BRIEF REPORT

Plasma Neurofilament Light Chain Is Elevated in Adaptor Protein Complex 4-Related Hereditary Spastic Paraplegia

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ABSTRACT: Background: Adaptor protein complex 4-associated hereditary spastic paraplegia (AP-4-HSP) is caused by pathogenic biallelic variants in *AP4B1*, *AP4M1*, *AP4E1,* and *AP4S1*.

Objective: The aim was to explore blood markers of neuroaxonal damage in AP-4-HSP.

Methods: Plasma neurofilament light chain (pNfL) and glial fibrillary acidic protein (GFAP) levels were measured in samples from patients and age- and sex-matched controls (NfL: n = 46 vs. n = 46; GFAP: n = 14 vs. n = 21) using single-molecule array assays. Patients' phenotypes were systematically assessed using the AP-4-HSP natural history study questionnaires, the Spastic Paraplegia Rating Scale, and the SPATAX disability score.

Results: pNfL levels increased in AP-4-HSP patients, allowing differentiation from controls (Mann-Whitney *U* test: *P* = 3.0e-10; area under the curve = 0.87 with a 95% confidence interval of 0.80–0.94). Phenotypic cluster analyses revealed a subgroup of individuals with severe generalized-onset seizures and developmental stagnation, who showed differentially higher pNfL levels (Mann-Whitney *U* test between two identified clusters: *P* = 2.5e-6). Plasma GFAP levels were unchanged in patients with AP-4-HSP.

Conclusions: pNfL is a potential disease marker in AP-4-HSP and can help differentiate between phenotypic subgroups. © 2023 International Parkinson and Movement Disorder Society.

Key Words: hereditary spastic paraplegia; adaptor protein complex-4; *SPG47*; *SPG50*; *SPG51*; *SPG52*; biomarker; neurofilament light; plasma; phenotypic clustering

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Adaptor protein complex 4-associated hereditary spastic paraplegia (AP-4-HSP) consists of four forms of childhood-onset HSP with a shared phenotype, caused by biallelic loss-of-function variants in the genes that encode the subunits of the AP-4 (*AP4B1*, *AP4M1*, *AP4E1*, and *AP4S1*, also referred to as *SPG47*, *SPG50*, *SPG51*, and *SPG52*).[1] AP-4-HSP has emerged as an important mimic of cerebral palsy and a prototypical form of complex HSP in children, presenting with features of a neurodevelopmental disorder (ie, global developmental delay, microcephaly, and early-onset seizures) and a progressive neurodegenerative disease (ie, progressive spasticity with loss of ambulation, extrapyramidal movement disorders, and bulbar dysfunction).[2-5] Although we have previously shown that cell-based functional assays, that is, in patient-derived fibroblasts, can establish AP-4 function and thus aid a diagnosis of AP-4-HSP, to date no readily available disease marker has been established.[6] With a growing number of potential treatment approaches in the development pipeline, including gene replacement therapy, blood biomarkers would present a significant step toward clinical trial readiness.[7]

Development of advanced ultrasensitive detection methods such as single-molecule array (SiMoA) has allowed for the reliable quantification of neuronal and glial proteins such as neurofilament light chain (NfL) and glial fibrillary acidic protein (GFAP) in blood.[8-10] Both NfL and GFAP have emerged as potential biomarkers of neuroaxonal damage and neurodegeneration and have been validated for a variety of neurological disorders, including Parkinson's disease, multiple sclerosis, and adult-onset HSP.[11-15]

Here, we investigate plasma NfL (pNfL) and GFAP (pGFAP) as potential markers of neuroaxonal damage in AP-4-HSP using a well-characterized cohort of 46 mostly pediatric patients enrolled in an ongoing natural history study (NCT04712812), paired with 46 age- and sex-matched controls.

Patients and Methods

Detailed information is available in the Supplementary Material.

Study Design and Participants

This study was approved by the Boston Children's Hospital (IRB-P00033016), and written consent was obtained from all participants. Patients were recruited from the AP-4-HSP International Registry (NCT04712812). Clinical information was collected using the AP-4-HSP Natural History Study Questionnaire, the Spastic Paraplegia Rating Scale (SPRS), and the SPATAX disability score (SPATAX).[16-18] Plasma samples from age- and sex-matched controls with no history of neurological disease (Table S1) were acquired from the Boston Children's Hospital PrecisionLink Biobank.[19] Demographic, genetic, and clinical information is provided in Table 1.

SiMoA Measurements

Plasma was stored at −80°C and not thawed until measurements were performed. pNfL and pGFAP measurements were performed in duplicates on the Simoa HD-X analyzer using the GFAP Discovery and NF-Light Advantage kits (Quanterix, Billerica, MA). pNfL levels were measured in 46 patient and 46 control samples, and pGFAP levels were measured in 14 patient and 21 control samples. Forty patient and 45 control samples (for pNfL), as well as all 12 patient and 20 control samples (for pGFAP), were included in the final analysis after quality control filtering. Details on outlier removal are available in the Supplementary Material. The following median intra-assay percentage coefficients of variation (CV) were observed: 3.59% (IQR [interquartile range] = 3.46) for patient and 4.73% (IQR = 6.15) for control samples when measuring pNfL and 3.62% (IQR = 3.56) for patient and 3.29% (IQR = 4.45) for control samples when measuring pGFAP.

Statistical Analyses

Normality of distributions was assessed using Shapiro–Wilk test and graphical methods. Medians and IQRs are reported for continuous variables. Differences between groups were evaluated using Mann-Whitney *U* test or Kruskal–Wallis test. Fisher's exact test was used for evaluating dependence between categorical variables. *P*-values were adjusted for multiple hypothesis testing using the Benjamini–Hochberg procedure.[20] Classification performance based on pNfL was assessed using receiver operating characteristic (ROC) analysis and area under the ROC curve (AUC). Spearman's rank correlation coefficient and multivariate linear regression analyses adjusted for age and sex were used to evaluate associations between pNfL and functional scores. To assess associations between pNfL and dichotomous clinical findings, a multivariate linear regression adjusted for age and sex was performed. Unsupervised clustering based on clinical data was conducted using Gower's distance and the partitioning around medoids (PAM) algorithm.[21,22] All statistical tests were two sided, and $P < 0.05$ was considered significant. Statistical analyses were performed in R (v4.2.2) and RStudio (v2022.12.03, RStudio, Inc., Boston, MA).

Results

Demographic, molecular, and clinical data of the cohort of 46 AP-4-HSP patients are summarized in Table 1. Core clinical manifestations were delayed motor development (100.0%, n = 45), intellectual

TABLE 1 *Demographic, genetic, and clinical data of the cohort and overview of regression analyses*

Group demographics		**AP-4-HSP patients**	**Matched controls**
Individuals (n)		46	46
Sex (M:F)		26:20	21:25
Median age at data collection (y)		7.5 (IQR = 9.4)	9.0 (IQR = 12.2)
Genetic information (n = 46)		**Core clinical features (n/N)**	
Affected gene (%)		Delayed motor development	45/45 (100.0%)
AP4B1	22 (47.8%)	Intellectual disability	30/30 (100.0%)
AP4E1	1 (2.2%)	Mild	3/30 (10.0%)
AP4M1	14 (30.4%)	Moderate	14/30 (46.7%)
AP4S1	9 (19.6%)	Severe	13/30 (43.3%)
Heterozygous:homozygous	21:25	Hypotonia progressing to hypertonia	42/46 (91.3%)
Coding impact (number of alleles)		Lower-limb spasticity	42/46 (91.3%)
Truncating:nontruncating[a]	49:43	Neonatal/infantile hypotonia	40/45 (88.9%)
Exon deletion (n)	2 (2.2%)	Babinski sign	17/20 (85.0%)
Frameshift (n)	25 (27.2%)	Stool incontinence	29/36 (80.6%)
Missense (n)	17 (18.5%)	Urinary incontinence	28/36 (77.8%)
Nonsense (n)	22 (23.9%)	Sensory deficit	27/35 (77.1%)
Splice site (n)	26 (28.3%)	Seizures	35/46 (76.1%)
		Febrile seizures	33/46 (71.7%)
		Generalized-onset seizures	22/46 (47.8%)
		Status epilepticus	22/46 (47.8%)
		Focal-onset seizures	16/46 (34.8%)
		Walking aid dependent	33 (75.0%)
		Lower-limb hyperreflexia	25 (71.4%)
		Lower-limb muscle weakness	30 (65.2%)

		Correlation		**Multivariate regression**	
Association of functional scores with pNfL	**n**	ρ	***P***	R^2_{adj}[b]	P_{adj}[b]
SPRS score	40	−0.03	0.846	0.10	0.785
Maximum SPRS score	40	0.01	0.977	0.10	0.651
SPRS spasticity subscore	40	−0.35	0.025	0.44	0.281
Maximum SPRS spasticity subscore	40	−0.35	0.025	0.39	0.267
SPATAX score	40	−0.04	0.821	0.05	0.834
Maximum SPATAX score	40	0.03	0.845	0.01	0.921

Abbreviations: AP-4-HSP, adaptor protein complex 4-associated hereditary spastic paraplegia; IQR, interquartile range; pNfL, plasma neurofilament light chain; SPRS, Spastic Paraplegia Rating Scale.
[a]Nonsense and frameshift variants as well as exon deletions were considered protein truncating.
[b]R^2 and *P* are age- and sex adjusted.

disability (100.0%, n = 30), lower-limb spasticity (91.3%, n = 46), and a positive Babinski sign (85.0%, n = 20). Patients had a median SPRS score of 31 (IQR = 12.25), a median SPRS spasticity subscore of 5 (IQR = 6.75), and a median SPATAX score of 5 (IQR = 3). Patients with AP-4-HSP had significantly higher median pNfL levels compared to age- and sex-matched healthy controls (6.26 pg/mL [IQR = 3.62 pg/mL, n = 40] vs. 3.48 pg/mL [IQR = 1.40 pg/mL, n = 45], Mann-Whitney *U* test, *P* = 3.0e-10; Fig. 1A).

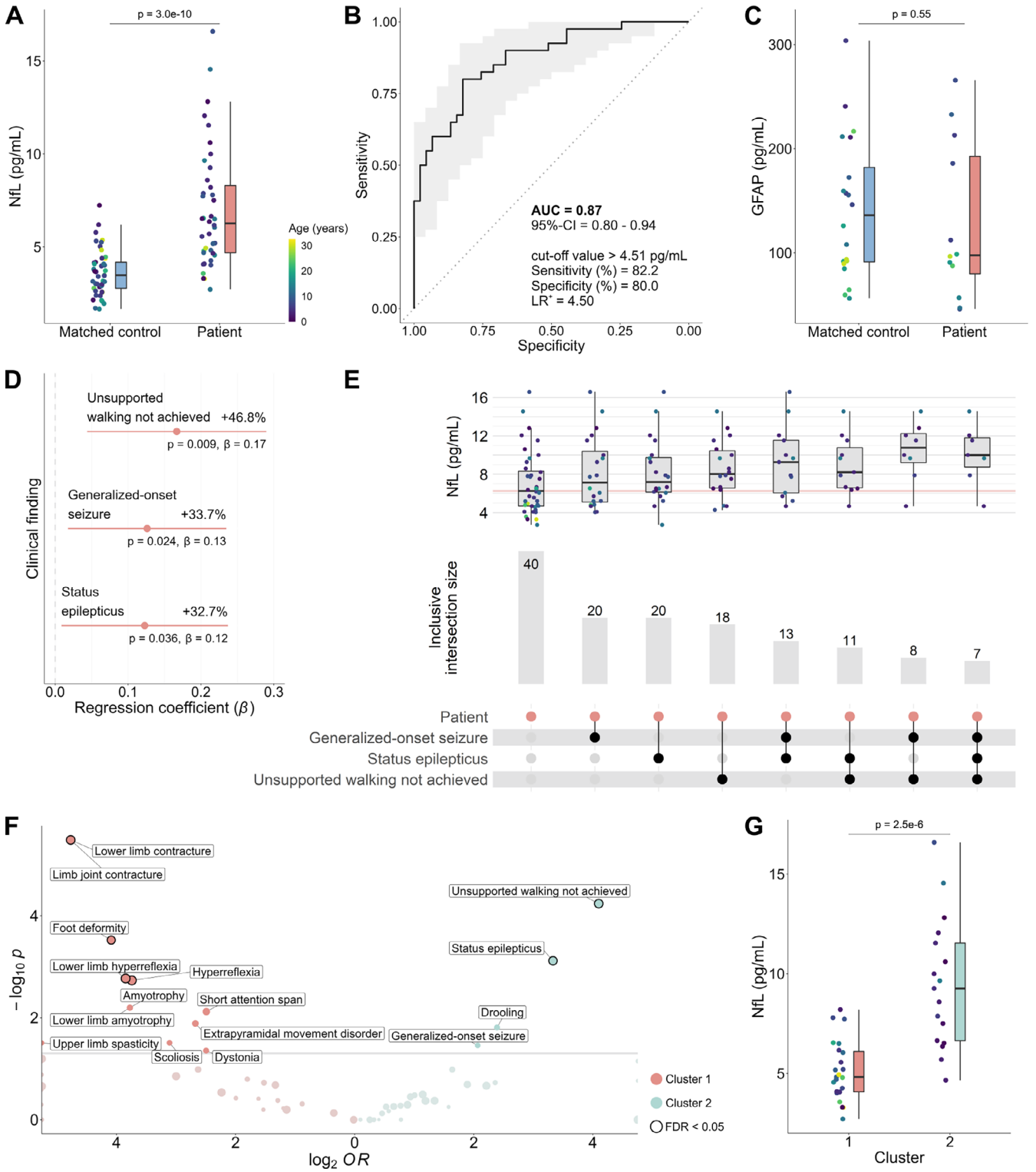

FIG. 1. Evaluation of plasma NfL (neurofilament light chain) and GFAP (glial fibrillary acidic protein) levels as disease markers in AP-4-HSP (adaptor protein complex 4-associated hereditary spastic paraplegia). (**A**) Comparison of pNfL levels in AP-4-HSP patients and age- and sex-matched controls. (**B**) Receiver operating characteristic (ROC) analysis for pNfL. ROC curve for the pooled pNfL levels of AP-4-HSP patients versus matched controls. (**C**) Comparison of pGFAP (plasma glial fibrillary acidic protein) levels in AP-4-HSP patients and age- and sex-matched controls. (**D**) Associations with dichotomous clinical findings for pNfL levels in patients using multivariate regression models adjusted for age and sex. Forest plot with respective regression coefficients (β), *P*-values, and percentage increase in pNfL levels in the case of symptom presence (using $\log_{10}$-backtransformed regression coefficients). (**E**) Upset plot annotated with respective pNfL levels showing the permutation of all possible inclusive intersections for patients presenting with a history of generalized-onset seizures, status epilepticus, and never having achieved unsupported walking. (**F**) Volcano plot showing the odds ratios and respective *P*-values for clinical findings when comparing patient clusters 1 and 2. *P*-values were adjusted for multiple hypothesis testing using the Benjamini–Hochberg procedure. Dot sizes represent relative frequencies of the respective clinical findings within the patient cluster. (**G**) Comparison of pNfL levels in patients assigned to clusters 1 and 2. [Color figure can be viewed at wileyonlinelibrary.com]

No significant differences in pNfL levels were observed when patients were stratified for affected gene or coding impact (Fig. S1). Interestingly, pNfL levels showed a weak inverse correlation with age in patients ($\rho = 0.36$, $P = 0.024$) but not in controls ($P = 0.45$; Fig. S1). To assess the potential value of pNfL as a diagnostic disease marker, we performed an ROC analysis, which revealed moderate performance in differentiating patients and controls (AUC = 0.87, 95% confidence interval [CI] = 0.80–0.94; Fig. 1B). Maximizing the Youden index yielded a cutoff value of >4.51 pg/mL to distinguish between groups (sensitivity = 82.2%, specificity = 80.0%, positive likelihood ratio = 4.5; Fig. 1B). In contrast to pNfL, pGFAP levels were not significantly different between patients and controls (97.7 pg/mL [IQR = 112.8 pg/mL, n = 12] vs. 136.1 pg/mL [IQR = 90.8 pg/mL, n = 20], Mann-Whitney *U* test, $P = 0.55$; Fig. 1C).

Next, we sought to explore the associations between pNfL levels and clinical manifestations and disease severity. Given the well-characterized positive correlation of clinical scores, such as the SPRS and SPATAX with age in AP-4-HSP patients,[2,14] and the significant association between NfL levels and sex and age that have been previously reported,[12,23,24] we decided to adjust subsequent analyses for age and sex. Although no significant associations between pNfL levels and absolute SPRS or SPATAX scores were observed when adjusting the models for age and sex (Table 1), we found a significant association with three clinical symptoms. Presence of "unsupported walking never achieved" ($P_{adj} = 0.009$, $\beta = 0.17$ [95% CI = 0.04–0.29], $R_{adj}^2 = 0.23$), "generalized-onset seizures" ($P_{adj} = 0.024$, $\beta = 0.13$ [95% CI = 0.02–0.23], $R_{adj}^2 = 0.19$), and "history of status epilepticus" ($P_{adj} = 0.036$, $\beta = 0.12$ [95% CI = 0.01–0.24], $R_{adj}^2 = 0.17$) resulted in an increase of 46.8%, 33.7%, and 32.7% pNfL levels, respectively (using $\log_{10}$-backtransformed β; Fig. 1D). To account for potential additive effects not captured by our regression models, we additionally examined pNfL levels in patients who presented with two or more of the three aforementioned phenotypes. This revealed a trend towards higher pNfL concentrations in patients with two or three of these manifestations (eg, median: 10.0 pg/mL [IQR = 3.0, n = 7] in patients with all three symptoms vs. 6.3 pg/mL [IQR = 3.6, n = 40] in the entire patient cohort; Fig. 1E).

Considering the potential presence of latent phenotypic subgroups, which might significantly differ in pNfL levels, we decided to employ PAM, an unsupervised clustering algorithm suitable for use with mixed-type data sets. Phenotypic clustering uncovered two distinct subgroups (cluster 1, n = 27; cluster 2, n = 19). Whereas cluster 1 mainly included older patients with signs of advanced corticospinal tract dysfunction (median age: 10.0 years [IQR = 7.5]), reflected by higher frequencies of secondary musculoskeletal manifestations, that is, contractures (85.2%, OR [odds ratio] = 27.3 [95% CI = 5.0–220.1], $P_{adj} = 1.3e\text{-}4$), foot deformities (71.4%, OR = 17.0 [95% CI = 2.7–198.0], $P_{adj} = 5.8e\text{-}3$), and hyperreflexia (91.7%, OR = 13.7 [95% CI = 2.0–161.9], $P_{adj} = 0.02$), patients assigned to cluster 2 were on average younger (median age: 3.6 years [IQR = 2.8]) and presented with a severe phenotype, characterized by profound motor delay in the form of "unsupported walking never achieved" (84.2%, OR = 17.1 [95% CI = 3.4–123.5], $P_{adj} = 1.5e\text{-}3$) and a history of status epilepticus (79.0%, OR = 10.1 [95% CI = 2.3–56.9], $P_{adj} = 0.01$; Fig. 1F; Fig. S2). The difference in age distributions suggests that the defining symptoms of cluster 2 are indeed absent from cluster 1 and not merely representative of natural disease progression. Median pNfL levels were significantly lower in cluster 1 compared to cluster 2 patients (4.82 pg/mL [IQR = 2.01 pg/mL, n = 23] vs. 9.27 pg/mL [IQR = 4.89 pg/mL, n = 17], Mann-Whitney *U* test, $P = 2.5e\text{-}6$; Fig. 1G). To control for the differences in age and sex distributions between clusters, a multivariate regression adjusting for the two covariates was performed. This confirmed a significant positive association between assignment to cluster 2 and pNfL levels ($P = 2.3e\text{-}5$, $\beta = 0.25$ [95% CI = 0.15–0.36], $R_{adj}^2 = 0.44$), translating to 78% higher pNfL levels in cluster 2 compared to cluster 1 patients.

Discussion

In this study, we examined plasma NfL and GFAP levels as potential disease markers in AP-4-HSP patients. We report increased pNfL levels in patients compared to age- and sex-matched controls. Although no correlation between pNfL levels and established measures of disease severity and function (SPRS, SPRS spasticity subscore, and SPATAX score) was observed, pNfL was associated with certain clinical findings, indicative of developmental stagnation and generalized-onset seizures. Unsupervised clustering identified a subgroup of individuals of young age with a severe seizure phenotype and inability to walk, who had differentially higher pNfL levels.

The present study identifies pNfL as a potential disease marker in patients with AP-4-HSP. pGFAP levels, although measured only in a relatively small number of patients, were found to be unchanged, providing further evidence that AP-4-HSP is a disease driven by axonal injury rather than progressive demyelination.

Our study has several limitations. First, the sample size was relatively small, particularly when accounting for the relatively large age range, which limits predictive strength, in particular, in older individuals

(>15 years of age). Second, a longitudinal assessment of pNfL levels, including samples collected prior to the onset of significant spasticity, is needed to confirm our cross-sectional data. Third, despite the availability of age-matched controls in our study, the age-dependent reference range in larger and more diverse populations of healthy children remains to be established.

Our findings highlight the important role of comprehensive phenotyping when evaluating potential biomarkers and the need to include matched controls, particularly in the pediatric age group. The former is particularly relevant for neurological disorders with profound phenotypic pleiotropy, such as HSP.[25-28] Our study provides a data set that adds to the few available data for pNfL in neurologically healthy children.[29-33] In summary, by establishing a baseline of elevated pNfL levels in patients with AP-4-HSP, this first cross-sectional study provides the basis for future longitudinal measurement of pNfL, which will help determine whether pNfL is a prognostic biomarker sensitive to disease progression and disease modifying intervention. This step towards clinical trial readiness is important as it might inform the therapeutic window, disease stratification, and outcome measures in safety monitoring emerging gene replacement therapies for AP-4-related HSP.[34]

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Supplementary Information

Study Design and Participants

This study was approved at Boston Children's Hospital (IRB-P00033016), and written consent was obtained. Patients with a genetically confirmed diagnosis of AP-4-HSP were recruited from the AP-4-HSP International Registry (ClinicalTrials.gov identifier: NCT04712812). Clinical information was collected using the AP-4-HSP Natural History Study Questionnaire, the Spastic Paraplegia Rating Scale (SPRS) and the SPATAX Disability score (SPATAX).[5,16-18] SPRS spasticity subscores were derived from SPRS scores as previously described.[35] Briefly, the subscore included SPRS items 7 to 10 (ie, spasticity of hip adductor muscles, spasticity of knee flexion, weakness of hip abduction, and weakness of foot dorsiflexion), with a maximum score of 16. Probands were examined by board-certified pediatric neurologists either at the Boston Children's Hospital Movement Disorders Program or at the recruitment site indicated in Table S1. For patients who were initially not seen at Boston Children's Hospital, consensus on the clinical findings was reached by all examining neurologists. Plasma samples from age- and sex-matched controls with no history of neurological disease were acquired from the Boston Children's Hospital PrecisionLink Biobank.[19] Detailed demographic, genetic, and clinical descriptions are provided in Table 1 and Table S1.

Plasma Collection and Single-Molecule Array Measurements

Plasma samples were obtained by standard venipuncture and collected in lithium-heparin-coated BD Vacutainer PST tubes (BD 367962) for probands or EDTA-containing tubes (BD 366643) in matched controls. The tube coatings have previously been validated and shown not to differentially affect NfL quantification.[36] Samples were centrifuged at 2000*g* for 10 minutes at room temperature, aliquoted in cryovials (0.5 ml per vial), and stored at −80°C. Plasma samples were checked for hemolysis, and samples with severe hemolysis were excluded from further analyses. The pre-analytical interval was <3 days for all samples, well within the window of stability for NfL.[37] Plasma NfL and GFAP measurements were performed in duplicates at the Massachusetts General Hospital Clinical and Translational Research Unit (CTRU) biomarker core on a Simoa HD-X analyzer using the GFAP Discovery and NF-Light Advantage kits (Quanterix). A dilution factor of 1:4 was used. Prior to analysis, plasma samples were thawed on ice and centrifuged at 10,000*g* for 10 minutes at 4°C to pellet any remaining debris. pNfL levels were measured in all patients (n = 46) and controls (n = 46), and pGFAP levels were measured in a subset of patients (n = 14) and controls (n = 21). Multiple strategies were employed to identify outliers in the data set, including Rosner's test and graphical means. This revealed six and one outliers in the pNfL data set for patients and controls, respectively. For pGFAP levels, two outliers were identified in the patient and one in the control cohort. These were attributed to pre-analytic factors such as hemolysis or debris. Phenotypic data of patients whose samples were identified as outliers were carefully examined for distinctive features (eg, higher disease-related scores, clinical findings indicative of a more severe course of disease or faster disease progression), which revealed no clinical correlates for the deviations in the measurements. Therefore, we decided to exclude the outlier values from any downstream analyses. The following median intra-assay percentage coefficients of variation (CV) were observed: 3.59% (IQR = 3.46) for patient and 4.73% (IQR = 6.15) for control samples when measuring pNfL and 3.62% (IQR = 3.56) for patient and 3.29%

(IQR = 4.45) for control samples when measuring pGFAP.

Statistical Analyses

Normality of distributions was assessed using Shapiro–Wilk test and graphical methods, and correspondingly, medians and IQRs are reported for continuous variables. Differences between groups were evaluated using Mann-Whitney *U* test or Kruskal–Wallis test for more than two groups and Fisher's exact test for evaluating dependence between categorical variables. After Fisher's exact test, *P*-values were adjusted for multiple hypothesis testing using the Benjamini–Hochberg procedure, and odds ratios and 95% CIs were estimated using a conditional maximum likelihood estimation.[20] The diagnostic value of pNfL and pGFAP was assessed using ROC analysis and the AUC. Cutoff values were calculated as the concentrations maximizing Youden's index. Association between pNfL and functional scores was assessed using Spearman's rank correlation coefficient and by performing a multivariate linear regression analysis adjusting for age and sex.[24] To assess the association between pNfL and clinical findings, a multivariate linear regression adjusting for age and sex was carried out. pNfL concentrations were $\log_{10}$-transformed to meet model assumptions. Unsupervised clustering based on clinical data was performed using the PAM algorithm. Before cluster analysis, variables with more than 20% missing values were excluded, numerical variables were *z*-scaled, and a dissimilarity matrix using Gower's distance was calculated.[21,22] The optimal number of clusters *k* was determined by calculating the average silhouette width and total within-cluster sum of squares, which in both cases indicated two latent clusters. The following variables were used for unsupervised clustering: prenatal/neonatal complications, NICU (neonatal intensive care unit) stay, developmental regression, motor regression, gross motor regression, fine motor regression, progressive cognitive deficits, unsupported sitting never achieved, supported walking never achieved, unsupported walking never achieved, nonverbal, motor stereotypies, stereotypic laughter, aggressive behavior, self-injurious behavior, short attention span, neonatal/infantile hypotonia, hypotonia progressing to hypertonia, upper-limb spasticity, lower-limb spasticity, upper-limb muscle weakness, lower-limb muscle weakness, amyotrophy, upper-limb amyotrophy, lower-limb amyotrophy, upper-limb contracture, lower-limb contracture, scoliosis, hyperreflexia, extrapyramidal movement disorder, dystonia, ataxia, hypokinesia, rigidity, postural instability, cerebellar signs, nystagmus, dysarthria, drooling, dysphagia, seizures, febrile seizures, focal-onset seizures, generalized-onset seizures, focal-onset seizure with impaired awareness, secondary generalized seizures, generalized tonic–clonic seizures, typical absence, status epilepticus, walking aid dependency, wheelchair dependency, urinary retention, foot deformity, ophthalmoplegia, visual impairment, ptosis, SPRS score, maximum SPRS score, SPRS spasticity subscore, maximum SPRS spasticity subscore, SPATAX score, maximum SPATAX score, age at which developmental delay was first noticed, age at which unsupported sitting was achieved, and age at which supported walking was achieved. All statistical tests were two sided, and $P < 0.05$ was considered significant. Where appropriate, *P*-values were annotated using the scientific E notation. Statistical analyses were performed in R (v4.2.2) and RStudio (v2022.12.03, RStudio, Inc., Boston, MA).

Data Availability Statement

The data and code that support the findings of this study are available from the corresponding author upon reasonable request. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Data

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