The clinical and molecular spectrum of *ZFYVE26*-associated hereditary spastic paraplegia: SPG15

Afshin Saffari,[1,2] Melanie Kellner,[3,4] Catherine Jordan,[1] Helena Rosengarten,[1] Alisa Mo,[1] Bo Zhang,[1,5] Oleksandr Strelko,[1,6] Sonja Neuser,[7] Marie Y. Davis,[8,9] Nobuaki Yoshikura,[10] Naonobu Futamura,[11] Tomoya Takeuchi,[12] Shin Nabatame,[13] Hiroyuki Ishiura,[14] Shoji Tsuji,[15,16] Huda Shujaa Aldeen,[17] Elisa Cali,[17] Clarissa Rocca,[17] Henry Houlden,[17] Stephanie Efthymiou,[17] SYNaPS Study Group,[17] Birgit Assmann,[2] Grace Yoon,[18] Bianca A. Trombetta,[19] Pia Kivisäkk,[19] Florian Eichler,[20] Haitian Nan,[21] Yoshihisa Takiyama,[21,22] Alessandra Tessa,[23] Filippo M. Santorelli,[23] Mustafa Sahin,[1,6] Craig Blackstone,[24] Edward Yang,[25] Rebecca Schüle[3,4] and Darius Ebrahimi-Fakhari[1,26,27,28]

ABSTRACT

In the field of hereditary spastic paraplegia (HSP), progress in molecular diagnostics needs to be translated into robust phenotyping studies to understand genetic and phenotypic heterogeneity and to support interventional trials. *ZFYVE26*-associated hereditary spastic paraplegia (HSP-*ZFYVE26*, SPG15) is a rare, early-onset complex HSP, characterized by progressive spasticity and a variety of other neurological symptoms. While prior reports, often in populations with high rates of consanguinity, have established a general phenotype, there is a lack of systematic investigations and a limited understanding of age-dependent manifestation of symptoms. Here we delineate the clinical, neuroimaging and molecular features of 44 individuals from 36 families, the largest cohort assembled to date. Median age at last follow-up was 23.8 years covering a wide age range (11-61 years). While symptom onset often occurred in early childhood (median: 24 months, IQR=24), a molecular diagnosis was reached at a median age of 18.8 years (IQR=8), indicating significant diagnostic delay. We demonstrate that most patients present with motor and/or speech delay or learning disabilities. Importantly, these developmental symptoms preceded the onset of motor symptoms by several years. Progressive spasticity in the lower extremities, the hallmark feature of HSP-*ZFYVE26*, typically presents in adolescence and involves the distal lower limbs before progressing proximally. Spasticity in the upper extremities was seen in 64%. We found a high prevalence of extrapyramidal movement disorders including

cerebellar ataxia (64%) and dystonia (11%). Parkinsonism (16%) was present in a subset and showed no sustained response to levodopa. Cognitive decline and neurogenic bladder dysfunction progressed over time in most patients. A systematic analysis of brain MRI features revealed a common diagnostic signature consisting of thinning of the anterior corpus callosum, signal changes of the anterior forceps, and non-specific cortical and cerebellar atrophy. The molecular spectrum included 45 distinct variants, distributed across the protein structure without mutational hotspots. Spastic Paraplegia Rating Scale (SPRS) scores, SPATAX Disability Scores and the 4-Stage Functional Mobility Score showed moderate strength in representing the proportion of variation between disease duration and motor dysfunction. Plasma neurofilament light chain levels were significantly elevated in all patients (Mann-Whitney-U test, $p<0.0001$) and were correlated inversely with age (Spearman's rank correlation coefficient $r=-0.65$, $p=0.01$). In summary, our systematic cross-sectional analysis of HSP-*ZFYVE26* patients across a wide age-range, delineates core clinical, neuroimaging and molecular features and identifies markers of disease severity. These results raise awareness to this rare disease, facilitate an early diagnosis and create clinical trial readiness.

Author affiliations:

1 Department of Neurology, Boston Children's Hospital, Harvard Medical School, Boston, MA, USA

2 Division of Child Neurology and Inherited Metabolic Diseases, Heidelberg University Hospital, Heidelberg, Germany

3 Department of Neurodegenerative Diseases, Hertie Institute for Clinical Brain Research and Center of Neurology, University of Tübingen, Tübingen, Germany

4 German Center for Neurodegenerative Diseases (DZNE), Tübingen, Germany

5 ICCTR Biostatistics and Research Design Center, Boston Children's Hospital, Harvard Medical School, Boston, MA, USA

6 Rosamund Stone Zander Translational Neuroscience Center, Boston Children's Hospital, Harvard Medical School, Boston, MA, USA

7 Institute of Human Genetics, University of Leipzig Medical Center, Leipzig, Germany

8 Department of Neurology, University of Washington, Seattle, WA, USA

9 VA Puget Sound Healthcare System, Seattle, WA, USA

10 Department of Neurology, Gifu University Graduate School of Medicine, Gifu, Japan

11 Department of Neurology, National Hospital Organization Hyogo-Chuo National Hospital, Ohara, Sanda, Japan

12 Japanese Red Cross Aichi Medical Center Nagoya Daiichi Hospital, Aichi, Japan

13 Department of Pediatrics, Osaka University Graduate School of Medicine, Suita, Osaka, Japan

14 Department of Neurology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

15 Department of Molecular Neurology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

16 Institute of Medical Genomics, International University of Health and Welfare, Chiba, Japan

17 Department of Neuromuscular Disorders, UCL Queen Square Institute of Neurology, University College London, London, UK

18 Divisions of Clinical and Metabolic Genetics and Neurology, Department of Paediatrics, The Hospital for Sick Children, University of Toronto, Toronto, Canada

19 Alzheimer's Clinical and Translational Research Unit, Department of Neurology, Massachusetts General Hospital, Boston, MA, USA

20 Department of Neurology, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

21 Department of Neurology, Graduate School of Medical Sciences, University of Yamanashi, Yamanashi, Japan

22 Department of Neurology, Fuefuki Central Hospital, Yamanashi, Japan

23 Department of Molecular Medicine, IRCCS Fondazione Stella Maris, 56128 Pisa, Italy

24 Movement Disorders Division, Department of Neurology, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

25 Division of Neuroradiology, Department of Radiology, Boston Children's Hospital, Harvard Medical School, Boston, MA, USA

26 Movement Disorders Program, Department of Neurology, Boston Children's Hospital, Harvard Medical School, Boston, MA, USA

27 The Manton Center for Orphan Disease Research, Boston Children's Hospital, Boston, MA, USA

3

1   28 Intellectual and Developmental Disabilities Research Center, Boston Children's Hospital,

2   Boston, MA, USA

3

4   Correspondence to: Darius Ebrahimi-Fakhari, MD, PhD

5   Department of Neurology, Boston Children's Hospital

6   300 Longwood Avenue, Boston, MA, 02215, USA

7   E-mail: darius.ebrahimi-fakhari@childrens.harvard.edu

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16

17   **INTRODUCTION**

18   Autosomal recessive forms of hereditary spastic paraplegia (HSP) are a large, heterogenous

19   group of rare, progressive disorders [1], often first presenting with non-specific symptoms during

20   childhood [2]. Molecular testing is typically not pursued until progressive spasticity becomes

21   evident. In recent years, the increasing availability of next-generation sequencing has enabled a

22   diagnosis in many patients with previously undefined forms of spastic paraplegia. This progress

23   in gene discovery and molecular diagnostics now needs to be translated into robust phenotyping

24   studies that provide a foundation for future interventional trials. Detailed cross-sectional

25   analyses, followed by longitudinal natural history studies, provide the necessary framework for

26   understanding genetic heterogeneity and phenotypic pleiotropy and hold the potential to

27   galvanize international collaboration and partnerships between patients, patient advocates,

1 physicians and researchers. This is of particular importance given the rare nature of many

2 autosomal recessive HSPs and the urgent need for natural history data to support the

3 development of novel molecular therapies.

4

5 *ZFYVE26*-associated hereditary spastic paraplegia (HSP-*ZFYVE26* or SPG15 [3]) is a rare form of

6 early-onset complex hereditary spastic paraplegia, characterized by progressive spasticity that

7 begins in the lower extremities and is associated with several symptoms resulting from central

8 and peripheral nervous system dysfunction [4]. While prior reports of small case series, often in

9 populations with high rates of consanguinity, have established a general phenotype [5-19], a timely

10 clinical diagnosis, counseling of families, and development of research protocols is limited by

11 the lack of systematic investigations of the clinical and molecular spectrum including an

12 assessment of the age-dependent manifestation of symptoms.

13

14 Here, we report a detailed cross-sectional analysis of clinical, radiographic, and molecular

15 features of 44 patients from 36 families with HSP-*ZFYVE26*. We compare our findings to

16 previously reported cases, affirm a core set of clinical and imaging features, describe early

17 disease manifestations and progression in a standardized manner, and provide general

18 recommendations for management and surveillance.

19

20 **SUBJECTS/MATERIALS AND METHODS**

21 *Clinical Characterization*

22 This study was approved by Institutional Review Boards at participating center (Boston

23 Children's Hospital IRB-A00033016-6, University of Tübingen IRB 423/2019BO1, IRCCS

5

Fondazione Stella Maris IRB-HSP-PBP-102/20, University of Yamanashi #734 & #953). Patients with bi-allelic variants in *ZFYVE26* were recruited from the International Registry for Early-Onset Hereditary Spastic Paraplegia (NCT04712812), the Treat-HSP network (NCT03981276) and the JAPSAC Consortium [20]. Only patients with variants classified as pathogenic or likely pathogenic according to the latest ACMG criteria [21] were included. A cross-sectional analysis of demographic, clinical and molecular data was conducted using a standardized questionnaire [22]. Rating scales including the Spastic Paraplegia Rating Scale (SPRS) [23] (n=28), the SPRS Spasticity Subscore (n=34) [22], Four Stage Functional Mobility Score (1 = unlimited walking; 2 = walking without aid but unable to run; 3 = walking with aid; and 4 = wheelchair-dependent, n=43) and the SPATAX Disability Score [24] (n=28) were applied. Brain MRI scans from 33 patients were scored and brain MRI scans from 15 patients were available for a detailed quantitative and qualitative analysis. Previously reported cases were ascertained through a systematic literature research, yielding 65 patients from 26 references which are summarized in Supplementary Figure 1 & Supplementary Table 1. For Supplementary Table 2, phenotypic information was translated into standardized Human Phenotype Ontology (HPO) terms (v1.7.15) [25].

Molecular Characterization

Bi-allelic variants in *ZFYVE26* were identified in all 44 patients (molecular diagnoses were made between January 2008 and November 2021), most commonly identified by exome sequencing (n=22), multi-gene panels (n=12), single gene testing (n=9) or chromosomal microarray (n=1). All variants were harmonized to the canonical Ensembl transcript ENST00000347230.9 (RefSeq NM_015346.4) of the GRCh38/hg38 human reference genome build using the Mutalyzer

6

1 Nomenclature Checker web tool [26] and VarSome [27]. Variants were classified according to

2 American College of Medical Genetics and Genomics (ACMG) criteria [21] using InterVar [28] and

3 Varsome [27] (Supplementary Table 3).

4

5 *Modeling of ZFYVE26 protein structure and reported variants*

6 Protein sequence and functional annotation of human ZFYVE26 were obtained from the

7 Universal Protein Resource (UniProt) database (UniProt ID: Q68DK2) [29] and recent publications

8 [30]. New exonic variants identified in this study, as well as previously reported pathogenic

9 variants, were annotated along the protein structure. CADD PHRED scores (version 1.6) [31] of all

10 possible nucleotide substitutions of the *ZFYVE26* transcript were computed and mapped to the

11 corresponding linear protein model using VarMAP [32,33].

12

13 *Quantification of neurofilament light chain levels*

14 Plasma samples were obtained by standard venipuncture and collected in lithium-heparin coated

15 BD Vacutainer PST tubes (BD #367962) for probands or EDTA-containing tubes (BD #366643)

16 in matched controls (Supplementary Table 4). Samples were centrifuged at 2,000×g within 30

17 minutes, aliquoted in cryovials and stored at -80°C. The pre-analytical interval was ≤3 days for

18 all samples, well within the window of stability for neurofilament light chain (NfL) [34]

19 (Supplementary Table 4). NfL plasma concentrations were measured using the HD-X NfL kit

20 (Quanterix #103186) on the SiMoA HD-X Analyzer (Quanterix). Plasma samples were

21 centrifuged at 10,000×g for 5 minutes and diluted 1:4 in sample buffer. All samples were run in

22 duplicates according to the manufacturer's protocol. Data was normalized across multiple

23 batches using two kit-provided controls and two pooled plasma controls to adjust for inter-batch

7

1 variability. The assay has a lower limit of quantification of 0.174 pg/mL, a limit of detection of

2 0.038 pg/mL (range 0.003-0.079 pg/mL), and a dynamic range in plasma of 0-1800 pg/mL.

3

4 *Statistical Analysis*

5 Statistical analysis was performed using *R* version 4.2.0 (2022-04-22) and *RStudio* (version

6 2022.02.2+485; RStudio, Inc.). Demographic data were summarized using frequency counts and

7 percentages of the total study population for categorical variables and with either mean and

8 standard deviation (SD) or median and interquartile range (IQR) for continuous variables,

9 depending on the distribution of data tested by visualization with histograms, quantile-quantile

10 plots and normality testing using the Shapiro-Wilk test. Sample sizes are indicated (*n*) for each

11 analysis. Linear regression analysis and, in case of repeated measures collected for some

12 individuals, linear mixed-effects regression analysis was conducted to quantify the extent to

13 which a dependent variable can be predicted from an independent variable. For linear regression

14 models, the adjusted coefficient of determination (R_{adj}^2) was reported. For linear mixed-effects

15 regression models that take into account several variance components, including fixed and

16 random effects, both the marginal coefficient of determination (R_m^2, variance explained by fixed

17 effects only) and the conditional coefficient of determination (R_c^2, variance explained by both

18 fixed and random effects) were reported, as defined by Nakagawa and Schielzeth [35,36]. To

19 determine correlation between quantitative imaging metrics, disease scores, or neurofilament

20 light chain levels, Spearman's rank correlation coefficient (*r*) was calculated. Mann-Whitney U

21 test was performed to test the difference in plasma neurofilament light chain levels.

22

23 *Data availability*

1 The authors confirm that the data supporting the findings of this study are available upon

2 reasonable request.

3

4

5 **RESULTS**

6 *Demographic and Anthropometric Features*

7 This study included 44 patients from 36 families with HSP-*ZFYVE26* (*Table 1 & Supplementary*

8 *Table 2*). Forty-one patients were reported for the first time and new, or follow up data, were

9 reported for three cases [6,37]. Demographic information of this cohort and previously reported

10 patients is summarized in *Table 1*. The ratio of male-to-female patients was 3.4:1. The median

11 age at last follow up was 23.8 years (range: 11-61 years). Most individuals were of European or

12 Asian background, likely reflecting the locations of the participating centers. Anthropometric

13 data at last follow-up showed a median height and weight corresponding to -0.6 SD (IQR=0.7)

14 and 1.4 SD (IQR=1.5) according to sex- and age-appropriate CDC growth charts. No

15 characteristic facial features were noted.

16

17 *First Clinical Symptoms*

18 There was no evidence for an increased prevalence of pre-, peri- or neonatal complications. Birth

19 weight was normal in all individuals. Median age at first reported symptom was 24 months

20 (IQR=24), while the age at molecular diagnosis of HSP-*ZFYVE26* was 18.8 years (IQR=8),

21 corresponding to a median diagnostic delay of 14.4 years (IQR=4.6) for those with sufficient

22 information available. The first reported symptom was mild developmental delay in 25/37 cases,

23 most commonly mild speech delay (23/36) (*Table 1*). Gross motor milestones were usually

9

achieved within expected time frames: Unsupported sitting was attained at a median age of 6

months (IQR=2) while unsupported walking was met at a median of 14 months (IQR=6). Six

individuals never achieved independent walking and were always dependent on assistance or

walking aids. Anecdotally, a history of mild learning disability was common in this cohort.

There were no reports of developmental regression in early childhood.

Spasticity and Motor Symptoms

The first reported motor symptom in early childhood consisted of 'balance problems' and

'clumsiness' in most cases. Spasticity in the lower extremities was eventually present in nearly

all patients (*Table 1, Videos 1-8*). Typically, this involved the ankles first, with subsequent

progression to the level of the knees and hips. Pyramidal signs (Babinski sign and ankle clonus)

were present in the majority of patients, often early into the clinical presentation. Spasticity

progressed to involve the upper extremities in a subset of individuals (61%, median age: 21.3

(IQR=10.6) years versus 32.7 (IQR=14.6) years in those with no upper extremity involvement).

Lower extremity spasticity was accompanied by weakness in the same distribution. Loss of

muscle bulk was present in a subset of patients (10/44), as were contractures of the ankles, knees,

or hips (11/37). This was accompanied by significant scoliosis in some (7/34). At last follow-up,

approximately 40% of patients were able to walk independently, about 30% patients required a

walking aid (cane or walker) and about 30% were dependent on a wheelchair. Pain in the lower

extremities, described as 'muscle cramps' or 'muscle spasms' was common and occurred early in

the disease course, particularly after long periods of standing, walking, or exercising. In the

majority of patients, muscle cramps after exertion or at night were clinically significant,

requiring treatment with pain medications, i.e., gabapentin, or muscle relaxants. Extrapyramidal

1 movement disorders were present in the majority of patients (29/44). A postural tremor was

2 present in 8/44 though this was often mild and of limited functional impact. Bradykinesia in

3 combination with rigidity or rest tremor were reported in 7/44, consistent with mild to moderate

4 parkinsonism. There was no significant or sustained benefit from levodopa (n=4). Dystonia, most

5 commonly focal limb or cervical dystonia, was present in about 10%. Ataxia was commonly

6 found, in 28/44, and signs of cerebellar dysfunction included dysarthria (n=30), dysmetria (n=9),

7 intention tremor (n=7), dysdiadochokinesia (n=14), and nystagmus (n=12). Bulbar dysfunction

8 with swallowing dysfunction was reported in four individuals with prolonged disease, but

9 associated comorbidities such as aspiration (n=3) and aspiration pneumonia (n=1) were rare

10 overall.

11

12 *Urinary Symptoms*

13 Symptoms of neurogenic bladder dysfunction were common, often first occurring around the

14 same time as motor symptoms though mild symptoms, such as increased urinary frequency or

15 mild urgency, preceded motor symptoms in some patients. Urinary urgency was prevalent

16 (20/38) whereas more severe symptoms of bladder or bowel dysfunction such as urinary

17 retention (3/40), urinary incontinence (15/41) and bowel incontinence (8/36) were less common.

18

19 *Sensory Symptoms*

20 Sensory symptoms were not systematically assessed in all patients. In the subset of patients with

21 information available, loss of vibration sense and reduced pain sensation in the distal lower

22 extremities were present. Nerve conduction studies were conducted in two individuals and

11

1   showed axonal sensory neuropathy in one and axonal sensorimotor neuropathy in the other

2   individual.

3

4   *Cognitive Symptoms*

5   Whereas speech delay and learning disabilities were present in early childhood, progressive

6   cognitive impairment occurred after onset of overt motor symptoms. Cognitive dysfunction was

7   rated as mild in 45% (median: 18.3 years (IQR: 5.8)), moderate in 24% (median: 21.1 years

8   (IQR: 11.2)), and severe in 21% (median: 32.6 years (IQR: 8.1)). Formal full-scale IQ testing

9   was available in five individuals showing a range between 40 and 82, though no longitudinal

10 data were available. While it was difficult to quantify cognitive symptoms, loss of previously

11 acquired skills was reported by 29/36 and some adult patients were dependent on help with all

12 activities of daily living.

13

14 *Other Symptoms*

15 Retinopathy was found in four cases, including one case with evidence of retinal macular

16 dystrophy on electroretinogram. No cases of optic nerve atrophy, cataract, ophthalmoplegia, or

17 ptosis were reported. No cases of clinically significant hearing impairment were present. One

18 patient in our cohort experienced seizures. No systemic or other organ-system related symptoms

19 were detected.

20

21 *Molecular Spectrum*

22 Consanguinity was infrequent compared to prior cohorts, occurring in 12/36 families (*Table 1*).

23 Three-generation family histories were consistent with an autosomal recessive disease but

12

1   otherwise did not yield any significant patterns. All variants were confirmed to be inherited from

2   an unaffected heterozygous parent. Forty-five distinct variants (14 in a homozygous and 31 in a

3   compound heterozygous state) were identified (*Fig. 1*, *Supplementary Table 3*), including one

4   459 nucleotide *out of frame* deletion of exon 22 (c.4373-81_4569+181) occurring *in trans* with a

5   multiexon deletion/insertion variant (c.3194_5070delinsAGCTTGCA) affecting exons 18-26 and

6   a   previously   reported   complex   chromosomal   rearrangement

7   (chr14:67316025_67319414del+g.67316025_67316026insTCTA+g.67319319_67319414inv)

8   resulting in a frameshift (p.(Arg1209fsTer1220)) [6]. The latter was identified in a homozygous

9   state in two siblings and a third reportedly unrelated individual from the same geographic region.

10   Recurrent variants in our cohort included the c.5621+1G>T (allele frequency (AF): 8),

11   p.(Ser1312Ter) (AF=8), and p.(Arg1209fsTer1120) (AF=6) variants. The majority of identified

12   variants were nonsense (n=22) or frameshift (n=12) variants, predicted to lead to a loss of protein

13   function due to nonsense-mediated mRNA decay. For the single- and multi-exon deletion

14   variants, as well as the additional canonical splice-site variants (c.363+1G>A, p.(0)?,

15   c.3019+1G>C, p.(0)?, and c.7128+1G>C, p.(0)?) the same pathogenic mechanism is postulated.

16   Three unique missense variants were detected in four individuals, one of which carried the

17   homozygous p.(Arg65Lys) variant, while three harbored an additional truncating variant *in trans*.

18   All missense variants were classified as likely pathogenic according to the latest ACMG criteria

19   (*Supplementary Table 3*). *In silico* analysis revealed CADD PHRED scores above the

20   recommended cut off (CADD PHRED > 20) for all missense variants underscoring their

21   pathogenicity (*Supplementary Table 3*).   Furthermore, two in frame deletion variants

22   (p.(Lys931SerfsTer19) and p.(Lys2248del)), classified as likely pathogenic, and the complex

23   p.(Leu1065_Lys1690delinsGlnLeuAla) delins variant were present in our cohort. Visualization

1 of the spectrum of *ZFYVE26* variants, including previously published disease-causing variants,

2 showed a broad distribution across the primary protein structure without mutational hotspots for

3 missense variants (*Fig. 2*). No disease-causing variants mapped to the Zinc finger or FYVE

4 domains, while a single previously reported frameshift variant (p.(Trp2234CysfsTer5)) localized

5 to the Leucine Zipper domain. Modeling of CADD PHRED scores for all theoretically possible

6 missense variants suggested a high level of intolerability for genomic variation, particularly in

7 the C-terminal region of the ZFYVE26 protein.

8

9 *Neuroimaging Findings*

10 Thirty-three patients underwent brain MR imaging (median age at last scan: 19 years (IQR=10).

11 The most common qualitative findings included: 1) A thin corpus callosum, particularly of the

12 anterior parts (100%, Fig. 2A&B); 2) T1-hypointense and T2-FLAIR hyperintense signal

13 changes in the region of the forceps minor of the corpus callosum consistent with an "ears of the

14 lynx sign" [16,38] (76%, Fig. 2C&D), 3) non-specific T2- or FLAIR-hyperintense signal changes in

15 the periventricular white matter (94%, Fig. 2C&D); 4) cerebral atrophy (34%, Fig. 2D) and

16 cerebellar atrophy (34%, Fig. 2E). A Venn diagram summarizing these core features is shown in

17 *Fig. 2F*. An in-depth qualitative and quantitative analysis of 15 brain MRI scans available at

18 Boston Children's Hospital confirmed thinning of the corpus callosum with a thickness of the

19 genu under the 3rd percentile for age in all patients. Diffuse, patchy dysmyelination was seen in

20 the periventricular and periatrial white matter in all cases with 12/15 cases showing a

21 characteristic "ears of the lynx" configuration. In contrast to other forms of autosomal-recessive

22 HSP with a thin corpus callosum, such as AP-4-associated HSP [38], the anterior commissure was

23 fully formed in 12/15. The periventricular white matter was mildly to moderately depressed in

1 8/15 patients which tended to affect posterior more than anterior regions. Exploratory analysis of

2 correlations between neuroimaging findings and clinical features and motor function scores

3 revealed an inverse correlation of the thinnest periatrial white matter diameter [38] with age, as a

4 surrogate of disease duration (Fig. 2F, r = -0.63, P = 0.0206), the SPRS (Fig. 2G, r = -0.75, P =

5 0.0197) and the 4-Stage Functional Mobility Score (Fig. 2H, r = -0.66, P = 0.0144). Cerebral

6 gray matter volume was reduced in 5/15 patients, while cerebellar atrophy was present in 6/15.

7 No cases of pontine atrophy were found in this sub-cohort of mostly younger patients. Eight

8 patients underwent total spinal cord imaging with no abnormalities identified.

9

10 *Clinical Rating Scales and Disease Progression*

11 The mean SPRS score was 25.2 ± 13.3 (SD), with a mean spasticity subscore (items 7-10) of

12 7.3±4.3 (SD). Both scores showed moderate strength in representing the proportion of the

13 variation of disease severity from age as a surrogate for disease duration (R_{m}^2 = 0.51, R_{c}^2 = 0.90

14 and R_{m}^2 = 0.47, R_{c}^2 = 0.93, P < 0.0001, respectively), confirming the clinical impression of

15 progressive corticospinal tract dysfunction (Fig. 3A & B). The level of ambulation, as measured

16 on the Four Stage Functional Mobility Score, delineated several clusters, indicating that the

17 SPRS score elevation is largely driven by motor disability (Fig. 3A&B). In the few patients with

18 longitudinal assessments available (n=8 for longitudinal SPRS scores; n=28 for the SPATAX

19 disability score), we found a trajectory of rapid progression. Disease progression over time was

20 also evident in the progression of the SPATAX disability score (mean: 3.2 ± 1.7), R_{m}^2 = 0.63, R_{c}^2

21 = 0.82, P < 0.0001) (Fig. 3C & D). The distribution of SPATAX disability scores on a cohort

22 level is shown in Figure 3C. Individual trajectories and inter-individual differences in the rate of

23 progression are highlighted in Figure 3D. Both the total SPRS and the SPATAX disability score

15

did not show a ceiling effect. In contrast, the predictive value of the Four Stage Functional Mobility Score was limited, likely due to a scale attenuation effect ($R_{adj}^2 = 0.25$, $P = 0.0004$, Fig. 3E). A systematic analysis of the age-dependent manifestation and progression of core clinical features is summarized in Figure 2F. This shows a sequence ranging from developmental symptoms (mainly speech delay, stuttering, and learning disabilities) in early childhood to a first occurrence of motor symptoms in adolescence. The latter consisted of 'clumsiness' early on, followed by gait impairment and lower limb spasticity towards the second decade of life. In this subcohort (n=15), dependency on walking aids or a wheelchair developed soon after onset of lower limb spasticity (at a media age of 17 and 20 years, respectively).

Plasma neurofilament light chain levels

Neurofilament light chain levels are an emerging biomarker in several neurodegenerative diseases [39], including HSP [39-41]. Plasma neurofilament light chain (NfL) levels were measured in 15 patients (median age: 19.5 years (IQR=10.7)) and 15 healthy age controls, mostly matched for age and sex (median age: 20.6 years (IQR=10.4), Supplementary Table 4). NfL levels were significantly increased in HSP-*ZFYVE26* patients (median: 47.0 pg/ml (IQR=25.7) vs. 3.2 pg/ml (IQR=1.9) in controls, $P < 0.0001$, Mann-Whitney U test, Fig. 4A). Interestingly, while NfL levels remained relatively stable in controls across the age-spectrum covered in this study, NfL level in HSP-*ZFYVE26* patients showed an inverse correlation with age, with larger elevation in younger compared to older patients ($r = -0.65$, $P = 0.01$, Fig. 4B). No correlation with neuroimaging findings or motor scores could be established (not shown).

Treatment

1   Symptomatic treatment consisted of physical and occupational therapy, baclofen, botulinum

2   toxin injections to treat spasticity and dystonia, and gabapentin for pain associated with muscle

3   cramps.

4   Levodopa trials were conducted in four individuals but did not lead in any significant

5   improvement of Parkinsonian features.

6

7   *Core clinical features of HSP-ZFYVE26*

8   A systematic analysis of the HSP-*ZFYVE26* associated disease spectrum, including all previously

9   reported cases (n=65), delineates a set of core clinical features (Box 1 & Fig. 5). A summary of

10 all pertinent clinical features using Human Phenotype Ontology terminology is provided in

11 *Supplementary Table 2*. Our findings delineate speech delay and learning disability as core

12 clinical features of HSP-*ZFYVE26* (Fig. 5A). While developmental delays were likely

13 underappreciated in prior reports, more than 60% of our patients showed delayed acquisition of

14 speech and/or learning disabilities during childhood. In most cases, developmental delay was

15 mild and did not result in further diagnostic investigations. Gait impairment, as a result of

16 evolving spastic diplegia, ataxia or dystonia, remains the hallmark symptom of HSP-*ZFYVE26*,

17 and a major contributor to morbidity and reduced quality of life. Onset is typically gradual and,

18 in all cases, eventually prompted a referral to a neurologist and genetic testing. Cerebellar

19 dysfunction, mostly consisting of ataxia and dysarthria, was present in more than 60% of our

20 cohort, and seems to have been underappreciated in prior studies (Figure 5B). Parkinsonism was

21 present in a subset of patients and was associated with significant impact on quality of life. Brain

22 MRI findings were frequently summarized in the literature, in many cases without detailed

23 description or quantitative assessments. Our analysis demonstrates that a characteristic

1 neuroimaging signature, including highly prevalent findings of thinning of the corpus callosum,

2 an 'ears of the lynx' sign and mild to moderate brain atrophy can accelerate the diagnostic

3 process (Fig. 5C).

4

5

6 **DISCUSSION**

7 We report cross-sectional data on the clinical and molecular characteristics of 44 patients with

8 molecularly confirmed HSP-*ZFYVE26* (SPG15), the largest cohort assembled to date. For the

9 first time, we provide a detailed assessment of the age-dependent manifestation of clinical

10 symptoms and evaluate several metrics of disease severity and progression.

11

12 A review of first clinical symptoms led to the recognition that most patients have a history of

13 speech delay and learning disability; findings that preceded progressive motor symptoms by

14 several years but generally did not lead to a referral to a pediatric neurologist or further

15 diagnostic testing. The reported median age of first developmental concerns was 24 months,

16 while the reported onset of gait or motor impairment was 14.5 years (IQR=3.5). Initial motor

17 symptoms were often subtle and described as "clumsiness", "frequent tripping", or "stiff legs",

18 particularly during tasks such as running. A concern for HSP was typically raised when

19 progressive gait impairment became apparent, resulting in a molecular diagnosis at a median age

20 of 18.8 years (IQR=8). Pyramidal signs were often present upon first neurologic evaluation and

21 spasticity would first be noted in the distal legs. In most patients, the spastic diplegia progressed

22 over the course of several years, though anecdotally not in a linear fashion but more commonly

23 in episodic periods of decline. The reported average age of loss of independent ambulation was

18

1   17 years (IQR=5.5). A third of patients ultimately became dependent on a wheelchair, at a mean

2   age of 20 years (IQR=5).

3

4   Beyond pyramidal dysfunction, most patients experienced a slow decline of academic or

5   cognitive abilities, though the rate of this decline was difficult to establish. Most patients had

6   mild intellectual disability at the time of last evaluation. Extra-pyramidal movement disorders in

7   HSP-*ZFYVE26* often included ataxia, usually accompanied by other cerebellar signs such as

8   dysarthria, intention tremor or nystagmus. Dystonia or parkinsonism were less common.

9   Parkinsonism, predominately consisted of bradykinesia, hypomimia, and a rest tremor, and none

10   of the patients treated with levodopa showed any substantial improvement. Signs of a peripheral

11   neuropathy, i.e. loss of deep tendon reflexes in the lower extremities, were present in about 40%

12   of patients, though sensory or autonomic function were not systematically assessed in all

13   patients.

14

15   The average SPRS score was 25.2, indicating moderate pyramidal dysfunction, with a significant

16   association of higher scores with age, as a surrogate for disease duration. The SPRS spasticity

17   subscore does not depend on ambulation and hence was included in a sub-analysis. This sub-

18   score mirrored the total score with a similar correlation with age. The SPATAX disability score

19   showed a comparable age-dependent evolution but also highlights the inter-individual rates of

20   progression. This loss of motor function over time was also reflected in high rates of patients

21   with need for assisted ambulation or wheelchair-dependence. However, signs of bulbar

22   dysfunction or other complications such as contractures or progressive scoliosis were rare.

1   Neurogenic bladder dysfunction was more common in our cohort compared to published cases,

2   likely reflecting that this symptom is often under-recognized.

3

4   Patients with HSP-*ZFYVE26* showed a significant elevation of plasma NfL levels compared to

5   age- and sex-matched controls across the evaluated age-spectrum. NfL levels inversely

6   correlated with age, with the highest levels coinciding around the age at onset of motor

7   disability. We speculate that a rise in plasma NfL could possibly precede overt motor decline.

8   Anecdotally several patients showed a relatively rapid clinical decline in the first 1-2 years

9   following onset of spasticity, with a slower progression thereafter, possibly mirroring the trend

10 seen in NfL levels. No correlations with neuroimaging findings or motor function scores were

11 identified. Despite showing a robust separation of patients and controls, the moderate size of our

12 cohort precludes definitive conclusions about the value of NfL levels as a longitudinal biomarker

13 of disease activity. Larger cohorts and longitudinal measurements of NfL levels will be helpful

14 to evaluate the value as biomarkers for disease severity, individual disease trajectories and the

15 ability to reflect dynamic changes under current or future therapies.

16

17 We conclude that core features of HSP-*ZFYVE26*, present in the majority of patients, include

18 spastic diplegia, pyramidal signs, gait impairment, delayed speech development, cognitive

19 decline, and ataxia. While many of these findings are shared with other forms of autosomal-

20 recessive HSP, including HSP-*SPG11*, which is closely linked on a molecular level, there seems

21 to be a relatively uniform timeline of clinical symptoms in HSP-*ZFYVE26*.

22

1   Based on this data and our clinical experience, we recommend a multidisciplinary approach to

2   the treatment and surveillance of symptoms in HSP-*ZFYVE26*. Depending on the symptoms

3   present, this may include input from neurology, physiatry, orthopedics, genetics, physical

4   therapy, speech and language pathology, gastroenterology and nutrition, ophthalmology and

5   primary care [4]. Beyond the treatment of spasticity with associated musculoskeletal complications

6   or of extra-pyramidal movement disorders, symptomatic management of speech impairment,

7   neurogenic bladder dysfunction, and chronic constipation can improve health-related quality of

8   life. Based on our experience, the prevalence of clinically-relevant retinopathy in HSP-*ZFYVE26*

9   is rare, in contrast to prior reports. Retinopathy-related changes may be detectable on dedicated

10 studies and may present in an age-dependent manner.

11

12 The combination of pyramidal dysfunction, extra-pyramidal movement disorders such as ataxia,

13 cognitive decline, retinopathy and peripheral neuropathy in some, suggest a wide-spread

14 neurodegenerative process involving the central and potentially peripheral nervous system. This

15 hypothesis is supported by our review of neuroimaging findings. In addition to the more specific

16 findings of a thin corpus callosum and the signal changes of the anterior forceps of the corpus

17 callosum, we found non-specific atrophy of the cerebral cortex and cerebellum in a subset. While

18 the neuroimaging spectrum of HSP-*ZFYVE26* remains to be defined quantitatively in larger

19 longitudinal cohorts, this supports the wide-spread involvement of different brain areas.

20

21 Molecular testing was usually pursued when progressive spasticity or the combination of a thin

22 corpus callosum and spastic diplegia were noted. More patients were diagnosed via exome

23 sequencing than multigene panels, which reflect the shift from panel to broader exome

1   diagnostics as well as its broad use in the research setting. Similar to other genetic diseases,

2   exome sequencing for HSP [42] overcomes several limitations of multigene panels and can reach a

3   diagnostic yield of up to 50% [43]. Analysis of structural variants is important since at least 6% of

4   HSP-associated variants may be small, large or complex structural changes [43]. Disease-causing

5   structural variants in 4/44 cases in our cohort of HSP-*ZFYVE26* confirm their relevance. Most

6   *ZFYVE26* variants identified are single nucleotide variants with predicted truncating effects (stop

7   gained, frameshift or affecting canonical splice site) which supports a loss-of-function

8   mechanism. Contrary to other observations in other HSPs, disease-associated missense variants

9   in *ZFYVE26* do not affect the known functional domains of ZFYVE26 or show any clustering as

10   a hint to a possible molecular mechanism. Identification of crucial interaction sites and

11   functional domains is needed to better understand the molecular impact of missense variants.

12   Thirty percent of families in our cohort harbored homozygous variants, a number driven in part

13   by consanguinity. Compound-heterozygous variants were identified in non-consanguineous

14   families. Recurrent but rare pathogenic alleles within one population or geographic region are

15   suggestive for the theory of "Clan Genomics" [44]. No clear genotype-phenotype correlations were

16   apparent, likely reflecting the small sample size.

17

18   This study has several limitations. Patient recruitment, though representing multiple centers in

19   North America, Europe and East Asia, occurred at tertiary care centers only, possibly introducing

20   a bias towards more severely affected cases. A possible difference to prior published cohorts

21   exists in that most patients in our cohort are located in countries with advanced health care

22   services, whereas prior reports have often focused on cohorts from regions of the world with

23   high rates of consanguinity, which often includes low- and middle-income countries. In our

1 cohort, the age of onset of developmental symptoms was reported by the patients' parents, which

2 may result in inaccurate estimates or recall bias. In addition, since the absence of a given clinical

3 symptom may not be consistently assessed in the literature, our summary of previous reports was

4 based on the total number of reported individuals, irrespective of whether a given symptom was

5 mentioned or not. The prevalence of certain core symptoms might therefore be underestimated.

6 While the cross-sectional data presented here allow for a delineation of core clinical features and

7 an approximation of morbidity across the age spectrum (Fig. 3F), longitudinal natural history

8 studies, beginning in the early oligosymptomatic stages, are needed to define individual disease

9 progression and understand areas of greatest clinical need. The heterogeneity of clinical

10 manifestations, and likely also of disease progression, discovered in our analysis will inform the

11 choice of outcome parameters for longitudinal studies by helping to prioritize quantitative

12 metrics that are likely to change over the timeframe typical of interventional trials.

13

14 Earlier and wide-spread use of next generation sequencing based molecular diagnostics,

15 preferably exome or genome sequencing combined with copy number variant analyses, including

16 for chief complaints such as developmental delay, spasticity or ataxia, will result in an earlier

17 diagnosis of patients with HSP-*ZFYVE26*. Detailed and careful additional phenotyping,

18 following a molecular diagnosis based on a single or set of presenting symptoms, however, will

19 become even more important given the recognition of significant clinical heterogeneity and

20 relevant correlations between genotype and phenotype. Recurrent variants in populations which

21 are underrepresented in public sequencing databases hamper reliable calculation of incidences.

22 As molecular mechanism-based therapies are being developed, an earlier diagnosis and better

understanding of the full clinical spectrum of rare forms of HSP will be necessary to design interventional trials.

Hospitals Biomedical Research Centre. The BCH Intellectual and the Developmental Disabilities Research Center is supported by the National Institutes of Health (BCH IDDRC, NIH P50 HD105351).

COMPETING INTERESTS

M.S. reports grant support from Novartis, Biogen, Astellas, Aeovian, Bridgebio, and Aucta. He has served on Scientific Advisory Boards for Novartis, Roche, Regenxbio, SpringWorks Therapeutics, Jaguar Therapeutics and Alkermes. D.E.F. received speaker honoraria from the Movement Disorder Society, publishing royalties from Cambridge University Press and reports research funding through a joint research agreement with Astellas Pharmaceuticals Inc. The other authors report no conflict of interest.

SUPPLEMENTAL MATERIAL

Supplementary material is available at *Brain* online.

APPENDIX 1

Members of the SYNAPS Study Group who have contributed to this project include: Faisal Zafar, and Nuzhat Rana. Further details are supplied in the Supplementary material.

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1 **FIGURE LEGENDS**

2 **Figure 1 Molecular spectrum of HSP-*ZFYVE26* (SPG15).** (A). *Upper panel:* Schematic of the

3 ZFYVE26 primary protein structure. Novel variants identified in our cohort are labeled in blue,

4 previously reported variants are labeled in black. Coding impacts are color coded and missense

5 variants are annotated above the protein structure, while all other variants are depicted below.

6 *Lower panel:* CADD PHRED scores for all possible missense variants in *ZFYVE26* were

7 computed and mapped to the linear protein structure. A generalized additive model was used to

8 predict the tolerance for genetic variation across the protein (blue line). The recommended cut

9 off value for deleteriousness (CADD PHRED = 20) is marked by a red line.

10

11 **Figure 2 Analysis of the neuroimaging spectrum of HSP-*ZFYVE26* (SPG15) delineates**

12 **several core features.** Key neuroimaging findings in HSP-*ZFYVE26* include thinning of the

13 corpus callosum which predominantly affects the anterior parts (A; sagittal T_1-weighted image of

14 an 18-year-old patient). (B&C) White matter signal changes include a classic 'ears of the lynx'

15 sign as well as diffuse periventricular white matter signal changes (B, axial T_2-FLAIR image of

16 an 18-year-old patient). Cerebral volume loss and enlarged lateral ventricles are seen in a subset

17 of patients (C, axial T_2-FLAIR image of a 22-year-old patient). (D) Cerebellar volume loss is

18 uncommon and usually presents with mild prominence of the cerebellar fissures (coronal T_2-

19 weighted image of an 18-year-old patient). (E) Venn diagram of key MRI findings in HSP-

20 *ZFYVE26*. This consists of (1) thinning of the corpus callosum, (2) abnormal signal of the

21 forceps minor consistent with an 'ears of the lynx' appearance, (3) cerebral volume loss and (4)

22 cerebellar volume loss. (F-H) Correlation analysis of MRI findings and clinical characteristics or

23 motor function scores. Red lines represent linear regression lines. Periventricular white matter,

29

approximated using the thinnest periatrial white matter diameter, inversely correlates with age, the Spastic Paraplegia Rating Scale (SPRS) and the 4-Stage Functional Mobility Score as an indicator of motor impairment and associated complications.

Figure 3 Clinical rating scales reflect disease progression in HSP-*ZFYVE26*. Standardized assessment of disease severity using the (A) SPRS score, (B) SPRS spasticity subscore, (C&D) SPATAX disability score (0 = no functional handicap, 1 = no functional handicap but signs on examination, 2 = mild, able to run, walking unlimited, 3 = moderate, unable to run, limited walking without aid, 4 = severe, walking with one stick, 5 = walking with two sticks or four-wheel walker, 6 = unable to walk, requiring wheelchair, 7 = confined to bed) and (E) the Four Stage Functional Mobility score (1 = unlimited walking; 2 = walking without aid but unable to run; 3 = walking with aid; and 4 = wheelchair-dependent). The number of individuals included in the respective analyses are indicated below the graphs. Data were modeled using linear regression analysis and, in case of repeated measures collected for some individuals, linear mixed-effects regression analysis. For linear regression models, the adjusted coefficient of determination (R_{adj}^2) and for linear mixed-effects regression models the marginal coefficient of determination (R_m^2) were reported. *P* values are depicted for each analysis. (F) Assessment of age-dependent presentation of clinical signs. Age at first report of the respective symptoms is shown (box plots shows the median and IQR, whiskers indicate minimum and maximum values).

Figure 4 Plasma neurofilament light chain levels are elevated in HSP-*ZFYVE26*. Plasma neurofilament light chain (NfL) levels were measured in 15 HSP-*ZFYVE26* patients and mostly age- and sex-matched controls. (A) Absolute NfL levels show a robust separation of patients and

30

1   controls (Mann-Whitney U test, $P < 0.0001$). (B) Linear model of NfL levels across the age

2   spectrum of the cohort. Variant coding impacts are coded by shape; circles indicate individuals

3   with bi-allelic truncating variants, triangles represent individuals with one truncating variant in

4   *trans* with a missense variant and squares illustrate healthy controls. Correlation analysis was

5   performed using Spearman's rank correlation coefficient.

6

7   **Figure 5 Core clinical features of HSP-*ZFYVE26* in this cohort compared to previously**

8   **reported cases.** Bar plots present the frequency of core clinical features. Individuals with HSP-

9   *ZFYVE26* described in this study (n=44) are depicted in green, while reported cases from the

10   literature are illustrated in orange (n=65).

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Table 1 Synopsis of demographic, clinical and neuroimaging findings in HSP-*ZFYVE26* (SPG15) in the present cohort and in previously reported patients

	This cohort (*n* = 44)[a]	Previously reported cases (*n* = 65)
Demographic Data		
Sex (Male : Female)	34:10 (77%:23%); *n* = 44	26:30 (46%:54%); *n* = 56
Ethnicity/Origin	European: 66% (29/44)	European: 36% (17/48)
	Middle Eastern: 14% (6/44, 4 Turkish, 2 Arab)	Middle Eastern: 19% (9/44, 4 Turkish, 4 Arab, 1 Iranian)
	East Asian: 11% (5/44)	East Asian: 10% (5/48)
	North African: 5% (2/44)	North African: 29% (14/48)
	Indian subcontinent: 2% (1/44)	Indian subcontinent: 4% (2/48)
	Hispanic 2% (1/44)	Hispanic: 2% (1/48)
Consanguinity	34% (12/35, NA = 1)	35% (23/65)
Development and Cognitive Symptoms		
Developmental delay	68% (25/37, NA = 7)	11% (7/65)
Delayed speech development	64% (23/36, NA = 8)	3% (2/65)
Delayed motor development	21% (7/33, NA = 11)	2% (1/65)
Cognitive impairment	89% (34/38, NA = 6)	62% (40/65)
Progressive cognitive decline	69% (25/35, NA = 9)	23% (15/65)
Motor Symptoms		
Spasticity	Lower limbs: 98% (43/44)	Lower limbs: 65% (42/65)
	Upper limbs: 64% (28/44)	Upper limbs: 22% (14/65)
Muscle wasting	Lower limbs: 23% (10/44)	Lower limbs: 31% (20/65)
	Upper limbs: 16% (7/44)	Upper limbs: 9% (6/65)
Pyramidal signs	98% (43/44)	82% (53/65)
Contractures	30% (11/37, NA = 7)	2% (1/65)
Ataxia	64% (28/44)	8% (5/65)
Dysarthria	68% (30/44)	42% (27/65)
Nystagmus	27% (12/44)	18% (12/65)
Dystonia	11% (5/44)	5% (3/65)
Postural tremor	18% (8/44)	11% (7/65)
Parkinsonism	16% (7/44)	8% (5/65)
Level of ambulation	I: 9% (4/43, *NA = 1*)	I: 6% (3/48)
	II: 30% (13/43, *NA = 1*)	II: 13% (6/48)
	III: 28% (12/43, *NA = 1*)	III: 33% (16/48)
	IV: 33% (14/43, *NA = 1*)	IV: 48% (23/48)
Musculoskeletal Symptoms		
Foot deformity	28% (11/39, NA = 5)	22% (14/65)
Scoliosis	21% (7/34, NA = 10)	9% (6/65)
Peripheral and Autonomic Symptoms		
Peripheral polyneuropathy	38% (10/26, NA = 18)	46% (30/65)
Urinary urgency / incontinence	54% (22/41, NA = 3)	26% (17/65)
Ocular and Auditory Symptoms		
Retinopathy	11% (4/35, NA = 9)	18% (12/65)
Hearing impairment	0% (0/37, NA = 7)	2% (1/65)
Brain MRI findings		
Thin corpus callosum	100% (33/33)	54% (35/65)
'Ears of the lynx' sign	76% (16/21, NA = 12)	12% (8/65)
Periventricular white matter abnormalities	94% (30/32, NA = 1)	42% (27/65)
Cerebral atrophy	34% (11/32, NA = 1)	23% (15/65)

Cerebellar atrophy	34% (11/32, NA = 1)	*NA*
Rating scale		
Spastic Paraplegia Rating Scale score	25.2 ± 13.3 (range 4–48, *n = 28*)	22.5 ± 9.7 (range 8–28, *n = 4*)

1 [a]Three previously reported patients [6,37] were counted towards the present cohort given the availability of additional and follow-up data. NA =
2 not available.
3

1   **Box 1 Core clinical and neuroimaging features of HSP-*ZFYVE26* (SPG15)**

Gait disturbance HP:0001288   92% (84/91)

Spasticity HP:0001257

- Spastic diplegia HP:0001264   78% (85/109)

- Abnormal pyramidal sign HP:0007256   88% (100/114)

Delayed speech and language development HP:0000750   25% (25/101)

Cognitive impairment HP:0100543   72% (74/103)

Mental deterioration HP:0001268   40% (40/100)

Ataxia HP:0001251   30% (33/109)

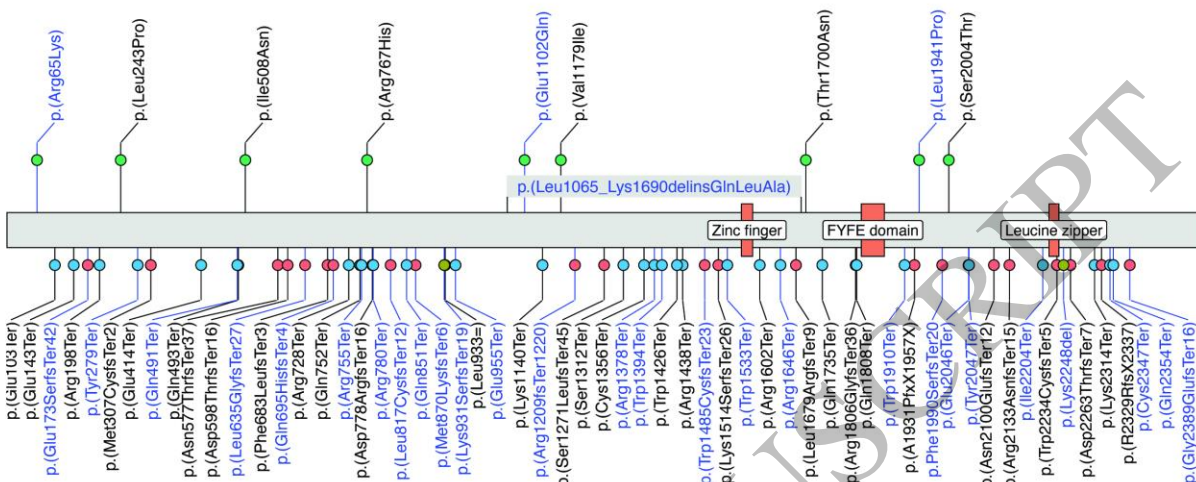Thin corpus callosum HP:0033725   70% (69/98)

Ears of the Lynx sign   28% (24/86)

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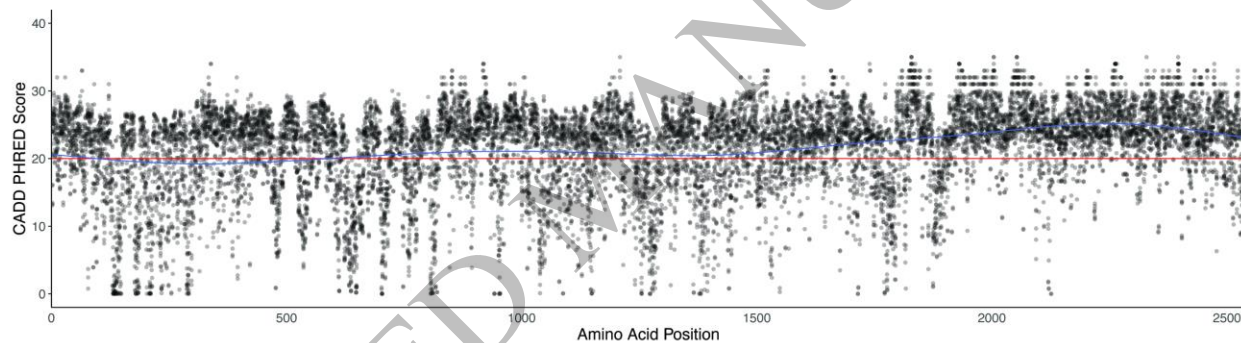
Figure 1
165x131 mm (.70 x DPI)
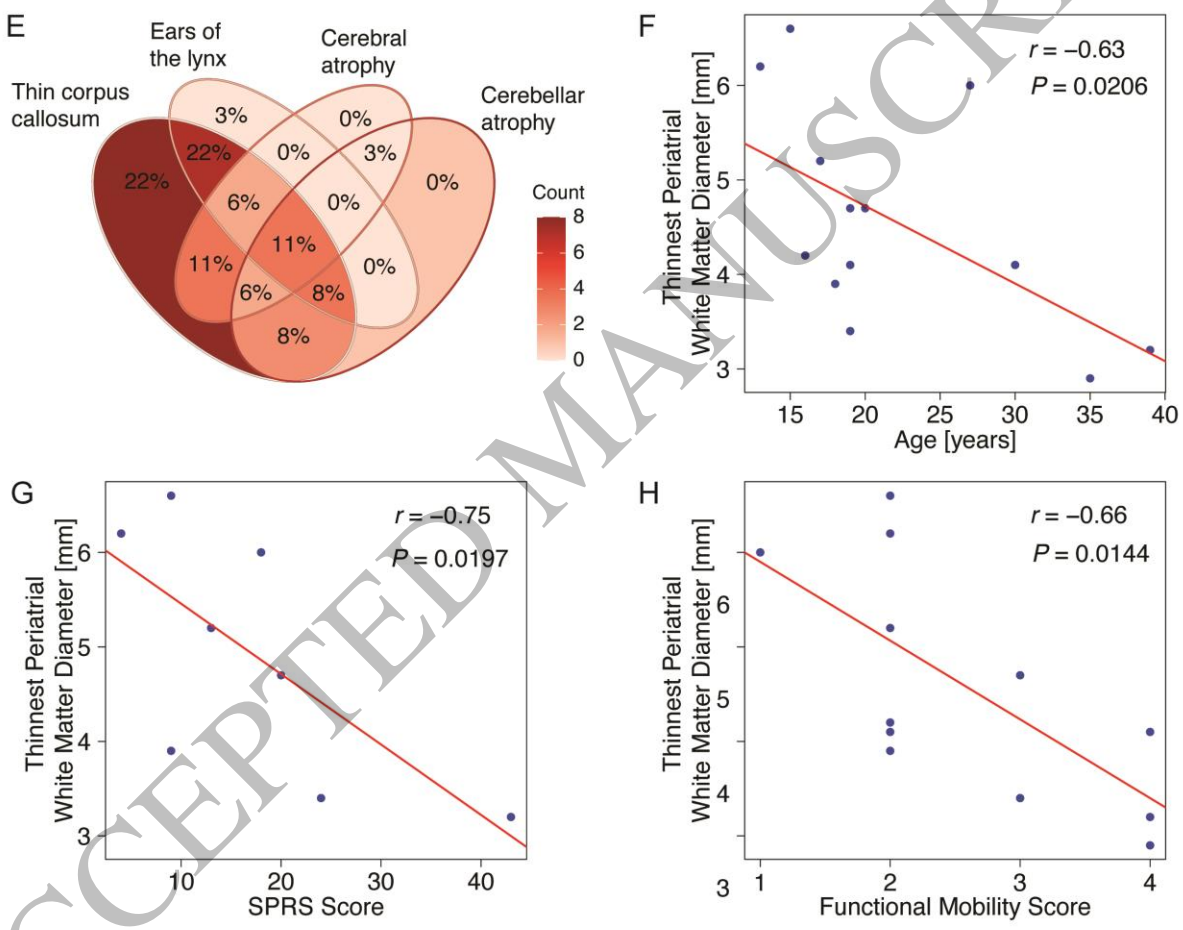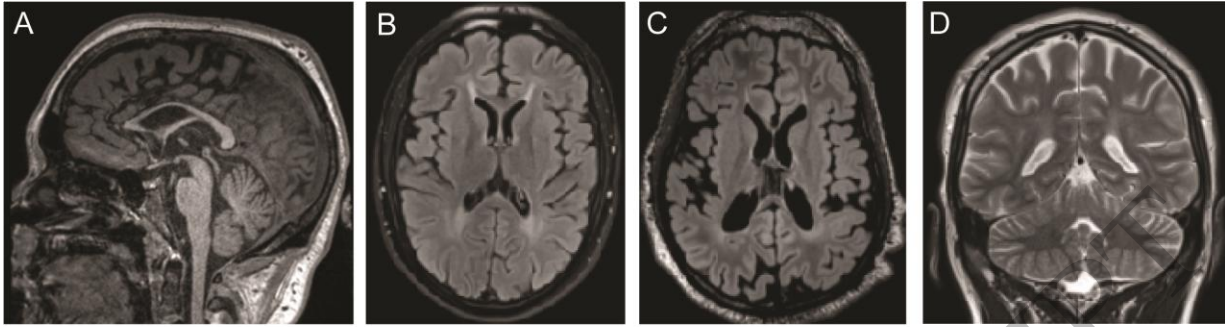
Figure 2
165x174 mm (.70 x DPI)
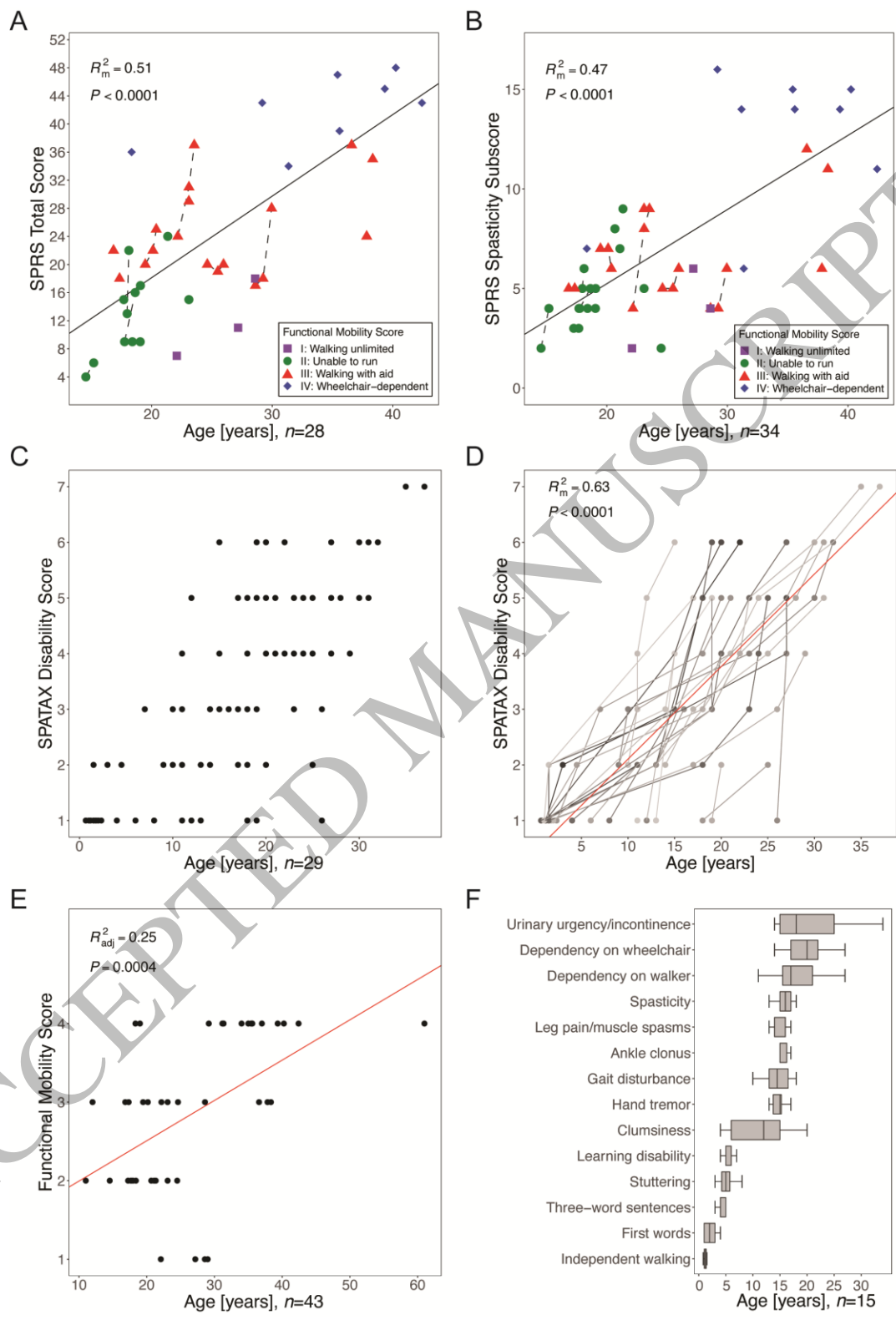
Figure 3
155x229 mm (.70 x DPI)
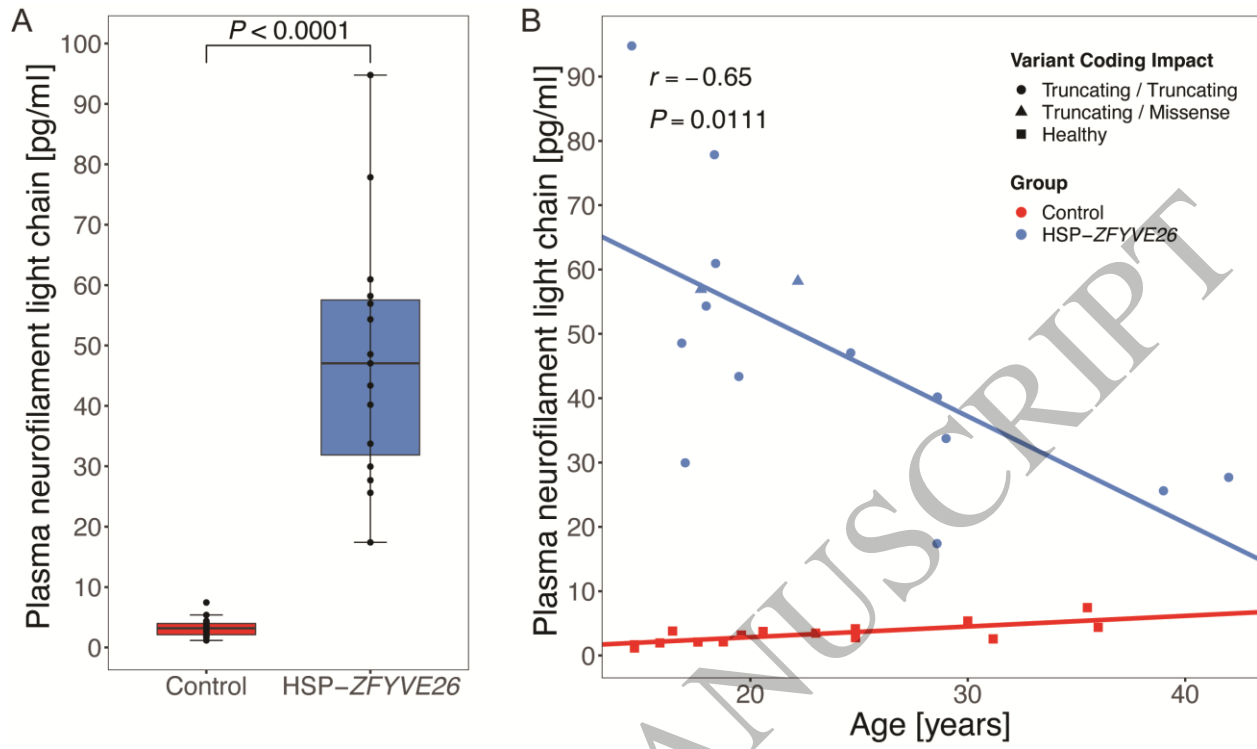
Figure 4
165x99 mm (.70 x DPI)

A Development and Cognition

Developmental delay
Delayed motor development
Delayed speech development
Cognitive impairment
Progressive cognitive decline

100% 50% 0% 50% 100%

B Motor Symptoms

Pyramidal signs
Spasticity: Lower limbs
Spasticity: Upper limbs
Muscle wasting: Lower limbs
Muscle wasting: Upper limbs
Contractures
Dysarthria
Ataxia
Nystagmus
Dystonia
Postural tremor
Parkinsonism

100% 50% 0% 50% 100%

C MRI Findings

Thin corpus callosum
White matter abnormality
Ears of the lynx sign
Brain atrophy

100% 50% 0% 50% 100%

HSP-*ZFYVE26* Literature

Figure 5
165x88 mm (.70 x DPI)