivière RC, Nguyen MD, Ribeiro-da-Silva A, Julien JP. Reduced number of unmyelinated sensory axons in peripherin null mice. J neurochem 2002;81:525–532.
8. Helfand BT, Mendez MG, Pugh J, et al. A role for intermediate filaments in determining and maintaining the shape of nerve cells. Mol Biol Cell 2003;14:5069–5081.
9. Hedlund E, Karlsson M, Osborn T, et al. Global gene expression profiling of somatic motor neuron populations with different vulnerability identify molecules and pathways of degeneration and protection. Brain 2010;133:2313–2330.
10. Millecamps S, Robertson J, Lariviere R, et al. Defective axonal transport of neurofilament proteins in neurons overexpressing peripherin. J Neurochem 2016;98:926–938.
11. Beaulieu JM, Nguyen MD, Juliena JP. Late onset death of motor neurons in mice overexpressing wild-type peripherin. J Cell Biol 1999;147:531–544.
12. Terao E, Janssens S, van den Bosch de Aguilar PH, et al. In vivo expression of the intermediate filament peripherin in rat motoneurons: modulation by inhibitory and stimulatory signals. Neuroscience 2000;101:679–688.

13. Corbo M, Hays AP. Peripherin and neurofilament protein coexist in spinal spheroids of motor neuron disease. J Neuropathol Exp Neurol 1992;51:531–537.
14. Gros-Louis F, Larivière R, Gowing G, et al. A frameshift deletion in peripherin gene associated with amyotrophic lateral sclerosis. J Biol Chem 2004;279:45951–45956.
15. Corrado L, Carlomagno Y, Falasco L, et al. A novel peripherin gene (PRPH) mutation identified in one sporadic amyotrophic lateral sclerosis patient. Neurobiol Aging 2011;32:552.
16. Brooks BR, Miller RG, Swash M, Munsat TL. El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. ALS Other Motor Neuron Disord 2009;1:293–299.
17. Kimura F, Fujimura C, Ishida S, et al. Progression rate of ALSFRS-R at time of diagnosis predicts survival time in ALS. Neurology 2006;66:265–267.
18. Andreasson U, Perret-Liaudet A, van Waalwijk van Doorn LJ, et al. A practical guide to immunoassay method validation. Front Neurol 2015;6:179.
19. Therneau TM, Grambsch PM. Modeling survival data: extending the Cox Model, 1st ed. New York: Springer-Verlag, 2000.
20. Kassambara A, Kosinski M, Biecek P. Survminer: drawing survival curves using 'ggplot2'. R package version 0.4.8. https://CRAN.R-project.org/package=survminer
21. Wickham H, Averick M, Bryan J, et al. Welcome to the Tidyverse. J Open Source Softw 2019;4:1686. https://doi.org/10.21105/joss.01686
22. Kassambara A. ggpubr: 'ggplot2' Based Publication Ready Plots. R package version 0.4.0. 2020. https://CRAN.R-project.org/package=ggpubr
23. Yuan A, Sasaki T, Kumar A, et al. Peripherin is a subunit of peripheral nerve neurofilaments: implications for differential vulnerability of CNS and peripheral nervous system axons. J Neurosci 2012;32:8501–8508.
24. Hama T, Hirayama M, Hara T, et al. Discrimination of spinal and bulbar muscular atrophy from amyotrophic lateral sclerosis using sensory nerve action potentials. Muscle Nerve 2012;45:169–174.
25. Wurster CD, Steinacker P, Günther R, et al. Neurofilament light chain in serum of adolescent and adult SMA patients under treatment with nusinersen. J Neurol 2020;267:36–44.
26. Lombardi V, Querin G, Ziff OJ, et al. Muscle and not neuronal biomarkers correlate with severity in spinal and bulbar muscular atrophy. Neurology 2019;92:e1205–e1211.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. (A) Kaplan–Meier Plots of the survival probability relative to time (months) from onset to death (or tracheostomy or last follow-up) grouped by PRPH concentrations in serum for ALS group. 1°q indicates first quartile, corresponding to a value from 1.546 to 3.569 ng/mL; 2°q, 3.569–5.376 ng/mL; 3°q, 5.376–7.422 ng/mL; 4°q, 7.422–13.913 ng/mL. (B) The forest plot shows measures of the Cox proportional hazards regression analysis of effect of PRPH concentration in serum, sex, ΔFS, and age at onset on overall survival.

Figure S2. (A) Kaplan–Meier Plots of the survival probability relative to time (months) from onset to death (or tracheostomy or last follow-up) grouped by log[NfL] concentrations in CSF for ALS group. 1°q indicates first quartile, corresponding to a value from 320.695 to 1768.3 pg/mL; 2°q, 1768.3–4357.803 pg/mL; 3°q, 4357.803–6429.1 pg/mL; 4°q (in violet), 6429.1–19387.100 pg/mL. (B) The forest plot shows measures of the Cox proportional hazards regression analysis of effect of log[NfL] concentration in CSF, sex, ΔFS, and age at onset on overall survival.

Table S1. The table shows the number of patients at risk, the number of events (death or tracheostomy) and the cumulative survival rate relative to each time point event for serum PRPH in ALS group.

Table S2. The table shows the number of patients at risk, the number of events (death or tracheostomy) and the cumulative survival rate relative to each time point event for CSF-NfL in ALS group.

