

ARTICLE

Massive sequencing of 70 genes reveals a myriad of missing genes or mechanisms to be uncovered in hereditary spastic paraplegias

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Hereditary spastic paraplegias (HSP) are neurodegenerative disorders characterized by lower limb spasticity and weakness that can be complicated by other neurological or non-neurological signs. Despite a high genetic heterogeneity (> 60 causative genes), 40–70% of the families remain without a molecular diagnosis. Analysis of one of the pioneer cohorts of 193 HSP families generated in the early 1990s in Portugal highlighted that *SPAST* and *SPG11* are the most frequent diagnoses. We have now explored 98 unsolved families from this series using custom next generation sequencing panels analyzing up to 70 candidate HSP genes. We identified the likely disease-causing variant in 20 of the 98 families with *KIF5A* being the most frequently mutated gene. We also found 52 variants of unknown significance (VUS) in 38% of the cases. These new diagnoses resulted in 42% of solved cases in the full Portuguese cohort (81/193). Segregation of the variants was not always compatible with the presumed inheritance, indicating that the analysis of all HSP genes regardless of the inheritance mode can help to explain some cases. Our results show that there is still a large set of unknown genes responsible for HSP and most likely novel mechanisms or inheritance modes leading to the disease to be uncovered, but this will require international collaborative efforts, particularly for the analysis of VUS.

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INTRODUCTION

Hereditary spastic paraplegias (HSP) are a clinically and genetically heterogeneous group of rare neurodegenerative disorders. HSP have a variable age at onset and are mainly characterized by progressive lower limb spasticity and weakness. These hallmarks are caused by degeneration of the motor axons that mostly affects the distal ends of the long central nervous system tracts.¹ Their estimated prevalence ranges from 1–10/100 000 depending on the geographic localization² and is 4.1/100 000 in Portugal.³

These disorders are historically divided on clinical grounds into pure and complex forms, according to the absence or presence of additional neurological and extraneurological features, such as neuropathy, cognitive impairment, cerebellar ataxia and epilepsy. Consequently, ancillary tests such as brain/spinal cord magnetic resonance imaging, electroneuromyography and others can show various abnormal patterns. Genetically, all classical modes of inheritance have been described, with 79 SPG-associated loci and more than 60 identified genes, a number that is constantly increasing.^{4–10} Moreover, at least 11 additional genes have also been associated with spastic paraplegia, some of them very recently.^{11,12} Although a considerable number of genes have already been identified, most of the families worldwide

have not been extensively screened so far and remain without a molecular diagnosis, as was the case of 68.9% families in Portugal, before this study. Indeed, among the HSP a considerable number of genes and variants are apparently orphan, affecting single families.

Identification of the responsible genes has a great impact on patients and their families as it reveals the cause of the disease, and may also be important to better understand how these variants arise (founder effects and susceptibility chromosomes, among others) and how they lead to specific phenotypes. This study aimed to provide a molecular diagnosis for the Portuguese families identified through a population-based survey³ using targeted next generation sequencing (NGS) to screen these patients for the presence of variants in 70 genes already associated with HSP or candidate genes, covering all classical modes of inheritance.

MATERIALS AND METHODS

Patients

In Portugal, 193 families with hereditary spastic paraplegia were identified in a national, population-based survey.^{3,13} After exclusion of the most common genes in most of the families and isolated cases (*ATL1*, *SPAST* and *REEP1* in families with dominant inheritance, and *SPG11*, *ZFYVE26* and *CYP7B1* in the recessive ones), there were still 68.9% (133/193) of the families without a

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molecular diagnosis, 62% (98/159) if we consider only the families with DNA available for testing. Taking that into consideration, 98 unsolved families (ie, with DNA available) were selected to be screened for variants in known genes using one patient per family.

Among these 98 Portuguese families, 38 showed a probable recessive inheritance, 44 a dominant inheritance and 16 were isolated cases. Pure and complex forms were present and age at onset in the whole cohort ranged from less than 1 year to 65 years. Written informed consent for genetic testing was obtained from all tested individuals or their respective legal guardian and the genetic analyses were approved by the ethical committees of ICBAS (Portugal) and of the Paris-Necker Hospital (France).

HSP panels

Two sequential custom NGS panels were developed at ICM, France, to search for variants in HSP-related genes (Supplementary Table S1). This strategy was favored to exome sequencing for ethical concerns regarding the risk of secondary or incidental findings in HSP diagnosis in agreement with our local ethics committees and for the cost effectiveness given the number of genes involved in HSP that could carry a mutation. The first panel covered 34 genes among which 31 were known to be responsible for dominant and recessive HSP forms and the other three (*ALS2*, *SACS* and *SETX*) were implicated in overlapping phenotypes.^{14–16} The 34 genes corresponded to 531 regions with a total length of 109 768 base pairs. In order to increase achieving a molecular diagnosis, a second panel was later designed that covered 70 genes. This added 30 newly identified genes⁵ to the previous panel as well as three genes (*FBXO7*, *GJA1* and *SAMHD1*) causing overlapping phenotypes^{17–19} and three candidate genes that encode partners of known HSP proteins (*AP5B1*, *AP5M1* and *AP5S1*). This panel covered a total length of 210 363 bases corresponding to 1001 regions. These two panels targeted exonic regions and at least 20 intronic bases at the exon-intron boundaries.

Genetic analysis was performed using a customized Roche/Nimblegen capture followed by NGS in the MiSeq apparatus (Illumina, San Diego, CA, USA). The procedure consisted of four major steps: (1) library preparation, where we used the Illumina TruSeq DNA LT Sample Preparation kit v2-Set A (Illumina) according to the manufacturer's protocol for the first panel and the Kapa HTP Library Prep Kit Illumina (Roche, Basel, Switzerland) with its corresponding protocol for the second panel; (2) double capture using the Roche NimbleGen SeqCap EZ Reagent Kit and SeqCap EZ Library (Roche) using the SeqCap EZ Library SR User's Guide protocol; (3) massive parallel sequencing using the Illumina MiSeq Benchtop Sequencer; and (4) data analysis using the CLC Bio-Genomics Workbench 6.5.1 software (<https://www.qiagen-bioinformatics.com/>). After the alignment with the *Homo sapiens* (hg19) reference sequence, data were filtered taking into consideration that the variants had to: (i) be present in the target regions, (ii) cause a change at the protein level (nonsense, amino acid change or a splicing effect), (iii) be present in the local database with a frequency below 15% and, (iv) if known in the databases, its minor allele frequency had to be below 1% for autosomal recessive inheritance and 0.2% for autosomal dominant transmission. All filtered variants were further analyzed using Alamut v.2.9.0 software (Interactive Biosoftware, La Rochelle, France) for functional effect prediction with SpliceSiteFinder, MaxEntScan, NNSPLICE, GeneSplicer, Human Splicing finder, Polyphen-2, SIFT, MutationTaster, Align GVGD and UMD-Predictor.

Twelve patients per experiment were sequenced using the first panel, while 24 were analyzed simultaneously using the second panel. The first 25 Portuguese patients tested were sequenced using the first panel and the remaining 73 patients were screened with the second panel.

In order to detect rearrangements, we analyzed the coverage of all the regions with an in-house algorithm based on the analysis of the coverage. In the genes where a suggestive alteration in dosage was detected, multiplex ligation-dependent probe amplification or quantitative real-time PCR was applied to confirm the deletion/duplication.

All the variants that were not excluded after filtering and segregation analysis (whenever possible) were submitted to ClinVar (<http://www.ncbi.nlm.nih.gov/clinvar/>). ClinVar accession numbers are SCV000574442–SCV000574511.

Sanger sequencing

We confirmed the presence of the variants identified by NGS checking, by Sanger sequencing, the region where the variant was present, including additional family members when DNA was available. Primers were designed using Primer3Plus software (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>). The PCR amplification was done using the DreamTaq DNA Polymerase Kit (ThermoFisher Scientific, Waltham, MA, USA) or when necessary, AccuPrime GC-Rich DNA polymerase (Invitrogen, Carlsbad, CA, MA, USA), followed by sequencing by GATC BioTech using BigDye chemistry in an ABI3730 sequencer (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using SeqScape 2.6 software (Applied Biosystems).

Multiplex ligation-dependent probe amplification

In order to confirm the presence of large gene rearrangements in the *SPG11* gene we performed multiplex ligation-dependent probe amplification using the SALSA MLPA kit P306 (MRC Holland, Amsterdam, Netherlands) according to manufacturer's instructions and analyzed the resulting fragments on an ABI 3130xl Genetic Analyzer using 500-LIZ (Applied Biosystems), as a size standard, and GeneMarker v1.90 (SoftGenetics, State College, PA, USA).

RESULTS

Study design

A total of 98 index cases of Portuguese families without molecular diagnosis were screened for variants using either the first or the second custom sequencing panel. The mean coverage varied from 126 to 801 reads: 419 reads per base with 94% of the regions with a coverage $\geq 30\times$ with the first panel and 362 reads with 97% of the regions with at least $30\times$ of coverage with the second panel. All the variants considered relevant (rare, predicted deleterious, expected to have an impact given the suspected mode of inheritance) were confirmed by Sanger sequencing and segregation analysis was performed in all the available family members. The regions with less than 30-fold coverage were screened by Sanger sequencing in autosomal recessive cases in which only one heterozygous potentially disease-causing variant was identified. Relevant insufficiently covered exons in patients with a compatible phenotype were also sequenced by Sanger (ie, exon 1 of *SPG7* in patients with cerebellar atrophy and/or optic atrophy). An in-house index of coverage was calculated for each exon and for each patient to search for genomic rearrangements.

Genetic characterization

A molecular diagnosis of HSP was confirmed in 20.4% (20/98) of the cases. The coverage analysis was responsible for the identification of one large homozygous deletion encompassing exons 12 to 14 in *SPG11* that was confirmed by multiplex ligation-dependent probe amplification (Table 1, Supplementary Figure S1) and was considered to affect spatacsin function. Additionally, after filtering of the NGS data, we found 139 nucleotide variants present in 74 families with minor allele frequency below 1.5% that potentially affected protein function according to at least one prediction software. These variants were divided in: (i) Disease-causing variants (17 variants in 14 families), (ii) Likely disease-causing variants (6 variants in 5 families), (iii) variants of unknown significance – VUS (52 variants in 37 families) and (iv) Excluded variants (64 variants in 45 families) by several criteria detailed in Figure 1. In the remaining 24 families, no disease-causing variants were found after filtering.

We considered 24 (of the 140) variants to be affecting protein function in 20 families (Table 1, Figure 2 and Supplementary Figure S1). Most ($n=18$) were classified as disease-causing variants because they were already reported as disease-causing variants, caused an early stop codon (nonsense and frameshift) or were large gene rearrangements. The other ones ($n=6$) were considered as likely

Table 1 Disease-causing and likely disease-causing variants found in 20 families

Family code	Inheritance mode ^a	Mutated gene	Accession.Version numbers	Exon ^b	cDNA	Predicted consequence	State	N° software ^c	Segregation tested	Reference	ClinVar accession number
<i>(a) Disease-causing variants</i>											
SR88	Recessive	<i>C19orf12</i>	NM_001031726.2	E2	c.157G>A	p.(Gly53Arg)	Hom	3/3	No	20 in NBIA	SCV000574442
SR97	Recessive	<i>CYP7B1</i>	NM_004820.4	E4	c.914dup	p.(Met305Ilefs*16)	Het	frameshift	Yes	This study	SCV000574443
				E3	c.825T>A	p.(Tyr275*)	Het	nonsense	Yes	21	SCV000574444
S93	Recessive	<i>CYP7B1</i>		E6	c.1456C>T	p.(Arg486Cys)	Hom	3/5	Yes	21	SCV000574445
SR84	Recessive	<i>GBA2</i>	NM_020944.2	E17	c.2618G>A	p.(Arg873His)	Het	4/5	Yes	22	SCV000574446
				E12	c.1888C>T	p.(Arg630Trp)	Het	4/5	Yes	23	SCV000574447
SR75	Recessive	<i>SPG7</i>	NM_003119.2	E11	c.1454_1462del	p.(Arg485_Glu487del)	Hom	Inframe deletion	Yes	24	SCV000574448
SR16	Recessive	<i>SPG11</i>	NM_025137.3	E4	c.733_734del	p.(Met245Valfs*2)	Hom	frameshift	Yes	25	SCV000574449
SR82	Recessive	<i>ZFYVE26</i>	NM_015346.3	E34	c.6296dup	p.(Asn2100Gluifs*12)	Hom	frameshift	Yes	26	SCV000574450
S31	Dominant	<i>KIF5A</i>	NM_004984.2	E8	c.610C>T	p.(Arg204Trp)	Het	4/5	Yes	27	SCV000574451
S41	Dominant	<i>KIF5A</i>		E10	c.839G>A	p.(Arg280His)	Het	5/5	Yes	28	SCV000574452
S61	Dominant	<i>NIPA1</i>	NM_144599.4	E4	c.316G>A	p.(Gly106Arg)	Het	4/5	Yes	29	SCV000574453
S26	Classified as dominant	<i>SPG7</i>	NM_003119.2	E10	c.1447C>T	p.(Gln483*)	Hom	nonsense	No	This study	SCV000574454
SR98	Isolated case	<i>KIF1A</i>	NM_001244008.1	E2	c.31C>T	p.(Arg111Trp)	Het <i>de novo</i>	4/5	Yes	This study	SCV000574455
SR4	Isolated case	<i>SPAST</i>	NM_014946.3	E8	c.1133T>A	p.(Leu378Gln)	Het <i>de novo</i>	4/5	Yes	30	SCV000574456
C13	Isolated case	<i>SPG7</i>	NM_003119.2	E11	c.1529C>T	p.(Ala510Val)	Het	5/5	No	31	SCV000574457
				E3	c.376G>C	p.(Glu126Gln)	Het	3/5	No	32	SCV000574458
SR17	Isolated case	<i>SPG11</i>	NG_008885.1 (NM_025137.3)	E12-14	c.(2244+1_2245-1)(2620+1_2621-1)del	r.?: p.?	Hom	deletion	Yes	This study	SCV000574459
<i>(b) Likely disease-causing variants</i>											
S75	Recessive	<i>ERLIN2</i>	NM_007175.6	E12	c.899A>T	p.(Asp300Val)	Hom	5/5	Yes	This study	SCV000574460
SR6	Recessive	<i>SACS</i>	NM_014363.5	E8	c.[1640C>T;1634G>T]	p.([Pro547Leu;Trp545Leu])	Hom (both)	2/5; 4/5	Yes	This study	SCV000574461
S27	Dominant	<i>KIF5A</i>	NM_004984.2	E10	c.833C>T	p.(Pro278Leu)	Het	5/5	Yes	This study	SCV000574462
S58	Dominant	<i>KIF5A</i>		E10	c.868G>C	p.(Asp290His)	Het	4/5	Yes	This study	SCV000574463
SR99	Isolated case	<i>REEPI</i>	NM_001164730.1	E3	c.79G>C	p.(Ala27Pro)	Het	4/5	Yes	This study	SCV000574464

^aThe inheritance mode is the one assumed by pedigree analysis.

^bExon Number according to Alamut software v2.9.0 that used the NCBI RefSeq numbering scheme (along with simple systematic numbering) on the GRCh37 (hg19) assembly.

^cNumber of Software predicting a functional effect of all the prediction software for the missense variants; Het, Heterozygous; Hom, Homozygous; NBIA, Neurodegeneration with Brain Iron Accumulation.

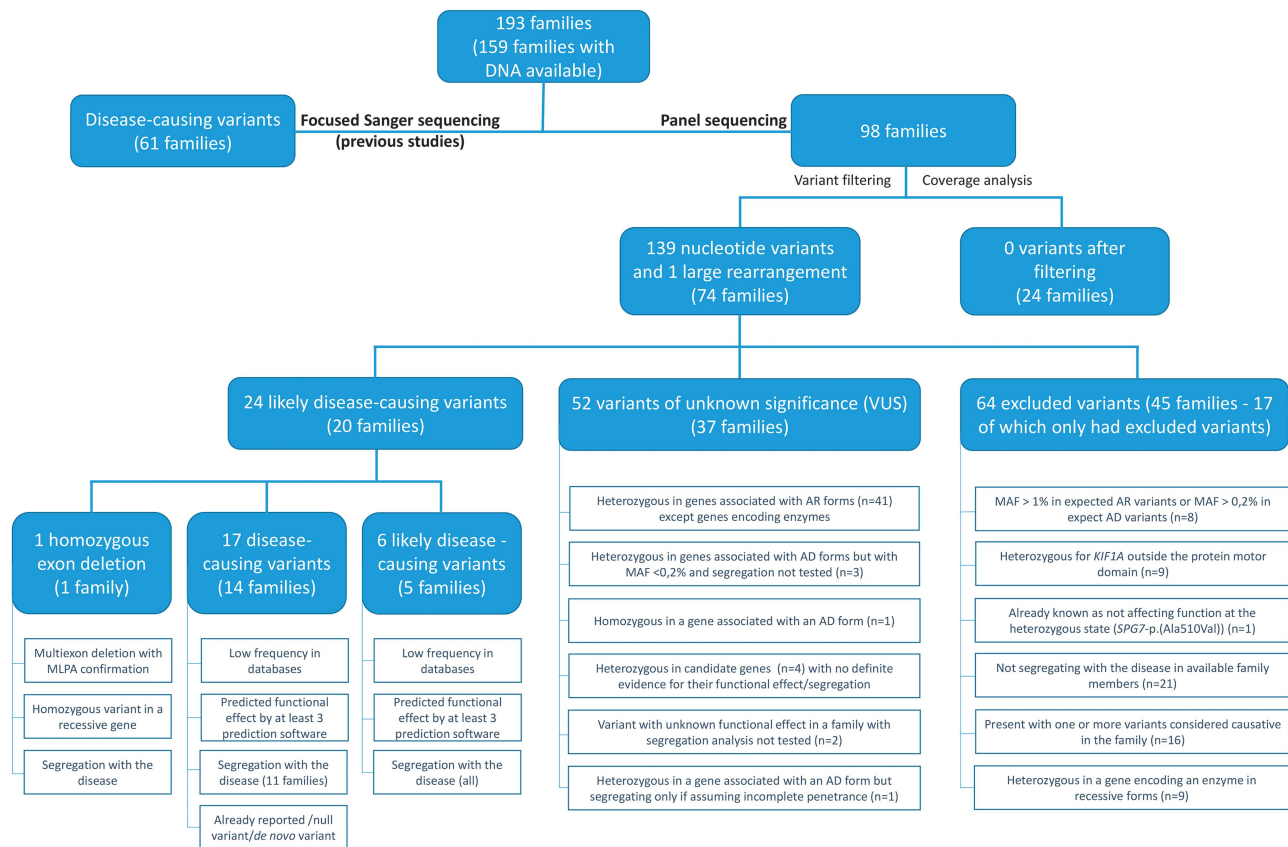


Figure 1 Schematic illustration of the Portuguese cohort with the criteria applied for filtering and classification of the variants identified in the 98 families.

disease-causing because we did not perform functional studies to prove it. All of them had low frequency in databases and a predicted deleterious effect (Table 1) and additionally, in 16 of these families, we could demonstrate their segregation with the disease (Figure 2 and Supplementary Figure S1), which included all the families with likely disease-causing variants.

Among the 52 VUS (Table 2), three are illustrative of the difficulties we faced in the interpretation of their biological relevance (Supplementary Figure S2): a homozygous missense variant in a gene causing a recessive form where family members were not available for segregation analysis (family SR26); a variant in a gene causing a dominant form, of interest only if we consider incomplete penetrance (family SR49) and a variant in the homozygous state in a gene causing a dominant form that segregated with the disease (family S88). In addition, in families SR65 and S86 we found compound heterozygous variants in two different genes known to interact at the protein level (Supplementary Figure S2). In both cases, the variants were found in two different members or interactors of the adaptor protein complex 5, but neither their cosegregation nor their effect at the cellular level could be analyzed, which would represent a prerequisite to prove these potential cases of digenism.

In this study, we report 10 new disease-causing variants among the 24 variants affecting protein function, of which seven are new missense variants (Figure 2), one is a nonsense variant, one a frameshift variant and the last is the deletion of three consecutive exons in *SPG11* (Table 1).

The yield of mutational results varied according to inheritance mode (Figure 3). We found the disease-causing variants in nine of the

38 recessive families (23.7%) and in six of the 44 families with dominant transmission (13.6%). Of note, one family with a homozygous *SPG7* variant was initially considered as dominant (Family S26, Supplementary Figure S1) because of a suspected family history of the disease in the father, who however was not examined and not tested. Five of the 16 isolated cases were also explained at the molecular level (31.3%), including one confirmed *de novo* variant in *KIF1A* (Family SR98, Figure 2). Segregation analysis could not be performed in three families, but all of them had variants already described as disease-causing in the literature.

Clinical characterization

The phenotype of the patients was mostly similar to that of previously reported series. A detailed phenotypic description of the families is presented in Table 3. For example, in the two families carrying *SPG11* variants, the clinical picture included early age at onset and a complex phenotype with cognitive impairment in both families and neuropathy in one of them. Magnetic resonance imaging was not available to check for the presence of a thin corpus callosum. In another family with a homozygous *ZFYVE26* variant and an SPG11-like phenotype, thin corpus callosum was overt. Similarly to published cases, a pure form of the disease was observed in (i) the case with a *de novo* *SPAST* variant (SR4, Supplementary Figure S1), (ii) the patient presenting a variant in *REEP1* (SR99), (iii) the two families with *CYP7B1* variants (families S93 and SR97, Supplementary Figure S1) and the patient with an *NIPA1* variant (S61, Supplementary Figure S1). On the contrary, in our *KIF5A* families (S41, S31, S27, S58, Figure 2 and Supplementary Figure S1), pure and complex forms were found, in

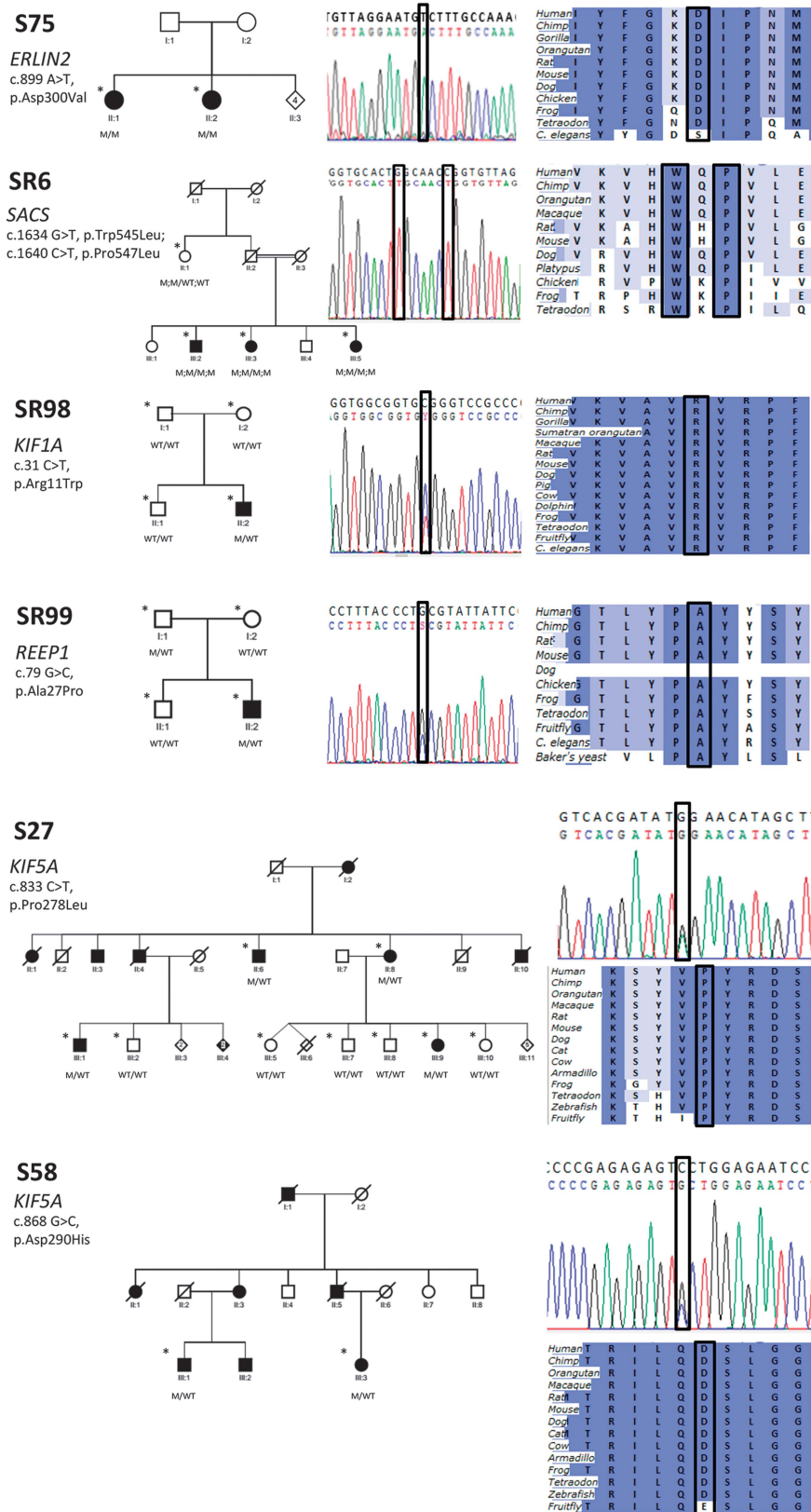


Figure 2 Pedigree with segregation analysis, electropherogram and conservation of the seven novel missense variants. A black circles and squares indicate affected individuals with HSP; an asterisk in a symbol indicates family members with available DNA. Electropherogram with the position of the missense variant boxed. Conservation of the region of the altered amino acid (boxed).

Table 2 Variants of unknown significance (VUS) found in 37 families that remained without a molecular diagnosis

Family code	Chr	Genomic coordinate (GRCh37)	Gene	Accession.Version numbers	Exon ^a	Coding change	Predicted change	State	MAF EA	N ^b software ^b	ClinVar accession number	Reason for exclusion
SR1	9	135202274	SETX	NM_015046.5	E10	c.4711G>A	p.(Val1571Met)	Het	0.081	1/5	SCV000574465	Heterozygous variant in a recessive gene
SR5	7	99702495	AP4M1	NG_016312.1 (NM_004722.3)	E8	c.607-2A>G	r.; p.?	Het	0.081	5/5	SCV000574466	Heterozygous variant of a recessive gene
SR12	1	110169006	AMPD2	NM_001257360.1	E6	c.650G>C	p.(Arg217Pro)	Het	0.081	2/5	SCV000574467	Heterozygous variant of a recessive gene
SR20	5	10256222	CCT5	NM_012073.4	E4	c.487G>A	p.(Glu163Lys)	Het	0.081	2/4	SCV000574468	Heterozygous variant of a recessive gene
SR23	13	23912386	SACS	NM_014363.5	E10	c.5629C>T	p.(Arg1877*)	Het	0.081	nonsense	SCV000574469	Heterozygous variant of a recessive gene
SR15	15	44890903	SPG11	NM_025137.3	E22	c.3818A>G	p.(Lys1273Arg)	Het	0.110	1/5	SCV000574470	Heterozygous variant of a recessive gene
SR26	3	39108050	WDR48	NM_001346225.1	E4	c.280T>G	p.(Ser94Ala)	Hom	0.500	1/5	SCV000574471	Segregation could not be tested in an inconclusive variant
SR29	2	198359436	HSPD1	NM_002156.4	E6	c.551A>G	p.(Asn184Ser)	Het	0.081	2/5	SCV000574472	Dominant with MAF
SR49	11	62459902	BSC12	NM_001122955.3	E6	c.809G>T	p.(Arg270Leu)	Het	0.081	5/5	SCV000574473	Dominant, incomplete penetrance?
SR54	1	110169006	AMPD2	NM_001257360.1	E6	c.650G>C	p.(Arg217Pro)	Het	0.081	2/5	SCV000574467	Heterozygous variant of a recessive gene
SR60	2	202591249	ALS2	NM_020919.3	E19	c.3206G>A	p.(Gly1069Glu)	Het	0.073	5/5	SCV000574474	Heterozygous variant of a recessive gene
SR65	11	65546763	AP5B1	NM_138368.4	E2	c.1201C>T	p.(Leu401Phe)	Het	0.499	3/4	SCV000574475	Candidate gene
SR65	11	65545875	AP5B1	NM_138368.4	E2	c.2089T>C	p.(Phe697Leu)	Het	0.499	2/4	SCV000574476	Candidate gene
SR65	7	4825294	AP5Z1	NM_014855.2	E9	c.1111G>A	p.(Ala371Thr)	Het	0.012	3/5	SCV000574477	Heterozygous variant of a recessive gene
SR66	7	4824586	AP5Z1	NM_014855.2	E7	c.838A>C	p.(Thr280Pro)	Het	0.140	2/5	SCV000574478	Heterozygous variant of a recessive gene
SR71	5	149677373	ARSI	NM_001012301.3	E2	c.1114G>A	p.(Asp372Asn)	Het	0.012	1/5	SCV000574479	Heterozygous variant of a recessive gene
SR76	2	171709291	BSC12	NM_001122955.3	E7	c.895G>A	p.(Val418Phe)	Het	0.012	5/5	SCV000574480	Dominant with MAF
SR81	9	95491490	BICD2	NM_001003800.1	E2	c.269A>G	p.(Lys90Arg)	Het	0.581	1/5	SCV000574482	Heterozygous variant of a recessive gene
SR92	9	95491490	BICD2	NM_001003800.1	E2	c.269A>G	p.(Lys90Arg)	Het	0.581	1/5	SCV000574482	Heterozygous variant of a recessive gene
S19	16	89619540	SPG7	NM_003119.2	E14	c.1933T>A	p.(Ser645Thr)	Het	0.070	2/5	SCV000574483	Heterozygous variant of a Recessive Gene
S20	17	4925675	KIF1C	NM_006612.5	E22	c.2299G>A	p.(Gly767Arg)	Het	0.245	4/5	SCV000574484	Heterozygous variant of a recessive gene
S20	11	62458862	BSC12	NM_001122955.3	E7	c.895G>A	p.(Ala299Thr)	Het	0.012	5/5	SCV000574480	Dominant with MAF
S21	17	4908241	KIF1C	NM_006612.5	E13	c.1252G>T	p.(Val418Phe)	Het	0.430	4/5	SCV000574485	Heterozygous variant of a recessive gene
S21	17	4925967	KIF1C	NM_006612.5	E22	c.2591G>A	p.(Arg864His)	Het	0.047	4/5	SCV000574486	Heterozygous variant of a recessive gene
S22	13	35793497	MAG	NM_002361.3	E7	c.1117A>C	p.(Ser373Arg)	Het	0.012	4/5	SCV000574487	Heterozygous variant of a recessive gene
S22	13	23929378	SACS	NM_014363.5	E8	c.1373C>T	p.(Thr458Ile)	Het	0.337	2/4	SCV000574488	Heterozygous variant of a Recessive Gene
S24	1	114442497	AP4B1	NG_031901.1 (NM_006594.3)	E6	c.1114 +29G>C	r.; p.?	Het	0.020	4/5	SCV000574489	Heterozygous variant of a recessive gene
S32	11	63885330	FLRT1	NM_013280.4	E2	c.1591G>A	p.(Asp531Asn)	Het	0.012	5/5	SCV000574490	Heterozygous variant of a Recessive Gene
S35	19	35802850	MAG	NM_002361.3	E10	c.1646C>T	p.(Ser549Leu)	Het	0.012	2/5	SCV000574491	Heterozygous variant of a recessive gene
S37	2	202582890	ALS2	NM_020919.3	E24	c.3746T>C	p.(Phe1249Ser)	Het	0.012	4/5	SCV000574492	Heterozygous variant of a recessive gene
S47	12	57881856	MARS	NG_034077.1 (NM_004990.3)	E1	c.-18C>T	r.; p.?	Het	0.020	3/5	SCV000574493	Heterozygous variant of a recessive gene
S47	15	23086364 [^]	MIPAI	NM_144599.4	E2	c.42_47dup	p.(Ala15_Ala16dup)	Het	0.465	Inframe insertion	SCV000574494	Segregation could not be tested in an inconclusive variant
S44	11	65546173	AP5B1	NM_138368.4	E2	c.1791G>C	p.(Gln597His)	Het	0.012	4/4	SCV000574495	Candidate gene
S47	9	95482883	BICD2	NM_001003800.1	E4	c.761A>G	p.(Lys254Arg)	Het	0.012	3/5	SCV000574496	Heterozygous variant of a recessive gene
S47	X	73641849	SLC16A2	NM_006517.3	E1	c.599C>A	p.(Ser200Tyr)	Het	0.012	1/4	SCV000574497	Heterozygous variant of a recessive gene
S47	13	23904298	SACS	NM_014363.5	E10	c.13717A>C	p.(Asn4573His)	Het	0.465	3/4	SCV000574498	Heterozygous variant of a recessive gene

Table 2 (Continued)

Family code	Chr	Genomic coordinate (GRCh37)	Gene	Accession.Version numbers	Exor ^b	Coding change	Predicted change	State	MAF EA	N ^c software ^b	ClinVar accession number	Reason for exclusion
S51	11	65546763	AP5B1	NM_138368.4	E2	c.1201C>T	p.(Leu401Phe)	Het	0.499	3/4	SCV000574475	Candidate gene
	17	4926868	KIF1C	NM_006612.5	E23	c.2734C>T	p.(Arg912Trp)	Het	0.128	3/5	SCV000574499	Heterozygous variant of a recessive gene
S52	19	35790493	MAG	NM_002361.3	E5	c.452C>T	p.(Ala151Val)	Het	0.430	3/5	SCV000574500	Heterozygous variant of a recessive gene
S55	9	135203756	SETX	NM_015046.5	E10	c.3229G>A	p.(Asp1077Asn)	Het	0.093	3/5	SCV000574501	Heterozygous variant of a recessive gene
S56	7	4830418	AP5Z1	NM_014855.2	E16	c.2053C>T	p.(Arg685Cys)	Het	0.012	4/5	SCV000574502	Heterozygous variant of a recessive gene
S62	19	35790493	MAG	NM_002361.3	E5	c.452C>T	p.(Ala151Val)	Het	0.430	3/5	SCV000574500	Heterozygous variant of a recessive gene
S64	5	149677031	ARSI	NM_001012301.3	E2	c.1456C>T	p.(Arg486Cys)	Het	0.012	4/5	SCV000574503	Heterozygous variant of a recessive gene
	14	68264796	ZFYVE26	NM_015346.3	E11	c.2183G>A	p.(Arg728Gln)	Het		1/5	SCV000574504	Heterozygous variant of a recessive gene
S73	1	110170804	AMPD2	NM_001257360.1	E11	c.1342G>A	p.(Val448Met)	Het	0.012	2/5	SCV000574505	Heterozygous variant of a recessive gene
	9	135203159	SETX	NM_015046.5	E10	c.3826C>G	p.(Gln1276Glu)	Het	0.023	1/5	SCV000574506	Heterozygous variant of a recessive gene
S74	7	4830379	AP5Z1	NM_014855.2	E16	c.2014G>A	p.(Glu672Lys)	Het	0.012	4/5	SCV000574507	Heterozygous variant of a recessive gene
S76	16	89611173	SPG7	NM_003119.2	E10	c.1442C>T	p.(Thr481Met)	Het		5/5	SCV000574508	Heterozygous variant of a recessive gene
S86	7	4821291	AP5Z1	NM_014855.2	E3	c.272G>A	p.(Arg91Gln)	Het		3/5	SCV000574509	Heterozygous variant of a recessive gene
	15	44914091	SPG11	NM_025137.3	E14	c.2486T>G	p.(Val829Gly)	Het		3/5	SCV000574510	Heterozygous variant of a recessive gene
S88	12	57960979	KIF5A	NM_004984.2	E7	c.572G>A	p.(Arg191His)	Hom		5/5	SCV000574511	Homozygosity in a dominant gene

^aExon Number according to Alamut software v2.9.0 that used the NCBI RefSeq numbering scheme (along with simple systematic numbering) on the GRCh37 (hg19) assembly.
^bNumber of Software predicting a functional effect of all the prediction software for the missense and possible splice-causing variants; Chr, Chromosome; MAF EA, Minor Allele Frequency in the European American population from Exome Variant Server (<http://evs.gs.washington.edu/EVS/>); Het, Heterozygous; Hom, Homozygous.

agreement with Goizet and colleagues.²⁸ We also identified three families with *SPG7* variants (Supplementary Figure S1) with late-onset HSP and a complex phenotype: families SR75 and S26 also presenting ataxia and the sporadic case CI2 that has minor cerebellar atrophy at brain magnetic resonance imaging which confirms that the involvement of the cerebellum is part of the *SPG7* core phenotype. In family SR84 (Supplementary Figure S1), presenting two known variants in the *GBA2* gene, one affecting the catalytic site and one destabilizing the protein by disturbance of the charge balance,³³ patients had an onset in the expected range (infancy or childhood) and a complex phenotype with cerebellar signs and ataxic gait (Table 3).

Interestingly, we confirmed the higher occurrence of cases with heterozygous vs homozygous/compound heterozygous (*SPG30*) variants in *KIF1A*. One patient in the SR98 family (Figure 2) carried a novel heterozygous *de novo KIF1A* variant located in the motor domain and presented a complex phenotype with mental retardation and neuropathy and an early age at onset, in the first year of life.

On the other hand, the clinical features of some of our patients extended the phenotypic spectrum of some clinico-genetic entities. This was the case of the family with the *SACS* variants (SR6, Figure 2 and Table 3), in which patients presented with spasticity and peripheral neuropathy but without ataxia, as in another single case recently reported in an Italian family.¹⁵ In the family with a *C19orf12* variant (family SR88, Supplementary Figure S1), the phenotype was similar to *SPG43* but also presenting cerebellar ataxia, mental retardation and dementia. Interestingly, the same variant was described in patients with neurodegeneration with brain iron accumulation type 4 (NBIA4),²⁰ extending the spectrum of phenotypes associated with this variant. Brain imaging data were not available for this patient, so we could not check for the presence of brain iron accumulation. Lastly, patients of the S75 family (Figure 2) showing *ERLIN2* variants had an early onset, at 8 and 19 years old, however with a pure phenotype, contrary to the complex phenotype previously described.³⁴

DISCUSSION

Genetic diagnostic yield

The identification of the genetic cause for HSP in only 20.4% of this cohort shows that there is still a large set of genes responsible for spastic paraplegia to be uncovered or that novel inheritance modes, as in the case of *KIF1A*,³⁵ should be taken into account. Although most of these families had been previously screened for *SPG3*, *SPG4* and *SPG31* in the families with dominant forms³⁶ and for *SPG11*, *SPG15* and *SPG5* in the families with recessive transmission (unpublished data), we expected a higher frequency of diagnosed families since we were testing almost all the known genetic causes of HSP, including all the genes identified by Novarino and colleagues.³⁷ This frequency is very similar to that found in a previous study covering far fewer genes in patients where only *SPAST* (*SPG4*) variants were excluded.³⁸ On the other hand, our results are also very similar and comparable to a study where exome sequencing identified disease-causing variants in eight of 48 HSP families (16.6%) and potentially disease-causing variants in another eight families where previous screening for the most likely genes had been performed.³⁹ Taking into account the full cohort, we have found the responsible gene in 42% of the Portuguese families (81/193), or in 51% if we only take into account the families with available DNA (81/159), a frequency similar to a Greek study where only 16 HSP genes were screened.⁴⁰ The most frequently mutated genes are (Figure 4): *SPAST* (*SPG4*) with a frequency of 18% (28/159) and *SPG11* with a frequency of 13% (20/159). Interestingly, we found a high frequency of *KIF5A* variants, present in four out of 44 families

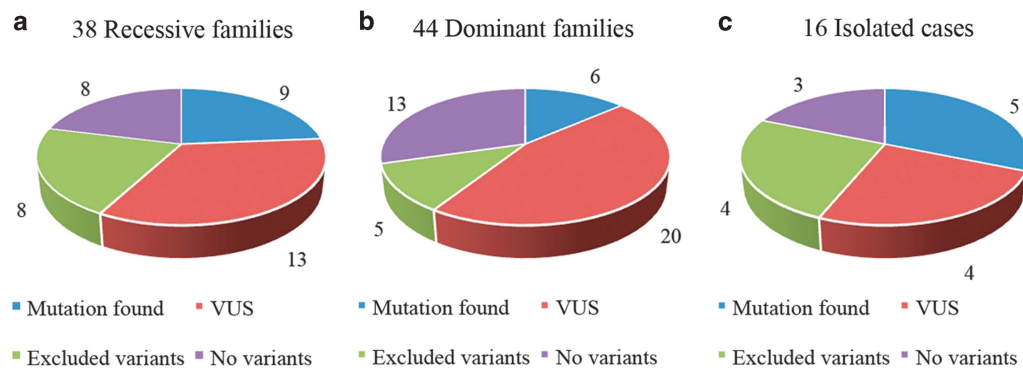


Figure 3 Distribution of the number of families in each class of variants identified. (a) Recessive families, (b) Dominant families, (c) Isolated cases.

with dominant inheritance (9.1%) and in 4 of the 159 families (2.5%) becoming the third most frequently mutated gene in dominant forms. This high occurrence was also found in the Greek population where it was the second cause of disease in families with dominant transmission⁴⁰ and in the study by Warrenburg *et al* where it was found as the most frequently mutated gene (after exclusion of the most likely genes).³⁹ Most of the other genes tested in our cohort have accounted for single families until now, as is the case of *REEP2* (*SPG72*)⁴¹ and in the case of a family that was negative in this screening but after exome analysis proved to segregate *ALDH18A1* (*SPG9*) variants.⁷ In line with our results showing that most of the new HSP genes are rarely involved, the yield of positive results obtained with the two panels, the second containing 30 additional HSP genes, was very similar. We cannot exclude that a variant in the 30 additional genes added to the second panel may explain some of the 17 cases without diagnosis (of the 25) tested with the first panel. This is however very unlikely given (1) the low relative frequencies of these genes,^{5,37} (2) their involvement in recessive forms while 10 of 17 of our patients were associated with dominant inheritance of the disease, (3) and because seven of these cases processed under exome sequencing more recently do not carry mutations in the genes present in the second panel (unpublished data).

Our relatively high percentage of diagnosis among the isolated cases (5 out of 16) is also an interesting finding that shows how important it is to test these patients that frequently are not considered in genetic analyses due to the absence of additional family members to confirm diagnosis.

This study also demonstrates that a panel strategy is also capable of detecting large rearrangements, as proved with the identification of a large deletion, although at the homozygous state, encompassing exons 12–14 in *SPG11* in family SR17. This was possible by the analysis of the coverage, where we compared the mean coverage of all patients tested with each other taking into account the average coverage of each one.

Towards an unbiased approach

Our results also highlight the importance of testing all families for the same set of genes, regardless the presumed inheritance mode. This is important as sometimes the transmission mode is unclear in the pedigree because of its size or censured consanguinity, and because there is an increasing number of HSP genes associated with different modes of transmission (eg, *BICD2*, *KIF1C* and *KIF1A*), sometimes associated with a different disease presentation (eg, *SPG7*). We have some examples in our cohort of families with an apparent recessive mode of transmission (including sporadic cases) in whom we found a

variant in a gene associated with a dominant form. This can happen when the disease is caused by a 'de novo' variant, as in families SR4 (Supplementary Figure S1) and SR98 (Figure 2), and also in late-onset families in which the disease could have been missed in the older generations. The reverse is also true since we report one case with a homozygous variant in *SPG7*, usually associated with a recessive form, found in a family classified as dominant (S26, Supplementary Figure S1). This could likely be explained by censured consanguinity or by the presence of older individuals reported as affected but with a different neurological condition. Family SR98, with a variant in the motor domain of *KIF1A*, is also an example of a gene that can have recessive or dominant inheritance depending on the localization of the variant. It was recently shown by Lee and colleagues that *de novo* heterozygous variants in the motor domain of *KIF1A* can be disease-causing³⁵ and that this type of variants are more common than the homozygous ones, which has been later confirmed in several studies^{39,42,43} including our own. In family SR99 (Figure 2), we found a new variant (p.Ala27Pro) in *REEP1* that, despite being present in the unaffected father, is likely the disease-causing variant because incomplete penetrance was already described for variants in this gene and because the same amino acid was affected in another HSP case.⁴⁴

Need for international collaborative efforts

Our results also highlight the difficulty of reaching a conclusion as to the causative nature of the variants in 37% of the cases. Large screening studies, like ours, result in a large quantity of data that has to be interpreted, a task that is not always easy even with all the available software. This is especially difficult in HSP since the disease can have different inheritance modes. An example of the difficulty in interpretation without functional data is illustrated by family SR6 (Figure 2), where we found two new variants, within six bases of distance, that segregate together, and we were not able to demonstrate which one was deleterious, although the predictions on their effect on protein function is stronger in one of them.

It is highly likely that in our 37 families with VUS, some of these VUS could be variants with new inheritance forms for already known genes. This could be the case in family SR88, where we found a *KIF5A* variant present at the homozygous state in patients in two generations of a family with no reported consanguinity (Supplementary Figure S2). This might represent the first autosomal recessive case due to *KIF5A* variants, but this cannot be ascertained in the absence of functional evidence or additional cases segregating in autosomal recessive pedigrees. We also found some variants in candidate genes selected by function (interactors) for which we were not able to conclude as to

Table 3 Clinical characteristics of the 20 families with an established molecular diagnosis

Family code	Individual No. (gender)	Gene	Disease-causing variant ^a	Inheritance	Phenotype	Age at onset (years)		Age at examination (years)		Disease duration (years)		Spasticity		Spasticity		Babinski sign	Sphincter disturbances	Dysarthria	Cognitive impairment	Other signs	MRI	
						(years)	(date)	(years)	(date)	(years)	(date)	LL (0-4)	UL (0-4)	LL (0-4)	UL (0-4)							LL (0-5)
SR88	IV:1 (M)	C19orf12	c.157G>A; c.157G>A	Recessive	Complex	8	17 (2004)	9		3	1	2	4	4	4	4	Yes	NA	Yes and dysphagia	Yes (1) and dementia	Motor neuropathy, cerebellar ataxia, bilateral optic atrophy, proximal spastic tetraparesis, deterioration, distal atrophy, pes cavus	NA
SR97	II:2 (F)	CYP7B1	c.914dup; c.825T>A	Recessive	Pure	10	36 (2006)	26		NA	0	4	5	5	5	5	Yes	NA	No	No	Pes cavus, gait ataxia	NA
	II:3 (M)					10	26 (2006)	16		NA	0	4	5	5	5	5	Yes	NA	No	No	Pes cavus, gait ataxia	NA
S93	IV:7 (M)	CYP7B1	c.1456C>T; c.1456C>T	Recessive	Pure	40	73 (2007)	33		4	0	NA	NA	NA	NA	NA	Yes	Mild	NA	NA	No	NA
SR84	IV:13 (F)					55	67 (2007)	12		NA	0	NA	NA	NA	NA	NA	Yes	NA	NA	NA	NA	NA
	II:2 (F)	GBA2	c.2618G>A; c.1888C>T	Recessive	Complex	7	38 (2007)	46		4	0	4	0	0	0	0	Yes	Slight	No	No	Dysphagia, cerebellar signs, ataxic gait, postural tremor, distal LL hypoesthesia	NA
SR75	II:4 (M)					12	23 (2007)	31		4	0	4	0	0	0	0	Yes	No	No	No	NA	NA
	IV:2 (M)	SPG7	c.1454_1462del; c.1454_1462del	Recessive	Complex	22	34 (1999)	12		NA	NA	Yes	NA	NA	NA	NA	NA	NA	Yes	No	Progressive ataxia at onset	Cerebellar atrophy (cortical and diffuse)
SR16	III:4 (F)	SPG11	c.733_734del; c.733_734del	Recessive	Complex with neuropathy	18	37 (1993)	19		4	3	2	2	2	2	2	NA	Yes	Yes (pseudobulbar)	Yes	NA	NA
	II:6 (M)					18	32 (1996)	14		4	2	2	3	3	3	3	Yes	No	Yes (Pseudobulbar and dysphagia)	Yes	NA	NA
SR82	II:7 (M)					18	NA	NA		NA	NA	NA	NA	NA	NA	NA	NA	No	NA	No	NA	NA
	V:1 (M)	ZFYVE26	c.6296dup; c.6296dup	Recessive	Complex	14	25 (2004)	11		4	NA	4	5	5	5	5	Yes	Yes	Yes	Yes (1)	Abnormal TOC, bilateral cortical atrophy	NA
S31	IV:8 (F)	KIF5A	c.610C>T	Dominant	Pure	14	51 (2006)	37		NA	0	4	5	5	5	5	Yes	NA	No	No	NA	NA
S41	IV:2 (F)	KIF5A	c.839G>A	Dominant	Pure	20	64 (2008)	44		4	0	4	NA	NA	NA	NA	Yes	NA	NA	NA	NA	NA
	V:5 (F)					20	34 (2008)	12		1	0	NA	NA	NA	NA	NA	Yes	NA	NA	NA	NA	NA
S61	V:1 (F)	NIPA1	c.316G>A	Dominant	Pure	10	19 (2008)	9		0	0	NA	NA	NA	NA	NA	Yes	NA	NA	NA	NA	NA
	II:5 (F)					9	58 (2005)	49		4	0	NA	NA	NA	NA	NA	Yes	NA	NA	NA	NA	NA
	III:2 (M)					5	32 (2005)	27		3	0	NA	NA	NA	NA	NA	Yes	NA	NA	NA	NA	NA
	IV:1 (F)					2	12 (2005)	10		NA	NA	NA	NA	NA	NA	NA	Yes	NA	NA	NA	NA	NA
S26	IV:3 (M)	SPG7	c.1447C>T; c.1447C>T	classified as dominant	Complex	40	55 (1998)	15		2	0	NA	NA	NA	NA	NA	Yes	NA	NA	NA	Slight cerebellar atrophy	NA
SR98	IV:5 (M)					40	60 (2008)	20		1	0	4	5	5	5	5	Yes	NA	NA	NA	Ataxia	NA
	IV:2 (M)	KIF1A	c.31C>T	Isolated case	Complex	1	13 (2007)	12		NA	0	5	5	5	5	5	No	NA	No	Yes (2)	Neuropathy	Normal
SR4	IV:3 (M)	SPAST	c.1133T>A	Isolated case	Pure	23	48 (1995)	25		NA	NA	NA	NA	NA	NA	NA	Yes	No	NA	No	Pes cavus	NA
O13	II:1 (M)	SPG7	c.1529C>T; c.376G>C	Isolated case	Complex	57	60 (2006)	3		1	0	5	5	5	5	5	No	No	No	No	Slight cerebellar atrophy	NA

Table 3 (Continued)

Family code	Individual No. (gender)	Gene	Disease-causing variant ^a	Inheritance	Phenotype	Age at examination		Disease duration		Spasticity LL (0-4)	Spasticity UL (0-4)	Weakness LL (0-5)	Weakness UL (0-5)	Babinski sign	Sphincter disturbances	Dysarthria	Cognitive impairment	Other signs	MRI
						onset (years)	in years (date)	in years (date)	(years)										
SR17	III:3 (M)	SPG11	c.(2244+1_2245-1)_(2620+1_2621-1)del; c.(2244+1_2245-1)_(2620+1_2621-1)del	Isolated Case	Complex	13	25 (1993)	12		3	0	4	5	Yes	No	No	Yes (1)	Pes cavus	NA
S75	II:1 (F)	ERLIN2	c.899A>T; c.899A>T	Recessive	Pure	8	31 (2001)	NA		NA	NA	NA	NA	NA	NA	NA	NA	No	NA
SR6	IV:6 (M)	SACS	c.[1640C>T; 1634G>T]; c.[1640C>T; 1634G>T]	Recessive	Complex	6	55 (1996)	47		3	0	4	0	Yes	No	No	No	Pes cavus	NA
S27	III:7 (M)	KIF5A	c.833C>T	Dominant	Complex	21	74 (2008)	53		1	0	4	5	NA	NA	No	No mental retardation, dementia probably of other etiology	Pes cavus	NA
III:9 (F)						43	71 (2008)	28		0	0	2	5	NA	NA	Yes	No	Ataxia and extrapyramidal signs, parkinsonian syndrome only akimetic without spasticity	NA
IV:3 (F)							47 (2008)			2	0	5	5	No	No	No	No		NA
S58	III:2 (M)	KIF5A	c.868G>C	Dominant	Pure	25	54 (2003)	29		NA	NA	NA	NA	NA	NA	NA	No	Hammer toes	NA
III:6 (F)						18	44 (2004)	26		NA	0	4	5	Yes	No	No	No		NA
SR99	IV:2 (M)	REEP1	c.79G>C	Isolated case	Pure	2	16 (2010)	14		2	0	5	5	Yes	No	No	No		Normal

Abbreviations: M, male; F, female; NA, not available; MRI, magnetic resonance imaging. For spasticity a scale of 0-4 according to the Modified Ashworth Scale was used. Weakness was graded from 0 to 5 using the Medical Research Council Scale for Muscle Strength. ^aAccession.Version numbers available on Table 1.

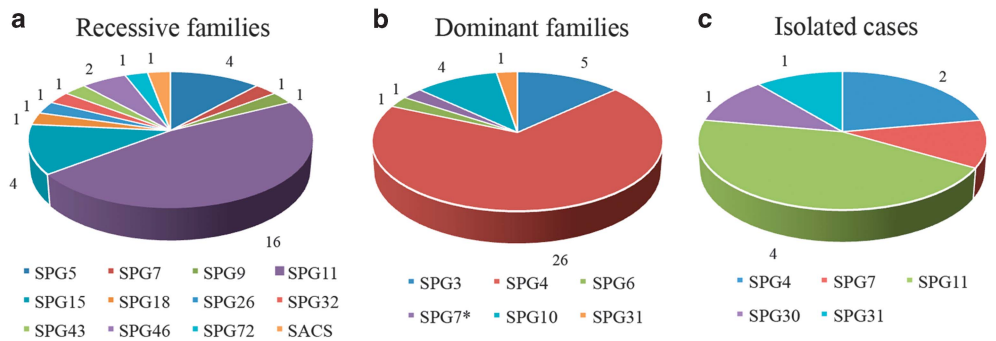


Figure 4 Distribution of the disease-causing variants found in 81 families from the full cohort of Portuguese cases.[#] (a) Recessive families, (b) Dominant families, (c) Isolated cases. *Case described in this paper of a family classified with dominant inheritance but with a homozygous *SPG7* variant. # Include mutations found in previously screened genes.^{7,36,41}

their causative effect due to the insufficient number of family members to validate their co-transmission (families SR65 and S86, Supplementary Figure S2), and the absence of cell lines to demonstrate the disruption of the AP5 complex by biochemical experiments.⁴⁵ We observed a variant in a gene associated with dominant transmission, *BSCL2*, that is in a conserved region, is predicted to affect function by five software and is in the same domain as the two published variants (with reports of incomplete penetrance) but, since it is present in the non-affected mother and there are no other known carriers, we were not able to conclude as to the causative effect of this variant (case SR49, Supplementary Figure S2). Absence of family members to confirm segregation of a homozygous variant in the *WDR48* was also an issue in family SR26 (Supplementary Figure S2). This family presents a complex phenotype with motor neuropathy that is in accordance with the reported clinical features,³⁷ although with a later age at onset (20 and 22 years old vs 1-year-old), and only functional studies will allow to prove its causative role. Therefore, the availability of relatives to test segregation still remains crucial to conclude on a causative variant, and diagnosis in HSP should not rely only on the clinical/genealogical presentation because the clinical phenotype and full mutational spectrum associated with each gene are continuously expanding.⁵

In conclusion, this comprehensive study of a large cohort of 98 families of homogeneous origin (only Portuguese families) allowed us to conclude on the frequency of all the less common HSP genes. Also, it highlights the fact that although a high number of genes have been already identified in this condition, a large percentage of cases remain without molecular diagnosis, some of them due to the absence of extensive screening. Gene panel strategy is a cost-effective way to screen HSP families and probably a small panel like ours with 34 genes could give a diagnosis to almost half of the families if used as a first approach. The use of an extensive panel of genes like our second panel, including the more recent and less frequently mutated genes such as *VCP*^{39,46} would only slightly increase the number of diagnosis. This suggests that performing exome sequencing in the families without diagnosis in the most frequent genes (34) is probably a good strategy in HSP.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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