



Identification of genetic modifiers in Hereditary Spastic Paraplegias due to SPAST/SPG4 mutations

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Sorbonne Université
Institut du Cerveau et de la Moelle Épinière
École Doctorale Cerveau-Cognition-Comportement

Thèse de doctorat en Neurosciences

Identification of genetic modifiers in
Hereditary Spastic Paraplegias due to
SPAST/SPG4 mutations

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Table of contents

Abbreviations	3
Preface	4
Introduction	5
Hereditary Spastic Paraplegias (HSPs)	5
Clinical background	5
Genetic background	8
Physiopathology and neuropathology	15
Diagnosis and treatment	17
Spastic Paraplegia type 4	18
Clinical and genetic background	18
Spastin, a microtubule severing protein	20
The pursuit of genetic modifiers	23
Learning from other disorders...	23
...And what about SPAST-HSP modifiers?	28
Objectives	31
Patients and methods – Part 1: Modifiers identification	33
Patients	33
SPAST-HSP patient cohort	33
Genotyped cohort	34
Exomed cohort	35
RNA-sequencing cohort	36
Sequencing, quality control and analysis settings	38
Genome-wide genotyping	38
Whole Exome Sequencing (WES)	40
RNA sequencing	41
Material and methods – Part 2: Modifiers validation	44
Drosophila mutant lines	44
RT-qPCR	44
Western Immunoblotting	44
Results – part 1: better alone than in bad company?	45
SPAST-HSP cohort analysis	45
Genome-wide linkage analysis	74
Genome-wide association analysis	76
Whole Exome Sequencing (WES) analysis	96
RNA-sequencing	99
Results – part 2: unity is strength!	101
Discussion	105

Conclusions and perspectives	113
<i>Parodi L, Fenu S, Stevanin G and Durr A. Hereditary spastic paraplegia: More than an upper neuron disease. Rev Neurol (Paris). 2017 May;173(5):352-360.</i>	117
<i>Parodi L, Coarelli G, Stevanin G, Brice A and Durr A. Hereditary ataxias and paraplegias: genetic and clinical update. Curr Opin Neurol. 2018 Aug;31(4):462-471.</i>	127
<i>Parodi L, Rydning SL, Tallaksen C and Durr A. Spastic Paraplegia 4. GeneReviews®[Internet].Seattle WA: University of Washington, Seattle; 1993-2019.</i>	139
References	161

Abbreviations

HSPs: Hereditary Spastic Paraplegias

SPAST-HSPs/SPG4-HSPs: Spastic Paraplegias caused by *SPAST* mutations

SPGs: Spastic Paraplegia Genes

SPG4: Spastic Paraplegia Type 4

ADHSPs: Autosomal Dominant HSPs

ARHSPs: Autosomal Recessive HSPs

SCA: Spino Cerebellar Ataxias

HD: Huntington's Disease

PD: Parkinson's Disease

AD: Alzheimer's Disease

MS : Multiple Sclerosis

NGS: Next Generation Sequencing

GWAS: Genome Wide Association Studies

WES: Whole Exome Sequencing

MAF: Minor Allele Frequency

eQTLs: expression Quantitative Trait Loci

PBLs : Primary Blood Lymphoblasts

Preface

Hereditary Spastic Paraplegias (HSPs) are a group of rare, inherited, neurodegenerative disorders that arise following the progressive degeneration of the corticospinal tracts, leading to lower limbs spasticity, the disorder hallmark. HSPs are characterized by an extreme complexity underlying both clinical and genetic features, leading to a widening of the associated phenotype, often in overlap with other neurological disorders. The observed heterogeneity is not restrained to the already broad combination of clinical signs manifested by HSP patients, but also to other disorder features, such as age at onset and severity. This variability is typically observed among HSP patients carrying mutations in *SPAST*, the most frequently mutated HSP gene. Indeed, after collecting clinical data of multiple *SPAST*-HSP families, it was possible to observe a clear age at onset and severity heterogeneity, even among related patients sharing the same causative mutation. To dig deeper into the causes underlying the observed variability, *in primis* into the presence of variants acting as phenotypic modifiers, we decided to combine different NGS strategies to carry out the analysis from different angles. Surprisingly, after overcoming multiple challenges, we managed to obtain different candidate genes/variants possibly acting as age at onset modifiers, as well as to shed light on *SPAST*-HSP clinical and genetic background.

Introduction

Hereditary Spastic Paraplegias (HSPs)

Clinical background

“ (...) These are the conclusions made by Strümpell: 1. A slow and progressive degeneration of the spinal cord develops under the influence of a congenital malformation. 2. This lesion is generally familial and apparently it is more frequent among men. 3. The first signs of the illness start frequently between 20 and 30 years, in the form of spasmodic motor troubles of the lower extremities. (...) 4. Generally, the disorder leads to an actual *spasmodic* paraplegia. The pyramidal tracts sections that refer to the upper extremities, to the tongue, the lips, are subsequently and more rarely affected than the sections that respond to the lower limbs. 5. Generally, the pyramidal tracts lesions seem to associate to a mild degeneration of other systems (cerebellar

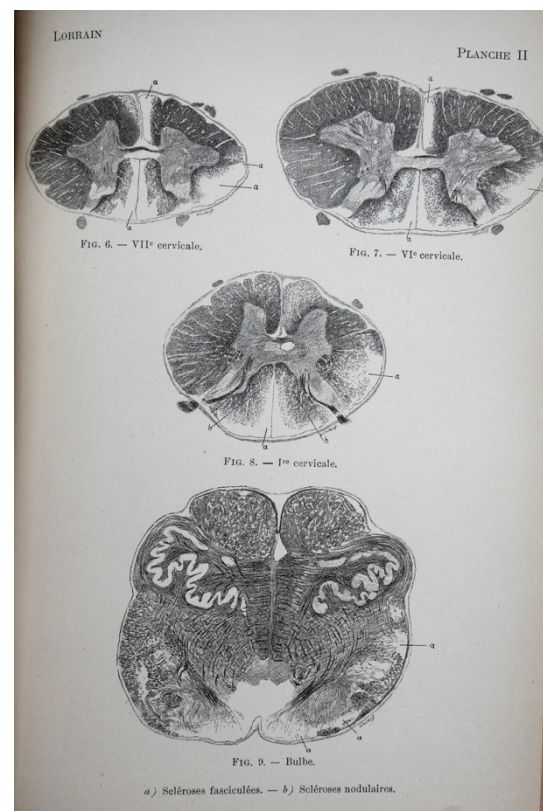
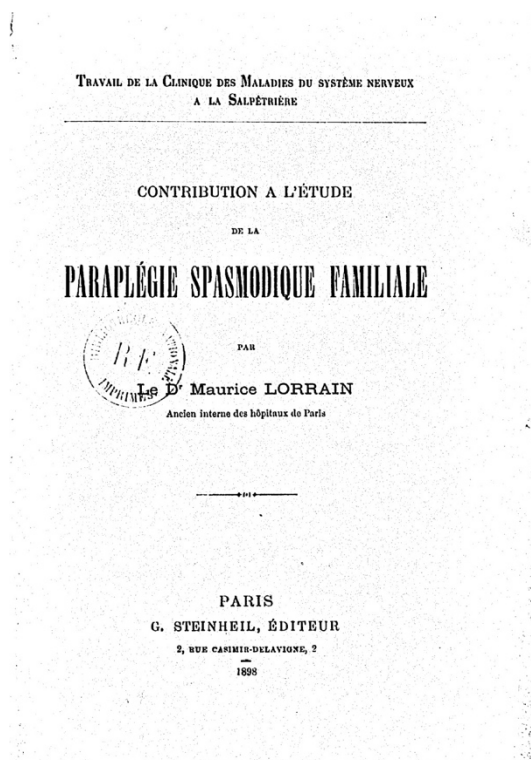


Figure 1. Original manuscript of Dr. Maurice Lorrain. Source: gallica.bnf.fr/Bibliothèque nationale de France.

tract, Goll's tract). From a clinic point of view, troubles affecting the sense of temperature and mild vesical troubles make these associations possible."

With this concise but straightforward summary Dr Maurice Lorrain reports in his thesis, "Contribution to the study of Familial Spastic Paraplegia" (1898) (Figure 1), what had previously been observed by Pr Adolf Strümpell. As first described by the two clinicians at the end of the 19th century, Hereditary Spastic Paraplegias (HSPs) or Strümpell-Lorrain disease, arises following a progressive distal axonopathy that mainly involves the corticospinal tracts, therefore resulting in spasticity of the lower limbs when walking, the disorder hallmark.

HSPs global prevalence, at the latest estimate, resulted being 1-5: 100'000, depending on the country and taking in consideration that epidemiologic data are still missing from large world's areas (Ruano *et al.*, 2014).

To distinguish HSPs presenting almost exclusively with pyramidal signs, from forms characterised by additional neurological and non-neurological features, a new classification was proposed in the '80s by Professor Anita E Harding. Pure HSPs were described as presenting with pyramidal signs predominantly affecting the lower limbs, therefore causing spasticity, weakness and, in some cases, sphincter disturbances (Harding, 1983). At neurological examination, pure HSPs show increased lower-limb muscle tone (especially in the hamstrings, quadriceps, gastrocnemius-soleus and adductors) and weakness (in the iliopsoas, hamstrings and tibialis anterior), as well as hyperreflexia, extensor plantar responses and attenuated vibratory sensation in the ankles.

The presence of additional neurological symptoms defines HSPs complex forms. Dystonia and other extrapyramidal features, such as cognitive disability and/or

deterioration, optic atrophy, cataract and hearing impairment, are some of the many symptoms that can be associated to HSPs core features. A complex HSP is for instance represented by spastic ataxia, in which cerebellar ataxia and dysarthria, major Spino Cerebellar Ataxias (SCA) features, are associated with core HSP symptoms (Parodi *et al.*, 2017). A correct definition of pure HSP can be therefore truthful only after investigating and ensuring the absence of additional signs not clinically evident (Dürr *et al.*, 1994).

The already complex and extremely heterogeneous HSPs clinical background has furthermore been complicated by the introduction of Next Generation Sequencing (NGS) techniques. NGS introduction in everyday diagnostic process has rapidly led to a broadening of HSPs clinico-genetic aspects, therefore allowing the identification of multiple overlaps between HSPs and other neurological disorders, including in particular SCAs. The recent discovery of a remarkable number of genes that, if mutated, produce hybrid phenotypes, ranging from a more pure ataxia to a pure spastic paraparesis, led to the introduction of a new concept of spastic ataxia phenotypic spectrum, rather than referring to SCAs and HSPs as two separate diseases. Moreover, HSPs core features can be observed as a novel clinical manifestation in patients affected by Charcot-Marie-Tooth disease or Parkinson's disease, amongst others (Sambuughin *et al.*, 2015; van de Warrenburg *et al.*, 2016).

The extreme symptoms variety is not the only feature contributing to HSPs clinical heterogeneity. Both age at onset and disease progression are indeed extremely variable among HSPs patients, even when the same causative mutation is shared. High intrafamilial variability is frequently observed in HSP familial nuclei: mutation carriers may experience early onset and rapid progression or be asymptomatic, strongly suggesting the existence of yet unidentified modifying factors (Parodi *et al.*, 2017).

Genetic background

Linkage analysis was the first strategy that allowed the identification of genomic regions harbouring HSPs causative genes. The subsequent introduction of NGS techniques progressively revolutionized the disorder's genetic diagnosis. The combination of NGS and the use of screening panels including genes linked to the onset of HSPs, or allelic diseases, greatly increased the power of genetic diagnosis, steadily increasing the number of new candidate genes. Yet, despite these advances, the difficulty in connecting an observed phenotype with a specific candidate gene, as well as the occasional uncertainty surrounding the inheritance pattern, make the research of HSPs genetic cause particularly arduous, leaving 20% of familial and 52% of sporadic HSP patients without a genetic diagnosis (Lo Giudice *et al.*, 2014; Novarino *et al.*, 2014; Ruano *et al.*, 2014). To date 79 loci and 65 corresponding Spastic Paraplegia Genes (SPGs) have been linked to HSP onset (Table 1, A-E). Disease-causing mutations are transmitted through all the known inheritance patterns, even if they are mostly inherited through dominant and recessive transmissions. Autosomal-Dominant HSPs (ADHSPs) mostly lead to a pure form of the disorder and are predominantly caused by mutations affecting *SPAST*/SPG4, *ATL1*/SPG3A, *REEP1*/SPG31 and *KIF5A*/SPG10, responsible altogether for the 57% of ADHSP cases. A more complicated phenotype is observed in autosomal recessive HSPs (ARHSPs), with mutations impairing *KIAA1840*/SPG11, *CYP7B1*/SPG5A, SPG7 and *ZFYVE26*/SPG15, accounting for almost 34% of ARHSPs onset (Klebe *et al.*, 2015; Tesson *et al.*, 2015). Rare forms of HSP include X-linked and maternally inherited HSPs. Currently, five loci have been linked to X-linked HSPs, that mostly arises following mutations in *L1CAM*/SPG1 and *PLP1*/SPG2, both leading to a complicated HSP (Bonneau *et al.*, 1993; Jouet *et al.*, 1994). To date, only one

gene encoded by the mitochondrial genome has clearly been related to HSP onset; complicated spastic paraplegia was indeed observed in a family harbouring mutations in *MT-ATP6*, coding for a component of the adenosine triphosphate synthase complex (Verny *et al.*, 2011).

Multiple inheritance patterns have been observed in patients carrying mutations in *KIF1C*/SPG58 and *REEP2*/SPG72, leading to HSP onset when present in both heterozygous or homozygous state (Caballero Oteyza *et al.*, 2014; Esteves *et al.*, 2014). Similarly, mutations in *ALDH18A1* resulted responsible for SPG9A-HSP when inherited through a dominant transmission and for SPG9B-HSP, when present in a homozygous state (Coutelier *et al.*, 2015; Panza *et al.*, 2016).

A direct consequence of the large number of genes and loci involved in HSPs is the extreme variety that characterises SPG-encoded proteins and their roles in the cellular environment. Amongst others, axon pathfinding and preservation, myelination, endoplasmic reticulum maintenance, lipid metabolism, endosomal dynamics and intracellular transport are only a few of the cellular functions covered by SPGs-encoded proteins (Blackstone, 2012).

Table 1. A) Spastic paraplegia genes.

SPG locus	Gene	Protein	Inheritance	Frequency	Additional Clinical Signs	Protein function
SPG1	L1CAM	Neural cell adhesion molecule 1	X-linked	1.1% familial	MASA, CRASH syndromes	Cell adhesion and signaling
SPG2	PLP1	Myelin proteolipid protein	X-linked	Rare	Cerebellar signs, intellectual disability, seizures	Major myelin protein
SPG3A	ATL1	Atlastin-1	AD	6.8% familial, 3.5% sporadic	-	ER morphogenesis, BMP signaling, LD regulation
SPG4	SPAST	Spastin	AD	37.6% familial, 18.8% sporadic	-	Microtubule severing, ER morphogenesis, endosomal traffic, BMP signaling, LD biogenesis, cytokinesis
SPG5	CYP7B1	25-hydroxycholesterol 7-alpha hydroxylase	AR	5.1% familial, 7.9% sporadic	Cerebellar ataxia, optic atrophy	Cholesterol metabolism
SPG6	NIPA1	Magnesium transporter NIPA1	AD	Rare	-	Endosomal traffic, Mg ²⁺ transport, BMP signaling
SPG7	-	Paraplegin	AR	7% familial	Cerebellar ataxia, optic atrophy	Mitochondrial m-AAA ATPase
SPG8	KIAA0196	Strumpellin	AD	Rare	-	Endosomal traffic, cytoskeletal (actin) regulation
SPG9A/B	ALDH18A1	Delta-1-pyrroline-5-carboxylate synthase	AD/AR	Extremely rare	Psychomotor retardation, intellectual disability, cataract, cutis laxa, gastroesophageal reflux, dysmorphisms	-
SPG10	KIF5A	Kinesin heavy chain isoform 5A	AD	3.4% familial	Intellectual disability, extrapyramidal signs	Microtubule-based motor protein, anterograde axon transport
SPG11	KIAA1840	Spatacsin	AR	16.2% familial, 7.3% sporadic	Cognitive decline, cerebellar signs, extrapyramidal signs, retinal degeneration	Endosomal traffic, lysosomal biogenesis, autophagy
SPG12	RTN2	Reticulon-2	AD	Rare	-	ER morphogenesis
SPG13	HSPD1	Mitochondrial heat shock protein	AD	Extremely rare	-	Mitochondrial chaperonin
SPG14	-	-	AR	Extremely rare	Mild intellectual disability	-
SPG15	ZFYVE26	Spastizin	AR	4% familial	Cerebellar ataxia, retinal degeneration	Endosomal traffic, lysosomal biogenesis, autophagy
SPG16	-	-	X-linked	Extremely rare	-	-

Table 1. B) Spastic paraplegia genes.

SPG locus	Gene	Protein	Inheritance	Frequency	Additional Clinical Signs	Protein function
SPG17	BSCL2	Seipin	AD	Rare	-	LD biogenesis at ER
SPG18	ERLIN2	Erlin-2	AR	Rare	Psychomotor developmental delay, joints contractures	ER-associated degradation, lipid-raft associated
SPG19	-	-	AD	Extremely rare	Scoliosis	-
SPG20		Spartin	AR			Endosomal traffic, BMP signaling, cytokinesis, LD turnover
SPG21	ACP33	Masparidin	AR	Rare	Mast syndrome: cognitive decline, cerebellar signs	Endosomal traffic (late)
SPG22	SLC16A2	Monocarboxylate transporter 8	X-linked	Rare	Allan-Herndon-Dudley syndrome with severe psychomotor retardation, thyrotoxicosis	Thyroid hormone (T3) transporter
SPG23	DSTYK	Dusty protein kinase	AR	Rare	Pigmentary abnormalities	Cell death regulation
SPG24	-	-	AR	Extremely rare	-	-
SPG25	-	-	AR	Extremely rare	Disk herniations	-
SPG26	B4GALNT1	Beta-1,4-N-acetylgalactosaminyltransferase 1	AR	Rare	Cerebellar ataxia, intellectual disability, dystonia	Ganglioside biosynthesis
SPG27	-	-	AR	Extremely rare	-	-
SPG28	DDHD1	Phospholipase DDHD1	AR	Rare	Scoliosis	Phosphatidic acid metabolism, membrane traffic
SPG29	-	-	AR	Extremely rare	Para oesophageal hernia	-
SPG30	KIF1A	Kinesin-like protein KIF1A	AD/AR	Rare	Cerebellar ataxia, psychomotor developmental delay	Microtubule-based motor protein, axon transport
SPG31	REEP1	Receptor expression-enhancing protein 1	AD	4.9% familial	Peripheral neuropathy	ER morphogenesis, microtubule interactions, LD regulations
SPG32	-	-	AR	Extremely rare	Mental retardation, cerebellar and cortical atrophy, pontine dysraphism	-

Table 1. C) Spastic paraplegia genes.

SPG locus	Gene	Protein	Inheritance	Frequency	Additional Clinical Signs	Protein function
SPG33	ZFYVE27	Protrudin	AD	Rare	-	ER morphogenesis, endosome interactions
SPG34	-	-	X-linked	Extremely rare	-	-
SPG35	FA2H	Dihydroceramide fatty acyl 2-hydroxylase	AR	Rare	Dystonia, cerebellar signs, cognitive decline, brain iron accumulation, seizures	Myelin lipid hydroxylation
SPG36	-	-	AD	Extremely rare	-	-
SPG37	-	-	AD	Extremely rare	-	-
SPG38	-	-	AD	Extremely rare	Silver syndrome	-
SPG39	PNPLA6	Neuropathy target esterase	AR	Rare	Intellectual disability	Phospholipid homeostasis, BMP signaling
SPG41	-	-	AD	Extremely rare	-	-
SPG42	SLC33A1	Acetyl-coenzyme A transporter 1	AD	Extremely rare	-	Acetyl-CoA transporter, BMP signaling
SPG43	C19orf12	C19orf12	AR	Extremely rare	Neurodegeneration with brain iron accumulation	-
SPG44	GJC2	Gap junction gamma-2 protein	AR	Extremely rare	Cerebellar signs, seizures, mild intellectual disability	Intracellular gap junction channel
SPG45/65	NT5C2	Cytosolic purine 5'-nucleotidase	AR	Extremely rare	Psychomotor retardation, intellectual disability, ocular signs	IMP hydrolysis, purine/pyrimidine nucleotide metabolism
SPG46	GBA2	Non-lysosomal glucosylceramidase	AD	Rare	Cerebellar ataxia, intellectual disability, cataracts, infertility in males	Lipid metabolism
SPG47	AP4B1	AP-4 complex subunit beta-1	AR	Rare	Intellectual disability, dysmorphisms	Endocytic adaptor protein complex
SPG48	AP5Z1	AP-5 complex subunit zeta-1	AR	Rare	Intellectual impairment	Endocytic adaptor protein complex
SPG49	TECPR2	Tectonin beta-propeller repeat-containing protein 2	AR	Rare	Intellectual disability, respiration troubles, gastroesophageal reflux	Autophagy
SPG50	AP4M1	AP-4 complex subunit mu-1	AR	Rare	Infantile hypotonia, intellectual disability, speech disorder	Endocytic adaptor protein complex
SPG51	AP4E1	AP-4 complex subunit epsilon-1	AR	Rare	Infantile hypotonia, intellectual disability, speech disorder	Endocytic adaptor protein complex

Table 1. D) Spastic paraplegia genes.

SPG locus	Gene	Protein	Inheritance	Frequency	Additional Clinical Signs	Protein function
SPG52	AP4S1	AP-4 complex subunit sigma-1	AR	Extremely rare	Neonatal hypotonia, intellectual disability, speech disorder, dysmorphism	Endocytic adaptor protein complex
SPG53	Vps37A	Vacuolar protein sorting-associated protein 37A	AR	Rare	Psychomotor retardation, intellectual disability	Retromer component
SPG54	DDHD2	Phospholipase DDHD2	AR	Rare	Psychomotor retardation, intellectual disability	Phosphatidic acid metabolism, membrane traffic
SPG55	C12orf65	–	AR	Rare	Intellectual disability, strabismus	Mitochondrial protein translation
SPG56	CYP2U1	Cytochrome P450 2U1	AR	Rare	Intellectual disability, subclinical axonal neuropathy	Long-chain fatty acid metabolism
SPG57	TFG	Trk-fused gene	AR	Extremely rare	Optic atrophy	ER morphology, vesicle biogenesis
SPG58	KIF1C	Kinesin-like protein KIF1C	AD/AR	Rare	Cerebellar ataxia, mild intellectual disability, chorea	Motor protein, retrograde Golgi-to-ER transport
SPG59	USP8	Ubiquitin carboxyl-terminal hydrolase 8	AR	Extremely rare	Mild intellectual disability	De-ubiquitination enzyme
SPG60	WDR48	WD repeat-containing protein 48	AR	Extremely rare	Nystagmus, peripheral neuropathy	Regulation of de-ubiquitination
SPG61	ARL6IP	ADP-ribosylation factor-like protein 6-interacting protein 1	AR	Extremely rare	Sensory/motor polyneuropathy	ER morphogenesis
SPG62	ERLIN1	Erlin-1	AR	Rare	Cerebellar signs, mild intellectual disability	ER-associated degradation, lipid-raft associated
SPG63	AMPD2	AMP deaminase 2	AR	Extremely rare	Short stature	Purine metabolism
SPG64	ENTPD1	Ectonucleoside triphosphate diphosphohydrolase 1	AR	Rare	Cerebellar signs, intellectual disability, delayed puberty	Purinergic transmission
SPG66	ARSI	Arylsulfatase I	AR	Extremely rare	Intellectual disability, sensory/motor polyneuropathy	Sulfate ester hydrolysis, hormone biosynthesis
SPG67	PGAP1	GPI inositol-deacylase	AR	Extremely rare	Intellectual disability	Transport of GPI-anchored proteins from ER to Golgi apparatus
SPG68	FLRT1	Leucine-rich repeat transmembrane protein FLTR1	AR	Extremely rare	Nystagmus, optic atrophy	Regulation of cell adhesion and fibroblast growth factor signaling
SPG69	RAB3GAP2	Rab3 GTPase-activating protein non-catalytic subunit	AR	Extremely rare	Psychomotor retardation, intellectual disability, deafness, cataracts	ER morphogenesis, exocytosis

Table 1. E) Spastic paraplegia genes.

SPG locus	Gene	Protein	Inheritance	Frequency	Additional Clinical Signs	Protein function
SPG70	MARS	Methionine–tRNA ligase, cytoplasmic	AR	Extremely rare	-	Cytosolic methionyl-tRNA synthesis
SPG71	ZFR	Zinc finger RNA-binding protein	AR	Extremely rare	-	-
SPG72	REEP2	Receptor expression-enhancing protein 2	AD/AR	Rare	-	ER morphogenesis, microtubule interactions
SPG73	CPT1C	Carnitine O-palmitoyltransferase 1, brain isoform	AD	Extremely rare	-	Lipid metabolism, ceramides
SPG74	IBA57	Putative transferase CAF17, mitochondrial	AR	Extremely rare	Optic atrophy	Mitochondrial iron-sulfur cluster assembly pathway
SPG75	MAG	Myelin-associated glycoprotein	AR	Extremely rare	Intellectual disability	Cell adhesion and signaling
SPG76	CAPN1	Calpain-1 catalytic subunit	AR	Rare	Cerebellar ataxia, peripheral neuropathy, dysarthria	Calcium-activated, non-lysosomal, thiol protease
SPG77	FARS2	Phenylalanine tRNA synthetase 2	AR	Rare	-	Mitochondrial protein translation
SPG78	ATP13A2	Probable cation-transporting ATPase 13A2	AR	Extremely rare	Cerebellar signs, intellectual disability	Endosomal and lysosomal traffic
SPG79	UCHL1	Exosome component 3	AR	Extremely rare	Spastic ataxia	RNA exosome complex
–	HACE1	E3 ubiquitin-protein ligase	AR	Extremely rare	Psychomotor retardation, seizures	Proteasomal degradation
–	LYST	Lysosomal-trafficking regulation	AR	Extremely rare	Cerebellar ataxia, sensorimotor demyelinating neuropathy	Endosomal traffic
–	TPP1	Tripeptidyl-peptidase 1	AR	Extremely rare	Mild intellectual disability, seizures, bulbar palsy, dystonia	Serine proteases
–	MT-ATP6		Mitochondrial	Extremely rare	Cerebellar signs, axonal neuropathy	Mitochondrial Complex V
–	BICD2	Protein bicaudal D homolog 2	AD	Extremely rare	-	Microtubule-based motor protein (dynein-mediated), axon transport
–	EXOSC3	Exosome complex component RRP40	AR	Rare	Cerebellar signs, intellectual disability	Exosome component
–	FAM134B	Reticulophagy receptor FAM134B	AR	Rare	Skeletal abnormalities, hyperhidrosis	Rho GTPases signaling

Physiopathology and neuropathology

Neuropathological examination gave a crucial contribution in the understanding of the mechanisms underlying HSPs neurodegeneration process. The majority of the analysed cases showed a marked degeneration of the lateral corticospinal tracts, progressively decreasing from lower lumbar to upper cervical level. Frequently, additional involvement of the uncrossed pyramidal tracts and increased degeneration of the fasciculus gracilis from lumbar to upper cervical level, were observed (Bruyn, 1992) (Figure 2, a-b).

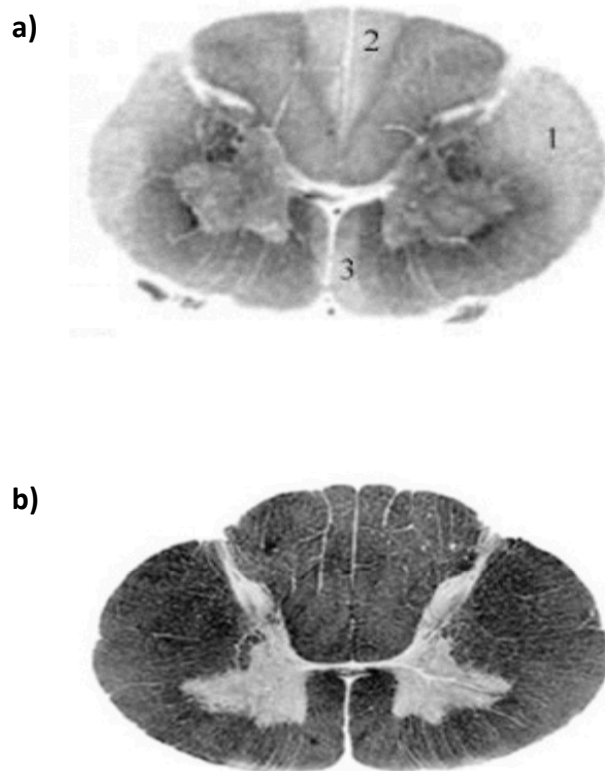


Figure 2. Spinal cord frontal section of an HSP-patient (a: 1. Lateral column; 2. Dorsal column; 3. Anterior column) and a control (b), clearly highlighting white matter atrophy and degeneration in HSP-patient section.

The degenerative process causing HSPs was hypothesized occurring through a “dying-back” phenomenon, involving the long ascending (sensory) and descending (corticospinal) tracts. The progressive degeneration, ascending from axons and reaching the cell body, led to cell death (Bruyn, 1992). The “dying back” process was furthermore confirmed by Deluca *et al* in the first quantitative study aimed at examining axons population, from lumbar to cervical levels, in HSPs patients. They observed a significant reduction in axons area and density throughout all the corticospinal tracts length while, in sensory tracts, axonal loss was more marked only in the upper regions of the spinal cord (Deluca *et al.*, 2004). This gradual retraction of UMN axons gradually impairs and dysregulates the synapse between UMNs and LMNs. Leg spasticity, weakness, hypertonia and hyperreflexia subsequently arise following the lack of communication between the two neuronal partners. This neurodegenerative process can affect either only UMNs, consequently damaging their connections with LMNs, or both UMNs and LMNs, as clearly observed in some forms of HSPs (Figure 3). The latter dual degeneration is reminiscent of ALS, with the notable difference of sacral neurons (Onuf’s neurons) preservation and normal bladder and rectal sphincter function up to the final stages of the disease. On the other hand, LMN degeneration alone gives rise to an HSP manifesting with marked muscle-wasting that can be either generalized or restricted to the lower limbs. An alternative HSPs classification could therefore take into account motor neurons affection, distinguishing into UMN-HSPs or UMN/LMN-HSPs (Parodi *et al.*, 2017).

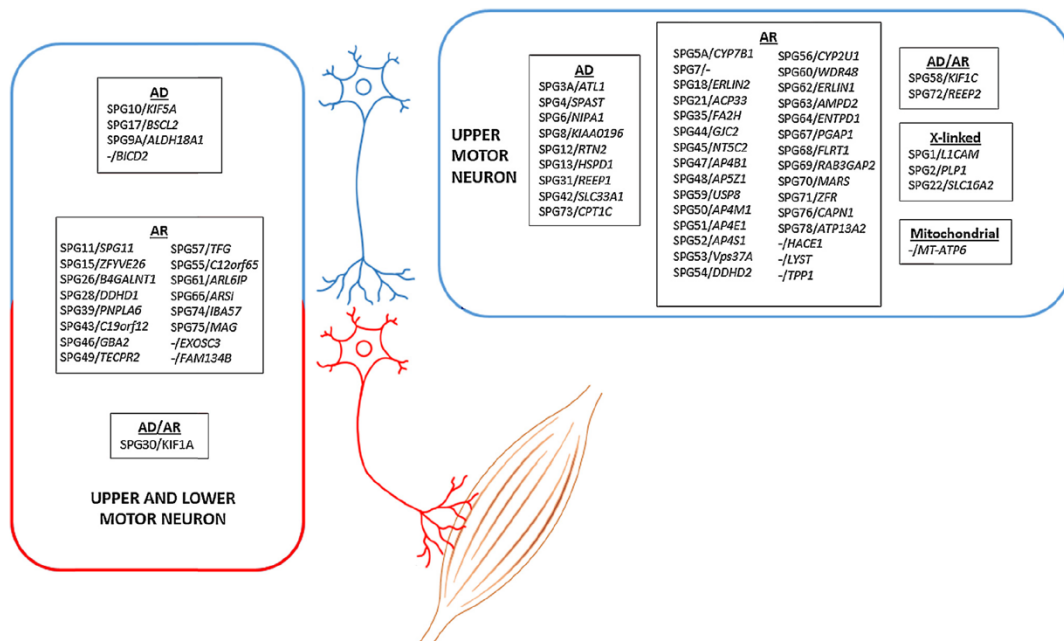


Figure 3. Spastic Paraplegia Genes (SPGs) grouped according to affected motor neurons and mode of inheritance (from Parodi *et al*, 2017).

Diagnosis and treatment

As already discussed, the clinical overlap of HSPs with other neurological disorders often makes the clinical diagnosis quite difficult. The association of gait spasticity with other neurological signs, a positive familial history and ancillary tests, such as brain and spinal cord magnetic resonance imaging, electromyography, nerve conduction studies and ophthalmological examination, are therefore crucial for an accurate patients' classification. Cerebrospinal fluid analysis may also be performed to differentiate HSPs from multiple sclerosis or to detect the presence of human-T cell leukaemia virus (HTLV-1), responsible for tropical spastic paraparesis. To enhance the diagnosis precision, specific plasma biomarkers can be measured to support the diagnosis of some HSP-related forms. These include increased levels of very long-chain fatty acids (VLCFA) in adrenoleukodystrophy caused by *ABCD1* gene mutations and cholesterol in cerebrotendinous xanthomatosis due to *CYP27A1* mutations (Parodi *et al.*, 2017).

It is important to underline that biomarkers identification is of crucial importance not only for its power in facilitating the diagnostic process, but especially for the progression of new treatments development. One example is given by the recent identification of 25-hydroxycholesterol (25-OHC) and 27-hydroxycholesterol (27-OHC) in SPG5 patients, that led to the testing of atorvastatin and chenodeoxycholic acid, resulting in the restoration of bile acids levels (Schöls *et al.*, 2017; Marelli *et al.*, 2018).

While waiting for the availability of innovative therapies, HSPs patients are treated with oral administration of antispastic drugs, as well as intramuscular Botox injections and intrathecal baclofen application (ITB), to reduce spasticity and prevent urinary urgency. Physiotherapy and rehabilitation are also recommended to improve strength and balance (Fink, 2013).

Spastic Paraplegia type 4

Clinical and genetic background

Spastic Paraplegia type 4 (SPG4) represents the most common AD-HSP, accounting for approximately 40% of familial and 10% of sporadic cases (Lo Giudice *et al.*, 2014). In early 90s, linkage studies allowed the identification of the 90-kb genomic region on chromosome 2 (2p22.3) harbouring SPG4-HSP causative gene, *SPAST*, comprising 17 exons and coding for spastin, a microtubule severing protein (Hazan *et al.*, 1994, 1999; Hentati *et al.*, 1994).

SPAST-HSP can be defined as a “pure” form, with gait impairment due to spasticity and weakness, and unsteadiness due to posterior cord impairment (decreased vibratory sensation) (Parodi *et al.*, 2017).

The cardinal clinical feature of *SPAST*-HSP is insidiously progressive bilateral-lower limb spasticity associated with brisk reflexes, ankle clonus, and bilateral extensor plantar responses. Sphincter disturbances are very frequent (77%), in particular urinary urgency and incontinence. Increased reflexes in the upper limbs may also occur (65%). A frequent additional feature is decreased, but not abolished, vibration sense at the ankles, occurring in 60% of affected patients. Moreover, around 50% of affected individuals have proximal weakness in the lower limbs (Parodi *et al.*, 2019).

Among the additional clinical features presented by *SPAST*-HSP patients, bladder dysfunction remains one of the most frequent problems and may be more frequent in individuals with *SPAST*-HSP (91.2% of affected patients) than in the overall HSP-affected population (Schneider *et al.*, 2019).

Cognitive deficits could appear late in the disease course and are not present in all affected members of a given family. When detected by neuropsychological testing, the impairment is often subtle, limited to executive dysfunction and without noticeable effect on daily living. The link between the subtle cognitive impairment and the disease is still undetermined (Tallaksen *et al.*, 2003; Erichsen *et al.*, 2009), and no definite correlation with the type of pathogenic variant in *SPAST* has been established (Parodi *et al.*, 2019).

Age at onset of symptoms ranges from infancy to the eighth decade and is indeed extremely variable, even among family members sharing the same pathogenic variant. It often shows a bimodal distribution, with the first peak before the first decade and a second one comprised between the third and fifth decades.

Disease severity generally worsens with the duration of the disease, although some individuals remain mildly affected all their lives. In general, after a long disease duration

(20 years), approximately 50% of patients need assistance for walking, and approximately 10% require a wheelchair. It is important to notice that, as in the case of age at onset (Loureiro *et al.*, 2013; Polymeris *et al.*, 2016; Chrestian *et al.*, 2017), even disease severity can be characterized by intrafamilial variability. Indeed, in *SPAST*-HSP carrier families, it is not uncommon to observe related carrier patients being asymptomatic after 70 years of age, along with severely affected patients, wheelchair-bound in their third decade (Fonknechten *et al.*, 2000).

Depression can be a frequently observed feature more present, for instance, among *SPAST*-HSP patients, when compared to SCA patients (du Montcel *et al.*, 2008).

Spastin, a microtubule severing protein

SPAST encodes spastin, a protein belonging to the AAA family (ATPases Associated with diverse cellular Activities) (Hazan *et al.*, 1999). Spastin hydrolyses ATP to sever microtubules and controls various aspects of microtubule dynamics, such as their length, number and motility (Errico *et al.*, 2002). Spastin is composed of four domains (Figure 4) necessary for its enzymatic activity, as well as for its interactions with intracellular partners. Through the N-terminal domain (starting from residue 1 to 87), spastin, together with two other SPG-encoded proteins *ATL1*/SPG3A and *REEP1*/SPG31, is involved in endoplasmic reticulum morphogenesis and lipid metabolism (Park *et al.*, 2010; Papadopoulos *et al.*, 2015). The microtubule interacting and trafficking domain (MIT), located between amino acids 116 and 194, allows interactions with two proteins belonging to the endosomal-sorting complex required for transport III (ESCRT-III) machinery, CHIMP1 and IST1, explaining the role of spastin in both cytokinesis and endosomal-tubule recycling (Reid *et al.*, 2005; Connell *et al.*, 2009; Allison *et al.*, 2013).

The two remaining domains, the microtubule-binding domain (MTBD), comprising amino acids 270 to 328, and the AAA ATPase cassette, from amino acid 342 to 616, are crucial for spastin-severing activity. Microtubules are severed by the energy derived from ATP hydrolysis following the assembly of six spastin subunits into a ring-shaped hexamer that binds to microtubules and introduction of tubulin C-terminus into the central pore (White *et al.*, 2007; Roll-Mecak and Vale, 2008) (Figure 4).

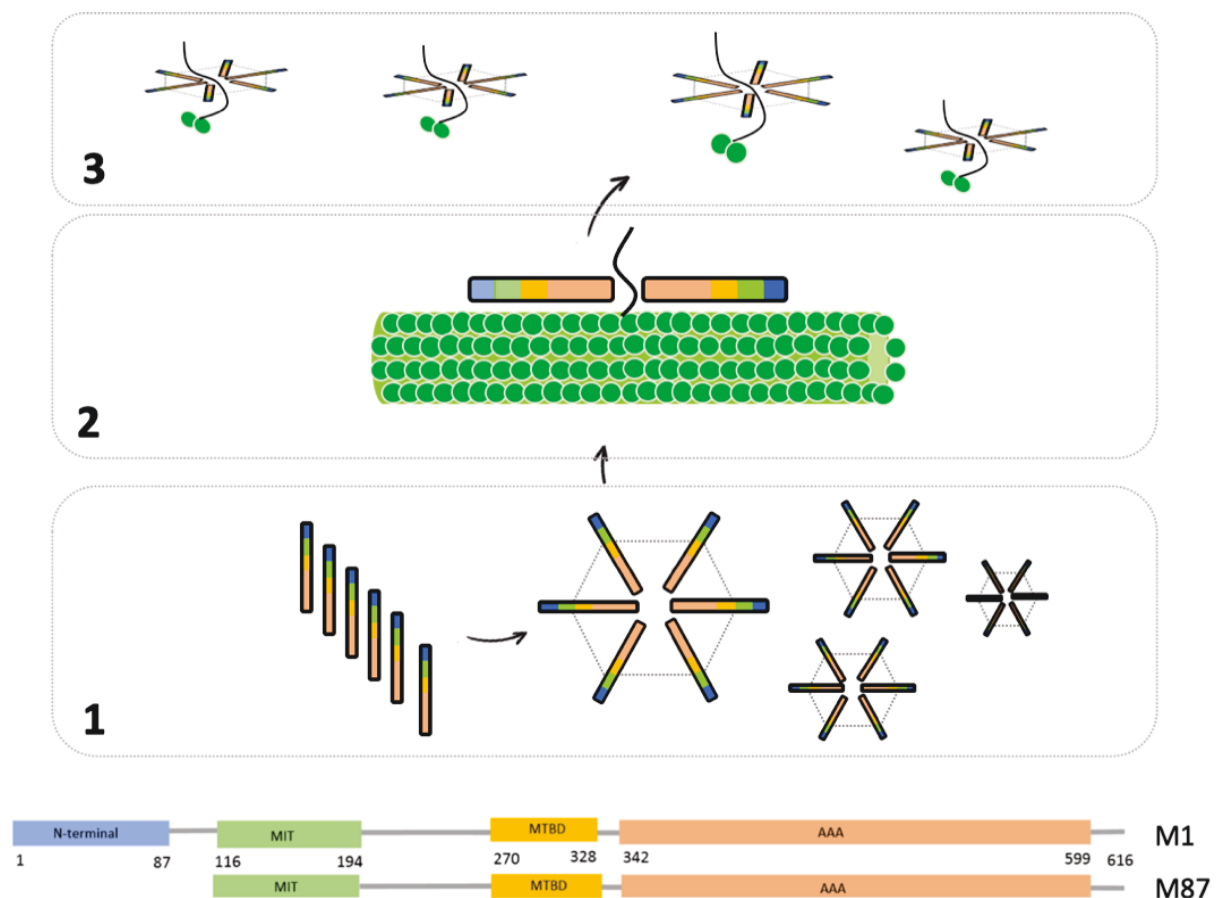


Figure 4. Spastin microtubules disassembling mechanism. First, 6 spastins assemble to form an hexamer-shaped ring (1). Using the MTBD domain, spastins' examers dock on microtubules (2). Following ATP hydrolysis, and after having introduced the C-terminal tail of a tubulin dimer into its central pore, spastin hexamers detach tubulins dimers, therefore disassembling microtubules (3).

Four spastin isoforms are produced by an alternative initiation codon and differential exon 4 splicing sites (Havlicek *et al.*, 2014). Spastin isoform M1 (68 kDa) and M87 (60 kDa) are produced by an alternative translation start site (Claudiani *et al.*, 2005;

Mancuso and Rugarli, 2008) and share all protein domains, except for the N-terminal domain, which is present only in the M1 isoform. Moreover, the human M87 isoform is expressed in both the spinal cord and the cerebral cortex, whereas spastin M1 is detectable only in the spinal cord (Solowska *et al.*, 2010).

Spastin loss of function, and consequent haploinsufficiency, has been proposed as the mechanism of disease causation, as most pathogenic mutations affects spastin functional domains, therefore causing the loss of large portions of the gene, or precluding the formation of a functional protein through mRNA-nonsense mediated decay due to large deletions or frameshift mutations (Bürger *et al.*, 2000; Depienne *et al.*, 2007). An alternative to the loss-of-function model was provided by the observation that *SPAST* pathogenic variants in the AAA cassette domain led to constitutive binding to microtubules, therefore suggesting a dominant-negative effect (Errico *et al.*, 2002). This abnormal spastin-microtubule interaction was observed leading to organelle transport impairments, possibly underlying the degeneration of the corticospinal axons (McDermott *et al.*, 2003). A further spastin pathogenic mechanism was recently proposed by Solowska *et al* (Solowska *et al.*, 2010, 2014), who generated a mouse model overexpressing human spastin and carrying a *SPAST* pathogenic missense mutation. After observing spastic-like tremors and gait impairments, as well as decreased microtubule stability, in adult homozygous mice, they concluded in favour of a gain (rather than loss) -of-function pathogenic mechanism (Qiang *et al.*, 2019). In conclusion, the debate concerning *SPAST*-HSP pathogenic mechanism remains open. It must be emphasized that the *SPAST* mutation spectrum, which mostly includes pathogenic mutations introducing premature termination codons, therefore leading to degradation

of the mRNA by nonsense-mediated decay, argues in favour of haploinsufficiency, rather than a dominant-negative effect (Parodi *et al.*, 2019).

The pursuit of genetic modifiers

Learning from other disorders...

As already discussed, the use of NGS approaches, including genes' panels, in everyday diagnostic process has allowed the broadening of the clinical/phenotypical spectrum within various disorders, therefore increasing the associated phenotypic variability. The wide phenotypical variability concerning disorders' symptoms or other features, such as age at onset and disorder's severity, observed among patients sharing pathogenic mutations couldn't be justified exclusively by the mere presence of the causative mutation itself. Indeed, the presence of additional genetic, or non-genetic factors (e.g. environmental, epigenetics), acting as modifiers was suggested as a possible explanation (Dipple and McCabe, 2000; Badano and Katsanis, 2002).

One of the most intriguing questions, common to different neurological disorders, focused on unravelling factors responsible for the age at onset variability, among affected patients sharing mutations in the same causative gene. Over the years, multiple strategies have been used to address this complex question, trying to dissect it in the smallest details.

Linkage analysis, extremely useful in the process of new pathogenic genes discovery, was one of the chosen strategies, especially given the strong genetic heritability often showed by age at onset. In the analysis of a cohort of patients affected by Huntington's Disease (HD), using age at onset as quantitative trait and adjusting for CAG-repeat

length, sib-pair (n = 629, 295 families) linkage analysis was helpful in identifying 2 loci on chromosomes 4, close to the HD locus, and 6 harbouring genes potentially involved in modifying HD age at onset (Li *et al.*, 2003). Similarly, Trinh *et al* performed linkage analysis after recruiting 41 Arab-Berber families (150 patients plus 103 unaffected family members), affected by Parkinson's Disease (PD) due to *LRKK2* p.(Gly2019Ser) mutation. This allowed to highlight a significant peak on chromosome 1, comprising different candidate genes. Association analysis within the linkage region pointed out, in both the discovery and an additional replication cohort, the presence of significant SNP on *DNM3* gene, therefore identified as PD age at onset modifier (Trinh *et al.*, 2016). In an attempt to identify genetic loci conferring risk for early onset Alzheimer's Disease (AD), Marchani *et al* performed linkage analysis in 265 affected patients carrying *PSEN2* causative mutations, highlighting two candidate regions on chromosome 1 and 17 (Marchani *et al.*, 2010). Fine-mapping of these regions in the same discovery cohort, as well as replication in 6 additional AD cohorts, finally allowed to converge on 2 genes. Furthermore, it allowed to identify variants harboured in the genes promoter regions, resulting acting as eQTLs, and associated to age at onset variations (Blue *et al.*, 2018). The progressive rise and rapid development of Genome Wide Association Studies (GWAS) definitely boosted the process of modifiers identification, especially when using large datasets. It is important to underline that, for a successful GWAS outcome, the selection of the phenotypic trait is an extremely important aspect to be considered. Indeed, most of GWAS approaches are designed to obtain the best and most reliable performances when quantitative traits, intended as measurable phenotypes having a normal distribution among the population, are analysed (Bush and Moore, 2012). Ideally, the perfect phenotypic trait should therefore be quantitative, strongly

associated to the disease trait and should, at the same time, share the same genetic architecture. Example is given by cerebrospinal fluid (CSF) amyloid-beta1-42 ($A\beta_{42}$) and phosphorylated tau ($p\tau_{181}$), two AD's biomarkers. GWAS analysis of such quantitative traits, defined as AD's endophenotypes, in AD patients cohorts had already allowed to identify genome-wide loci significantly associated with tau and $p\tau_{181}$, but also with AD's risk, tangle pathology and cognitive decline (Cruchaga *et al.*, 2013). Recently, focusing on the same traits, Deming *et al* analysed a cohort of 39'885 AD patients highlighting a locus associated with lower CSF $A\beta_{42}$ and earlier AD age at onset (Deming *et al.*, 2017).

Phenotypic traits such as age at onset, can therefore be considered as quantitative only when characterized by a normal distribution. As an example, residual age at onset of motor symptoms was calculated by Lee *et al* when analysing a cohort of HD patients carrying 40-53 CAG expansion on *HTT* gene. Since showing a normal distribution, it was used to perform quantitative GWAS analysis, highlighting the presence of different loci involved in hastening or delaying HD onset (Lee *et al.*, 2015). The results obtained were further replicated in a following GWAS study that, using similar settings in an European cohort of HD patients, allowed to identify an additional candidate locus on chromosome 3, responsible for delaying HD onset of 0.7 years (Lee *et al.*, 2017). In addition, significant association between age at onset and HD modifiers genes was furthermore observed when analysing a cohort of SCA patients, therefore highlighting a common genic background underlying age at onset variations in polyglutamine diseases (Bettencourt *et al.*, 2016).

A case-control study design was performed to identify disease onset and risk modifiers in Frontotemporal Lobar Degeneration (FTLD) affected patients carrying causative

mutations in the *GRN* gene. GWAS was first performed in a discovery cohort (382 *GRN*-FTLD, 1146 controls) and secondly in a replication cohort (67 *GRN*-FTLD, 1798 controls, 143 *GRN*-negative FTLD), pointing out the presence of variants significantly modifying disease risk but not of variants associated to age at onset variability (Pottier *et al.*, 2018). Signals suggestive of association ($p < 10^{-4}$) with age at onset variations were obtained by Poleggi *et al* when analysing a cohort of patients affected by Creutzfeldt-Jacob disease (CJD). Quantitative GWAS analysis was performed on 296 CJD-affected patients, detecting a SNP predicted having a role as eQTL, that was furthermore validated in CJD brain samples (Poleggi *et al.*, 2018).

Taking advantage of age at onset heritability, Hill-Burns *et al* implemented GWAS strategies to be applied to familial cohorts. Quantitative GWAS analysis of 431 familial-PD patients allowed to highlight the presence of two loci reaching significant p-values ($p < 10^{-8}$), furthermore replicated into 737 unrelated familial-PD patients (Hill-Burns *et al.*, 2016).

Besides neurodegenerative diseases, the process of genetic modifiers identification greatly improved the genetic and clinical characterization of many other different disorders including, amongst others, Marfan syndrome (Aubart *et al.*, 2018), Myelodysplastic syndromes (Danjou *et al.*, 2016), Heritable pulmonary arterial hypertension (Puigdevall *et al.*, 2019) and Duchenne muscular dystrophy (Vo and McNally, 2015). A key example is given by Cystic Fibrosis (CF). CF is a frequent (1 : 3000) and highly penetrant monogenic disorder caused by pathogenic mutations affecting *CFTR* gene (Ratjen *et al.*, 2015). CF patients present with impaired functioning of multiple organs, such as airways and lungs, *in primis*, but also the intestine, the pancreas, the hepatobiliary system, sweat glands and the reproductive system. The

overall phenotype presentation, in terms of tissue-specific susceptibility, is strictly related to *CFTR* mutations' nature, divided into five classes following their impact on the protein functioning (O'Neal and Knowles, 2018). Over the years, particularly through WES and GWAS association analyses, it was possible to show the presence of additional factors modifying the magnitude of effect linked to mutated *CFTR*, increasing the disorder complexity (Emond *et al.*, 2012, 2015; Corvol *et al.*, 2015) (Figure 5).

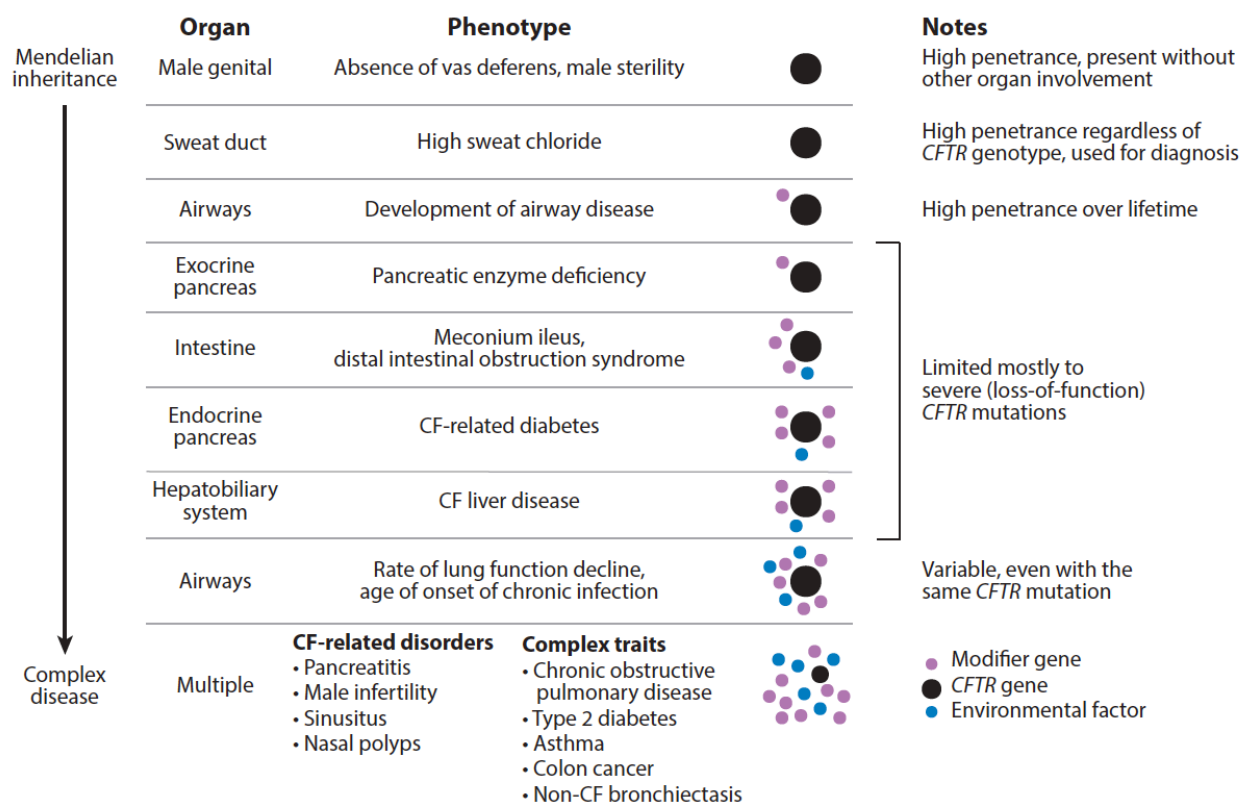


Figure 5. Impact of genetic and environmental modifiers on CF manifestation. The addition of modifying factors to the effects due to the *CFTR* mutation lead to an overall increased complexity of the CF phenotype (from O'Neal and Knowles, 2018).

The identification of various combination of genetic and environmental modifying factors responsible for the most complex CF presentations, allowed not only a more precise CF genetic and phenotypic classification, but also to adapt the treatment taking

into consideration the presence of specific modifiers (Blackman *et al.*, 2009; Strug *et al.*, 2016).

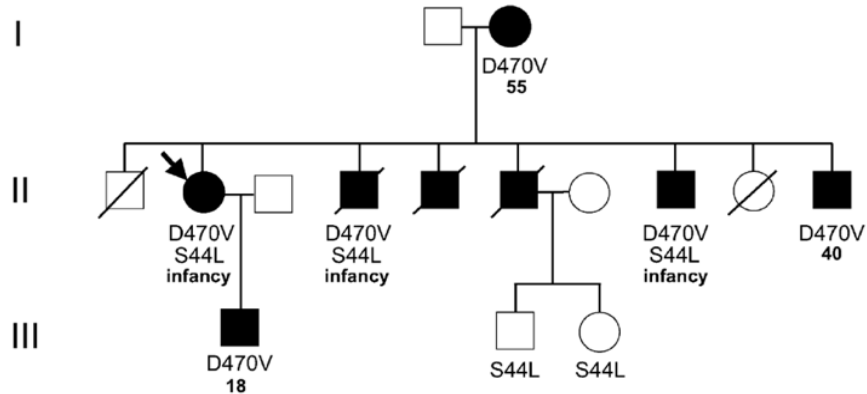
...And what about *SPAST*-HSP modifiers?

As previously mentioned, patients carrying *SPAST*-HSP causative mutations show a striking variability, when considering phenotypic traits such as age at onset and disorder severity. This phenomenon is particularly evident when focusing on carrier families. Indeed, quite often, related patients sharing the same causative mutation are characterized by great differences especially in terms of age at onset manifesting, for example, as a 69-years gap separating the disorder onset in two related patients (Parodi *et al.*, 2018). Thus, the presence of modifier factors is an extremely educated guess in order to explain the genetic bases of the observed phenotypic diversity.

To date, only 2 variants and specific deletions affecting directly *SPAST* gene sequence have been reported acting as age at onset/severity modifiers. When analysing three *SPAST*-HSP affected families, Svenson *et al* noticed that a previously detected missense variant altering *SPAST* exon 1, co-segregated independently (*in-trans*) with previously validated *SPAST*-HSP causative mutations. Interestingly, patients carrying both, the pathogenic mutation and the exon 1 variant, p.(Ser44Leu), were characterized by a disorder onset starting at infancy, as well as a greater disorder severity, when compared to relatives carrying only the *SPAST* pathogenic mutation (Figure 6, a). In addition, even a second adjacent polymorphism, p.(Pro45Glu), was observed acting as age at onset modifier, leading to a great age at onset lowering (Figure 6, b).

a)

Family 2512



b)

Family 1380

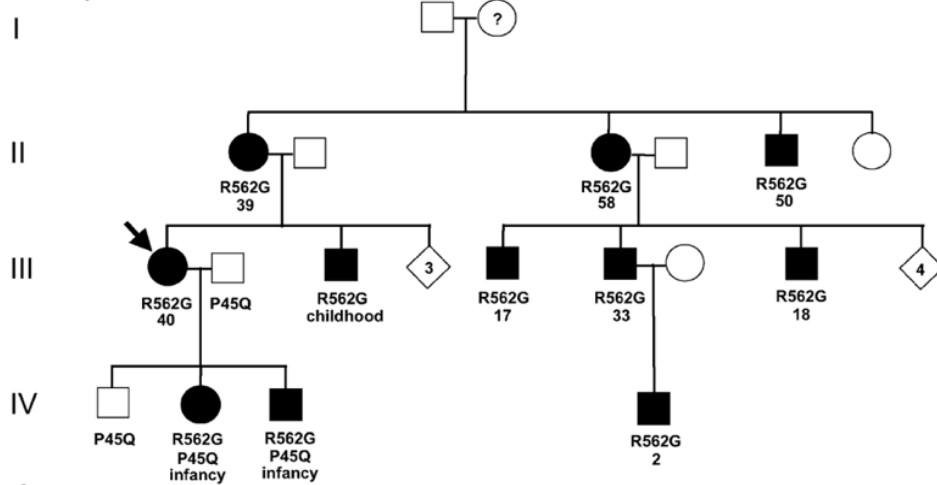


Figure 6. Familial segregation of *SPAST* intragenic modifiers (adapted from Svenson *et al*, 2004). a) The variant p.(Ser44Leu) co-segregated independently with a major pathogenic mutation, p.(D470V), leading to a decreased age at onset. b) Familial segregation of *SPAST* intragenic modifier, p.(P45Q). Patients carrying both, the polymorphism and the pathogenic mutation p.(R562G), were characterized by a lower age at onset.

Since potentially affecting a phosphorylated region, the authors hypothesized that these two polymorphisms may lead to impairments affecting spastins' interactions with microtubules (Svenson *et al.*, 2004). Since the association between the p.(Ser44Leu)

variant and a decreased age at onset has been subsequently observed in multiple cases (McDermott *et al.*, 2006; Parodi *et al.*, 2018), it is considered the most well-established *SPAST*-HSP age at onset modifier.

Recently, through a *SPAST*-HSP cohort analysis, a trend for an earlier age at onset associated to large *SPAST* deletions was observed (Chelban *et al.*, 2017). When digging the possibility that large deletions could have an impact on the expressed phenotype, Newton *et al* showed evidence that deletions act as age at onset modifiers when extending in the genomic region adjacent to *SPAST* gene, harbouring *DPY30* gene. The impact at the cellular level, observed after silencing *DPY30*, resulted highly similar to the consequences arising in *SPAST*-HSP cellular models (e.g. increased endosomal tabulation, defective endosomes-Golgi trafficking, abnormal lysosomal structures). This led to the demonstration that, through an epistatic mechanism, haploinsufficiency of *DPY30* could add further deleterious effects manifesting as an age at onset lowering in patients carrying large *SPAST* deletions (Newton *et al.*, 2018).

In addition to *SPAST* intragenic, or adjacent modifiers, *HSPD1*/SPG13 variant p.(Gly563Ala) was observed co-segregating with a major *SPAST* mutation and possibly associating to a lower disease onset (Hewamadduma *et al.*, 2008). However, even though this variant was detected segregating in other *SPAST*-HSP families (Svenstrup *et al.*, 2009), the previously observed correlation was not replicated and therefore, its role as age at onset modifiers is still under discussion.

Objectives

My PhD project lays its groundwork on the observation that *SPAST*-HSP patients are characterized by a striking inter and intra-familial phenotypic heterogeneity concerning, amongst other, symptoms' age at onset. The main goal of the project was therefore to identify genetic factors acting as modifiers and accounting for the observed variability.

To achieve the stated objectives, two main steps were necessary:

1- Assembling and analysis of *SPAST*-HSP patient cohort

Being a National Reference Centre for Rare hereditary neurological Disorders (CRMR Neurog n tique), the Piti -Salp tri re University Hospital and the diagnostic laboratory of its Genetic Department, were a fantastic source of both, clinical and genetic data, gathered since 1993, furthermore expanded taking advantage of the SPATAX network established in 2000 (<https://spatax.wordpress.com>). A cohort of 842 *SPAST*-mutated patients was therefore assembled and analysed, setting the bases for the subsequent phases of the study.

2- Patients Sequencing

In order to have the maximum chances at identifying genetic modifiers, different approaches were carried out simultaneously. It is important to underline that, even though multiple strategies were adopted, and performed at different time periods throughout the years, a common extreme-based study design was maintained during all the steps. That allowed to dissect different aspects of the subject by addressing multiple, but same-ended, questions. To build the most complete picture, data resulting from the different approaches used (genome-wide genotyping, RNA sequencing and Whole Exome Sequencing) were first analysed separately and then, whenever possible, combined.

3- Functional validation

Functional validation of potential candidate variants/genes was then carried out using diverse tools such as patients Primary Blood Lymphoblasts (PBLs) and *Drosophila Dspastin* RNAi lines and K467R mutants.

Patients and methods – Part 1: Modifiers identification

Patients

SPAST-HSP patient cohort

The cohort on which the entire study consists of 842 patients, all carriers of *SPAST* pathogenic mutations. Clinical information based on neurological examinations and genetic counselling were available for 636 patients.

Most of the patients had a familial history of the disorder (75.6%, 481/636), whereas 36 (5.7%) were sporadic cases. Reliable information concerning transmission of the disorder was unavailable for the remaining individuals (18.7%, 119/636).

Information gathered from neurological evaluations mainly revolved around clinical signs typically presented by HSPs-affected patients. Lower limb spasticity, pyramidal signs (increased reflexes and Babinski sign or extensor plantar reflex), upper limb increased reflexes, Hoffmann sign (equivalent to the Babinski sign in the lower limb), weakness and wasting, urinary disturbances, pes cavus or other foot deformation, decreased vibration sense at ankles, cognitive difficulties (either congenital intellectual deficiency and/or cognitive decline over time), constituted the clinical features reported.

Disorder severity was assessed using the SPATAX disability scale (0: no functional handicap; 1: no functional handicap but signs at examination; 2: mild, able to run, walking unlimited; 3: moderate, unable to run, limited walking without aid; 4: severe, walking with one cane; 5: walking with two canes; 6: unable to walk, requiring a wheelchair; 7: confined to bed).

The disability progression index (ratio of disability stage and disorder duration) at the last examination was used to evaluate progression of the disorder: the difference in severity between the last and first examination and the corresponding elapsed time were considered to assess progression.

Disorder penetrance was calculated as the number of affected patients divided by the total number of carriers. Age at onset could be determined for 547 patients.

IRB approved consent forms (under the promotion of INSERM with RBM 01-29, RBM 03-48) were signed by each participant included in the present study.

Genotyped cohort

Patients genotyping was carried out in order to perform linkage and GWAS analysis. Thirty-seven large *SPAST*-HSP carrier families characterized by wide intrafamilial phenotypical variability were therefore chosen to be submitted to linkage analysis, and both carrier patients and non-carrier relatives were sent to genotyping.

Concerning the subset of patients selected for GWAS, since aiming at identifying genetic factors influencing *SPAST*-HSP through an extreme-based study setting, only patients having a disorder onset equal or lower than 15 years of age (early onset patients) and higher or equal than 45 years of age (late onset patients) were selected from the *SPAST*-HSP cohort and submitted to genotyping (Figure 7, a).

Overall, the number of affected women and men sent to genotyping was comparable, even though men were slightly more represented (52%, n = 177 men versus 48%, n = 161 women) (Figure 7, b).

Altogether (linkage + GWAS), the genotyped cohort is composed by 448 individuals: 338 *SPAST* carriers, 29 of which still asymptomatic, and 110 non-carrier relatives.

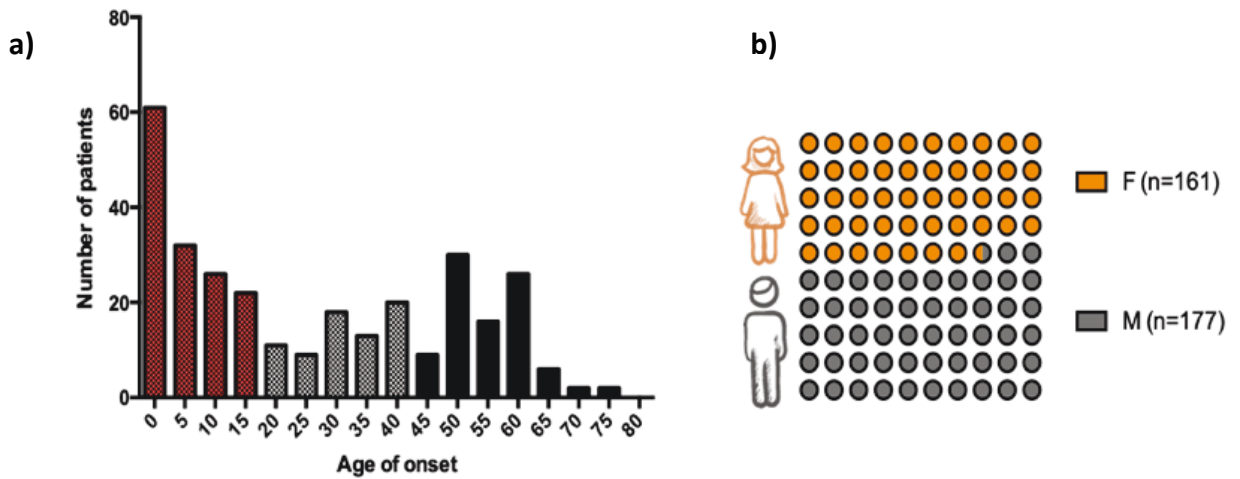


Figure 7. a) Age at onset distribution; early onset patients (AO \leq 15 yo) are represented by red bars, while black bars define late onset patients (AO \geq 45 yo). b) Sex proportion among genotyped patients.

Exomed cohort

Fifty-seven *SPAST*-HSP patients were submitted to Whole Exome Sequencing (WES). This patients' sub-group was formed by 48 *SPAST*-HSP affected patients, as well as 9 asymptomatic carriers. The majority of the exomed cohort ($n = 34$) was formed by parent-sibling couples selected among those characterized by an extreme discordance in terms of age at onset (Figure 8, a). The selected cohort was therefore mainly composed by early onset patients having a disorder onset starting before 15 years of age (70%, $n = 24$), with 13 patients having an onset between birth and 5 years (Figure 8, b). Women were slightly more present than men (58%, $n = 33$ women versus 42%, $n = 27$ men) (Figure 8, c).

Since WES was performed together with German collaborators (R. Schüle and L. Schols, University of Tübingen), as part of NeurOmics frameworks (<https://rd-neuromics.eu>), 16 German patients, chosen following the same criteria (discordant parent-sibling couples), were included in the exomed cohort.

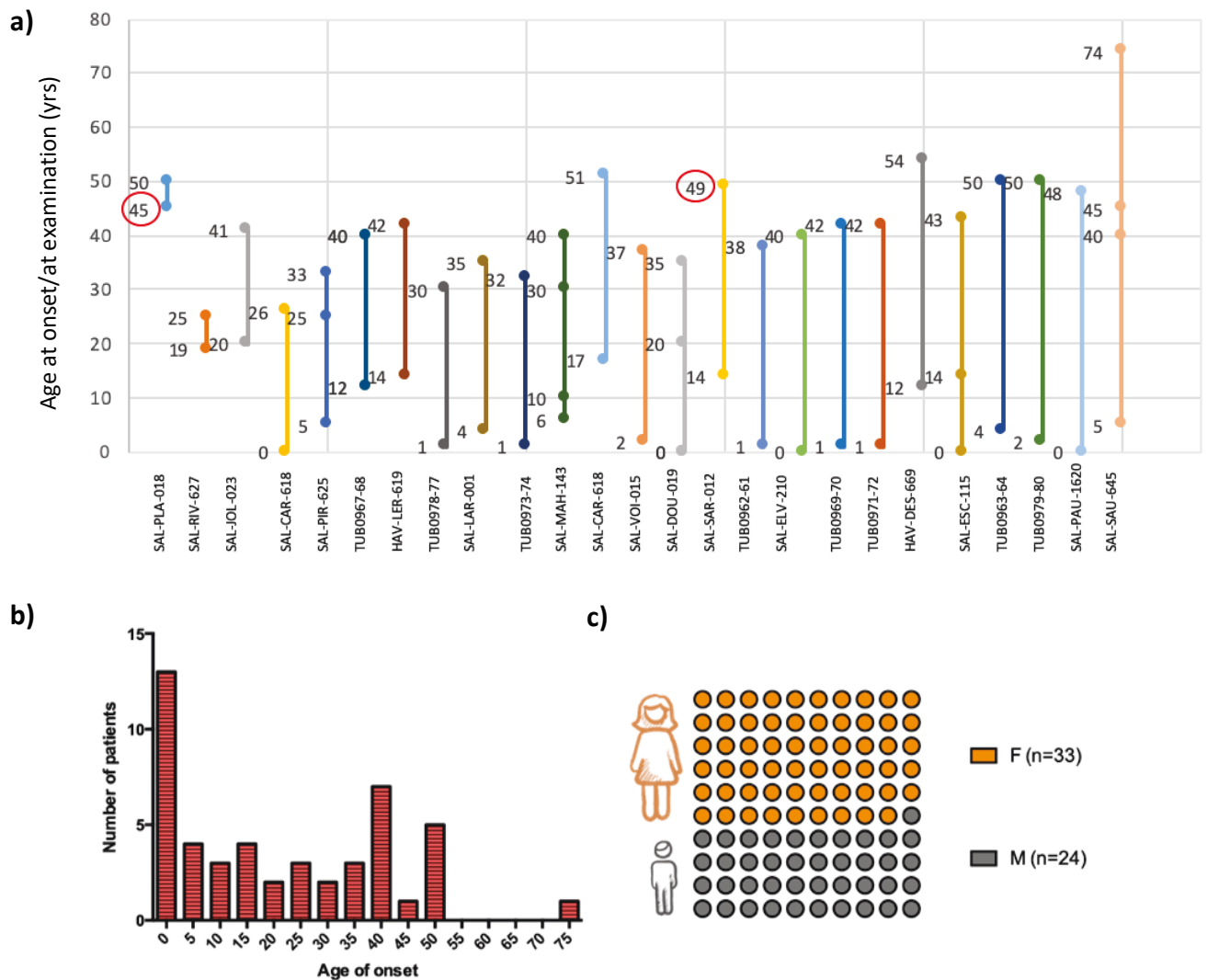


Figure 8. a) Age at onset of sequenced patients, grouped by family; age at examination (circled in red) is reported for patients carriers but still asymptomatic. b) Age at onset distribution and sex proportion (c) among exomed patients.

RNA-sequencing cohort

Thirty-nine *SPAST* patients characterized by discordant age at onset, including 1 asymptomatic carrier, were selected among the *SPAST*-HSP families and submitted to RNA-sequencing (Figure 9, a). Age at onset distribution of the selected cohort showed a bimodal distribution (Figure 9, b), and women represented the majority of the cohort (64%, $n = 25$ women versus 36%, $n = 14$ men) (Figure 9, c). To normalize genes

expression, RNAs belonging to matching controls (in terms of sex and age at sampling) were simultaneously sequenced. Finally, RNA was extracted from brain cortex of a deceased *SPAST*-HSP patient and sequenced together with a compatible control.

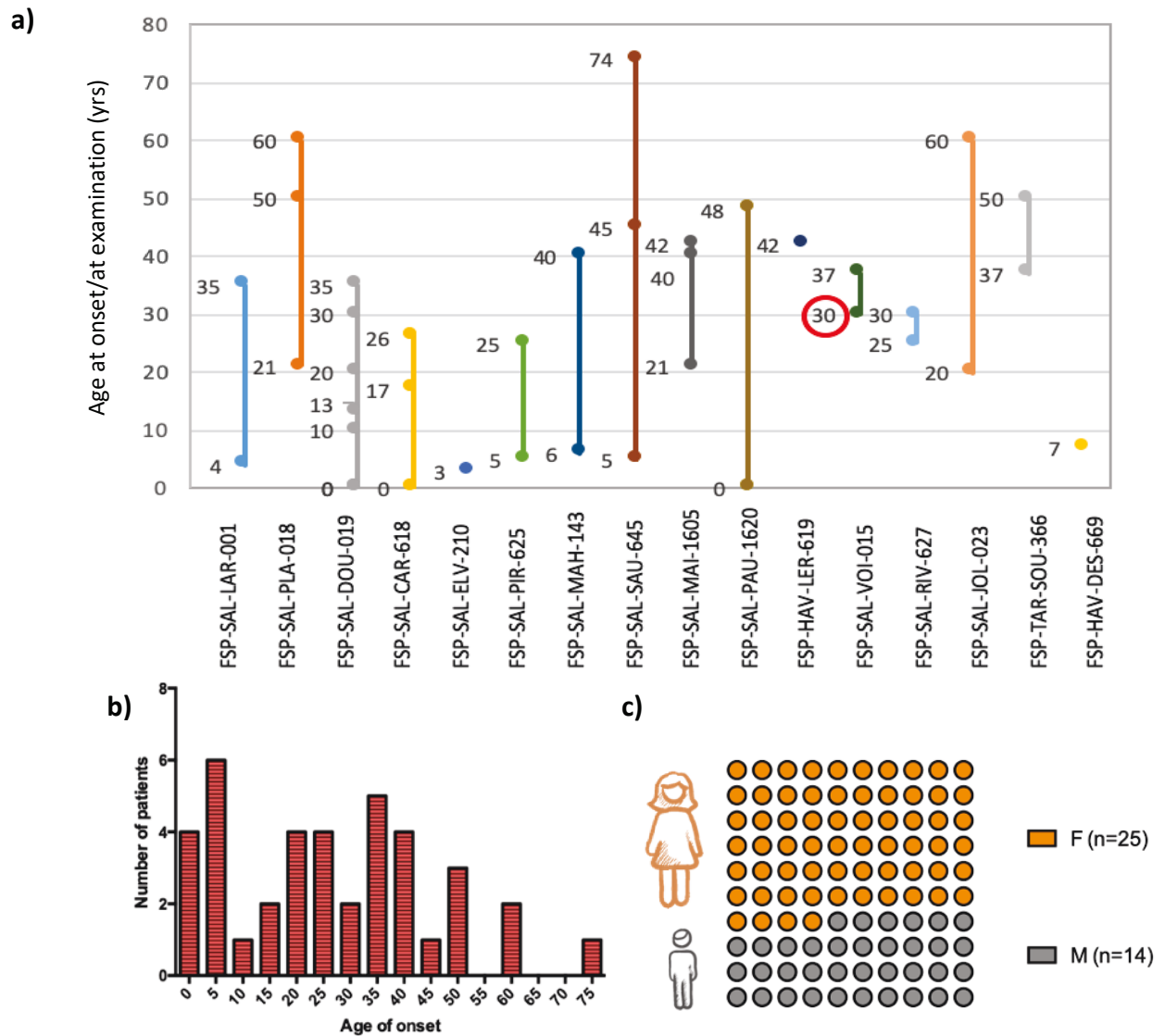


Figure 9. a) Age at onset of sequenced patients, grouped by family; age at examination (circled in red) is reported for one patient, carrier but still asymptomatic. Age at onset (b) and sex (c) distribution of the RNA-sequencing cohort.

Sequencing, quality control and analysis settings

Genome-wide genotyping

DNA samples belonging to the 448 selected patients were genotyped using the Infinium® Omni2.5Exome-8 v1.3 and v1.4 BeadChip arrays, provided by Illumina (San Diego, CA, USA). Quality control on the resulting genotype data was performed using PLINK version 1.9b6 (Chang *et al.*, 2015), after merging the markers in common between the two arrays' versions. Samples presenting discordant sex information or elevated missing data rates were excluded from the analysis, as well as uninformative SNPs, variants characterized by low call rates or failing Hardy-Weinberg equilibrium. To verify that the selected patients belonged to the same ethnic group, ancestry was predicted performing a PCA analysis using Peddy (Pedersen and Quinlan, 2017). A total of 1'701'047 SNPs and 434 samples passed the quality controls.

As stated before, data resulting from genome-wide genotyping were aimed at performing both linkage and GWAS analysis, therefore implying a different number of patients and of SNPs considered in the two approaches.

Linkage analysis

To perform genome-wide linkage analysis, only patients and unaffected relatives belonging to the 37 families were taken into consideration (n = 259). In order to take into account SNPs in linkage disequilibrium, data were submitted to pruning, therefore allowing to reach a final number of 41'444 SNPs ready to be analysed.

When used in the process of new genes' discovery, linkage analysis allows to follow the transmission of a "disease trait" common to all affected patients and absent in all unaffected relatives. To identify a locus potentially harbouring a gene acting as age at onset modifier, linkage analysis was set up defining as "affected" all the early onset

patients (age at onset ≤ 15 years, $n = 69$), while as “unaffected” all the late onset patients (age at onset ≥ 45 years, $n = 40$). Patients having an age at onset comprised between 15 and 45 years, as well as unaffected relatives were coded as “unknown”, in order to exclude them from the analysis but, at the same time, to take them into consideration to build familial structures.

Linkage analysis was then performed using MERLIN v1.1.2 (Abecasis *et al.*, 2002), using both a non-parametric and a parametric model. In the parametric setting, a dominant model was preferred and multiple disorder frequencies were tested ($f = 0.0046$, the MAF of p.(Ser44Leu) variant, and $f = 0.05$).

GWAS analysis

GWAS analysis was performed adopting an extreme-based setting. Starting from the overall cohort, only phenotypic extremes were submitted to GWAS, and early onset patients (age at onset ≤ 15 years, $n = 71$) were therefore compared to late onset patients (age at onset ≥ 45 years, $n = 63$). Given its binomial distribution in the overall SPAST-HSP population, age at onset could not be considered as a quantitative trait, and was therefore converted into a binary trait (1 = “late onset”, 2 = “early onset”). Moreover, to minimize the effects potentially caused by the mutation nature (Parodi *et al.*, 2018), only patients carrying a truncating (splice site, frameshift, deletion, nonsense) pathogenic mutation were included in the analysis.

Since the genotyped cohort was composed by both familial and sporadic cases, R package Popkin (Ochoa and Storey, 2019) was used to calculate a kinship matrix, allowing to take into consideration patients’ relatedness.

A single-variant association analysis (score tests) was then performed using a General Logistic Mixed Model (GLMM) provided by GMMAT v1.0.3 R-package (Chen *et al.*, 2019),

after adjusting for sex and relatedness. Manhattan plots and Q-Q plots were realized using the R-package qqman (Turner, 2014). Variants resulting from the association test were annotated using ANNOVAR (Wang *et al.*, 2010). RegulomeDB (<http://www.regulomedb.org>) as well as GTEx portal (<https://gtexportal.org>) were used to retrieve information about variants' role as transcription regulators or eQTLs.

Whole Exome Sequencing (WES)

Patients DNA was sequenced using Nexetera® Rapid Capture Expanded Exome (Illumina, San Diego, CA, USA), allowing to cover sequences extending up to 62Mb and comprising both coding exons, UTRs and miRNAs. FastQC software (Wingett and Andrews, 2018) was used to check bams files' quality, determining an 80%-average of covered bases. Given the structure of the exomed population, an extreme-based analysis was performed. GEMINI framework (Paila *et al.*, 2013) was used to filter WES results in order to select variants carried exclusively by early onset patients, and expected to be absent in late onset patients. Since WES patients cohort was mostly composed by discordant parents-offspring pairs, no specific age at onset thresholds were fixed, assigning each patient to the "early" or "late onset" category after comparing them to their discordant partner.

Since deciding not to make any *a priori* assumption concerning modifier variants frequency, no filters concerning variants' MAF were used during WES analysis. Furthermore, only variants shared by at least two patients and then detected in genes expressed in the central nervous system were selected using an in-house R script, according to Allen Brain Atlas portal (<http://portal.brain-map.org/>). Finally, candidate variants were annotated using ANNOVAR in order to select variants potentially having a

pathogenic effect (SIFT < 0.5, Polyphen2_HVAR>0.9, CADD_pred>12) that could explain the phenotype.

Two strategies were used to select the most interesting candidate variants. The first one took advantage of genes prioritization, performed by ENDEAVOUR (Tranchevent *et al.*, 2008, 2016), and based on similarities (e.g. concerning coding sequence, gene expression, functional annotation, literature, regulatory information) between the candidate gene and a training set of genes already known to be involved in HSPs onset or predicted interacting with spastin by BioGRID (<https://thebiogrid.org/>) and STRING (<https://string-db.org/>) databases. The second approach consisted in selecting variants among those most frequently carried among the selected patients. Sanger sequencing was then used to perform variants segregation in carrier families.

RNA sequencing

Total RNA was extracted from patients' Primary Blood Lymphoblasts (PBLs) using Maxwell® RSC simplyRNA Cells kit on Maxwell® RSC extractor (Promega). After tissues homogenization, RNeasy Lipid Tissue kit (Qiagen) was used to extract total RNA from the two samples deriving from brain cortex. RNA quality was checked at Agilent 4200 TapeStation System. RNAs were then sequenced using Illumina NextSeq® 500 High Output Kit v.2 (150 cycles, 400 million reads).

Quality control, as well as gene expression analysis among patients, were performed using GenoSplice Technology (www.genosplice.com). Sequencing, data quality, reads repartition (e.g. for potential ribosomal contamination), and insert size estimation were performed using FastQC (Wingett and Andrews, 2018), Picard tools (<http://broadinstitute.github.io/picard/>), Samtools (<http://samtools.sourceforge.net/>)

and RSeQC (Wang *et al.*, 2012). Reads were mapped using STARv2.4.0 (Dobin *et al.*, 2013) on the hg19 Human genome assembly. Gene expression regulation study was performed as already described (Noli *et al.*, 2015). For each gene annotated in the Human FAST DB v2016, reads aligning on constitutive regions (and therefore not prone to alternative splicing) were counted. Based on the read counts, normalization and differential gene expression were performed using DESeq2 R package (Love *et al.*, 2014). Genes were considered as expressed if their RPKM (Reads Per Kilobase Million) value resulted greater than 97% of the background RPKM value, based on intergenic regions. When comparing patients versus controls, to account for patients' heterogeneity, samples were paired according to sampling date, age at sampling and sex.

Results were considered as statistically significant when P-values ≤ 0.05 and fold-changes ≥ 1.5 .

Different approaches were used to analyse RNAseq results. To highlight genes dysregulated in a specific group of patients (e.g. early and late onset, missense or truncating mutation carriers), a paired analysis design was used. In a paired analysis workflow, comparisons are first made within the sample pair, and secondly among the different pairs included in the analysis. Multiple comparison therefore allow to give the proper weight to the resulting outcomes, enhancing truthful observations. Paired analysis was performed to detect genes differentially expressed in early versus late patients, performing a first analysis on 14 couples (28 patients, 14 early and 14 late onset), considering the median age at onset of the sequenced cohort, corresponding to 26 years of age, to define the early or late status. Moreover, in order to try to assess which genes were differentially expressed in the earlier stages of the disease, RNA expression of 9 pairs was analysed, including in the early onset category only patients

having a disorder onset starting before 10 years. To detect any possible effect due to the nature of the *SPAST* causative mutation, expression data of truncating versus missense mutation were analysed. As done for the WES analysis, the lists of differentially expressed genes resulting from the RNA-seq analysis was then examined especially focusing on genes coding for spastin interactors, HSPs candidate genes, as well as known HSPs causative genes.

In addition to paired analysis, normalized raw data were also exploited to assess the expression of specific target genes among controls, early onset patients (age at onset ≤ 20) and late onset patients (age at onset ≥ 40). Kruskal-Wallis test was used to make comparisons among the different groups and Dunn's test was used to correct for multiple comparisons.

Finally, RNA expression data deriving from brain cortex of a patient carrying a *SPAST* pathogenic frameshift mutation (p.Asn405LysfsX42), and having a disorder onset at 25 years, were analysed comparing the patient and his matching control, in a one-by-one comparison (reported in Brain publication, see p. 46).

Material and methods – Part 2: Modifiers validation

Drosophila mutant lines

Two *SPAST*-HSP *Drosophila* models were used in the validation phase. *Drosophila* lines carrying the *Dspastin* missense p.(Lys388Arg) mutation, as well as *Dspastin* RNAi lines (Trotta *et al.*, 2004), were available thanks to the collaboration with Dr. Genny Orso (University of Padua, Italy).

RT-qPCR

Total mRNA extracted from flies brain/total body was used for oligo-dT primed reverse transcription. qPCRs on resulting cDNAs were then performed, after housekeeping gene normalization (CG7263).

Western Immunoblotting

Proteins were extracted from patients' Primary Blood Lymphoblasts (PBLs) using a lysis buffer (1M Tris-HCl pH 7.5, 5M NaCl, 1% Triton X-100, Halt™ Protease Inhibitor Cocktail 100X (Thermo Fisher Scientific)). 30µg of extracted protein samples were run on NuPAGE™ 4-12% Bis-Tris protein gels (Thermo Fisher Scientific). Anti-SARS2 (PA5-31473, Thermo Fisher Scientific) and anti-Actin (ab3280, abcam) antibodies were used for protein detection. Protein signals were revealed using SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and ChemiDoc™ Touch Imaging System (Bio-Rad).

Results – part 1: better alone than in bad company?

This first Results' section reports the results obtained through the analysis of each approach used. Indeed, results were first analysed separately, especially to avoid overlooking any possible interesting result.

SPAST-HSP cohort analysis

This first part of the work, recently published in *Brain* under the title of “Spastic paraplegia due to *SPAST* mutations is modified by the underlying mutation and sex”, was aimed at the genetic and clinical characterization of the *SPAST*-HSP cohort available at the Pitié-Salpêtrière University Hospital. A total of 842 *SPAST*-HSP patients were included, therefore allowing to describe what resulted being the world's biggest *SPAST*-HSP cohort. The statistical power given by such amount of data collected enabled to describe more clearly some aspects of the disorder that, since considering smaller cohorts, hadn't been observed so distinctly before.

After merging information about patients' age at onset and pathogenic mutations nature (n = 547), it was clear that the mutation's nature had an impact on age at onset. Missense carrier patients showed a 10 years-earlier onset when compared to truncating mutations carriers allowing, for the first time, to establish a clear phenotype-genotype correlation. This finding also gave an explanation to the bimodal trend typical of *SPAST*-HSP age at onset distribution.

When focusing on disorder penetrance, it was also possible to highlight that women had a lower penetrance than men for the entire disorder course, leading us to conclude that both, the mutation nature and sex, modify *SPAST*-HSP onset.

Spastic paraplegia due to SPAST mutations is modified by the underlying mutation and sex

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*Appendix 1.

Hereditary spastic paraplegias (HSPs) are rare neurological disorders caused by progressive distal degeneration of the corticospinal tracts. Among the 79 loci and 65 spastic paraplegia genes (SPGs) involved in HSPs, mutations in *SPAST*, which encodes spastin, responsible for SPG4, are the most frequent cause of both familial and sporadic HSP. SPG4 is characterized by a clinically pure phenotype associated with restricted involvement of the corticospinal tracts and posterior columns of the spinal cord. It is rarely associated with additional neurological signs. However, both age of onset and severity of the disorder are extremely variable. Such variability is both intra- and inter-familial and may suggest incomplete penetrance, with some patients carrying mutations remaining asymptomatic for their entire life. We analysed a cohort of 842 patients with SPG4-HSP to assess genotype–phenotype correlations. Most patients were French (89%) and had a family history of SPG4-HSP (75%). Age at onset was characterized by a bimodal distribution, with high inter-familial and intra-familial variability, especially concerning first-degree relatives. Penetrance of the disorder was 0.9, complete after 70 years of age. Penetrance was lower in females (0.88 versus 0.94 in males, $P = 0.01$), despite a more diffuse phenotype with more frequent upper limb involvement. Seventy-seven per cent of pathogenic mutations (missense, frameshift, splice site, nonsense, and deletions) were located in the AAA cassette of spastin, impairing its microtubule-severing activity. A comparison of the missense and truncating mutations revealed a significantly lower age at onset for patients carrying missense mutations than those carrying truncating mutations, explaining the bimodal distribution of the age at onset. The age at onset for patients carrying missense mutations was often before 10 years, sometimes associated with intellectual deficiency. Neuropathological examination of a single case showed degeneration of the spinocerebellar and spinocortical tracts, as well as the posterior columns. However, there were numerous small-diameter processes among unusually large myelinated fibres in the corticospinal tract, suggesting marked regeneration. In conclusion, this large cohort of 842 individuals allowed us to identify a significantly younger age at onset in missense mutation carriers and lower penetrance in females, despite a more severe disorder. Neuropathology in one case showed numerous small fibres suggesting regeneration.

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Abbreviations: HSP = hereditary spastic paraplegia; SPG = spastic paraplegia gene

Introduction

Hereditary spastic paraplegias (HSPs) are inherited disorders caused by the progressive degeneration of the corticospinal tracts, leading to gait disorder and spasticity of the lower limbs. HSPs are characterized by the high heterogeneity of both their genetic background and clinical manifestation. The number of known HSP genes continues to increase, with 79 loci and 65 genes (spastic paraplegia genes, SPGs) (Parodi *et al.*, 2018) identified to date. Pathogenic mutations can manifest sporadically or be transmitted through all known inheritance patterns (Klebe *et al.*, 2015), mostly by autosomal-dominant and recessive transmission, accounting for an overall prevalence of 1–5:100 000 (Ruano *et al.*, 2014). HSPs have been historically classified into ‘pure’ and ‘complex’ forms based on symptoms: pure HSPs are defined by an upper motor neuron phenotype (spastic gait, lower-limb hypertonicity, hyperreflexia, extensor plantar responses, sphincter disturbances, and proximal weakness), whereas the presence of additional neurological or extra-neurological signs define the ‘complex’ or ‘complicated’ form of the disorder (Fink, 2014). Degeneration may also affect the lower motor neurons, associated with an ALS phenotype (Parodi *et al.*, 2017). The age at onset of HSPs is variable, ranging from early childhood to 70 years of age (Salinas *et al.*, 2008). Carriers may thus be asymptomatic for decades and the probability of being affected (penetrance) is age-dependent (Harding, 1981; Durr *et al.*, 1993).

SPG4 is due to heterozygous mutations of the *SPAST* gene and is the most frequent cause of both familial and sporadic HSP (Lo Giudice *et al.*, 2014). Located on chromosome 2p22.3, *SPAST* encodes spastin (Hazan *et al.*, 1999), a protein belonging to the AAA family. Spastin hydrolyses ATP to sever microtubules and controls various aspects of microtubule dynamics, such as their number, length, and motility (Errico *et al.*, 2002). Spastin is composed of four domains necessary for its enzymatic function and interaction with intracellular partners. It is involved in endoplasmic reticulum morphogenesis (Park *et al.*, 2010) and lipid metabolism (Papadopoulos *et al.*, 2015) through its N-terminal domain (starting from residue

1 to 87), together with other SPG-encoded proteins such as *ATL1/SPG3A* and *REEP1/SPG31*. The microtubule interacting and trafficking domain (MIT), located between amino acids 116 and 194, allows interactions with two proteins belonging to the endosomal-sorting complex required for transport III (ESCRT-III) machinery, CHIMP1 and IST1, explaining the role of spastin in both cytokinesis and endosomal-tubule recycling (Reid *et al.*, 2005; Connell *et al.*, 2009; Allison *et al.*, 2013). The two remaining domains, the microtubule-binding domain (MTBD), comprising amino acids 270 to 328, and the AAA ATPase cassette, from amino acid 342 to 616, are crucial for spastin-severing activity. Microtubules are severed by the energy derived from ATP hydrolysis following the assembly of six spastin subunits into a ring-shaped hexamer that binds to microtubules and introduction of the C terminus of tubulin into the central pore (White *et al.*, 2007; Roll-Mecak and Vale, 2008).

Four spastin isoforms are produced by an alternative initiation codon and differential exon 4 splicing (Havlicek *et al.*, 2014). Spastin isoform M1 (68 kDa) and M87 (60 kDa) are produced by an alternative translation start site (Claudiani *et al.*, 2005; Mancuso and Rugarli, 2008) and share all protein domains, except for the N-terminal domain, which is present only in the M1 isoform. Moreover, the human M87 isoform is expressed both in the spinal cord and cerebral cortex, whereas spastin-M1 is detectable only in the spinal cord (Solowska *et al.*, 2010).

SPG4-HSP has been defined as a ‘pure’ HSP, based on the neurological symptoms presented by affected patients, with the lesions being restricted to the corticospinal tract and the posterior column (Hazan *et al.*, 1999; Fonknechten *et al.*, 2000). The most frequent additional observations have concerned cognitive decline and white matter abnormalities by cerebral MRI (Tallaksen *et al.*, 2003; Nielsen *et al.*, 2004; Ribai *et al.*, 2008; Murphy *et al.*, 2009). SPG4-HSP is characterized by an age of onset ranging from childhood to the eighth decade of life. The presence of genetic modifiers underlies such high intra- and inter-familial variability, mostly affecting age of onset and severity. To date, the *SPAST* c.131C>T/p.(Ser44Leu) variant is

the most well established SPG4-HSP genetic modifier, leading to a much lower age at onset and the expression of a more severe phenotype in carriers when associated *in trans* with a *SPAST* mutation (Svenson *et al.*, 2004; McDermott *et al.*, 2006).

We investigated the factors that account for phenotypic variability in detail by analysing a cohort of 842 SPG4-mutated patients recruited through the diagnostic unit of the Pitié-Salpêtrière University Hospital or the SPATAX network (www.spatax.wordpress.com). Data concerning the specific mutations, longitudinal neurological signs, the age at onset, and disorder severity were used to assess phenotype-genotype correlations and identify genetic modifiers.

Materials and methods

Patients

This study included 842 HSP-affected patients, 529 from the SPATAX network and 313 that were referred to the clinical diagnostic laboratory of the Genetics Department (G.B. and Ch. De.) of the Pitié-Salpêtrière University Hospital. Clinical information based on neurological examinations and genetic counselling were available for 636 patients.

Most of the patients had a family history of the disorder (75.6%, 481/636), whereas 36 (5.7%) were sporadic cases. Information concerning transmission of the disorder was unavailable for the remaining individuals (18.7%, 119/636).

In addition to pyramidal signs in the lower limbs, we listed the frequency of increased reflexes of the upper limbs, Hoffmann sign, and weakness and wasting of the lower limbs, as well as urinary disturbances, pes cavus, a decreased sense of vibration at the ankles, and cognitive difficulties, including intellectual deficiency and cognitive decline with age. Disorder severity was assessed using the SPATAX disability scale (0: no functional handicap; 1: no functional handicap but signs at examination; 2: mild, able to run, walking unlimited; 3: moderate, unable to run, limited walking without aid; 4: severe, walking with one cane; 5: walking with two canes; 6: unable to walk, requiring a wheelchair; 7: confined to bed). In addition, the disability progression index (ratio of disability stage and disorder duration) at the last examination was used to evaluate progression of the disorder: the difference in severity between the last and first examination and the corresponding elapsed time were taken into account to assess progression. Rapidly and slowly progressing patients were above or below the median of the disability progression index, respectively, of 0.09 in the analysed follow-up cohort.

Penetrance was calculated as the number of affected patients divided by the total number of carriers.

All patients included in the present study gave their consent for DNA testing in the research setting and consent forms (RBM 01-29, RBM 03-48) were signed by each participant.

Mutation analysis

Genomic DNA was extracted from peripheral blood and both targeted sequencing and Sanger sequencing were performed to

detect mutations that potentially affect *SPAST* exons and portions of the neighbouring intronic regions. Multiplex ligation-dependent probe amplification (MLPA) was used to identify the extent of large deletions and intragenic *SPAST* rearrangements (SALSA MLPA probemix P165, MRC-Holland). MLPA results were analysed using either GeneMapper® (version 4, ThermoFisher) or Coffalyzer.net (MRC-Holland). Mutations were interpreted using Alamut 8.0 (Interactive Biosoftware, Rouen, France), which includes Align GVGD (<http://agvgd.hci.utah.edu/>), PolyPhen-2 [Human Diversity (HumDiv), and Human Variation (HumVar) prediction models, <http://genetics.bwh.harvard.edu/pph2/>], MutationTaster (<http://www.mutationtaster.org/>) and SIFT prediction test (<http://sift.jcvi.org/>). The Human Gene Mutation Database (<http://www.hgmd.cf.ac.uk/ac/index.php>) and the genome Aggregation Database (gnomAD, <http://gnomad.broadinstitute.org/>) were consulted for single nucleotide polymorphisms (SNPs).

Frameshift and nonsense mutations occurring in exon sequences (NM_14946.3) were considered to be deleterious. Missense mutations affecting highly conserved amino acids of the AAA domain and predicted to be deleterious by at least three *in silico* tools were considered to be deleterious. Missense mutations affecting a highly conserved amino acid outside the AAA domain and predicted to be deleterious by at least three *in silico* tools were considered to be deleterious only when supported by Sanger sequencing segregation analysis. Finally, *in silico* predictions of splice mutations were performed using five models: SpliceSiteFinder-like (Interactive Biosoftware), MaxEntScan (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html), NNSPLICE (http://www.fruitfly.org/seq_tools/splice.html), GeneSplicer (<http://www.cbcb.umd.edu/software/GeneSplicer>), and Human Splicing Finder (<http://www.umd.be/HSF3/HSF.shtml>).

Missense, splice-site, nonsense, and frameshift mutations were annotated using wANNOVAR (<http://wannovar.wglab.org/>) (Wang *et al.*, 2010; Yang and Wang, 2015), allowing the assessment of gnomAD frequencies and mutation CADD scores (Kircher *et al.*, 2014).

Statistical analysis

Chi-square tests of clinical data were performed using IBM SPSS Statistics (IBM Corp. Released 2013. IBM SPSS Statistics for Macintosh, Version 22.0. Armonk, NY: IBM Corp.). The means, standard deviations, Mann-Whitney tests (two-tailed), chi-square tests, and log-rank tests were computed using GraphPad Prism version 6.00 for Macintosh (GraphPad Software, La Jolla California USA, www.graphpad.com). R package ggplot2 was used for graphic representations (Wickham, 2009). The resulting *P*-values were considered to be statistically significant when <0.05 .

The FCOR package, part of S.A.G.E software packages (S.A.G.E. [2016] Statistical Analysis for Genetic Epidemiology, Release 6.4: <http://darwin.cwru.edu>), was used to calculate intra-familial correlations.

Neuropathological examination

A post-mortem neuropathological examination was performed on Patient FSP-625-011, who died from brainstem compression due to cerebral haemorrhage related to vesical cancer at age 59. The brain and spinal cord were available for examination. Horizontal sections of the spinal cord were prepared

and staining included haematin-eosin, Luxol fast blue for myelin, CD68 for microglia, and double labelling of myelin basic protein (MBP) and neurofilaments. The lateral column was cut sagittally, along the long axis of the spinal cord at the thoracic level, to examine the aspect of the fibres of the pyramidal tract.

Data availability

The data that support the findings of this study are available on request from the corresponding author. The data, processed in accordance with consent forms RBM 01-29 and RBM 03-48, are not publicly available due to the European General Data Protection Regulation (GDPR).

Results

Age of onset and intra-familial correlation

Age of onset could be determined for 547 patients and ranged from 0 to 77 years, with a mean age of 29 years (29.3 ± 18.6). Fifty-one mutation carriers did not report any age at onset because they felt asymptomatic (mean age at examination 37.3 ± 15.6 years, ranging from 6 to 70), but 28/51 had an extensor plantar reflex in the lower limbs. Only 23 mutations carriers, with a mean age at examination of 39.5 ± 16 years, ranging from 17 to 70 years, were asymptomatic and had no apparent pyramidal signs. Overall penetrance of the disorder was 0.9 and was complete at 70 years of age.

Index cases were distinguished from other family members to avoid possible bias and assess the distribution of age at onset. The mean age at onset was similar for the index cases (28.7 ± 17 , range 0 to 69, $n = 215$) and family members (27.3 ± 19 , range 0 to 77, $n = 213$; Mann-Whitney test, $P = 0.38$). The onset of SPG4-HSP followed a bimodal distribution among both probands and non-probands, characterized by a first peak extending from birth to the first decade and a second peak from the third to fifth decades (Fig. 1). The mean age at the last examination was 47 years (47 ± 17.8 , $n = 633$, range 1.5 to 95). Both the age at onset and at the last examination were available for 546 patients; the mean disorder duration (age at last examination minus the age of onset) was 18 years (18.5 ± 14.7 , range 6 months to 75 years).

Information retrieved from 84 pedigrees with at least two affected individuals with the known age at onset were used to estimate the intra-familial correlation of age at onset. The correlation was highly significant for the 113 sibling pairs ($r = 0.37$, $P = 0.0008$) and 109 parent-offspring pairs ($r = 0.34$, $P = 0.001$) analysed. The correlation was still significant for uncle-nephew pairs ($r = 0.27$, $n = 104$, $P = 0.042$), but lost significance between grandparents and grandchildren ($r = 0.40$, $n = 22$, $P = 0.105$). The correlations were affected by gender: the correlation was highly significant between brothers ($r = 0.58$, $n = 34$, $P = 0.0002$), did not

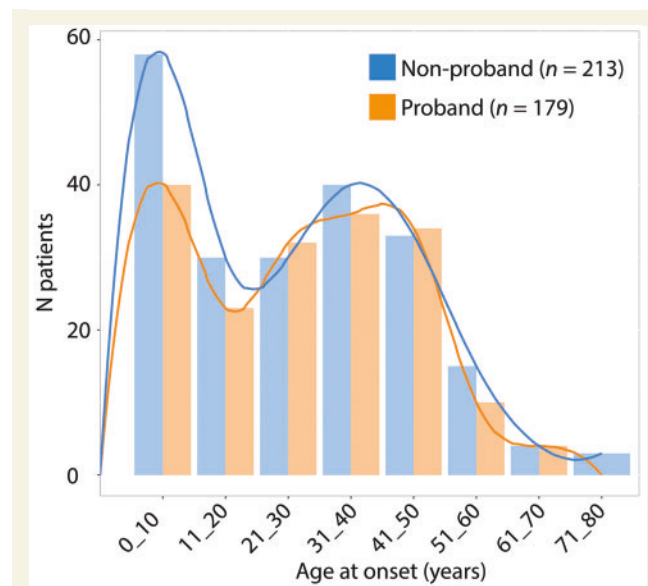


Figure 1 Distribution of the age at onset of the analysed cohort. Age at onset was compared among non-probands and probands. The resulting bimodal trend shows that the onset of SPG4-HSP occurs between birth and the first decade and between the third and fifth decades.

reach significance between sisters ($r = 0.39$, $n = 27$, $P = 0.059$), and was still lower for brother-sister pairs ($r = 0.23$, $n = 52$, $P = 0.187$).

The difference in the age of onset between members of the same family was 27 years on average (26.9 years ± 18.3 , $n = 28$) for the 84 families of our cohort, with a maximum of 69 years separating onset of the disorder for two related patients.

Clinical signs and severity

The mean level of disability of the overall cohort was 2.95 ± 1.6 ($n = 481$), with only one patient reaching the highest severity level of 7. The overall clinical presentation was spasticity in the lower limbs, more pronounced at gait than at rest, which was rarely severe. Half of the cohort showed proximal weakness and increased reflexes in the upper limbs (Table 1). Oculomotor abnormalities suggestive of cerebellar dysfunction (saccadic pursuit and/or nystagmus) were present in only 13.5% (15/111) of the cohort. Intellectual deficiency was present in 22 families and progressive intellectual disability was reported for only 17 patients (Table 1). The mean duration was 18 years (18.5 ± 14.7 , $n = 546$, range 0 to 75 years). The cohort was divided into two groups: cases of relatively low duration (below the mean) and cases of relatively long duration (above the mean). The long duration cases more often showed proximal weakness (131/201 versus 66/173; chi-square test, $P < 0.0001$), greater upper limb reflexes (146/196 versus 108/172; chi-square test, $P = 0.02$), and lower sense of vibration (133/188 versus 92/158;

Table 1 Clinical presentation of analysed SPG4-HSP patients

	All patients	Females	Males	P
<i>n</i>	636	302	334	-
Mean age at onset, years (<i>n</i>)	29.3 ± 18.6 (547)	30.8 ± 18.9 (252)	28 ± 18.3 (295)	0.13
Mean age at examination, years (<i>n</i>)	47.1 ± 17.8 (632)	48.3 ± 17.5 (302)	46 ± 18.2 (330)	0.11
Mean stage of disability (<i>n</i>)	2.9 ± 1.6 (481)	2.8 ± 1.6 (225)	3 ± 1.5 (256)	0.2
Mean disease duration (<i>n</i>)	18.5 ± 14.7 (546)	18.7 ± 16.3 (252)	18.7 ± 13.4 (294)	0.47
Gait spasticity, %	88 (367/417)	85.2 (161/189)	90.4 (206/228)	0.10
Severe, %	39.8 (146/367)	37.8 (61/161)	41.2 (85/206)	0.52
Spasticity at rest, %	79.4 (293/369)	73 (127/174)	85.1 (166/195)	0.003
Severe, %	26.3 (77/293)	23.6 (30/127)	28.3 (47/166)	0.42
Increased lower-limb reflexes, %	95 (410/431)	95.5 (193/202)	94.8 (217/229)	0.7
Extensor plantar reflex, %	83.4 (347/416)	83.5 (157/188)	83.3 (190/228)	0.96
Increased upper limb reflexes, %	65.4 (275/420)	74.6 (144/193)	57.7 (131/227)	0.0002
Hoffman sign, %	39.7 (95/239)	47.7 (51/107)	33.3 (44/132)	0.02
Proximal lower limb weakness, %	48.4 (202/417)	53.8 (99/184)	44.2 (103/233)	0.05
Pes cavus, %	22.4 (86/383)	24.7 (42/170)	20.7 (44/213)	0.34
Decreased sense of vibration at the ankles, %	61 (241/395)	66.5 (119/179)	56.5 (122/216)	0.04
Urinary symptoms, %	77 (168/218)	80.2 (77/96)	74.6 (91/122)	0.32
Intellectual impairment, %	4.2 (17/402)	5.5 (10/183)	3.2 (7/219)	0.26

P-values were assessed using a chi-square test.

chi-square test, $P=0.02$). Spasticity at rest (159/175 versus 116/143; chi-square test, $P=0.01$) was more severe for the long duration group, whereas the prevalence of gait spasticity was similar for the two groups.

Comparison according to sex

A comparison between males and females revealed no significant differences for age at onset or disorder severity. However, females showed greater upper limb reflexes and a higher frequency of Hoffmann sign than males, despite a similar mean duration of the disorder for both groups (Table 1). Apart from spasticity at rest, females appeared to express a more severe and diffuse disorder phenotype. In contrast, they were more often unable to indicate the age at onset and the proportion of asymptomatic carriers was higher for females than males, suggesting sex-linked penetrance. Penetrance of the disorder was indeed higher in males (0.94, 302/320 versus 0.88, 258/291; chi-square test, $P=0.01$) for the entire evolution of the disease ($P=0.01$, log-rank test) (Supplementary Fig. 1). The difference in penetrance was even higher when considering only early onset patients, examined before 30 years of age (0.91, 52/57 for males versus 0.7, 35/48 for females; chi-square test, $P=0.02$).

Nature and distribution of mutations

We detected a total of 266 different mutations, 134 were previously unreported (Supplementary Table 1). Missense mutations were the most frequent (33%, 87/266), followed by frameshift (24%, 65/266), splice-site (16%, 42/266), and nonsense (13%, 34/266) mutations and deletions (12%, 31/266). Duplications encompassing one or several

exons, even involving the 3' UTR in one patient, and small in-frame amino acid deletions (3%, 7/266) were less frequent. Most (77%, 205/266) pathogenic mutations clustered in the AAA cassette, between amino acids 342 and 599. The observed clustering was particularly evident for missense mutations, predominantly detected in the spastin AAA cassette (96.6%, 84/87) (Figs 2 and 3). Three missense mutations located outside the AAA cassette were classified as possibly pathogenic because they segregated with the disorder in the affected families (Families SAL-055, SAL-1143, SAL-1045). Deletions also tended to cluster in the C-terminus, mostly causing the loss of small portions of the gene, except for five large deletions, which in some cases led to the loss of the entire gene sequence.

We detected a phenocopy in one family (Family SAL-115): a 39-year-old male did not carry the mutation (c.1450_1451del/p.Gly484Cysfs*) responsible for the phenotype in 12 other affected family members. He had experienced vertigo, cramps, and pain from the age of 35. Examination revealed increased brisk tendon reflexes, indifferent plantar reflex, mild scoliosis, and normal MRI and conduction velocities on electromyography.

The GnomAD database was useful for assessing the frequency of the detected mutations in the general population. Only 11 of the 266 identified mutations were present in GnomAD (Supplementary Table 1), all at a frequency $<0.1\%$, providing further evidence in favour of their role in the pathogenicity of SPG4-HSP.

Phenotype–genotype correlation

We analysed the nature of the pathogenic mutation and its position to determine their possible influence on the

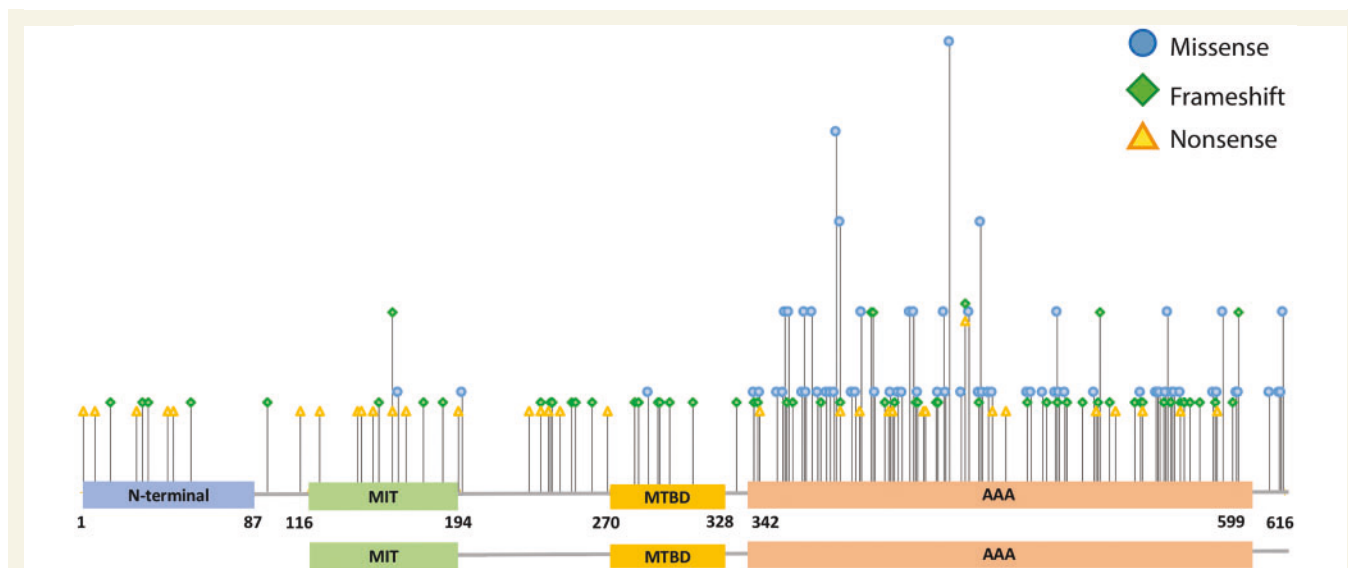


Figure 2 Distribution of missense, nonsense, and frameshift mutations. The height of the bars represents the number of different base changes affecting the same amino acid position of the gene sequence.

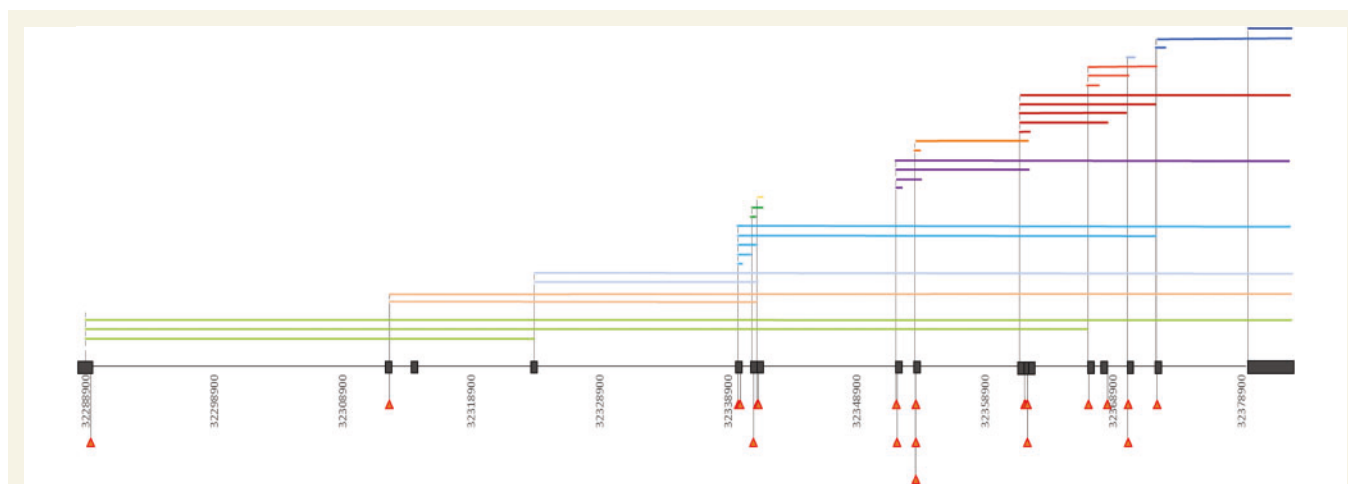
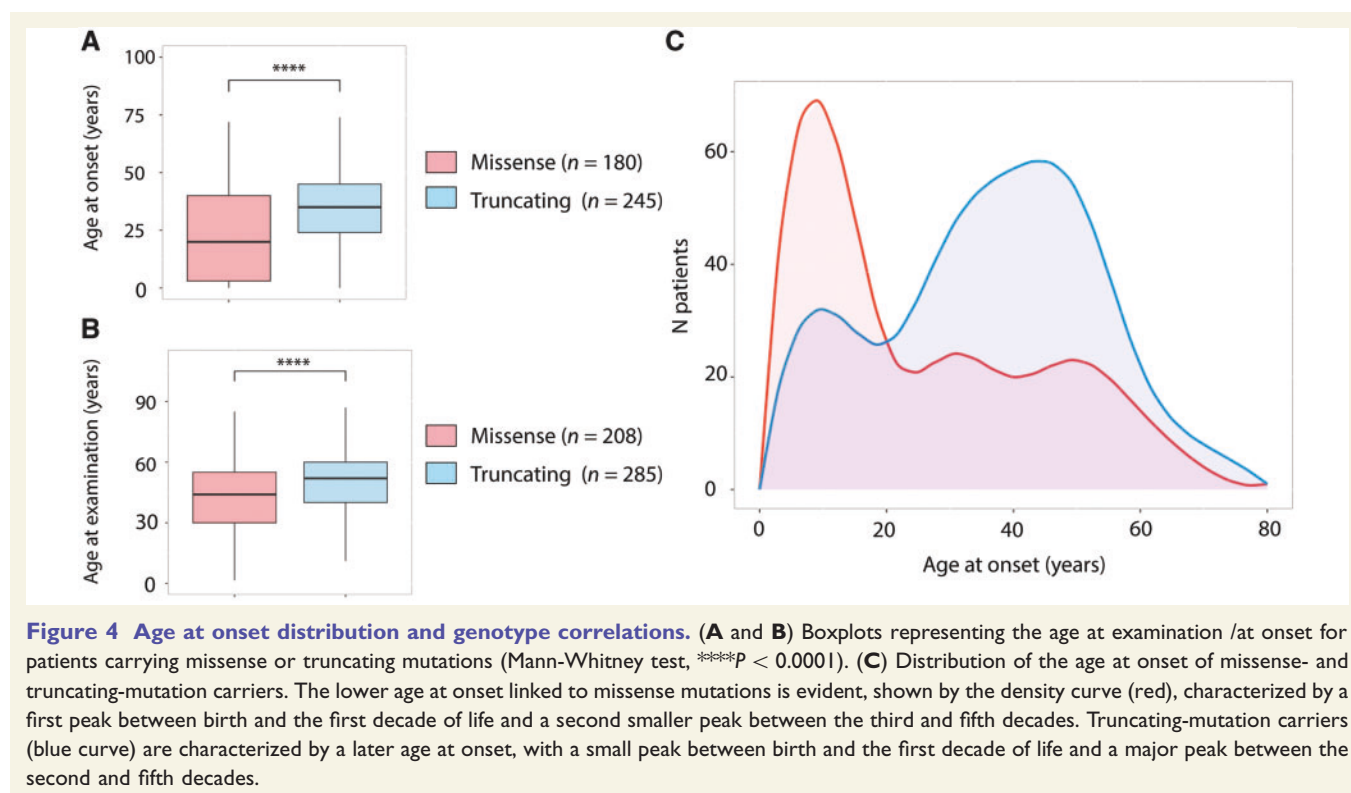


Figure 3 Distribution of deletions and splice-site mutations. SPAST exons are represented in black. Deletions are represented in the upper part of the figure by coloured horizontal bars, spanning the length of the deleted region. Splice-site mutations are represented in the lower part of the figure by red triangles.

clinical phenotype. The phenotype observed for mutations leading to a truncated protein (frameshift, nonsense, splice site, and small deletions) were compared to the phenotype of missense mutations. Only mutations affecting spastin after amino acid 342 were considered ($n=505$), as most pathogenic mutations altered the AAA cassette.

Individuals with missense mutations showed a much lower mean age at onset (23 ± 19.7 , $n=180$ versus 33.4 ± 16.7 , $n=245$; Mann-Whitney test, $P < 0.0001$) (Fig. 4A). The age at first clinical examination was also lower (41.8 ± 19.6 , $n=208$ versus 50.2 ± 15.5 , $n=285$; Mann-Whitney test, $P < 0.0001$) (Fig. 4B). The distribution

of the age at onset was bimodal: the clinical symptoms of most missense-mutation carriers first appeared before the second decade, whereas the first appearance of clinical symptoms of truncating-mutation carriers clustered between the second and sixth decades (Fig. 4C). There was no difference in the severity (3.1 ± 1.7 , $n=148$ versus 2.8 ± 1.5 , $n=224$; Mann-Whitney test, $P=0.11$) or duration (19.8 ± 15.7 , $n=180$ versus 17.7 ± 14.2 , $n=243$; Mann-Whitney test, $P=0.28$) of the disorder between missense- and truncating-mutation carriers. However, intellectual disability was significantly more frequent among missense mutation carriers (21.7% versus 4.7%; chi-square test $P < 0.0001$).



Factors influencing the disorder severity and progression

We assessed whether the severity of the disorder was influenced by the age at onset by dividing the cohort into early onset cases (below the median age of onset of 30 years) and late onset cases (above the median age of onset) ($n = 546$) and comparing the mean stage of disability at the latest examination. The disability for late onset cases was more severe than that for early onset cases. This was especially true when the duration of the disorder was between 11 and 30 years (3.2 ± 1.16 , $n = 86$ versus 3.8 ± 0.9 , $n = 88$; Mann-Whitney test, $P < 0.0001$) (Supplementary Fig. 2).

We performed a more comprehensive analysis of the progression of SPG4-HSP for 116 patients for whom several follow-up examinations were available. Patients with a slow course had a less severe outcome (4.2 ± 1.3 , $n = 61$ versus 3.3 ± 1.5 , $n = 54$; Mann-Whitney test, $P = 0.001$), which was not explained by their age at onset (slow course group 25.8 ± 17.4 , $n = 50$ versus fast course group 27.4 ± 16.4 , $n = 60$ Mann-Whitney test, $P = 0.6$) or genotypes (32% missense versus 68% truncating, $P = 0.7$). Patients with a more rapidly evolving disorder had a higher frequency of urinary incontinence and lower limb proximal weakness (82% versus 48%, $P = 0.01$ and 64.7% versus 34.5%, $P = 0.01$), and were more severely affected, as reflected by their disability scores.

The c.131C > T/p.(Ser44Leu) polymorphism

Eleven patients carried the *SPAST* exon 1 variant c.131C > T/p.(Ser44Leu) associated with a major pathogenic mutation. The S44L variant was associated with a significantly lower age at onset (11 ± 16.9 , $n = 11$ versus 29.3 ± 18.6 , $n = 547$; Mann-Whitney test, $P = 0.004$) and thus a lower age at the first examination (32 ± 22.2 , $n = 11$ versus 47.1 ± 17.9 , $n = 632$; Mann-Whitney test, $P = 0.02$). There was no difference in the severity of the disorder for patients with the S44L and those without (2.9 ± 1.6 , $n = 481$ versus 3.3 ± 0.94 , $n = 10$, Mann-Whitney test, $P = 0.46$). Patients with p.(Ser44Leu) and another *SPAST* mutation showed pure spastic paraplegia with increased tendon reflexes, a reduced sense of vibration at the ankles, a bilateral extension plantar response, and urinary urgency or incontinence. The phenotype was more complex in only two cases and accompanied by delayed psychomotor development and moderate or severe sphincter disturbance, probably due to the presence of a missense mutation and early onset (see above genotype-phenotype correlation). In one case, cerebral MRI revealed mild cerebellar atrophy.

Neuropathological examination

We were able to perform a post-mortem neuropathological examination of Case FSP-625-011 (with pre-mortem

consent of the patient). This male patient died from cerebral metastases of vesical cancer at 59 years of age. He had been afflicted with spastic paraplegia since the age of 25 years. He inherited the disorder from his mother, was the youngest of three brothers, and the only affected sibling. A *SPAST* frameshift mutation (c.1215_1219del/p.Asn405Lysfs*36) had been identified and segregated in the family. The stiffness of his legs progressed over the years and he needed a walking aid at 37, two canes at 42, and a wheelchair at 48 years of age. Evaluation by the Spastic Paraplegia Rating Scale (SPRS) gave a score of 43/52 at 55 and 49/52 at 58 years of age. The spasticity of the lower limbs was severe, with weakness and extensor plantar reflexes. Pyramidal signs were marked in the upper limbs. He had a bilateral Hoffmann sign. Mild urinary urgencies were noted since the age of 49. The sense of vibration disappeared at age 43 years of age. Eye gaze and the fundus were normal. No cerebellar or extrapyramidal signs were noted. Brain and medullar MRI were normal at 38 and 55 years of age. Conduction velocities were normal at the age of 59, except for left carpal compression signs. Haematuria led to the discovery of vesical cancer. The patient died 1 year later at the age of 59 due to brainstem compression caused by cerebral haemorrhage.

The brain weighed 1664g. The right hemi-brain was examined after formalin fixation and the left cryoprotected and sampled immediately post-mortem. Macroscopically, a temporal herniation had caused compression and haemorrhage of the midbrain. There was a large dilation of the lateral ventricles. We observed two metastases of 0.5 cm in diameter. The first was located in the frontobasal region and the second in the vermis of the cerebellum. Microscopically, the number of Betz cells in the motor cortex was low and there were a few neurofilament-positive-MBP-negative spheroids in the white matter; the cerebral cortex was otherwise normal. The thalamus, striatum, cerebellum, and dentate nucleus were normal. The pyramids were small at the level of the medulla oblongata. The posterior part of the lateral column of the spinal cord was pale in a region corresponding to the pyramidal tract. Such myelin pallor was also marked in the dorsal spinocerebellar tract. The cuneiform fasciculus in the posterior column of the spinal cord was also pale. MBP-neurofilament immunohistochemistry showed no demyelination, but degeneration of the affected tracts. We observed axon spheroids and abnormally large fibres with a sausage-like aspect and thin myelin layer in the pyramidal tract (Fig. 5A and B). Remarkably, a high density of very

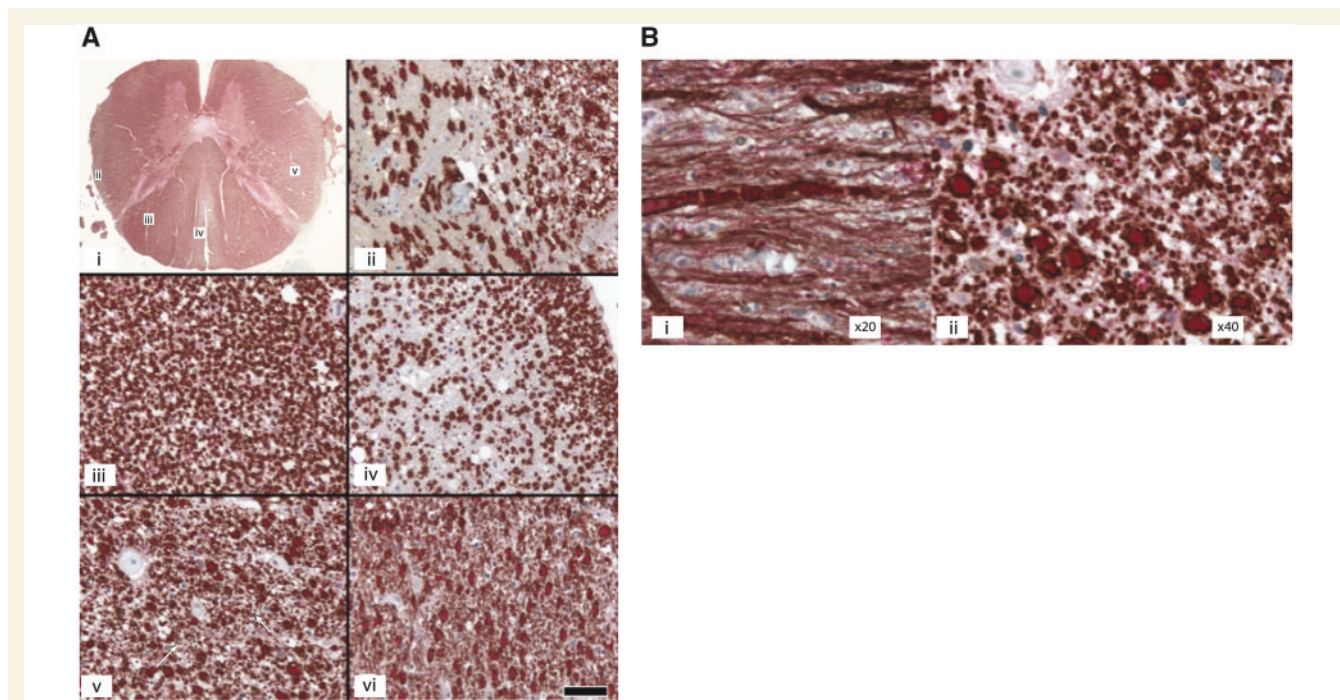


Figure 5 Spinal cord of a *SPAST* mutated patient. (A) Spinal cord of Patient FSP-625-01 I: alteration of the spinocerebellar and spinocortical tracts and posterior columns. Double labelling of myelin basic protein/neurofilaments (MBP/NF). (i) Transversal section of the spinal cord at the thoracic level. (ii–vi) Scale bar = 50 µm, MBP: brown/NF: red. (ii) Posterior spinocerebellar tract: loss of myelinated and unmyelinated fibres in the dorsal spinocerebellar tract. (iii) Fasciculus cuneiformis: sparing of the fasciculus cuneiformis. (iv) Fasciculus gracilis: loss of myelinated and unmyelinated fibres. (v) Cortico-spinal lateral tract at the thoracic level: numerous processes of small diameter among unusually large myelinated fibres, suggesting marked regeneration of the corticospinal tract. Arrows indicate clusters of small fibres. (vi) Lateral corticospinal tract at the level of the medulla oblongata: the number of small regenerating fibres appear to be as large in the medulla as at the thoracic level. (B) Pyramidal tract of the spinal cord. (i) Atypically large fibres with a sausage-like aspect and a poor myelin layer in longitudinal sections. (ii) Neurodegeneration and axon spheroids detected in transversal sections.

small fibres with thin myelin fibres was clearly shown by MBP-NF double immunohistochemistry on sagittal sections, suggesting regeneration of the corticospinal tract. Tau staining did not show the presence of tau-positive cells in any of the analysed regions. There were no amyloid- β deposits. The pars compacta of the substantia nigra and locus coeruleus showed mild neuronal loss. There were α -synuclein positive fibres in the dorsal nucleus of the vagus nerve and the locus coeruleus, substantia nigra, and amygdala. A diagnosis of mild brainstem-type Lewy body disease was made.

The metastases had the characteristics of a urothelial carcinoma.

Discussion

We assembled the largest SPG4-HSP cohort ($n=842$) to date, which allowed us to show that both the sex of the individual and the nature of the *SPAST* mutation act as modifiers of the HSP phenotype. The principal aim of this study was to search for factors that can account for the extremely high clinical variability of this disease, even when the same pathological variant is shared (Santorelli *et al.*, 2000). The age at onset ranged from early childhood to the seventh decade and followed a bimodal distribution, with the first peak before the first decade of life and the second lower peak between the second and fifth decades, similar to that observed in a Dutch SPG4 cohort (de Bot *et al.*, 2010). The underlying cause of such a bimodal distribution was missing from previous reports. Here, we show that the mutation type may explain the bimodal distribution, with the first-decade peak mainly due to missense mutations, whereas the second is most likely due to truncating mutations. However, it is important to underline the difficulty of assessing the precise age at onset, leading to a possible bias due to the wrong age or to the fact that early-onset patients are examined earlier. In addition, we could not include individuals for whom age at onset information was not available. Yet, the distribution of missense versus truncating mutations in this cohort was similar (30% versus 70%, $n=206$ compared to 33% versus 65%, $n=636$).

Among the 266 different mutations that we report here, most were located in the spastin AAA domain, in accordance with previous reports (Fonknechten *et al.*, 2000; Lindsey *et al.*, 2000; Svenson *et al.*, 2001; Meijer *et al.*, 2002; Charvin *et al.*, 2003; Shoukier *et al.*, 2009; Alvarez *et al.*, 2010; Loureiro *et al.*, 2013). Truncating mutations, with the loss of function of spastin as a possible consequence, comprised most of the mutations. Missense mutations clustered in the AAA cassette, whereas pathogenic frameshift and nonsense mutations were distributed throughout the gene. Missense- and truncating-mutation carriers showed no difference in disorder severity based on evaluation using a disability scale. Nevertheless, a notable exception was intellectual disability, which was more

frequent among missense carriers. Case reports of families that were included in our cohort had already suggested this possible association (Ribai *et al.*, 2008). In addition, we previously observed that missense-mutation carriers show greater cognitive decline (Tallaksen *et al.*, 2003).

In our cohort, we observed, for the first time, a correlation between the presence of a missense mutation that affects the AAA domain and an earlier age at onset when comparing missense and truncating mutation carriers. No clear genotype–phenotype correlation has been previously established for the possible association between the nature of the mutation and age of onset or severity of the disorder. Indeed, prior attempts have led to different conclusions (Fonknechten *et al.*, 2000; Svenson *et al.*, 2001; Proukakis *et al.*, 2003; Patrono *et al.*, 2005; Depienne *et al.*, 2007; Shoukier *et al.*, 2009; de Bot *et al.*, 2010; Loureiro *et al.*, 2013), possibly due to small sample size.

There is an ongoing scientific debate concerning the pathogenic mechanisms underlying the age at onset depending on the type of *SPAST* mutation. Loss of function and consequent haploinsufficiency have been the most plausible pathogenic mechanisms to explain SPG4-HSP onset, as truncating mutations are the most frequent cause of SPG4-HSP (Fonknechten *et al.*, 2000; Sauter *et al.*, 2002; Beetz *et al.*, 2006; Depienne *et al.*, 2007; Shoukier *et al.*, 2009; Alvarez *et al.*, 2010) and most are predicted to predominantly affect the enzymatic activity of the spastin domain, thus reducing its ATPase activity (Bürger *et al.*, 2000; Fonknechten *et al.*, 2000; Lindsey *et al.*, 2000; Molon *et al.*, 2004; Beetz *et al.*, 2006; Depienne *et al.*, 2007). However, missense mutations that affect the AAA domain were shown to lead to constitutive binding of spastin to microtubules, suggesting a dominant-negative mechanism (Errico *et al.*, 2002). Moreover, it was shown that missense mutations outside the AAA domain lead the M1 isoform of spastin to negatively interact with spastin-M87, diminishing its microtubule-severing activity (Solowska *et al.*, 2010). The observation of an earlier age at onset for missense carriers supports a loss-of-function mechanism, but does not exclude the possibility that a dominant-negative mechanism could be associated with specific missense mutations. Among the analysed cohort, this could be true for those patients (30%, 17/56) with an onset before 20 years of age who carry missense mutations already known to have a possible dominant-negative effect (Errico *et al.*, 2002). We were unable to link the severity of the disorder to the nature or position of the mutation, but confirmed that later onset was linked to faster progression (Fonknechten *et al.*, 2000). Multiple follow-up analyses showed that proximal weakness of the lower limbs and urinary incontinence were the symptoms most frequently associated with rapidly evolving disease.

The sex of the individual appeared to be a second factor that influences the age at onset in our cohort. Until now, no clear sex-related effect has been reported (Schüle *et al.*, 2016), but a higher prevalence in males has already been observed (Proukakis *et al.*, 2011). Females of our cohort

with a similar duration of the disorder had a more severe and diffuse form of the disorder, characterized by upper limb involvement. In contrast, the number of asymptomatic females was higher than asymptomatic males, leading to a lower penetrance estimate for females, especially before the third decade. It is possible that neurosteroid progesterone and oestrogens could protect females (Orlacchio *et al.*, 2005; Proukakis *et al.*, 2011). Based on our observations, protective factors could delay onset of the disorder, but once started, its evolution is more rapid and severe than in males. This is comparable to sex-linked differences observed in Alzheimer's disease, with affected females showing longer survival but a more severe disability (Sinforiani *et al.*, 2010).

The most extreme aspect of phenotypic variability was incomplete penetrance, i.e. individuals that do not express the disorder even though they carry the *SPAST* mutation. Fifty-one (8.3%) carriers declared themselves to be asymptomatic but only 23 were truly asymptomatic when examined. Overall penetrance was estimated to be 0.9, with half of the patients manifesting the disorder at 45 years of age and complete penetrance by the seventh decade. A highly significant correlation for the age at onset between sibling and parent-offspring pairs was less variable than between different families. The highest correlation was observed for same-sex sib-pairs, especially between brothers. This suggests that genetic factors may have a major impact on the age at onset. A sex-linked effect may also come into play, enhancing the correlation between siblings for the age at onset.

The phenotype in the overall cohort did not differ from the already reported features typical of SPG4-HSP (Durr *et al.*, 1993): a pure form of spastic paraplegia with evident but mildly severe spasticity at gait and proximal weakness, as well as increased upper limb reflexes in 65% of patients. Many studies tried to link additional phenotypical presentations to the core spastic phenotype such as dementia, intellectual deficiency, neuropathy, tremor but also Dandy Walker malformation in one family and one sporadic case (Durr *et al.*, 1993; Tallaksen *et al.*, 2003; McDermott *et al.*, 2006; Ribai *et al.*, 2008; Scuderi *et al.*, 2008; van de Warrenburg, 2008; Murphy *et al.*, 2009; Parodi *et al.*, 2017). In the latter, the malformation could be linked to the *SPAST* mutation in the described family, but also to another, yet unknown mutated gene that is in linkage disequilibrium with SPG4 or a modifier variant (Scuderi *et al.*, 2008; van de Warrenburg, 2008). Neuropathy and tremor are frequent neurological signs, and it is difficult to prove the link to *SPAST* variants and could be considered as coincidental. Subtle cognitive impairment was associated with SPG4, especially linked to missense variants (Tallaksen *et al.*, 2003) but without evidence in rare neuropathological cases reported to date (see below).

A known modifier, the *SPAST* c.131C>T/p.(Ser44Leu) variant (S44L) in exon 1, was present in 11 patients, in addition to a causative pathogenic *SPAST* mutation.

As expected, the age at onset was significantly earlier, as S44L was associated with a *SPAST* mutation, but the disorder was neither more severe nor associated with additional neurological features in this group of patients. These results confirm that S44L modifies the age at onset (Svenson *et al.*, 2004; McDermott *et al.*, 2006). S44L affects only the M1 spastin isoform. It has thus been proposed that S44L might act in a dominant-negative fashion on the M1 isoform, lowering the microtubule-severing activity of the M87 isoform (Solowska *et al.*, 2010). Further studies of the genetic modifiers of the evolution of the disorder are warranted.

Neuropathological examination showed myelin pallor in the pyramidal tract and dorsal column of the spinal cord. This was due to degeneration of the tracts (axons and myelin were affected) rather than demyelination. There were changes in the white matter of the motor cortex, indicating that the entire length of the pyramidal tract was affected. However, a number of Betz cells were still present, suggesting that the defect did not primarily involve the cell body. In contrast, the lesions predominated in the spinal cord, in agreement with the possibility of a dying-back mechanism. The small size of the pyramids (at the level of the medulla oblongata), raise the possibility of developmental hypoplasia preceding atrophy. The large number of thin axons in the pyramidal tracts suggests a regenerating process, although it is not currently possible to discard the alternative hypothesis of primary atrophy of the axons.

We did not find the tauopathy or amyloid- β accumulation reported in another SPG4-HSP post-mortem analysis (Wharton *et al.*, 2003).

In conclusion, pure loss-of-function mutations (truncating mutations) are associated with a later onset than missense mutations, which probably result in a dominant-negative effect in addition to the loss of function. Sex-linked factors could be protective in female carriers, leading to lower penetrance. Genetic modifiers are clearly implicated in such variability, since intra-familial correlations were stronger within nuclear families, particularly between siblings of the same sex. Further molecular studies should allow the identification of genes that modify the age at onset other than the p.(Ser44Leu) variant, for which effect was confirmed in this study.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

Appendix I

SPATAX network collaborators

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Supplementary material

Supplementary Tables. *SPAST* mutations detected in the analysed cohort.

Mutation	Amino acid change	Consequence	Transcript	dbSNP	gnomAD_exome_ALL	gnomAD_genome_ALL	CADD_raw	CADD_phred	Reference
c.G484A	p.(V162I)	Missense	NM_014946	rs141944844	0.002072	0.003886	2.437	19.06	(Braschinsky <i>et al.</i> , 2010)
c.C583G	p.(L195V)			-	-	-	4.697	24.6	(Crippa <i>et al.</i> , 2006)
c.G870C	p.(K290N)			-	4.07E-06	-	2.316	18.27	(Svenson <i>et al.</i> , 2001)
c.T1031G	p.(I344R)			-	-	-	5.986	27.8	-
c.A1040C	p.(E347P)			-	-	-	4.311	24	(Alvarez <i>et al.</i> , 2010)
c.A1068C	p.(E356D)			-	-	-	4.732	24.6	-
c.T1076A	p.(I359N)			-	-	-	6.58	31	-
c.C1078T	p.(L360F)			-	-	-	6.358	29.4	-
c.T1079C	p.(L360P)			-	-	-	5.413	26	(Elert-Dobkowska <i>et al.</i> , 2015)
c.C1085G	p.(S362C)			rs121908509	-	-	5.76	27	(Hazan <i>et al.</i> , 1999)
c.C1085T	p.(S362F)			-	-	-	6.256	28.9	-
c.A1105C	p.(T369P)			-	-	-	5.731	26.9	-
c.G1108A	p.(G370R)			-	-	-	6.934	33	(Fonknechten <i>et al.</i> , 2000)
c.G1109A	p.(G370E)			-	-	-	6.791	32	-
c.T1112A	p.(L371H)			-	-	-	6.64	32	-
c.C1121A	p.(P374H)			-	-	-	6.874	33	-
c.C1121T	p.(P374L)			-	-	3.23E-05	6.154	28.5	-
c.G1130C	p.(G377A)			-	-	-	5.828	27.2	(Proukakis <i>et al.</i> , 2011)
c.T1142G	p.(F381C)			-	-	-	6.519	31	(Fonknechten <i>et al.</i> , 2000)
c.C1148G	p.(P383R)			-	-	-	6.319	29.2	-
c.G1153A	p.(G385R)			-	-	-	6.882	33	-
c.T1158A	p.(N386K)			-	-	-	6.546	31	-
c.A1157G	p.(N386S)			rs121908514	-	-	4.817	24.8	(Orlacchio <i>et al.</i> , 2004)

Mutation	Amino acid change	Consequence	Transcript	dbSNP	gnomAD_exome _ALL	gnomAD_genome _ALL	CADD_raw	CADD_phred	Reference
c.G1164T	p.(K388N)	Missense	NM_014946	-	-	-	6	27.8	-
c.G1164C	p.(K388N)			-	-	-	-	-	-
c.A1163G	p.(K388R)			-	-	-	6.432	29.8	(Fonknechten <i>et al.</i> , 2000)
c.C1181A	p.(A394E)			-	-	-	7.492	34	-
c.G1186C	p.(A396P)			-	-	-	6.839	33	-
c.C1196T	p.(S399L)			-	-	-	7.775	35	(Meijer <i>et al.</i> , 2002)
c.A1216G	p.(I406V)			-	-	-	5.501	26.2	(Schickel <i>et al.</i> , 2006)
c.A1241C	p.(K414T)			-	-	-	6.109	28.3	-
c.G1252A	p.(E418K)			-	-	-	7.135	34	-
c.A1259C	p.(E420A)			-	-	-	6.027	27.9	-
c.A1270G	p.(R424G)			-	-	-	6.094	28.2	(White <i>et al.</i> , 2000)
c.G1271C	p.(R424T)			-	-	-	6.078	28.1	-
c.C1276G	p.(L426V)			-	-	-	6.167	28.5	(Fonknechten <i>et al.</i> , 2000)
c.C1276T	p.(L426F)			-	-	-	6.698	32	(Braschinsky <i>et al.</i> , 2010)
c.G1282C	p.(A428P)			-	-	-	6.388	29.5	(Balicza <i>et al.</i> , 2016)
c.T1313C	p.(I438T)			-	-	-	6	27.8	-
c.A1322G	p.(D441G)			rs121908512	-	-	6.478	30	(Bürger <i>et al.</i> , 2000)
c.G1324A	p.(E442K)			-	-	-	7.404	34	(Ribaï <i>et al.</i> , 2008)
c.G1330A	p.(D444N)			-	-	-	7.19	34	(Elert-Dobkowska <i>et al.</i> , 2015)
c.A1331C	p.(D444A)			-	-	-	6.331	29.3	-
c.A1331G	p.(D444G)			-	-	-	6.695	32	(Elert-Dobkowska <i>et al.</i> , 2015)
c.T1332A	p.(D444E)			-	-	-	5.945	27.6	-
c.T1332G	p.(D444E)			-	-	-	5.769	27	-
c.G1349A	p.(R450K)			-	-	-	6.454	29.9	-

Mutation	Amino acid change	Consequence	Transcript	dbSNP	gnomAD_exome_ALL	gnomAD_genome_ALL	CADD_raw	CADD_phred	Reference
c.G1360A	p.(E454K)	Missense	NM_014946	-	-	-	7.224	34	(Battini <i>et al.</i> , 2011)
c.A1361T	p.(E454V)			-	-	-	6.932	33	-
c.G1376C	p.(R459T)			-	-	-	6.453	29.9	(Patrono <i>et al.</i> , 2005)
c.C1378T	p.(R460C)			-	-	-	6.216	28.7	(Falco <i>et al.</i> , 2004)
c.G1379T	p.(R460L)			-	-	-	7.359	34	(Fonknechten <i>et al.</i> , 2000)
c.G1379C	p.(R460P)			-	-	-	7.427	34	-
c.T1382C	p.(L461P)			-	-	-	6.515	31	(Depienne <i>et al.</i> , 2005)
c.A1391C	p.(E464A)			-	-	-	6.233	28.8	(McCorquodale <i>et al.</i> , 2011)
c.C1396G	p.(L466V)			-	-	-	5.8	27.1	(Lu <i>et al.</i> , 2014)
c.G1450C	p.(G484R)			-	-	-	6.317	29.2	(Orsucci <i>et al.</i> , 2014)
c.A1456C	p.(T486P)			-	-	-	5.515	26.3	-
c.C1474T	p.(L492F)			-	-	-	5.467	26.2	(Alvarez <i>et al.</i> , 2010)
c.G1494T	p.(R498S)			-	-	-	4.565	24.4	(Elert-Dobkowska <i>et al.</i> , 2015)
c.C1495T	p.(R499C)			rs121908511	-	-	7.779	35	(Hazan <i>et al.</i> , 1999)
c.G1496A	p.(R499H)			-	-	-	7.481	34	(Park <i>et al.</i> , 2005)
c.T1499C	p.(F500S)			-	-	-	6.542	31	-
c.C1507T	p.(R503W)			rs864622162	4.08E-06	-	8.177	35	(Depienne <i>et al.</i> , 2005)
c.T1553C	p.(L518P)			-	-	-	6.535	31	-
c.A1625G	p.(D542G)			rs142053576	0.0004584	6.46E-05	4.639	24.5	(Brugman <i>et al.</i> , 2005)
c.C1649T	p.(T550I)			-	-	-	4.555	24.4	(Ivanova <i>et al.</i> , 2006)
c.C1652T	p.(A551V)			-	-	-	5.46	26.1	-
c.G1656C	p.(L552F)			-	-	-	5.385	26	-
c.G1663A	p.(D555N)			-	-	-	7.327	34	(Fonknechten <i>et al.</i> , 2000)
c.C1667T	p.(A556V)			-	-	-	6.699	32	(Fonknechten <i>et al.</i> , 2000)
c.C1667G	p.(A556G)			-	-	-	6.156	28.5	-

Mutation	Amino acid change	Consequence	Transcript	dbSNP	gnomAD_exome_ALL	gnomAD_genome_ALL	CADD_raw	CADD_phred	Reference
c.T1673C	p.(L558P)	Missense	NM_014946	rs869138742	-	-	6.373	29.5	-
c.G1675C	p.(G559R)			-	-	-	6.865	33	(Nanetti <i>et al.</i> , 2012)
c.G1685A	p.(R562Q)			-	-	-	5.862	27.3	(Meijer <i>et al.</i> , 2002)
c.A1735C	p.(N579H)			rs144594804	0.0003665	0.0004196	3.66	23.2	(Depienne <i>et al.</i> , 2005)
c.G1742C	p.(R581P)			-	-	-	4.482	24.2	-
c.A1751T	p.(D584V)			-	-	-	5.194	25.5	(Park <i>et al.</i> , 2005)
c.C1752A	p.(D584E)			-	-	-	4.252	23.9	-
c.A1773C	p.(K591N)			-	-	-	3.052	22.4	-
c.T1775A	p.(I592K)			-	-	-	6.278	29	-
c.A1823T	p.(N608I)			-	-	-	5.617	26.6	-
c.A1838G	p.(D613G)			-	-	-	5.498	26.2	-
c.A1840C	p.(T614P)			-	-	-	4.923	25	-
c.A1843T	p.(T615S)			-	-	-	1.191	11.7	-
c.C1844G	p.(T615S)			rs765941217	4.07E-06	-	1.996	16.19	-
c.415+1G>A	-	Splicing	-	-	-	5.067	25.3	(Depienne <i>et al.</i> , 2005)	
c.415+1G>T	-		-	-	-	4.602	24.4	(Depienne <i>et al.</i> , 2005)	
c.683-2A>G	-		-	-	-	4.697	24.6	(Fonknechten <i>et al.</i> , 2000)	
c.870+3A>G	-		-	-	-	-	-	(Lim <i>et al.</i> , 2010)	
c.1004+2T>G	-		-	-	-	5.083	25.3	(Fonknechten <i>et al.</i> , 2000)	
c.1004+5 G>T	-		-	-	-	-	-	-	
c.1005-2A>T	-		-	-	-	3.807	23.4	-	
c.1098+1G>T	-		-	-	-	4.256	23.9	(Fonknechten <i>et al.</i> , 2000)	
c.1099-1G>T	-		-	-	-	4.803	24.8	-	
c.1173+1G>A	-		-	-	-	5.675	26.7	(Fonknechten <i>et al.</i> , 2000)	
c.1173+1G>T	-		-	-	-	5.44	26.1	-	

Mutation	Amino acid change	Consequence	Transcript	dbSNP	gnomAD_exome_ ALL	gnomAD_genome_ ALL	CADD_ raw	CADD_phred	Reference
c.1245+1G>C	-	Splicing	NM_014946	-	-	-	5.504	26.2	(de Bot <i>et al.</i> , 2010)
c.1245+1G>A	-			-	-	-	5.938	27.6	(McDermott <i>et al.</i> , 2006)
c.1245+1G>T	-			-	-	-	5.594	26.5	(Yabe <i>et al.</i> , 2002)
c.1245+3G>C	-			-	-	-	-	-	-
c.1245+4_1245+5insA	-			-	-	-	-	-	-
c.1245+5G>T	-			-	-	-	-	-	-
c.1245+5G>A	-			-	-	-	-	-	-
c.1246-1G>T	-			-	-	-	5.048	25.2	-
c.1413+1G>T	-			-	-	-	5.351	25.9	(Patrono <i>et al.</i> , 2005)
c.1413+2T>A	-			-	-	-	5.162	25.4	(Chamard <i>et al.</i> , 2016)
c.1413+2T>G	-			-	-	-	5.09	25.3	(Nanetti <i>et al.</i> , 2012)
c.1413+3_1413+6delAAGT	-			-	-	-	-	-	(Lindsey <i>et al.</i> , 2000)
c.1413+5G>A	-			-	-	-	-	-	(Fonknechten <i>et al.</i> , 2000)
c.1414-1G>T	-			-	-	-	5.06	25.2	-
c.1493+18G>T	-			rs189961829	0.0029	0.0021	-	-	-
c.1493+2_1493+5delTAGG	-			-	-	-	-	-	(Chelban <i>et al.</i> , 2017)
c.1494-1G>A	-			-	-	-	5.334	25.8	-
c.1494-1G>C	-			-	-	-	4.946	25	-
c.1494-2A>G	-			-	-	-	4.8	24.8	(Alvarez <i>et al.</i> , 2010)
c.1536+1G>T	-			-	-	-	5.288	25.7	(Fonknechten <i>et al.</i> , 2000)
c.1616+2T>C	-			-	-	-	5.192	25.5	-
c.1616-2T>A	-			-	-	-	-	-	-
c.1617-2A>G	-			-	-	-	5.032	25.2	(Crippa <i>et al.</i> , 2006)
c.1687+1G>A	-			-	-	-	5.749	27	(Fonknechten <i>et al.</i> , 2000)

Mutation	Amino acid change	Consequence	Transcript	dbSNP	gnomAD_exome_ ALL	gnomAD_genome_ ALL	CADD_raw	CADD_phred	Reference
c.1687+2T>C	-	Splicing	NM_014946	-	-	-	5.01	25.1	-
c.1687+5G>C	-			-	-	-	-	-	
c.1688-1G>A	-			-	-	-	5.68	26.7	(Pantakani <i>et al.</i> , 2008)
c.1688-1G>C	-			-	-	-	5.384	26	(Mészárosová <i>et al.</i> , 2016)
c.1728+1G>A	-			-	-	-	5.842	27.3	(Fonknechten <i>et al.</i> , 2000)
c.G1155A	p.(G385G)			rs778305717	0.0000528	0.0000323	-	-	-
c.G1173A	p.(L391L)			-	-	-	-	-	-
c.G1321A	p.(D441N)			-	-	-	6.769	32	(Sauter <i>et al.</i> , 2002)
c.G3A	p.(M1?)			-	-	-	3.178	22.7	-
c.C19T	p.(R7X)	Nonsense		-	-	-	8.799	35	-
c.C84A	p.(C28X)			-	-	-	6.359	29.4	-
c.C131A	p.(S44X)			rs121908515	4.15E-06	-	7.696	35	(Kim <i>et al.</i> , 2014)
c.A139T	p.(K47X)			-	-	-	10.465	36	(Ishiura <i>et al.</i> , 2014)
c.G334T	p.(E112X)			-	-	-	10.825	36	(Hentati <i>et al.</i> , 2000)
c.C364T	p.(Q122X)			-	-	-	11.292	37	-
c.C421T	p.(Q141X)			-	-	-	11.947	38	(Patrono <i>et al.</i> , 2005)
c.G427T	p.(Q143X)			-	-	-	11.938	38	-
c.T447A	p.(Y149X)			-	-	-	10.06	36	(Luo <i>et al.</i> , 2014)
c.G475T	p.(G159X)			-	-	-	12.103	38	-
c.G496T	p.(G166X)			-	-	-	11.319	37	-
c.C577T	p.(Q193X)			-	-	-	13.294	41	(Fonknechten <i>et al.</i> , 2000)
c.A748T	p.(K250X)			-	-	-	12.517	39	(Fonknechten <i>et al.</i> , 2000)
c.703_706delinsT	p.(R235X)			-	-	-	-	-	-
c.716delT	p.(L239X)			-	-	-	-	-	-
c.C734G	p.(S245X)			-	-	-	11.797	38	(Lindsey <i>et al.</i> , 2000)

Mutation	Amino acid change	Consequence	Transcript	dbSNP	gnomAD_exome_ ALL	gnomAD_genome_ ALL	CADD_raw	CADD_phred	Reference
c.C807G	p.(Y269X)	Nonsense	NM_014946	-	-	-	10.389	36	(Fonknechten <i>et al.</i> , 2000)
c.C1039T	p.(Q347X)			-	-	-	12.445	39	(McDermott <i>et al.</i> , 2006)
c.A1162T	p.(K388X)			-	-	-	14.901	48	-
c.G1192T	p.(E398X)			-	-	-	15.781	52	-
c.C1238G	p.(S413X)			-	-	-	13.897	43	-
c.C1245G	p.(Y415X)			-	-	-	9.487	35	-
c.C1291T	p.(R431X)			rs786204126	-	-	12.751	40	(Fonknechten <i>et al.</i> , 2000)
c.1293_1294insT	p.(E432X)			-	-	-	-	-	-
c.1353_1354inv	p.(E452X)			-	-	-	-	-	-
c.1395del	p.(L466X)			-	-	-	-	-	(Fonknechten <i>et al.</i> , 2000)
c.C1417T	p.(Q473X)			-	-	-	14.152	44	(Loureiro <i>et al.</i> , 2009)
c.1473_1477del	p.(L492X)			-	-	-	-	-	-
c.A1555T	p.(Lys519X)			-	-	-	15.588	51	(Loureiro <i>et al.</i> , 2009)
c.1586del	p.(L529X)			-	-	-	-	-	-
c.G1627T	p.(G543X)			-	-	-	-	-	-
c.C1684T	p.(R562X)			-	-	-	11.916	38	(Fonknechten <i>et al.</i> , 2000)
c.C1741T	p.(R581X)			rs778023258	4.07E-06	-	13.627	42	(Patrono <i>et al.</i> , 2005)
c.14_41dup	p.(G15Wfs*42)	Frameshift		-	-	-	-	-	-
c.72_90dup	p.(P31Sfs*23)			-	-	-	-	-	-
c.80_98dup	p.(P34Ls*53)			-	-	-	-	-	(Mészárosová <i>et al.</i> , 2016)
c.167del	p.(P56Rfs*4)			-	-	-	-	-	-
c.283dup	p.(A95Gfs*41)			-	-	-	-	-	-
c.453dup	p.(G152Rfs*3)			-	-	-	-	-	(Fonknechten <i>et al.</i> , 2000)
c.474del	p.(G159Efs*2)			-	-	-	-	-	(Chelban <i>et al.</i> , 2017)
c.474dup	p.(G159Rfs*11)			-	-	-	-	-	-

Mutation	Amino acid change	Consequence	Transcript	dbSNP	gnomAD_exome_ ALL	gnomAD_genome_ ALL	CADD_raw	CADD_phred	Reference
c.523dup	p.(R175Kfs*5)	Frameshift	NM_014946	-	-	-	-	-	-
c.554_560del	p.(L185Wfs*9)			-	-	-	-	-	-
c.703del	p.(R235Efs*4)			-	-	-	-	-	-
c.719del	p.(T240Nfs*14)			-	-	-	-	-	-
c.719_720insAA	p.(H241Nfs*14)			-	-	-	-	-	-
c.752_753del	p.(T251Sfs*14)			-	-	-	-	-	-
c.757dup	p.(M253Nfs*13)			-	-	-	-	-	(Fonknechten <i>et al.</i> , 2000)
c.781del	p.(S261Qfs*18)			-	-	-	-	-	(Fonknechten <i>et al.</i> , 2000)
c.843_846dup	p.(G283Ifs*9)			-	-	-	-	-	(Proukakis <i>et al.</i> , 2011)
c.852_862del	p.(A285Sfs*2)			-	-	-	-	-	(Fonknechten <i>et al.</i> , 2000)
c.883del	p.(T295Qfs*20)			-	-	-	-	-	-
c.884_900del	p.(N296Ffs*8)			-	-	-	-	-	-
c.900dup	p.(P301Yfs*10)			-	-	-	-	-	-
c.936dup	p.(D313Rfs*6)			-	-	-	-	-	(Alvarez <i>et al.</i> , 2010)
c.1003_1004del	p.(N335Wfs*5)			-	-	-	-	-	-
c.1031_1032insG	p.(I344Mfs*23)			-	-	-	-	-	-
c.1036_1045del	p.(G346Wfs*15)			-	-	-	-	-	-
c.1082_1085del	p.(P361Lfs*2)			-	-	-	-	-	-
c.1088dup	p.(R364Efs*3)			-	-	-	-	-	-
c.1132_1143delins ACCCTCCCAG	p.(L378Tfs*15)			-	-	-	-	-	-
c.1160_1169del	p.(Lys388Trpfs*5)			-	-	-	-	-	-
c.1212_1213del	p.(F404Lfs*38)			-	-	-	-	-	-
c.1212_1213dupTT	p.(N405Ifs*3)			-	-	-	-	-	-
c.1215_1219del	p.(N405Kfs*36)			-	-	-	-	-	(Fonknechten <i>et al.</i> , 2000)
c.1231dup	p.(L411Ffs*32)			-	-	-	-	-	-

Mutation	Amino acid change	Consequence	Transcript	dbSNP	gnomAD_exome_ ALL	gnomAD_genome_ ALL	CADD_raw	CADD_phred	Reference
c.1246_1250del	p.(V416Rfs*25)	Frameshift	NM_014946	-	-	-	-	-	-
c.1281del	p.(F427Lfs*11)			-	-	-	-	-	(Lindsey <i>et al.</i> , 2000)
c.1281dupT	p.(A428Cfs*15)			-	-	-	-	-	(Magariello <i>et al.</i> , 2010)
c.1308dup	p.(I437Yfs*6)			-	-	-	-	-	-
c.1312del	p.(I438Ffs*3)			-	-	-	-	-	-
c.1348_1352del	p.(E452Gfs*5)			-	-	-	-	-	(Depienne <i>et al.</i> , 2005)
c.1353_1357del	p.(E452Gfs*5)			-	-	-	-	-	-
c.1377_1378delACinsCTA	p.(R459Sfs*2)			-	-	-	-	-	-
c.1451del	p.(G484Cfs*3)			-	-	-	-	-	(Fonknechten <i>et al.</i> , 2000)
c.1480del	p.(E494Rfs*36)			-	-	-	-	-	-
c.1496del	p.(R499Lfs*31)			-	-	-	-	-	-
c.1510dup	p.(V504Gfs*8)			-	-	-	-	-	-
c.1535_1536del	p.(E512Dfs*7)			-	-	-	-	-	(Chelban <i>et al.</i> , 2017)
c.1559del	p.(N520Ifs*10)			-	-	-	-	-	-
c.1561_1564del	p.(L521Yfs*8)			-	-	-	-	-	(Fonknechten <i>et al.</i> , 2000)
c.1560_1561insTT	p.(L521Ffs*10)			-	-	-	-	-	(Fonknechten <i>et al.</i> , 2000)
c.1575del	p.(G526Efs*4)			-	-	-	-	-	-
c.1616_1616+1insCA	p.(R539Sfs*3)			-	-	-	-	-	-
c.1624_1625ins CCTTCCCTTCCTCAGAATGACTG	p.(D542Afs*7)			-	-	-	-	-	-
c.1626_1627insTG	p.(G543Vfs*8)			-	-	-	-	-	-
c.1670del	p.(A557Dfs*8)			-	-	-	-	-	-
c.1684_1685insTT	p.(R562Ffs*4)			-	-	-	-	-	(Fonknechten <i>et al.</i> , 2000)
c.1699del	p.(E567Nfs*3)			-	-	-	-	-	-
c.1715_1716insC	p.(M572Ifs*5)			-	-	-	-	-	-
c.1738dup	p.(I580Nfs*5)			-	-	-	-	-	-

Mutation	Amino acid change	Consequence	Transcript	dbSNP	gnomAD_exome_ALL	gnomAD_genome_ALL	CADD_raw	CADD_phred	Reference
c.1767_1774delins TAAAAAAAG	p.(L589Ffs*42)	Frameshift	NM_014946	-	-	-	-	-	-
c.1774dup	p.(I592Nfs*39)			-	-	-	-	-	-
c.1775del	p.(I592Kfs*10)			-	-	-	-	-	-
c.1-?_415+?del	ex1	Deletion		-	-	-	-	-	(Depienne <i>et al.</i> , 2007)
c.1-?_682+?del	ex1-4			-	-	-	-	-	(Depienne <i>et al.</i> , 2007)
c.1-?_1536+?del	ex1-13			-	-	-	-	-	-
c.416-?_1098+?del	ex2-7			-	-	-	-	-	-
c.416-?_1851+?del	ex2-17			-	-	-	-	-	(Erichsen <i>et al.</i> , 2007)
c.587-?_1851+?del	ex4-17			-	-	-	-	-	(Depienne <i>et al.</i> , 2007)
c.683-?_870+?del	ex5			-	-	-	-	-	(Erichsen <i>et al.</i> , 2007)
c.683-?_1004+?del	ex5-6			-	-	-	-	-	(Depienne <i>et al.</i> , 2007)
c.683-?_1098+?del	ex5-7			-	-	-	-	-	(C. Beetz <i>et al.</i> , 2006)
c.683-?_1781+?del	ex5-17			-	-	-	-	-	(Park <i>et al.</i> , 2005)
c.871-?_1004+?del	ex6			-	-	-	-	-	(Depienne <i>et al.</i> , 2007)
c.871-?_1098+?del	ex6-7			-	-	-	-	-	(Alvarez <i>et al.</i> , 2010)
c.1099-?_1173+?del	ex8			-	-	-	-	-	(Shoukier <i>et al.</i> , 2009)
c.1099-?_1245+?del	ex8-9			-	-	-	-	-	(Svenstrup <i>et al.</i> , 2009)
c.1099-?_1493+?del	ex8-12			-	-	-	-	-	(Depienne <i>et al.</i> , 2007)
c.1099-?_1851+?del	ex8-17			-	-	-	-	-	(Depienne <i>et al.</i> , 2007)
c.1174-?_1245+?del	ex9			-	-	-	-	-	(Depienne <i>et al.</i> , 2007)
c.1174- ?_1493+ ?del	ex9-12			-	-	-	-	-	(Depienne <i>et al.</i> , 2007)
c.1246-?_1493+?del	ex10-12			-	-	-	-	-	(C Beetz <i>et al.</i> , 2006)

Mutation	Amino acid change	Consequence	Transcript	dbSNP	gnomAD_exome_ALL	gnomAD_genome_ALL	CADD_raw	CADD_phred	Reference	
c.1246-?_1616+?del	ex10-14	Deletion	NM_014946	-	-	-	-	-	-	
c.1246-?_1687+?del	ex10-15			-	-	-	-	-	-	
c.1246-?_1728+?del	ex10-16			-	-	-	-	-	(Depienne <i>et al.</i> , 2007)	
c.1246-?_1851+?del	ex10-17			-	-	-	-	-	(Sulek <i>et al.</i> , 2013)	
c.1494-?_1536+?del	ex13			-	-	-	-	-	(Depienne <i>et al.</i> , 2007)	
c.1494-?_1687+?del	ex13-15			-	-	-	-	-	(Lu <i>et al.</i> , 2014)	
c.1494-?_1728+?del	ex13-16			-	-	-	-	-	(C Beetz <i>et al.</i> , 2006)	
c.1617-?_1687+?del	ex15			-	-	-	-	-	-	
c.1688-?_1728+?del	ex16			-	-	-	-	-	(Depienne <i>et al.</i> , 2007)	
c.1688-?_1851+?del	ex16-17			-	-	-	-	-	(Depienne <i>et al.</i> , 2007)	
c.1729-?_1851+?del	ex17			-	-	-	-	-	(Depienne <i>et al.</i> , 2007)	
c.1-?_1851+?del	spg4			-	-	-	-	-	(Depienne <i>et al.</i> , 2007)	
c.1210_1212delTTT	p.(Phe404del)			-	-	-	-	-	(Park <i>et al.</i> , 2005)	
c.-17_-15dup	-			-	-	-	-	-	-	
c.416-23_419del	-			-	-	-	-	-	-	
c.1209_1211del	p.(F404del)			-	-	-	-	-	-	
c.1257_1268del	p.(E420_V423del)			-	-	-	-	-	-	
c.1441_1446del	p.(L481_V482del)			-	-	-	-	-	-	
c.1661_1666del	p.(K554_A556delinsT)			-	-	-	-	15.244	50	-
c.1691_1702del	p.(L564_E567del)			-	-	-	-	-	-	-
Duplication_ex4-7				-	-	-	-	-	-	
Duplication_ex5-16				-	-	-	-	-	-	
Duplication_ex16				-	-	-	-	-	(Vandebona <i>et al.</i> , 2012)	

Supplementary figures

Figure 1. Survival curve representing disease penetrance as the percentage of affected patients at the same age at examination. The blue curve, representing the penetrance for men, is significantly higher throughout the duration of disease than that representing the penetrance of women (red curve) (Log-rank test, $p = 0.01$).

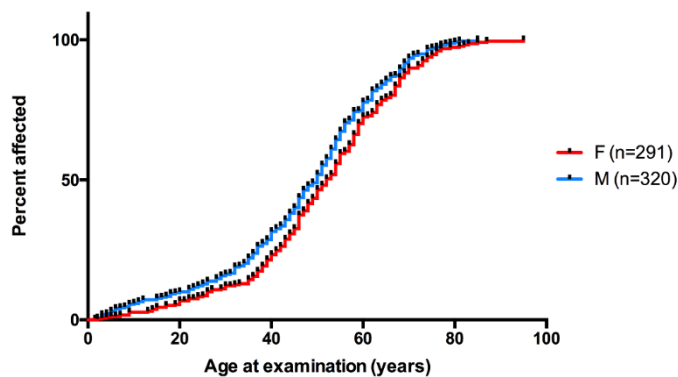
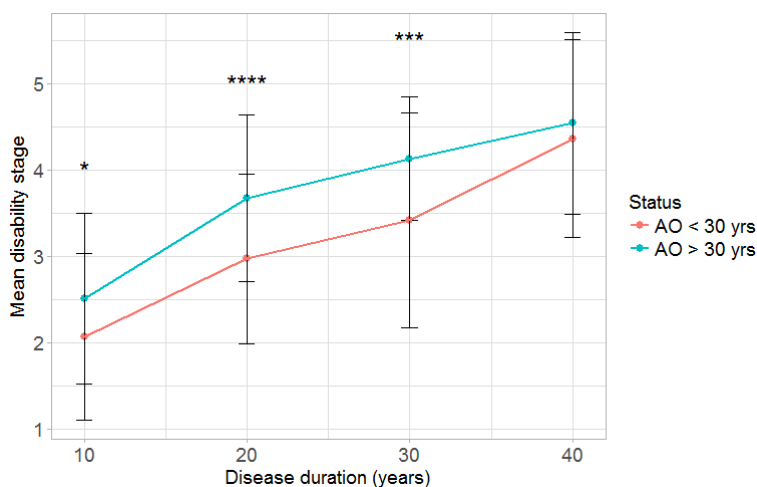


Figure 2. Comparison of disease severity between early and late onset patients. Patients for whom the onset of disease occurred after 30 years of age (blue line) are characterized by a more severe phenotype, determined by the mean of the stage of disability for same duration of disease, than those for whom the onset of disease occurred before 30 years of age (red line) (Mann-Whitney test, $*P = 0.01$, $****P < 0.0001$, $***P = 0.0008$).



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Genome-wide linkage analysis

To dig deeper into age at onset heritability issue, linkage analysis was performed on 37 families, following the transmission of the “early age at onset” trait and therefore comparing 69 early and 40 late onset *SPAST*-HSP patients.

Parametric linkage analysis was first performed setting the disorder frequency at 0.0046, representing the MAF of the known *SPAST* intragenic modifier p.(Ser44Leu), and a dominant inheritance mode.

A unique peak having a LOD score > 2 was detected on chromosome 1 (Figure 10, a), encompassing a 1.250 kb-genomic region (Figure 10, b).

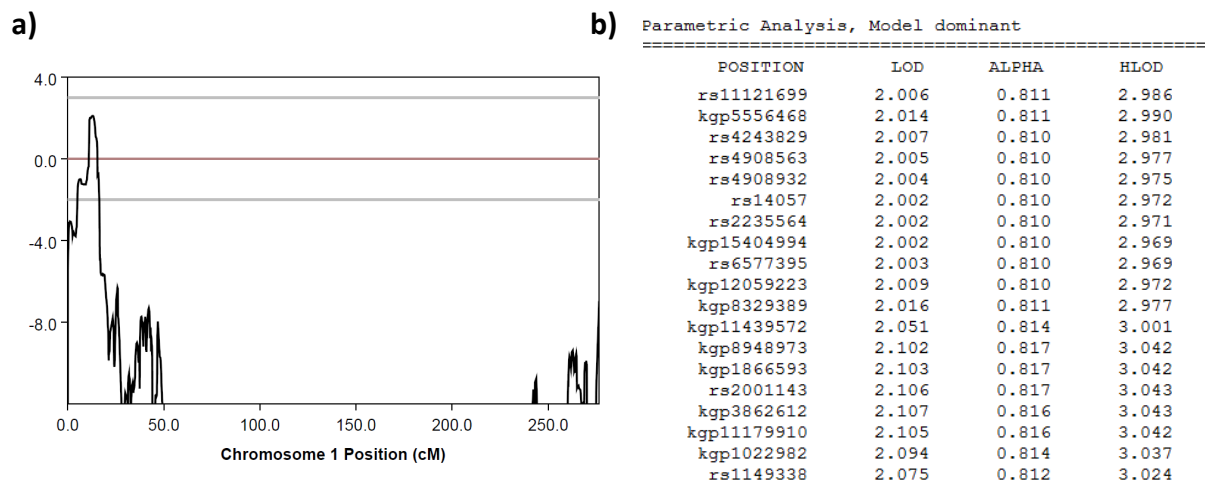


Figure 10. Linkage analysis results using freq = 0.0046. a) Linkage peak having LOD score > 2, on chromosome 1. b) List of markers defining the linkage region.

When increasing the disease frequency at 0.05 no additional spikes were detected while, as expected, the LOD score of the previously detected peak increased, reaching a maximum LOD score of 3.8 (Figure 11, a), significantly proving the presence of genetic linkage. The region encompassed by markers having a LOD score > 3, and therefore in significant linkage, harbours 26 coding genes (Figure 11, b).

Non-parametric analysis was also performed, allowing to reach a LOD score suggestive of linkage in a genomic region overlapping the one detected through parametric linkage

analysis (Figure 12). This finding further confirmed the presence of a region segregating with the trait “early age at onset” on chromosome 1.

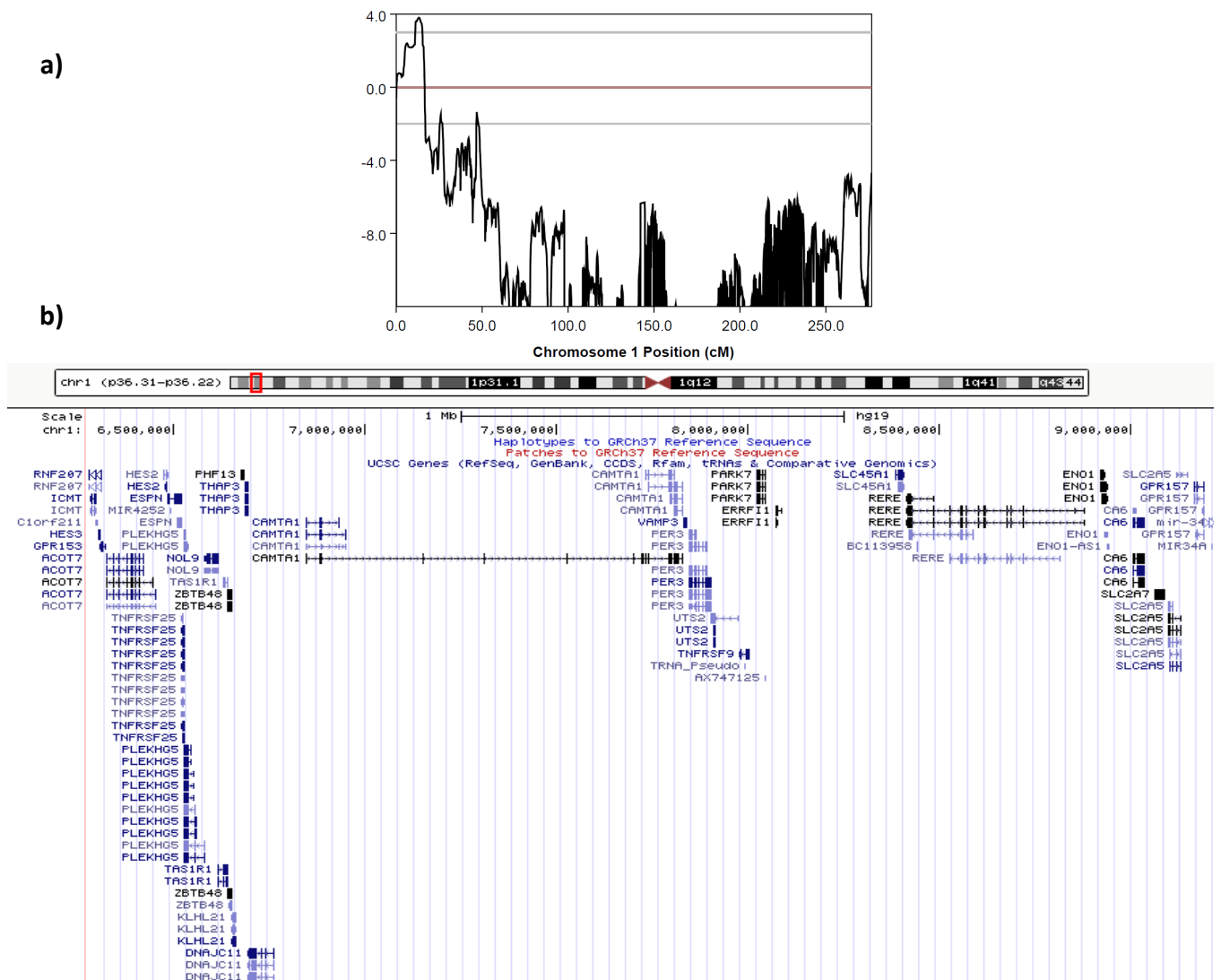


Figure 11. Linkage analysis results using freq = 0.05. a) Linkage peak having LOD score > 2, on chromosome 1. b) List of markers defining the linkage region. c) Genes harbored in the linkage region, as predicted by UCSC genome browser (<https://genome.ucsc.edu>).

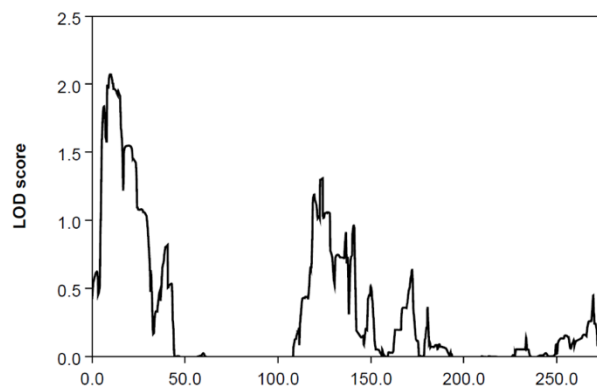


Figure 12. Linkage analysis results using non-parametric settings. A single peak on chromosome 1 reached LOD score > 2, indicative of suggestive linkage. The resulting region overlaps the region detected through parametric linkage analysis.

Genome-wide association analysis

As an alternative strategy to uncover age at onset modifiers, a GWAS analysis was performed using a phenotypic extreme-based design. Early onset patients were therefore compared to late onset patients, allowing to highlight the presence of a group of SNPs suggestively associated to age at onset variations and modifying the expression of *SARS2*, a mitochondrial aminoacyl-tRNA synthetase. The SNPs eQTL role was validated on lymphoblasts of early and late onset patients carrying, respectively, the ALT/ALT or REF/REF genotype. Indeed, as expected, western immunoblot analysis highlighted the presence of higher *SARS2* levels in early onset patients, when compared to those having a late onset, as well as to controls. Moreover, the two *SARS2* homologs, *SeRS2* and *SLIMP*, resulted overexpressed in *Drosophila Dspastin* RNAi and K467R mutant lines.

All the preliminary results obtained until now, as well as results' discussion, are included in the following manuscript, "SARS2 modifies age at onset in Spastic Paraplegia type 4". To conclude on *SARS2* role as *SPAST*-HSP modifier, further validation experiments are currently ongoing. In order to observe a possible phenotype improvement deriving from *SeRS2* down-regulation, *Drosophila SeRS2* RNAi lines are being crossed with *Dspastin* mutants. Both, behavioral tests and mitochondria morphology analyses will be performed. iPSCs-derived neurons are being produced from early and late onset patients carrying the ALT/ALT and REF/REF genotype, therefore allowing to evaluate different potential pathological aspects deriving from *SARS2* differential expression.

SARS2 modifies age at onset in Spastic Paraplegia type 4

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Abstract

The most frequent cause of Hereditary Spastic Paraplegia (HSP) is caused by variants in *SPAST/SPG4* gene. Inherited through an autosomal dominant pattern, *SPAST*-HSP manifests through lower limbs spasticity and deep sensory loss and is characterized by an extreme age at onset variability, observed even among related patients. In addition to the established onset-genotype correlations we aimed at identifying *SPAST*-HSP age of onset genetic modifiers. We submitted 134 *SPAST*-HSP patients characterized by discordant ages at onset to GWAS analysis. Early and late onset patients comparison highlighted the presence of a group of SNPs suggestive for association ($p\text{-value} < 10\text{E-}05$) and acting as eQTLs, dysregulating the expression of *SARS2*, a mitochondrial aminoacyl-tRNA synthetase. Increased *SARS2* protein levels were detected in early onset patients carrying the ALT/ALT genotype for the candidate SNPs, validating their role as eQTLs. *SARS2 Drosophila* homologs, *SeRS2* and *SLIMP*, resulted overexpressed in *Dspastin* Rnai lines and K467R mutants, presenting with abnormal mitochondrial morphology. Thus *SARS2* overexpression leads to earlier age at onset, through possible mitochondrial dysfunction. Therefore, we propose *SARS2* as a new *SPAST*-HSP age at onset modifier.

Introduction

Hereditary Spastic Paraplegias (HSPs) are rare inherited neurodegenerative disorders that arise following progressive corticospinal tracts degeneration, and manifest with progressive lower limbs spasticity, the disease hallmark. A wide heterogeneity characterizes both HSPs genetic and clinical backgrounds, with 79 loci (65 genes) involved and responsible for the great variety of symptoms associated, often in overlap with other neurological disorders¹. Among HSPs causative genes, named Spastic Paraplegia Genes (SPGs), *SPAST/SPG4* is the most

frequently mutated in both familial and sporadic cases². Spastin, the encoded protein, belongs to the AAA ATPases family and operates as a microtubule-severing protein, being therefore responsible for their length, number and motility^{3,4}. To date, more than 260 mutations including missense and truncating mutations (splice site, frameshift, nonsense, deletions and duplications) predominantly impairing spastin enzymatic activity, have been found responsible for *SPAST*-HSP onset⁵.

SPAST-HSP patients present mainly with gait impairment, caused by lower limb spasticity, as well as unsteadiness due to posterior column impairment with deep sensory loss. One third of *SPAST*-HSP patients present with sphincter disturbances and, occasionally, with intellectual deficiency of cognitive impairment⁶. Great inter- and intra-familial variability concerning both age at onset and clinical severity exists among *SPAST*-HSP patients, even among related patients sharing the same causative mutation. Age at onset ranges from birth to the seventh decade, and shows a bimodal distribution, with a distinguished group before age 15⁶. Recently, after retrieving clinical and genetic data of 842 *SPAST*-HSP patients, we were able to show that both, the nature of *SPAST* causative mutation and sex, had an impact on the disorder age of onset⁵. In particular, we observed earlier onset was associated with missense mutations compared to age at onset of truncating mutation carriers, as well a higher proportion of asymptomatic women⁵. Specific mutations were already shown acting as *SPAST*-HSP genetic modifiers: Svenson *et al* identified two adjacent missense variants on *SPAST* exon 1, c.131C>T/p.(Ser44Leu) and c.134C>A/p.(Pro45Glu) that co-segregated independently (*in-trans*) with a major causative mutation. Interestingly, patients carrying both, the causative mutation and the exon 1 variant, were characterized by a lower age at onset, as well as by greater severity⁷. In addition, starting from the hypothesis that deletions could decrease age at onset⁸, Newton *et al* showed that *SPAST* deletions extending into the adjacent genomic

region harbouring *DPY30* gene, acted as age at onset modifiers through an epistatic mechanism due to *DPY30* haploinsufficiency⁹. To identify still unravelled onset modifiers, we performed a GWAS analysis on a cohort of *SPAST*-HSP patients characterized by discordant ages at onset, followed by functional validation in *Drosophila Dspastin* mutants.

Patients and methods

Patients

Patients carrying a *SPAST* pathogenic mutation were selected among the 842 belonging to the *SPAST*-HSP cohort previously recruited at the Pitié-Salpêtrière University Hospital⁵. To identify age at onset modifiers, GWAS analysis was performed comparing either “early onset” patients, having an disorder starting before 15 years old and “late onset” individuals manifesting symptoms after 45 years of age. Both familial and sporadic cases were included. In order to minimize a possible effect caused by the mutation nature⁵, only patients carrying a truncating pathogenic mutation (splice site, frameshift, nonsense, deletion) were selected. Consent forms (RBM 01-29, RBM 03-48) were signed by each participant included in the present study.

Genotyping and quality control

DNA samples belonging to the selected patients were genotyped using the Infinium® Omni2.5Exome-8 v1.3 and v1.4 BeadChip arrays, provided by Illumina (San Diego, CA, USA). Quality control on the resulting genotype data was performed using PLINK version 1.9b6¹⁰, after merging the markers in common between the two arrays’ versions. Samples presenting discordant sex information or elevated missing data rates were excluded from the analysis, as well as uninformative SNPs and variants characterized by low call rates or failing Hardy-

Weinberg equilibrium test. To verify that the selected patients belonged to the same ethnic group, ancestry was predicted performing a PCA analysis using Peddy¹¹. A final number of 1'701'047 SNPs passed the quality controls and were therefore used in the GWAS analysis.

Association analysis

Given its bimodal distribution⁵, age of onset was converted into a binary trait (1 = age of onset ≥ 45 , 2 = age of onset ≤ 15) to run association test. Since the analysed cohort was composed by both familial and sporadic cases, R package Popkin¹² was used to produce a kinship matrix accounting for relatedness on pruned data (--indep-pairwise 50 5 0.2).

A single-variant association analysis (score tests) was performed using a General Linear Mixed Model (GLMM) provided by GMMAT v1.0.3 R-package¹³, after adjusting for sex and relatedness. Manhattan plots and Q-Q plots were realized using the R-package qqman. Variants resulting from the association test were annotated using ANNOVAR¹⁴. RegulomeDB (<http://www.regulomedb.org>) as well as GTEx portal (<https://gtexportal.org>) were used to retrieve information about variants' role as transcription regulators or eQTLs.

Drosophila lines and RT-qPCRs

Drosophila Dspastin mutant lines used in this study were produced through RNA interference and site-directed mutagenesis, as previously reported^{16,17}. *Drosophila Dspastin* RNAi line and *Dspastin* K467R mutants, as well as wild type controls, were used to assess *Slimp* and *Sers2* expression levels, *SARS2 Drosophila* homologs. Total mRNA extracted from brain/total body was used for oligo-dT primed reverse transcription. qPCRs on resulting cDNAs were then performed to assess *Slimp* and *Sers2* expression levels, after housekeeping gene normalization (CG7263).

Immunoblot analysis

Proteins were extracted from Primary Blood Lymphoblasts (PBLs) belonging to late (n = 20) and early (n = 7) onset patients carrying the REF/REF or ALT/ALT genotype for the suggestive SNPs on chromosome 19. Extraction was also performed on healthy individuals (n = 4), used as controls. Lysis buffer (1M Tris-HCl pH 7.5, 5M NaCl, 1% Triton X-100, Halt™ Protease Inhibitor Cocktail 100X (ThermoFisher Scientific)) was used for protein extraction. 30µg of lysate were ran on NuPAGE™ 4-12% Bis-Tris protein gels (Thermo Fisher Scientific). Anti-SARS2 (PA5-31473, Thermo Fisher Scientific) and anti-Actin (ab3280, abcam) antibodies were used for immunoblotting. Protein signals were revealed using SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and ChemiDoc™ Touch Imaging System (Bio-Rad).

Statistical analysis

Means, standard deviations, Mann-Whitney (two-tailed) and t-student tests were computed using GraphPad Prism version 6.0 for Macintosh (GraphPad Software, La Jolla California USA, www.graphpad.com). P-values were considered to be statistically significant when <0.05 .

Results

GWAS analysis on age of onset extremes

The single-variant test performed on the 134 selected patients, including 71 early onset and 63 late onset patients, highlighted the presence of 3 variants reaching $P < 10E-06$ and resulting suggestive for association with an earlier age of onset in the analysed patients (Fig. 1).

Following ANNOVAR annotation, the top-scoring locus ($P < 7.1E-06$), rs11147552 on

chromosome 13, resulted located on LINC00457, a long intergenic non-protein coding RNA. No additional information indicative of any predicted pathogenic effect were available. The second more suggestive SNP ($P < 8.7E-06$), rs10775533, was detected on chromosome 19. This SNP was found to be in linkage disequilibrium ($r^2 > 0.85$) with 5 other SNPs detected in the adjacent genomic region, all characterized by a P-value suggestive for association ($P \leq 3.3E-05$) at single-variant test (Fig. 2, Table 1). Annotation highlighted that this group of SNPs fell into intronic or 3' UTR regions of *NFKB1B*, *CCER2*, *SARS2* and *FBXO17* genes (Table 1). When checking on RegulomeDB for any possible involvement in transcription regulation mechanisms, all resulted located in a transcription factor binding site. In addition, GTEx portal query pointed out a possible role as cis-eQTLs, influencing *SARS2* gene expression. On average, for all the SNPs queried, an increase of 0.4 of the normalized genic expression was predicted being associated to the Alt/Alt allele combination. Moreover, as expected by the setup of the single-variant test, a significative earlier age of onset characterized all the patients carrying the Alt/Alt allele combination for every SNP belonging to the LD-block (Table 2).

The third locus reaching a suggestive P-value ($P < 8.8E-06$), rs7694367 on chromosome 4, was detected in an intergenic region comprised between genes *FSTL5* and *NAF1*. Both RegulomeDB and GTEx queries did not report any information about its possible functional role.

SARS2 protein levels in eQTLs carriers

Primary Blood Lymphoblasts (PBLs) of early and late onset patients, carrying respectively alt/alt or ref/ref alleles for the candidate SNPs, were used to verify SARS2 protein levels and, consequently, to validate their role as eQTLs. Early onset patients resulted characterized by significantly higher SARS2 levels when compared to late onset patients (3.2 ± 1.5 versus $1.2 \pm$

0.5; $p = 0.038$, paired t-test) (Fig. 3a), after normalizing using controls PBLs (Fig. 3b). Those findings resulted being in agreement with the predicted eQTLs effect on *SARS2* gene expression, therefore confirming that in early onset patients carrying the alt/alt allelic combination, *SARS2* was up-regulated, while in late onset patients carrying the ref/ref combination, *SARS2* was down-regulated.

SARS2 homologs expression in *Drosophila Dspastin* mutated lines

RNA expression of *SeRS2* and *SLIMP*, *Drosophila SARS2* homologs, was assessed on *Dspastin* RNAi lines as well as on mutants carrying *Dspastin* K467R missense mutation. *SeRS2* and *SLIMP* resulted up-regulated in *Dspastin* RNAi lines as well as in K467R mutants (Fig.4a, b).

Discussion

Phenotypic variability in genetic diseases is not easy to explain and thought to be due to a combination of genetic and external modifying agents. We report the first GWAS study in *SPAST*-HSP loss of function mutations carriers characterized by discordant age of onset. The analysis allowed us to identify a group of SNPs on chromosome 19, suggestive for association with age of onset and acting as *SARS2* eQTLs. Patients carrying the ALT/ALT allelic combination for the identified SNPs belonged to the early age at onset group, and had increased *SARS2* protein levels in lymphocytes, corresponding to the SNPs eQTL effect. Coding for a seryl-tRNA synthetase, *SARS2* belongs to the Aminoacyl-tRNA Synthetase (ARS) protein family. ARSs are a group of nuclear-encoded proteins responsible for the conjugation of each amino acid to the corresponding tRNA, crucial for proteins' translation process. In addition to the ARS allowing the cytosolic translations, some ARSs are imported from the cytosol into the mitochondria (mt-ARSs), where they contribute to the translation of mitochondrial-encoded

proteins¹⁸. Pathogenic mutations affecting mt-ARSs have already been implicated in the onset of a wide range of disorders, mostly inherited through an autosomal recessive pattern, impairing the correct functioning of different organs and manifesting as encephalopathies, leukodystrophies, cardiomyopathies but also responsible for Perrault and MASA syndromes onset¹⁹. Recently, homozygous mutations in two mt-ARSs, *DARS2* and *FARS2*, have been reported leading to complex forms of HSP^{20,21}. Since belonging to the mt-ARS, *SARS2* is imported into the mitochondrial matrix where it catalyses the attachment of a serine to the correspondent tRNAs, tRNA^{Ser(UGY)} and tRNA^{Ser(UCN)}²². *SARS2* homozygous mutations have been linked to HUPRA syndrome onset, a complex multisystem disorder characterized by progressive renal failure, pulmonary hypertension and tubulopathy²³. Moreover, in a recent case report, Linnankivi *et al* described a patient carrying an homozygous *SARS2* splice site mutation, not presenting with HUPRA syndrome but manifesting an early onset progressive spastic paraparesis²⁴. Since recessive mutations affecting mt-ARSs were mostly reported as leading to protein loss of function, not much is known on pathogenic effects associated to mt-ARSs overexpression, used only to rescue the pathogenic phenotype^{25,26}. Nevertheless, it was possible to observe that overexpression of mitochondrial alanyl-tRNA synthetase, encoded by *AARS2*, led to enhanced mitochondrial protein translation²⁷. To understand possible effects caused by *SARS2* upregulation, we assessed its expression in fly *Dspastin* RNAi lines and K467R mutants. As for human ARSs, two isoforms, *SeRS* and *SeRS2* are responsible for tRNA^{Ser} aminoacylation in *Drosophila* cytoplasm and mitochondria²⁸. When focusing on *SeRS2* functioning, Guitart *et al* identified *SLIMP*, a *SeRS2* paralog that localizes in mitochondria but lacks the enzymatic activity²⁹. It was recently shown that *SeRS2* and *SLIMP* stably interact, forming an heterodimer involved in both protein synthesis and influencing mitochondrial respiration³⁰. Both *SeRS2* and *SLIMP* resulted overexpressed in *Dspastin* mutants, indicating a

possible contribution to the expressed pathogenic phenotype.

SARS2 overexpression could trigger earlier onset in already impaired *SPAST*-HSP cellular background through different mechanisms. An enhanced mitochondrial translation could cause proteins' accumulation, leading to the unbalancing of the mitochondrial matrix protein homeostasis and possibly inducing mitochondrial stress, as already observed in various neurodegenerative disorders³¹. Moreover, it could also lead to an augmented ATP production and correspondent ROS accumulation, potentially leading to oxidative stress, and finally resulting in precipitated cell death³². In alternative, unbalanced *SARS2* levels could have an impact on its proofreading activity, increasing substrates misrecognition and impairing correct protein synthesis, as already observed following ARSs overexpression³³.

A further consequence linked to an increase of tRNA^{Ser} synthesis due to *SARS2* overexpression, could be the depletion of free serine at the mitochondrial matrix. Serine has a crucial role in different aspects of cell survival and proliferation, especially in mitochondria where its presence is fundamental for the correct functioning of serine-derived folate metabolism³⁴. Indeed, serine availability was shown having a strong impact on mitochondrial functioning, its depletion leading to mitochondrial fragmentation³⁵.

Finally, an important aspect is that patients analysed in the present study carry *SPAST* loss of function mutations, already shown to strongly impair mitochondria retrograde axonal transport in iPSCs-derived neurons^{36,37}, essential to bring damaged mitochondria back to the soma for lysosomal degradation³⁸.

In conclusion, in view of all this, it is likely that the presence of stuck mitochondria, damaged by *SARS2* overexpression, could have an impact on the overall neuronal wellness, potentially accelerating neurodegeneration by worsening the mitochondrial retrograde transport and therefore leading to a younger age at onset, making *SARS2* a *SPAST*-HSP age of onset modifier.

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Fig. 1 GWAS analysis on age at onset extremes spastic paraplegia due to loss of function mutations carriers in the *SPAST* gene. Manhattan and quantile-quantile plots resulting from score test performed comparing early (age at onset ≤ 15 years) versus late onset patients (age at onset ≥ 45 years). SNPs rs11147552 on chromosome 13, rs10775533 on chromosome 19 and rs7694367 on chromosome 4 resulted suggestive for association (p -value $< 10E-5$).

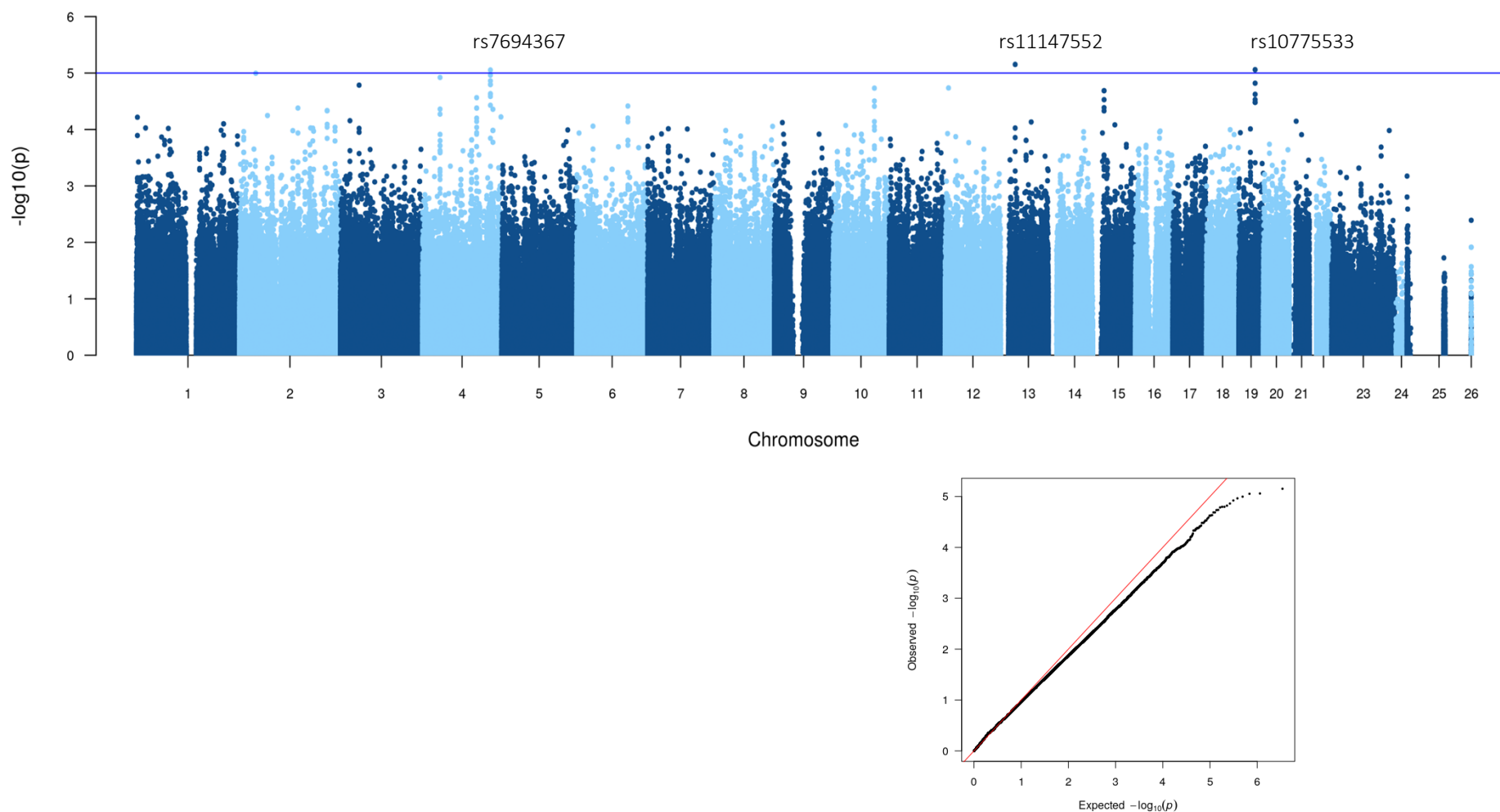
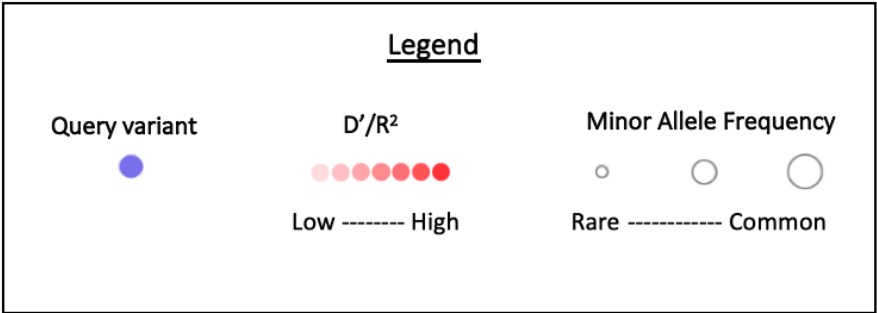
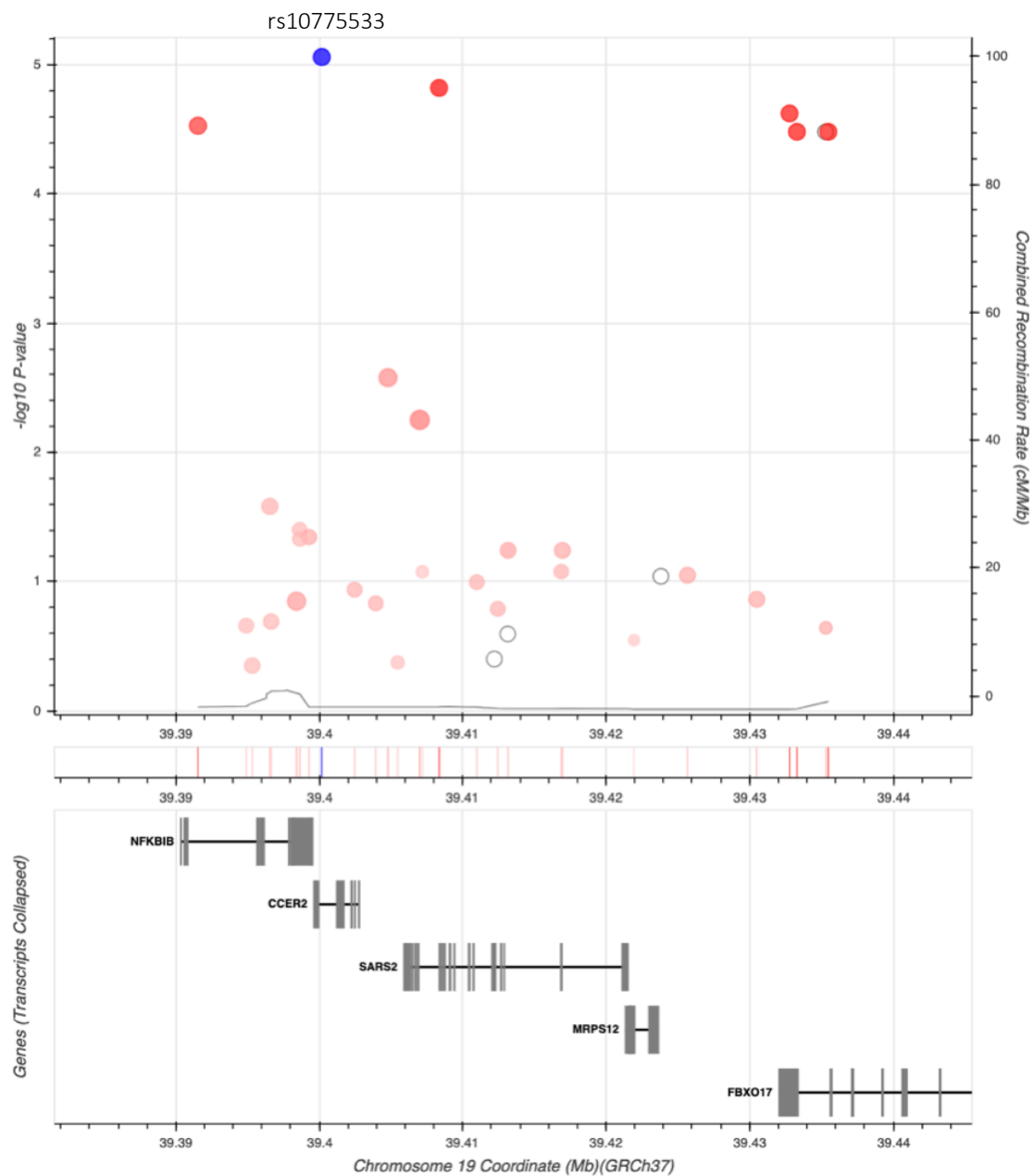


Fig. 2 LDlink regional plot for top SNPs on chromosome 19. The 6 SNPs in linkage disequilibrium with rs10775533 (queried SNP) are represented as well as corresponding gene transcripts.



Chr	Position (bp)	Ref	Alt	rsID	Gene	P-value (score test)	MAF	R ²
19	39400175	G	C	rs10775533	LOC643669	8.7E-06	0.39	1
19	39408360	G	A	rs7508411	SARS2	1.5E-05	0.38	0.98
19	39432783	C	T	rs575	FBXO17	2.4E-05	0.38	0.97
19	39391547	G	A	rs4803006	NFKBIB	3E-05	0.36	0.86
19	39433299	G	A	rs8113389	FBXO17	3.3E-05	0.38	0.97
19	39435485	A	G	rs10416055	FBXO17	3.3E-05	0.38	0.96

Table 1 Suggestive SNPs in LD on chromosome 19. For each SNP, chromosome, detected genomic position, reference and alternative alleles, dbSNP rsID, gene symbol, P-value resulting from the single-variant association test, MAF and R² are reported.

rsID	Genotype	Normalized expression	Average age of onset	P-value
rs10775533	GG	-0.2	42.2 ± 21.6 (n=48)	< 0.0001
	CC	0.44	14.1 ± 20.3 (n=19)	
rs7508411	GG	-0.17	42.2 ± 21.6 (n=48)	< 0.0001
	AA	0.46	14.2 ± 20.9 (n=18)	
rs575	CC	-0.17	41.8 ± 22.1 (n=50)	< 0.0001
	TT	0.47	14.2 ± 20.9 (n=18)	
rs4803006	GG	-0.15	41.3 ± 21.8 (n=52)	0.001
	AA	0.36	15.3 ± 21.7 (n=16)	
rs8113389	GG	-0.17	41.4 ± 22.1 (n=49)	0.0001
	AA	0.47	14.2 ± 20.9 (n=18)	
rs10416055	AA	-0.17	41.4 ± 22.1 (n=49)	0.0001
	GG	0.47	14.2 ± 20.9 (n=18)	

Table 2 Phenotypes associated to the suggestive SNPs. Median of SARS2 normalized expression values (as reported on GTEx portal), average age of onset and P-values resulting from Mann-Whitney test, are reported for the Ref/Ref and Alt/Alt genotypes of each SNP belonging to the LD-group on chromosome 19.

a



b

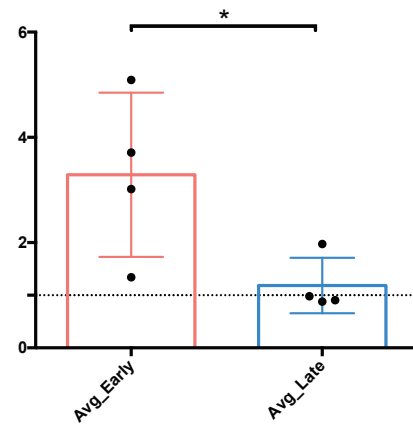


Fig. 3 SARS2 western immunoblots. (a) SARS2 protein signals in early, late onset patients with spastic paraplegia due to loss of function mutations carriers in the *SPAST* gene, as well as in controls. Actin C4 was used as loading control. (b) Western blots quantification; SARS2 protein signals obtained in early, late patients and in controls were first normalized using the corresponding actin signals. Then, average early and late SARS2 signals were normalized on average SARS2 controls signals, and finally compared through a t-test ($p = 0.038$).

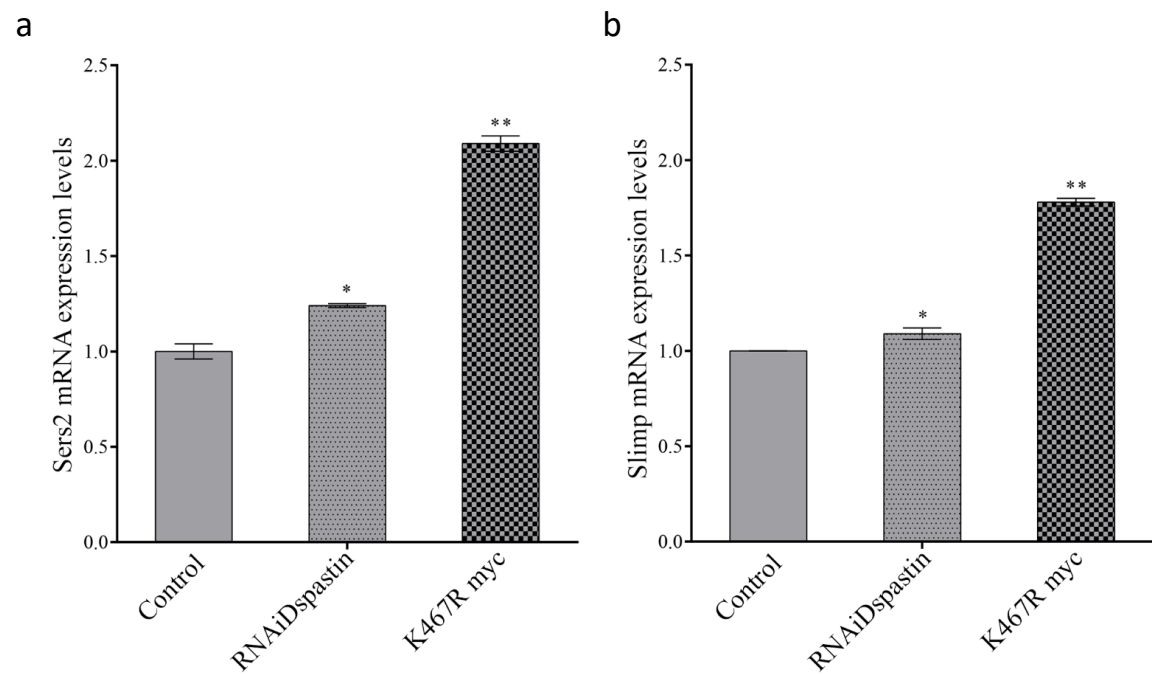


Fig. 4 Expression analysis of *SARS2 Drosophila* homologs, *Sers2* and *Slimp*. mRNA levels were assessed using *Dspastin* RNAi and *Dspastin* K467R mutant lines, as well as in controls. Both *Sers2* (a) and *Slimp* (b) resulted significantly over-expressed in the tested lines, when compared to controls.

Whole Exome Sequencing (WES) analysis

Mostly aiming at unravelling variants responsible for the discordant onset observed especially in parent-sibling pairs, WES analysis was performed on 57 patients, testing different approaches.

A total of 58 variants were left after genes' prioritization (known/candidate HSPs genes, *SPAST* interactors), when focusing on variants shared exclusively among early onset patients and absent in the late onset ones. Only the TOP-5 variants resulting from the analysis (Table 2) were selected to be submitted to Sanger sequencing segregation analysis.

Variant	Chromosome	Position	MAF (gnomAD)	Allele	Gene	Patients											He
1_10397567_A_G (rs2297881)	chr1	10397567	0.03	A/G	KIF1B						FSP-HAV- LER- 619-010		SAL-CAR- 618-014			TUB096 2	3
3_18068587_2_C_T (rs201616183)	chr3	180685872	0.0001	C/T	FXR1	SAL-CAR- 618-20						SAL-CAR- 618-038	SAL-CAR- 618-014				3
12_1233455_09_G_T (rs34149579)	chr12	123345509	0.03	G/T	HIP1R			SAL-PIR- 625-13	SAL-PIR- 625-7	HAV-DES- 669-016					FSP-SAL- ESC- 115-036		4
13_3563293_3_A_G	chr13	35632933	-	A/G	NBEA			SAL-PIR- 625-13	SAL-PIR- 625-7								2
16_8959834_0_C_G	chr16	89598340	-	C/G	SPG7	SAL-MAH- 143-23								FSP-SAL- MAH- 143-022			2

Table 2. WES analysis results. TOP 5 variants resulting from the comparison early vs late. Only heterozygous variants (He) resulted from the analysis.

One variant c.1016C>G/p.(Ala339Gly), detected on SPG7, was identified in two early onset patients belonging to the same family. After Sanger sequencing, this variant resulted carried by the totality of the early onset patients and absent in late onset patients of the family. On the contrary, all the other variants resulting from the analysis did not strictly associate to an earlier phenotype, being detected also in late onset patients or absent in early onset patients.

When looking for the candidate variant having the highest frequency among early onset patients, variant c.1763A>G/p.(Tyr588Cys) on *SHANK2* gene popped up (Table 3).

Variant	Chromosome	Position	Gene	Allele	Patients						He
11_70333498_T_C (rs62622853)	chr11	70333498	SHANK2	T/C	SAL-CAR- 618-20	SAL-DOU- 19-19	SAL-DOU- 19-20	SAL-CAR- 618-038	SAL-CAR- 618-014	FSP-SAL- VOI-015- 005	6

Table 3. WES analysis results. SHANK2 heterozygous (He) variant rs62622853 resulted being the most frequently carried among early onset patients.

When validating its association with early onset in the 3 carrier families, it was possible to highlight that, overall, the variant was not carried by 41% (7/17) of early onset patients. On the contrary, it was present in 25% (3/12) of the late onset patients, and therefore not associated with an earlier age at onset.

The *SPAST* intragenic modifier variant, p.(Ser44Leu) was detected in two patients having a disorder onset at birth. It was absent in all the other family members having a later onset, confirming its strong impact on age at onset (Figure 13, a). In addition, *HSPD1* variant p.(Gly563Ala), was detected in two non-related early onset patients. The segregation analysis showed a weak association between the variant's presence and an age at onset lowering, especially since not carried by early onset patients and vice-versa (Figure 13, b-c).

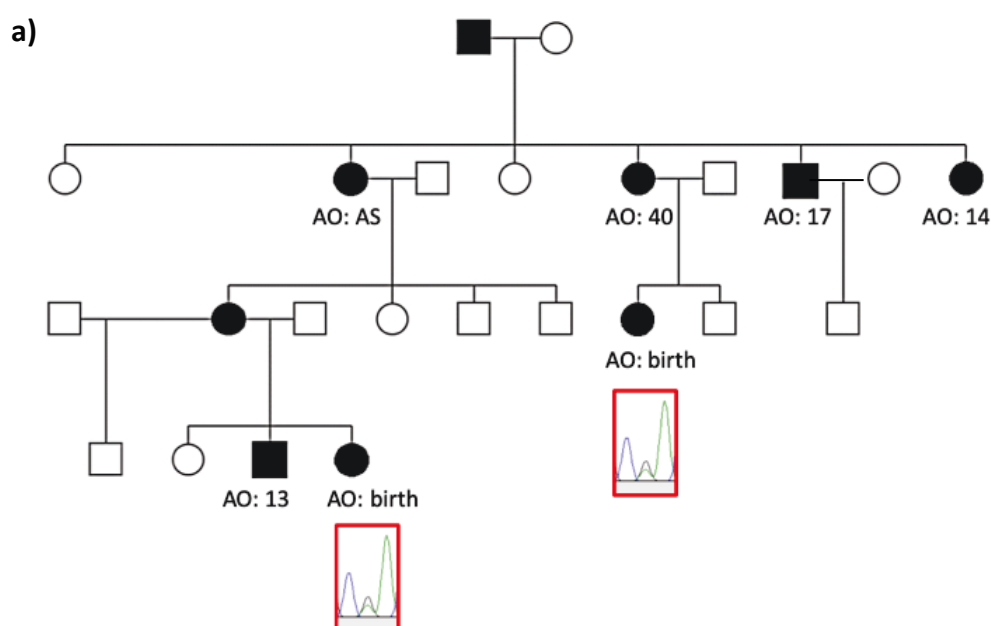


Figure 13. Segregation of *SPAST* p.(Ser44Leu) and *HSPD1* p.(Gly563Ala) candidate modifiers. a) *SPAST* variant p.(Ser44Leu) was carried only by two patients having a disorder onset at birth. (AO: Age of Onset; AS: Asymptomatic at clinical examination)

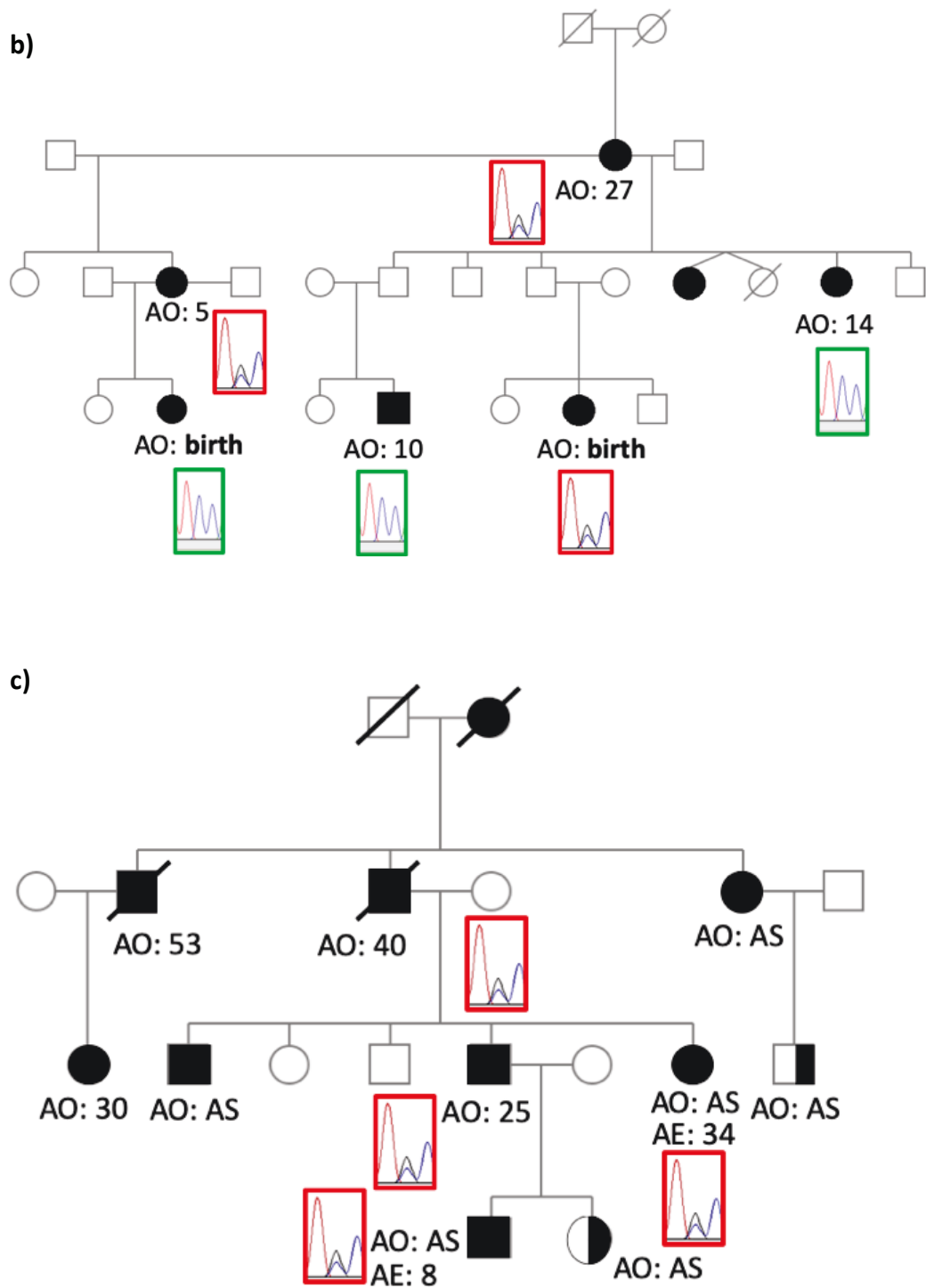


Figure 13. Segregation of *SPAST* p.(Ser44Leu) and *HSPD1* p.(Gly563Ala) candidate modifiers. b,c) *HSPD1* p.(Gly563Ala) variant did not show a strong association with an earlier age at onset. (AO: Age of Onset; AS: Asymptomatic at clinical examination)

RNA-sequencing

To begin, paired analysis was performed to highlight any differential gene expression among early and late onset patients groups.

When defining early and late onset patients on the basis of median age at onset (26 years of age), 6 genes belonging to the analysed categories (known/candidate HSPs genes, *SPAST* interactors) resulted differentially expressed among the 14 analysed pairs. All resulted up-regulated, since being more expressed in early onset patients as regarding to the late ones (Table 4).

Gene Symbol	Regulation	Fold-Change	P-Value	Gene Name	Expression late	Expression early	Category
NUP43	up	2,17	3,61E-03	nucleoporin 43	150,90	341,27	SPAST interactor
DDX17	up	1,97	1,43E-02	DEAD-box helicase 17	6663,36	13678,87	Candidate HSP gene
FBXO41	up	1,79	3,36E-02	F-box protein 41	160,94	297,93	Candidate HSP gene
ARHGEF6	up	1,70	3,04E-02	Rac/Cdc42 guanine nucleotide exchange factor 6	1667,52	2907,87	Candidate HSP gene
C19orf12	up	1,59	1,53E-02	chromosome 19 open reading frame 12	707,76	1136,63	Candidate HSP gene
GBP3	up	1,52	2,68E-02	guanylate binding protein 3	789,77	1214,30	Candidate HSP gene

Table 4. Genes resulting differentially regulated at RNA-sequencing in the analysis early versus late (median age at onset). Results were filtered in order to keep only those belonging to new/candidate HSPs gene, *SPAST*-interacting gene categories.

When setting 10 as early onset threshold, only 18 differentially regulated genes were detected. None of them resulted being part of the HSP background or a spastin interactor.

To establish any effect due to the nature of the *SPAST* causative mutation, the transcriptome of patients carrying a truncating mutation was compared to that of those carrying a missense mutation. The analysis highlighted the presence of only 1 gene, *FLOT1*, resulting upregulated in truncating mutations carriers (Table 5).

Gene Symbol	Regulation	Fold-Change	P-Value	Gene Name	Expression missense	Expression loss of function	Category
FLOT1	up	1,54	3,75E-02	flotillin 1	4471,56	6992,74	SPAST interactors

Table 5. Genes resulting differentially regulated at RNA-sequencing in the analysis truncating versus missense mutation carriers. Results were filtered in order to keep only those belonging to new/candidate HSPs gene, *SPAST*-interacting gene categories.

As expected, one-to-one comparison between the available brain sample with his paired control, resulted in a great amount of up/down-regulated genes belonging to the searched categories. When focusing on genes having the greater fold-change, only new candidate HSP genes were detected (Table 6).

Gene Symbol	Regulation	Fold-Change	P-Value	Gene Name	Expression in CTRL brain	Expression patient's brain	Category
MAP3K11	up	3,08	4,18E-03	mitogen-activated protein kinase kinase kinase 11	821,95	2580,94	Candidate HSP gene
KIF5B	up	2,90	1,18E-02	kinesin family member 5B	12092,40	35747,16	Candidate HSP gene
ENC1	down	59,68	1,02E-06	ectodermal-neural cortex 1	18286,98	218,44	Candidate HSP gene
KCNAB2	down	26,09	4,54E-13	potassium voltage-gated channel subfamily A regulatory beta subunit 2	5598,55	199,35	Candidate HSP gene
DNM1	down	25,80	1,38E-05	dynamins 1	21668,37	693,48	Candidate HSP gene
RTN4RL2	down	16,52	7,09E-05	reticulon 4 receptor-like 2	4581,02	239,64	Candidate HSP gene
CHN1	down	13,21	5,17E-04	chimerin 1	46800,74	3070,83	Candidate HSP gene
MOAP1	down	10,44	7,90E-14	modulator of apoptosis 1	43556,76	4071,82	Candidate HSP gene

Table 6. Genes resulting differentially regulated at RNA-sequencing when comparing samples extracted from one patient and one control brain cortex. Results were filtered in order to keep only those belonging to new/candidate HSPs gene, *SPAST*-interacting gene categories.

Results – part 2: unity is strength!

With the exception of GWAS analysis, that allowed to identify a strong candidate, the other approaches, when considered separately, did not allow to draw any convincing conclusion, leaving us with lists of candidate genes/variants. Thanks to the common design (early and late onset contraposition) shared by all the different methods, it was possible to finally combine all the results obtained in the different phases and time periods of the study. The significative and promising results obtained through linkage analysis were therefore considered as the “starting point”, and were then combined with transcriptome and WES results, as reported in this second part of the Results’ section.

In order to reduce the number of candidate genes detected in the linkage region on chromosome 1, results obtained from the totality of the analyses performed using RNA sequencing data were re-analysed. Any gene belonging to the selected region was found to be up/downregulated when using the paired analysis approach. When comparing normalized expression data of early (age at onset ≤ 20), late (age at onset ≥ 40) and control samples, Kruskal-Wallis tests highlighted the presence of differences in *KLHL21*, *THAP3*, *VAMP3* and *ERRFI1* expression (Figure 14, Table 7), all resulting being overexpressed in late onset patients when compared to those in which the disease started earlier.

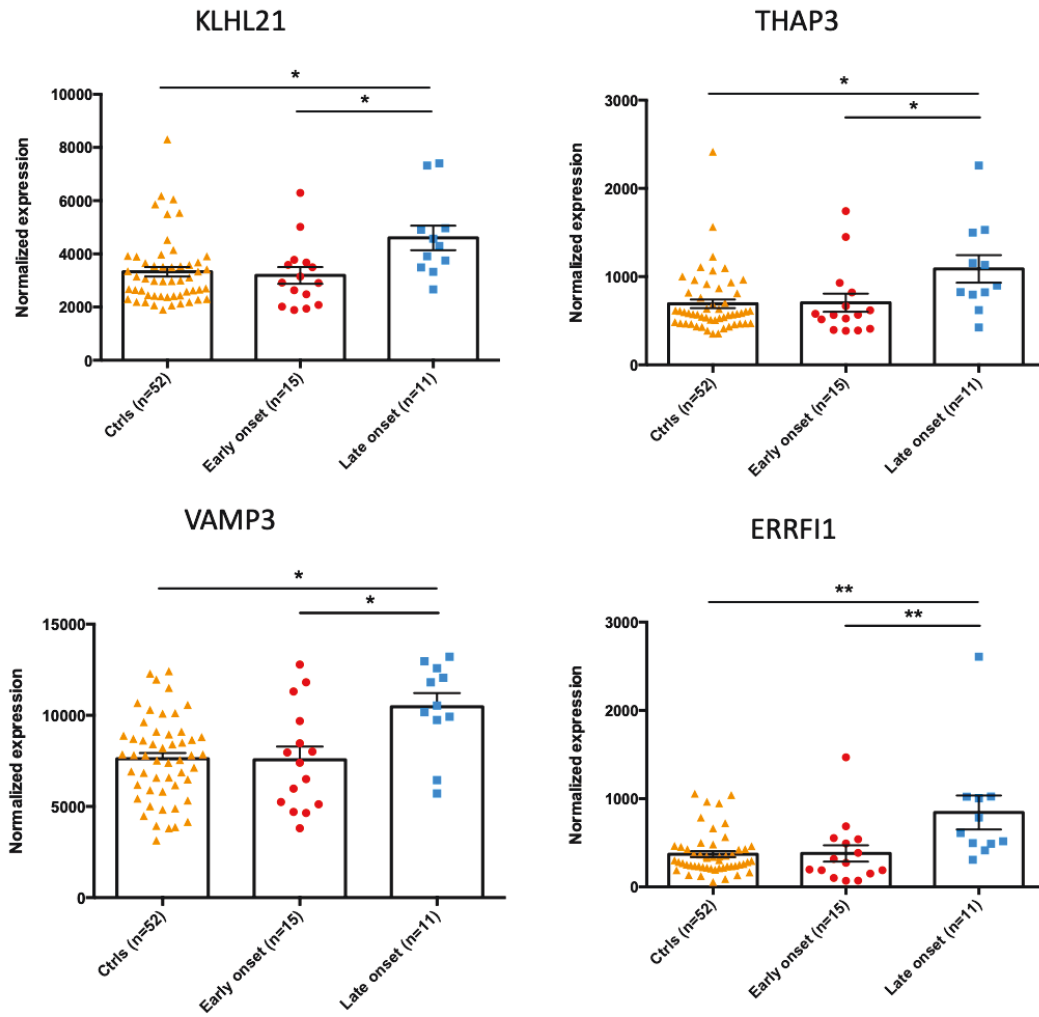


Figure 14. Linkage region genic expression. Expression levels of genes belonging to chromosome 1 linkage region were compared among early, late onset and controls. Kruskal-Wallis test was used to highlight significant differences. Dunn's test correction for multiple testing was then applied, and p-values < 0.05 were considered as significant.

Gene	Comparisons	Mean rank difference	P-value	
KLHL21	Early vs Late	-29	0.02	*
	Early vs Controls	-2.9	0.9	
	Late vs Controls	26.1	0.01	*
THAP3	Early vs Late	27.3	0.04	*
	Early vs Controls	-2.8	1	
	Late vs Controls	24.4	0.03	*
VAMP3	Early vs Late	29	0.02	*
	Early vs Controls	-1.9	0.88	
	Late vs Controls	27.1	0.01	*
ERRFI1	Early vs Late	34.6	0.005	**
	Early vs Controls	-3.9	0.82	
	Late vs Controls	30.7	0.003	**

Table 7. Kruskal-Wallis comparisons results. Gene expression differences among early, late and control samples were observed through rank comparisons. P-values were obtained using Dunn's test, after correcting for multiple comparisons.

To further investigate any possible impact due to the differential regulation of these genes, expression of their interactors was determined using the same analysis setting. Among the 25 proteins predicted interacting with the 4 candidate genes, expression of only two genes interacting with *VAMP3*, *SNAP23* and *STX4*, resulted differ significantly among early and late onset patients, after Kruskal-Wallis and Dunn's tests (Figure 15). In particular, late onset patients resulted having lower *SNAP23* expression levels and higher *STX4* levels, when compared to early onset patients (Table 8).

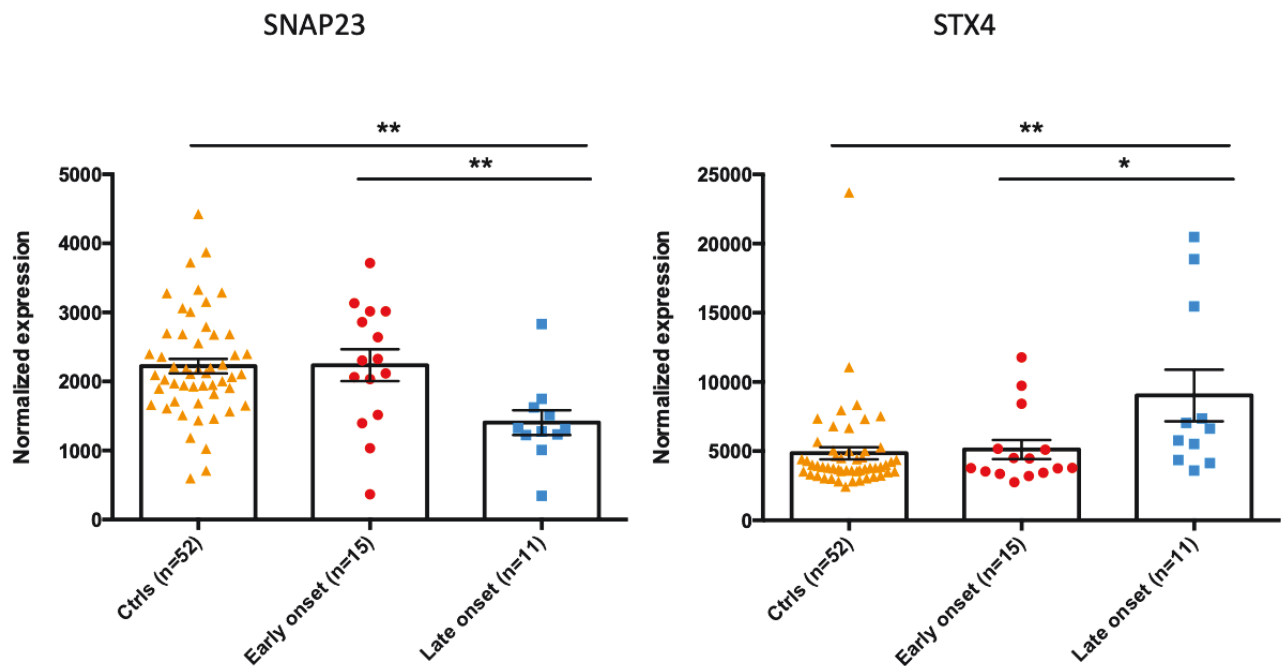


Figure 15. VAMP3 interactors genic expression. Expression levels of genes predicted interacting with VAMP3 was compared among early, late onset and controls. Kruskal-Wallis test was used to highlight significative differences. Dunn's test correction for multiple testing was then applied, and p-values < 0.05 were considered as significative.

Gene	Comparisons	Mean rank difference	P-value	
SNAP23	Early vs Late	-27.2	0.005	**
	Early vs Controls	1.8	0.7	
	Late vs Controls	-25.3	0.002	**
STX4	Early vs Late	20.2	0.04	*
	Early vs Controls	2.4	0.7	
	Late vs Controls	22.6	0.008	**

Table 8. Kruskal-Wallis comparisons results. Gene expression differences among early, late and control samples were observed through rank comparisons. P-values were obtained using Dunn's test, after correcting for multiple comparisons.

Unfortunately, when turning to WES results, looking for SNPs that might be responsible for the observed differential expression, none resulted associated with age at onset fluctuations.

Discussion

In this research work we used different approaches with the sole aim of identifying *SPAST*-HSP age at onset genetic modifiers. To achieve our goal we had to overcome different obstacles, predominantly linked to the nature of *SPAST*-HSP. Even though *SPAST* is the most frequently mutated SPG, it still remains part of a group of extremely rare disorders that account for a global prevalence of 1-5 : 100'000 (Ruano *et al.*, 2014). Since it is well established that, in the majority of cases, the most reliable and truthful results come from the analysis of great amount of patients (Lee *et al.*, 2015, 2017; Moss *et al.*, 2017; Pottier *et al.*, 2018), our first challenge consisted in the assembling of a *SPAST*-HSP cohort large enough to serve our goal. This first step was made much easier by the fact that the Pitié-Salpêtrière University Hospital and its Genetic Department's diagnostic unit are a National reference centre for rare disorders, such as HSPs. Moreover, we had access to SPATAX, a national database started in 1993 and organized in an electronic database in 2000, recently updated as REDCAP in 2016, containing both genetic and clinical data for different HSPs and SCAs. After retrieving information from all the available sources, online database or paper files from Hospital's archives, we were able to build a cohort of 842 *SPAST*-HSP patients. The cohort assembly and characterization resulted fundamental for two main reasons: first, it allowed to recapitulate the total number of *SPAST*-HSP patients in terms of samples availability (e.g. DNA, PBLs and brain samples), and therefore ready to be sequenced, as planned in the next steps of the study. Second, its characterization allowed to make assumptions that were essential for the design of the strategy used to achieve our objective. Indeed, the analysis of what resulted being the world's biggest *SPAST*-HSP cohort enabled us to definitely confirm some features typical of the disorder, that had previously been

proposed but never verified due to lack of patients, as well as to make new assumptions. We confirmed that *SPAST*-HSP age at onset distribution is characterized by a bimodal trend, as already observed (de Bot *et al.*, 2010), but at the same time we managed to explain the underlying cause, identifying for the first time a clear genotype-phenotype correlation. The observation that early onset patients predominantly carried missense mutations, while late onset patients mostly carried truncating mutations, clearly indicated that *SPAST* mutations' nature had an impact on age at onset, possibly due to a different mechanism underlying the pathogenic outcome. Moreover, we observed that women were significantly less affected than men throughout the entire disorder duration. Surprisingly, they resulted more severely affected than men once the disorder had started, probably implying the existence of some protective factors for onset but unable to act against its severity once the disorder arisen.

These observations were the starting point for the search of additional *SPAST*-HSP age at onset modifiers, using both a familial and an extreme-based approach. Thus, we selected patients and families characterized by extremely discordant age at onset, and analysed them using different -omics technologies. Early and late onset patients were therefore submitted to Whole Genome Genotyping, Whole Exome Sequencing and RNA sequencing. The decision to use such a variety of approaches was mainly due to the fact that we wanted to investigate the presence of genetic modifiers at multiple levels, and at the same time, through the combination of different strategies, we would be able to compensate for the sometimes limited amount of patients included.

Among the three sub-cohorts of patients (genotyped, WES, RNAseq), the one submitted to genotyping was the larger one, consequently allowing to draw the most robust conclusions. Indeed, both GWAS and linkage analysis allowed to identify genomic

variants/regions potentially involved in age at onset variations. As it often happens in science, these unexpected final outcomes were the results of countless (and mostly failed) attempts, and a bit of luck. GWAS analysis was the most challenging obstacle to overcome for different reasons. The vast majority of GWAS tests give their best performances when analysing quantitative traits in case-control study assets (Diabetes Genetics Initiative of Broad Institute of Harvard and MIT, Lund University, and Novartis Institutes of BioMedical Research *et al.*, 2007; Hunter *et al.*, 2007; Sladek *et al.*, 2007; Wellcome Trust Case Control Consortium, 2007; Yeager *et al.*, 2007; Imielinski *et al.*, 2009; Repapi *et al.*, 2010). In our case, given the bimodal distribution of *SPAST*-HSP age at onset, we were forced to run our GWAS analysis on a binary trait. In addition, the analysis should have fit to a cohort of related patients, simultaneously allowing to take into consideration covariables, such as patients' sex. Moreover, as discussed above, great statistical power derives from great amount of patients, a condition hard to achieve in rare diseases such as *SPAST*-HSPs, and possibly preventing from reaching significant results. After trying out a great variety of tests not totally suitable for our needs, we finally found GMMAT, a package released in January 2019, that perfectly adapted to our demands, allowing to perform single-variant association analyses on binary traits, while taking in consideration covariables such as patients' relatedness and sex. Against our expectations, the analysis allowed the identification of a group of SNPs acting as *SARS2* eQTLs and associated to age at onset variations. Since already included into HSPs causative genes background, we decided to investigate on how *SARS2* dysregulation could act as *SPAST*-HSP modifier. We confirmed the SNPs eQTL role at the protein level, comparing *SARS2* levels in lymphoblasts of early and late onset patients, and highlighting an higher *SARS2* amount in the early onset group. We furthermore

checked the expression levels of *SARS2* homologs, *SeRS2* and *SLIMP*, in *Drosophila Dspastin* RNAi lines and K467R mutants, observing its overexpression in both lines. Overall, even though multiple elements converge in identifying *SARS2* expression dysregulation as an additional mechanism explaining *SPAST*-HSP age at onset variations, further experiments are needed to definitely conclude on its age at onset modifier effect.

Since the initial input that started this project derived from observations concerning the extreme age at onset differences among *SPAST*-HSP carrier families, we took advantage of genotyping data availability to perform a family-based linkage analysis. We therefore included 37 large families, selected amongst those in which the greatest age at onset variability was observed. Linkage analysis using both a parametric or non-parametric strategy, highlighted a unique peak on chromosome 1 harbouring 26 potential candidate genes. To reduce the candidate genes list, we combined the results obtained through WES and RNA sequencing. Among the two approaches, results deriving from the analysis of RNA sequencing normalized raw data allowed to make the most interesting assumptions. Among the 26 genes harboured in the chromosome 1 linkage peak, only four resulted differentially expressed among early and late onset patients, drastically reducing the candidates list. We then extended the analysis to interactors, since considering the possibility that the effect of a genic dysregulation might have an impact on other proteins belonging to the same pathways. The expression of only two *VAMP3* interactors, *SNAP23* and *STX4*, resulted different among early and late onset patients. *VAMP3*, *SNAP23* and *STX4* code for proteins that belong to the SNARE complex family, fundamental components of the membrane fusion machinery. Among other things, SNARE proteins are involved in synaptic transmission, being responsible for synaptic

vesicles and membrane fusion, and more generally in the cellular exocytic processes (Han *et al.*, 2017). To allow membranes fusion, vesicular-associated R-SNAREs, such as VAMP3, assemble with target membrane Q-SNAREs, for instance SNAP23 and STX4, therefore promoting fusion through membranes rapprochement (Fasshauer *et al.*, 1998; Feldmann *et al.*, 2011). When focusing on SNARE complexes mediating myelin membrane traffic in oligodendrocytes, Feldmann *et al* identified the VAMP3-STX4-SNAP23 interaction as responsible for delivering to the cell surface the major myelin ProteoLipid Protein (PLP). More specifically, VAMP3 allowed fusion of PLP-containing vesicles from the endoplasmic reticulum to the oligodendrocyte plasma membrane where fusion took place, following the interaction with STX4 and SNAP23 (Feldmann *et al.*, 2011). PLP is the most abundant protein in CNS myelin sheets, where it is essential to maintain both myelin structural and functional integrity (Greer and Pender, 2008). Extensive myelination is crucial to keep neurons undamaged and healthy, the link between neurodegeneration and demyelination being already well established (Ettle *et al.*, 2016). Indeed, several studies on different neurodegenerative disorders, such as Multiple Sclerosis, AD and HD (De Stefano *et al.*, 1998; Bartzokis, 2004; Bartzokis *et al.*, 2007), showed the impact of oligodendroglial and myelin dysfunction on axonal and neuronal neurodegeneration, pointing out the fundamental role of myelin regeneration on neuronal survival (Wilkins *et al.*, 2010; Ettle *et al.*, 2016). In view of all this, *VAMP3* and *STX4* overexpression, as observed in late onset patients, could therefore be indicative of an increased myelination process protecting against neurodegeneration, and manifesting as a delay in terms of disorder onset.

Unlike *VAMP3* and *STX4*, both up-regulated, *SNAP23* resulted down-regulated in late onset patients. Besides being part of a SNARE complex, SNAP23 was shown being a

general regulator of membrane protein recycling, as well as a regulator of somatodendritic surface expression and membrane recycling of N-Methyl-D-Aspartate receptors (NMDAR)(Suh *et al.*, 2010). NMDARs number and activity have already been shown to be crucial, especially at extrasynaptic levels where increased NMDAR activity reduces neuronal survival (Hardingham *et al.*, 2002). Modifications of membrane recycling process or an impact on NMDARs membrane distribution could therefore be linked to *SNAP23* downregulation. It is important to underline that RNA sequencing was performed on patients' lymphoblasts and that, consequently, additional experiments need to be performed in order to confirm the hypothesis. Even in this case, *Drosophila* models could be helpful to observe any potential delay in the resulting phenotype onset, in presence of an up/down-regulation of VAMP3 and STX4 homologs. As already reported (Kerman *et al.*, 2015; Djelloul *et al.*, 2017), co-cultures of iPSCs-derived neurons and oligodendrocytes could be extremely useful to observe any impact on myelin formation due to SNAREs dysregulation. In addition, *VAMP3*, *STX4* and *SNAP23* 3'-UTR and promoter regions, as well as SNPs known to act as eQTLs could be sequenced, in order to highlight the presence of any variant potentially causing the observed changes in expression.

Surprisingly, association and linkage analysis allowed to identify two mechanisms potentially influencing *SPAST*-HSP onset, earlier after enhancing cellular stress, or later through increased myelination/slower transmission.

The fact that GWAS and linkage analyses results did not converge shall not alarm. First, the two analyses differed in terms of patients included, with GWAS analysis including both familial and sporadic cases and sharing only 40% (55/134) patients with linkage

analysis. Besides the difference due to the distinct patients' composition of each cohort, it should be highlighted the fact that linkage analysis and GWAS approached the same question differently. Linkage analysis is particularly reliable when studying high penetrant, rare phenotypes, segregating in families. We presumed that modifier transmission occurred either on the same chromosome or was frequent enough to happen at several instances. Linkage outputs a LOD score, that is the probability that a recombination event could happen by chance, not discriminating among affected and unaffected patients at a single SNPs level and therefore allowing to highlight genomic regions often containing dozens of candidate genes. On the contrary, GWAS analyses perform better in finding the association of low penetrant, frequent traits in a given population, and highlight the presence of SNPs associated to the analysed trait. When compared to GWAS, linkage is therefore less precise and powerful in the identification of multiple loci with modest contribution (Altmüller *et al.*, 2001). Moreover, signals indicative of association but falling outside a linkage region, have already proven to be truthful, and probably due to a lack of power in uncovering linkage in the surrounding region (Kitsios and Zintzaras, 2009). This could be our case, indeed, when focusing on patients submitted to both linkage and GWAS analyses, only six patients (2 early and 4 late onset) analysed using both approaches, resulted carrying *SARS2* variants, definitely not enough to be detected through a linkage analysis approach.

As already discussed, the number of patients analysed strongly influenced the possibility of concluding on the obtained results and that was, probably, the main reason why RNA sequencing and WES, if taken separately, were not so helpful. Beside the limited number of patients included, when focusing on RNA sequencing additional remarks should be made on the analysis settings. To identify genes that could characterize in terms of

expression the early and late onset patients groups, a paired analysis was performed. The analysis compared genes' expression first, within pair couples, and after on the overall pairs, implying that a specific gene resulted up/down-regulated only when retained after multiple comparisons. This allowed to highlight the main genes whose expression strongly differed among the various category considered. Through these settings, we aimed at the identification of the "ideal" candidate gene: strongly differently expressed in all the patients belonging to the same group. Thus, it is inevitable that a potential candidate, not shared among all grouped patients or not showing the same expression pattern among the different pairs, could have been missed. As for the other approaches, an increased number of patients included in the analysis could therefore be helpful to refine the analysis, giving the right "weight" to the detected genes and allowing to have a more complete overview on overall genes expression. Apart from all these considerations, RNA sequencing raw expression data were crucial to reduce the list of candidates resulting from linkage analysis, resulting in the identification of an additional potential *SPAST*-HSP modifying effect.

Unfortunately, the same cannot be said for WES, with the exception of SPG7 variant p.(Ala339Gly), that is absent from public databases and that resulted associated to an earlier onset in two carrier siblings. Replication will be crucial to conclude on its role as age of onset modifier. Nevertheless, it is important to underline that genotype-phenotype correlations have already been reported in SPG7-HSPs. The most frequent variant, p.(Ala510Val), when carried in a heterozygous state, resulted associated to a delayed disorder's onset, cerebellar ataxia at onset and a less complicated phenotype (less pronounced pyramidal syndrome) (Coarelli *et al*, 2019). Furthermore, additional variants such as p.(Leu78*) and p.(Arg485_Glu487del), were reported with a suggestion

of dominant inheritance (Sanchez-Ferrero *et al*, 2012; Coarelli *et al*, 2019) As for RNA sequencing, even for WES a larger number of patients included might have allowed to have more impact in the analysis. Indeed, association analyses on WES-deriving data have already allowed to identify genetic modifiers (Emond *et al.*, 2012, 2015; Sadovnick *et al.*, 2017). Even if tested (data not shown), burden tests and association analyses on our WES data did not allow to identify any suggestive candidate, probably due to a lack of power. Even when crossed with results deriving from the other approaches used, WES did not allow to identify any SNP associated to age at onset variations. This could be due to a limitation of the technique itself, since WES only partially covers UTRs regions and does not allow to detect variants harboured in intergenic regions, potentially acting as eQTLs and influencing genic expression of candidate genes. Speaking of NGS approaches potentially filling these gaps, Whole Genome Sequencing could be the perfect alternative, even if still excessively expensive.

Conclusions and perspectives

The identification of modifying factors is an extremely challenging, and at the same time fascinating, process that allowed to refine our knowledge about specific features characterizing various disorders. Success was achieved through the analysis of large cohort of patients, often resulting from the formation of multicentric consortia and consequent data sharing. Mainly due to lack of patients, modifiers identification in rare disorders is an even harder challenge. Nevertheless, our efforts prove that it is not impossible and that the lack of patients can be overcome through the combination of different approaches.

We obtained encouraging preliminary results supporting the genes/variants identified, but a lot still needs to be done in order to complete the validation process.

Results obtained through *Drosophila* lines testing, coupled to mitochondria morphology analysis, will be crucial for the validation of SARS2 impact on age at onset. Moreover, iPSCs-derived neurons are being produced, starting from lymphoblasts of patients carrying the SARS2 ALT/ALT or REF/REF genotype. To begin, impairments to a correct mitochondrial functioning (e.g. respiration, transport) will be checked.

Behavioural tests using *Drosophila* mutants overexpressing *VAMP3* and *STX4* homologs, as well as co-cultures of iPSCs-derived neurons and oligodendrocytes, will be useful to observe any changes due to a potential hypermyelination. Collaborations with the ICM teams “Myelin Plasticity and Regeneration” led by Drs Nait Oumesmar and Zujovic, and “Repair in Multiple Sclerosis: from biology to clinical translation” led by Profs Lubetzki and Stankoff, could definitely allow us to develop both molecular and clinical strategies to validate our hypothesis.

It is quite clear that, the possibility of replicating our results in other patient cohorts, would strongly empower our observations, conclusively confirming our candidate modifiers’ role. In this regard, collaborations with other clinical groups are envisioned through the TransNational HSP group, that brings together all the European members of SPATAX network.

In addition, to investigate the presence of environmental modifiers, we distributed to HSP patients a questionnaire. To date, 328 responses have been collected. They include 192 *SPAST*-HSP patient reports and the analysis, in collaboration with Dr. Sophie Tezenas du Montcel, will allow us to point out ameliorating and worsening factors characterizing

SPAST-HSP patients, potentially helping us in shedding a new light on additional aspects underlying *SPAST*-HSP age at onset variability.



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Motor neuron diseases

Hereditary spastic paraplegia: More than an upper motor neuron disease



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ABSTRACT

Hereditary spastic paraplegias (HSPs) are a group of rare inherited neurological diseases characterized by extreme heterogeneity in both their clinical manifestations and genetic backgrounds. Based on symptoms, HSPs can be divided into pure forms, presenting with pyramidal signs leading to lower-limb spasticity, and complex forms, when additional neurological or extraneurological symptoms are detected. The clinical diversity of HSPs partially reflects their underlying genetic backgrounds. To date, 76 loci and 58 corresponding genes [spastic paraplegia genes (SPGs)] have been linked to HSPs. The genetic diagnosis is further complicated by the fact that causative mutations of HSP can be inherited through all possible modes of transmission (autosomal-dominant and -recessive, X-linked, maternal), with some genes showing multiple inheritance patterns. The pathogenic mutations of SPGs primarily lead to progressive degeneration of the upper motor neurons (UMNs) comprising corticospinal tracts. However, it is possible to observe lower-limb muscle atrophy and fasciculations on clinical examination that are clear signs of lower motor neuron (LMN) involvement. The purpose of this review is to classify HSPs based on their degree of motor neuron involvement, distinguishing forms in which only UMNs are affected from those involving both UMN and LMN degeneration, and to describe their differential diagnosis from diseases such as amyotrophic lateral sclerosis.

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1. Introduction

Hereditary spastic paraplegias (HSPs) were first described in the late 1800s by German neurologist Adolf Strümpell through observations of degeneration of spinal cord nerve fibers in two

brothers presenting with gait disorders and spasticity in the lower limbs.

The latest estimate of the global prevalence of HSPs is 1–5/100,000 population, depending on the country [1], although there is still no information on its incidence in large parts of the world. HSPs refer to a very heterogeneous group of

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diseases, and the literature on HSPs highlights the extreme complexity that characterizes them, including both the observable set of clinical features in affected patients and their underlying genetic features.

The neurodegeneration that characterizes HSP patients is the result of a progressive distal axonopathy that mainly involves the corticospinal tracts, leading to spasticity of the lower limbs when walking, the hallmark of the disease. Moreover, a wide range of neurological and extraneurological features can be manifested by HSP patients that sometimes overlap with those of other diseases.

A high degree of genetic diversity underlies the observed phenotypic heterogeneity, with more than 70 loci and 50 genes involved in the onset of HSPs that can be inherited through autosomal-dominant and -recessive, X-linked and maternal modes of transmission [2].

No treatment is yet available to prevent or slow the neural degeneration. Drug therapy to alleviate spasticity, coupled with physiotherapy and rehabilitation, is therefore the only current strategy to ameliorate patients' quality of life (QoL).

2. Clinical classification and diagnosis

HSPs were initially classified into two groups, pure and complex (complicated), based on the clinical phenotype. In pure HSP forms, pyramidal signs predominantly affect the lower limbs, causing spasticity, weakness and, in some cases, sphincter disturbances [3]. The major features that define this pure form on neurological examination include increased lower-limb muscle tone (especially in the hamstrings, quadriceps, gastrocnemius-soleus and adductors) and weakness (in the iliopsoas, hamstrings and tibialis anterior), as well as hyperreflexia, extensor plantar responses and attenuated vibratory sensation in the ankles. Spasticity, usually more prominent when walking than at rest, allows the distinction between HSPs and multiple sclerosis.

Additional neurological symptoms define the complex forms of HSP, especially spastic ataxia, characterized by the association of cerebellar ataxia and dysarthria with core HSP symptoms. The presence of dystonia or other extrapyramidal features, such as cognitive disability and/or deterioration, optic atrophy, cataract and hearing impairment, among many other symptoms, are responsible for the wide clinical heterogeneity of these forms of the disease.

Furthermore, both the age at onset and disease progression are extremely variable among HSP patients, even among those with the same genetic background. High intrafamilial variability is often observed: mutation carriers may experience early onset and rapid progression or be asymptomatic, suggesting the influence of as yet unidentified modifying factors.

Because of the clinical overlap of HSPs with other neurological diseases, clinical diagnosis is sometimes difficult. The association of gait spasticity with other neurological signs, a positive familial history and ancillary tests, such as brain and spinal cord magnetic resonance imaging (MRI), electromyography (EMG), nerve conduction studies and ophthalmological examination, are therefore crucial for correct patient classification.

Cerebrospinal fluid (CSF) analysis may also be performed to differentially diagnose HSPs from multiple sclerosis or to detect the presence of human T-cell leukemia virus (HTLV)-1, responsible for tropical spastic paraparesis. In addition, specific plasma biomarker concentrations can be measured to support the diagnosis of some HSPs or HSP-related forms. These include increased levels of very long-chain fatty acids (VLCFA) in adrenoleukodystrophy (resulting from ABCD1 gene mutations) and cholestanol in cerebrotendinous xanthomatosis (due to CYP27A1 mutations), as well as 25- and 27-hydroxycholesterol in the spastic paraplegia type 5 (SPG5) gene (resulting from CYP7B1 mutations).

3. Genetics of HSPs

Linkage analysis was the first strategy to allow the identification of genomic regions harboring causative genes of HSPs. The subsequent introduction of next-generation sequencing (NGS) revolutionized the genetic diagnosis of HSPs: the combination of NGS and the use of screening panels of genes involved in HSPs, or allelic diseases, greatly increased the power of genetic diagnosis and is now steadily increasing the number of new candidate genes. Yet, despite these advances, the difficulty in connecting an observed phenotype with a specific candidate gene, and the occasional uncertainty surrounding the inheritance pattern, make research into the genetic causes of HSPs particularly arduous, leaving most HSP patients without a genetic diagnosis [1,4]. To date, 76 genomic loci and 58 corresponding genes have been linked to HSPs, highlighting the extreme heterogeneity in the mode of transmission of HSPs and the role played by SPG-encoded proteins.

Autosomal-dominant HSPs (ADHSPs) are linked to mutations in 19 SPG genes, leading mostly to the onset of a pure form of the disease. Among ADHSPs, SPG4/SPAST, SPG3A/ATL1, SPG31/REEP1 and SPG10/KIF5A are those most frequently mutated, and responsible for almost 57% of ADHSP cases.

A total of 57 loci and 52 genes are responsible for autosomal-recessive HSPs (ARHSPs), which often lead to more complicated phenotypes. SPG11/KIAA1840, SPG5A/CYP7B1, SPG7 and SPG15/ZFYVE26 account for almost 34% of causative mutations in ARHSP-affected patients [2,5]. However, the frequency of SACS mutations in patients with spastic ataxia is probably underestimated because of the misclassification of such patients as having either ataxia or an HSP based on the clinical picture [6].

Rare forms of HSP include X-linked and maternally inherited HSPs with five loci responsible for X-linked HSPs, most frequently due to mutations in SPG1/L1CAM and SPG2/PLP1, both leading mostly to a complicated form of HSP [7,8].

To date, only one gene encoded by the mitochondrial genome has been clearly shown to be responsible for HSP; complicated spastic paraplegia was indeed observed in a family harboring mutations of MT-ATP6, which codes for a component of the adenosine triphosphate (ATP) synthase complex [9].

Multiple inheritance patterns have been observed in patients carrying mutations in both SPG58/KIF1C and SPG72/REEP2, leading to HSPs when present in either a heterozygous

or homozygous state [10,11]. Similarly, mutations in *ALDH18A1* are responsible for HSP-SPG9A when inherited through a dominant pattern, and for HSP-SPG9B when in a homozygous state [12,13]. In HSP-SPG7, heterozygous carriers may exhibit only a partial phenotype (late-onset cerebellar ataxia or optic atrophy), leading to dominantly inherited disease [14].

A direct consequence of the large number of genes and loci involved in HSPs is the extreme variety that characterizes SPG-encoded proteins implicated in many processes, including axon pathfinding and preservation, myelination, maintenance of endoplasmic reticulum (ER), lipid metabolism, and endosomal dynamics and intracellular transport [15].

The pathological role played by SPG-encoded proteins has been further elucidated by the fact that SPG mutations are not only involved in HSPs, but are also associated with other neurodegenerative diseases. These include hereditary motor and sensory neuropathies, ataxias, amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy [5].

4. HSPs: UMN and LMN degeneration

Neurodegeneration in HSPs involves primarily sensory and corticospinal tract axons, and arises through a progressive 'dying-back' process starting from the distal ends of the axons [16]. The gradual retraction of UMN axons progressively impairs and dysregulates the synapse between UMNs and LMNs. Leg spasticity, weakness, hypertonia and hyperreflexia subsequently appear due to the lack of communication between the two neuronal partners.

However, such neurodegeneration can affect either only UMNs, consequently damaging their connections with LMNs, or both UMNs and LMNs, as clearly observed in some forms of HSP. The latter dual degeneration is reminiscent of ALS, with the notable difference of preservation of sacral neurons (Onuf's nucleus), and normal bladder and rectal sphincter function up to the final stages of the disease [17]. On the other hand, LMN degeneration alone gives rise to an HSP manifesting with marked muscle-wasting that can be either generalized or restricted to the lower limbs.

HSPs can therefore be classified according to which motor neurons are affected by neurodegeneration (Fig. 1, Tables 1–4), and the different degrees of motor neuron involvement.

4.1. SPG4/SPAST: neurodegeneration of only UMNs

Spastic paraplegia type 4 (SPG4) represents the most common autosomal-dominant form of HSP, accounting for approximately 40% of familial [18,19] and 10% of sporadic [20,21] cases. Linkage studies [22,23] have led to the identification of the 90-kb genomic region on chromosome 2 (2p22.3) harboring the SPG4/SPAST gene, comprising 17 exons.

HSP-SPG4 can be defined as a 'pure' form, with gait impairment due to spasticity and weakness, and unsteadiness due to posterior cord impairment (decreased vibratory sensation). Almost one-third of HSP-SPG4 patients are affected by sphincter disturbances [24], sometimes with both urinary and anal incontinence. Also, a thin corpus callosum, cerebellar atrophy and epilepsy have occasionally been reported [25,26], while cognitive impairment or dementia has been observed in association with SPG4 mutations, but limited to single

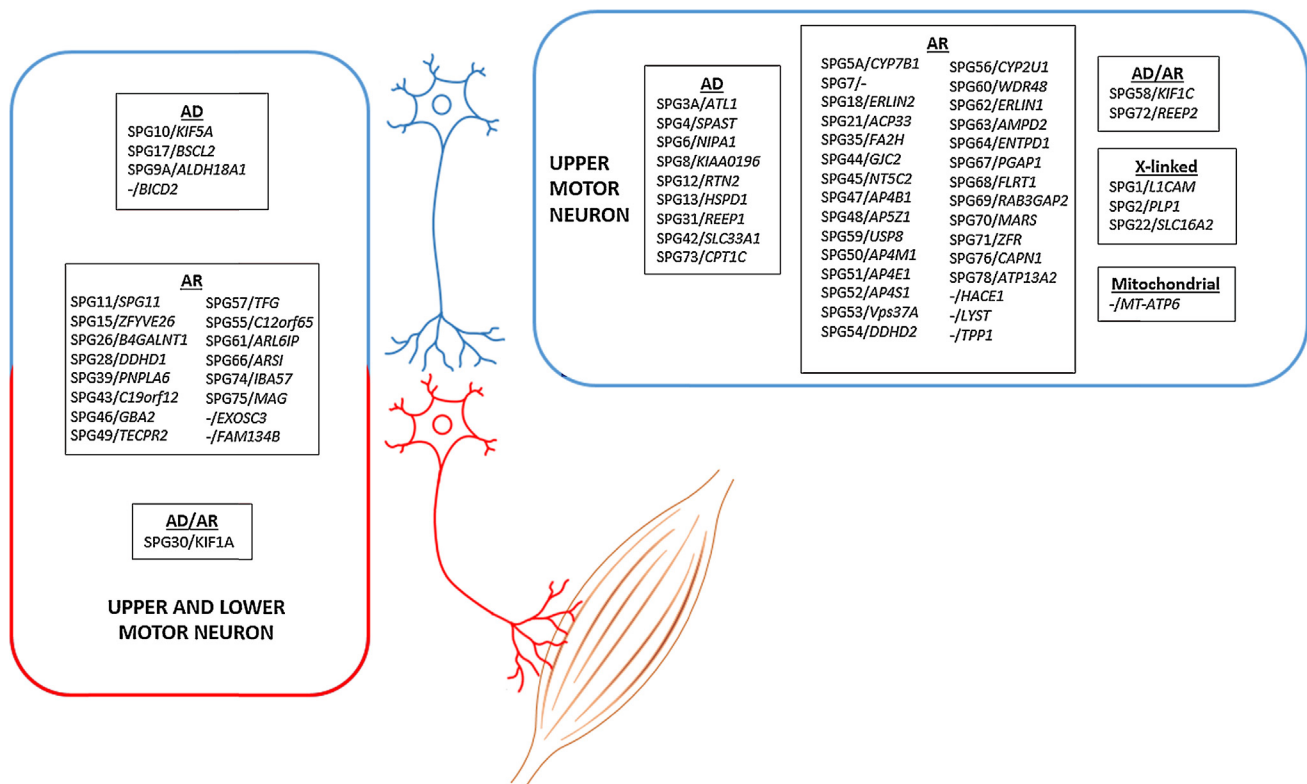


Fig. 1 – Spastic paraplegia genes (SPGs) grouped according to affected motor neurons and mode of inheritance.

Table 1 – Hereditary spastic paraplegias with primarily upper motor neuron signs.

Locus	Gene	Estimated frequency	Protein	Additional clinical signs
Autosomal-dominant				
SPG4	SPAST	Most frequent	Spastin	Pure or with peripheral neuropathy
SPG3A	ATL1	2nd most frequent	Atlastin-1	
SPG31	REEP1	Frequent	Receptor expression-enhancing protein 1	
SPG6	NIPA1	Rare	Magnesium transporter NIPA1	
SPG8	KIAA0196	Rare	Strumpellin	
SPG12	RTN2	Rare	Reticulon-2	
SPG13	HSPD1	Extremely rare	Mitochondrial heat shock protein	
SPG42	SLC33A1	Extremely rare	Acetyl-coenzyme A transporter 1	
SPG73	CPT1C	Extremely rare	Carnitine O-palmitoyltransferase 1, brain isoform	
Autosomal-recessive				
SPG7	-	Most frequent	Paraplegin	Cerebellar ataxia and/or cerebellar atrophy at MRI, optic atrophy
SPG5A	CYP7B1	2nd most frequent	25-hydroxycholesterol 7- α -hydroxylase	Pure or with cerebellar ataxia, optic atrophy
SPG18	ERLIN2	Rare	Erlin-2	Psychomotor developmental delay, joint contractures
SPG21	ACP33	Rare	Maspardin	Mast syndrome: cognitive decline, cerebellar signs
SPG35	FA2H	Rare	Dihydroceramide fatty acyl 2-hydroxylase	Dystonia, cerebellar signs, cognitive decline, brain iron accumulation, seizures
SPG47	AP4B1	Rare	AP-4 complex subunit beta-1	Intellectual disability, dysmorphism
SPG48	AP5Z1	Rare	AP-5 complex subunit zeta-1	Pure or with intellectual impairment

families [27]. Tallaksen et al. [28] evaluated cognitive function in 29 SPG4-mutation carriers and 29 controls from 10 families, but failed to demonstrate the presence of clinically defined cognitive impairment and instead found the presence of asymptomatic and subclinical cognitive impairment in SPG4 carriers compared with controls. The impairment primarily affected executive function, and correlated statistically with progression and severity of the disease, but not with patients' age. The impairment was also more severe in patients carrying a missense, rather than truncating, mutation [28].

Extreme inter- and intrafamilial heterogeneity in age at onset and disease progression are also characteristic of HSP-SPG4. The age at onset ranges from birth to the seventh decade and is indeed highly variable, even within families. It often shows a bimodal distribution, with the first peak before the first decade of life, and the second peak between the third and sixth decades. Disease progression can be either rapid or slow with variable penetrance, as revealed by the presence of asymptomatic mutation carriers, which is often observed in HSPs [29].

HSP-SPG4 motor disability mostly reflects progressive degeneration of UMNs, and is directly attributable to malfunction of the SPAST gene-encoded protein spastin. However, some patients may present with symptoms clearly attributable to LMN impairment [30–32].

Spastin is an ATPase that serves as a microtubule-severing protein, with a role to play in the regulation of several aspects of the microtubule network, such as their number and mobility. Spastin is detectable as two isoforms [33], characterized by differential cellular expression. Spastin M87 (60 kDa) can be detected in both the cerebral cortex and spinal cord, whereas spastin M1 (68 kDa) appears to be present in small quantities and only in the spinal cord [34]. Both isoforms are

formed by three functional domains: the AAA domain, necessary for ATPase activity; the microtubule-binding domain (MTBD); and the microtubule-interacting and -trafficking domain (MIT). In addition to these shared sequences, spastin M1 is characterized by the presence of a hydrophobic N-terminal domain, which allows interactions with ER and its consequent involvement, in association with two other SPG-related proteins—atlastin-1 (ATL1/SPG3A) and REEP1 (REEP1/SPG31)—in ER morphogenesis [35]. Furthermore, spastin is able to interact with lipid droplets through its N-terminal domain and is therefore also involved in lipid metabolism [36].

Given these multiple functions of spastin, impaired microtubule dynamics, organelle remodeling, axonal transport and axonal growth arise as a consequence of SPAST mutations [37–39]. In fact, more than 200 different pathogenic mutations have been detected along the gene sequence (with the exception of exon 4, which undergoes an alternative splicing process), including point mutations, frameshift mutations and large deletions, with missense mutations mainly clustered around the ATPase activity domain [40,41]. Missense mutations clearly involved in HSP-SPG4 are mostly detected in the spastin C-terminal region and impair its ATPase activity. The exceptions are two N-terminal missense mutations, c.131C > T/p.S44L and c.134C > A/p.P45Q, detected on SPAST exon 1. Through segregation studies, it has been shown that these two variants cosegregate independently with other SPAST mutations, leading to a drastic decrease in age at onset and, in some cases, expression of a more severe phenotype as well. Because these two polymorphisms are also detectable in healthy control populations, they are considered to be age-at-onset HSP-SPG4 intragenic modifiers [30,42].

Table 2 – Hereditary spastic paraplegias primarily affecting the upper motor neurons.

Locus	Gene	Estimated frequency	Protein	Additional clinical signs
Autosomal-recessive				
SPG50	AP4M1	Rare	AP-4 complex subunit mu-1	Infantile hypotonia, intellectual disability, speech disorder
SPG51	AP4E1	Rare	AP-4 complex subunit epsilon-1	Infantile hypotonia, intellectual disability, speech disorder
SPG54	DDHD2	Rare	Phospholipase DDHD2	Psychomotor retardation, intellectual disability
SPG56	CYP2U1	Rare	Cytochrome P450 2U1	Intellectual disability, subclinical axonal neuropathy
SPG62	ERLIN1	Rare	Erlin-1	Cerebellar signs, mild intellectual disability
SPG76	CAPN1	Rare	Calpain-1 catalytic subunit	Cerebellar ataxia, peripheral neuropathy, dysarthria
SPG53	Vps37A	Rare	Vacuolar protein sorting-associated protein 37A	Psychomotor retardation, intellectual disability
SPG64	ENTPD1	Rare	Ectonucleoside triphosphate diphosphohydrolase 1	Cerebellar signs, intellectual disability, delayed puberty
SPG45	NT5C2	Extremely rare	Cytosolic purine 5'-nucleotidase	Psychomotor retardation, intellectual disability, ocular signs
SPG52	AP4S1	Extremely rare	AP-4 complex subunit sigma-1	Neonatal hypotonia, intellectual disability, speech disorder, dysmorphism
SPG44	GJC2	Extremely rare	Gap junction gamma-2 protein	Cerebellar signs, seizures, mild intellectual disability
SPG59	USP8	Extremely rare	Ubiquitin carboxyl-terminal hydrolase 8	Mild intellectual disability
SPG60	WDR48	Extremely rare	WD repeat-containing protein 48	Nystagmus, peripheral neuropathy
SPG63	AMPD2	Extremely rare	AMP deaminase 2	Short stature
SPG67	PGAP1	Extremely rare	GPI inositol-deacylase	Intellectual disability
SPG68	FLRT1	Extremely rare	Leucine-rich repeat transmembrane protein FLRT1	Nystagmus, optic atrophy
SPG69	RAB3GAP2	Extremely rare	Rab3 GTPase-activating protein non-catalytic subunit	Psychomotor retardation, intellectual disability, deafness, cataracts
SPG70	MARS	Extremely rare	Methionine-tRNA ligase, cytoplasmic	
SPG71	ZFR	Extremely rare	Zinc finger RNA-binding protein	
–	HACE1	Extremely rare	E3 ubiquitin-protein ligase	Psychomotor retardation, seizures
–	LYST	Extremely rare	Lysosomal-trafficking regulator	Cerebellar ataxia, sensorimotor demyelinating neuropathy
SPG78	ATP13A2	Extremely rare	Probable cation-transporting ATPase 13A2	Cerebellar signs, intellectual disability
–	TPP1	Extremely rare	Tripeptidyl-peptidase 1	Mild intellectual disability, seizures, bulbar palsy, dystonia

Table 3 – Hereditary spastic paraplegias primarily affecting the upper motor neurons.

Locus	Gene	Estimated frequency	Protein	Additional clinical signs
Autosomal-dominant or -recessive				
SPG58	KIF1C	Rare	Kinesin-like protein KIF1C	Cerebellar ataxia, mild intellectual disability, chorea
SPG72	REEP2	Rare	Receptor expression-enhancing protein 2	
X-linked				
SPG1	L1CAM	Rare	Neural cell adhesion molecule 1	MASA/CRASH syndromes
SPG2	PLP1	Rare	Myelin proteolipid protein	Pure (late-onset cases) to complex forms with cerebellar signs, intellectual disability, seizures
SPG22	SLC16A2	Rare	Monocarboxylate transporter 8	Allan-Herndon-Dudley syndrome with severe psychomotor retardation, thyrotoxicosis
Mitochondrial				
–	MT-ATP6	Extremely rare		Cerebellar signs, axonal neuropathy

Table 4 – Hereditary spastic paraplegias affecting both upper and lower motor neurons.

Locus	Gene	Estimated frequency	Protein	Additional clinical signs
Autosomal-dominant				
SPG10	KIF5A	Most frequent	Kinesin heavy chain isoform 5A	Pure or with intellectual disability, extrapyramidal signs
SPG17	BSCL2	Rare	Seipin	
SPG9A	ALDH18A1	Extremely rare	Delta-1-pyrroline-5-carboxylate synthase	Pure or with psychomotor retardation, intellectual disability, cataract, cutis laxa gastroesophageal reflux, dysmorphism
–	BICD2	Extremely rare	Protein bicaudal D homolog 2	
Autosomal-recessive				
SPG11	SPG11	Most frequent	Spatacsin	Pure to complex forms with cognitive decline, cerebellar signs, extrapyramidal signs, retinal degeneration
SPG15	ZFYVE26	Frequent	Spastizin	Pure to complex forms with cerebellar ataxia, retinal degeneration
SPG26	B4GALNT1	Rare	Beta-1,4-N-acetylgalactosaminyltransferase 1	Cerebellar ataxia, intellectual disability, dystonia
SPG28	DDHD1	Rare	Phospholipase DDHD1	Scoliosis
SPG39	PNPLA6	Rare	Neuropathy target esterase	Pure or with intellectual disability
SPG46	GBA2	Rare	Non-lysosomal glucosylceramidase	Cerebellar ataxia, cataract, intellectual disability, infertility in males
SPG49	TECPR2	Rare	Tectonin beta-propeller repeat-containing protein 2	Intellectual disability, respiration troubles, gastroesophageal reflux
SPG55	C12orf65	Rare	–	Intellectual disability, strabismus
–	EXOSC3	Rare	Exosome complex component RRP40	Cerebellar signs, intellectual disability
–	FAM134B	Extremely rare	Reticulophagy receptor FAM134B	Skeletal abnormalities, hyperhidrosis
SPG43	C19orf12	Extremely rare	C19orf12	Neurodegeneration with brain iron accumulation (NBIA)
SPG57	TFG	Extremely rare	–	Optic atrophy
SPG61	ARL6IP	Extremely rare	ADP-ribosylation factor-like protein 6-interacting protein 1	Sensory/motor polyneuropathy
SPG66	ARSI	Extremely rare	Arylsulfatase I	Intellectual disability, sensory/motor polyneuropathy
SPG74	IBA57	Extremely rare	Putative transferase CAF17, mitochondrial	Optic atrophy
SPG75	MAG	Extremely rare	Myelin-associated glycoprotein	Intellectual disability
Autosomal-dominant or -recessive				
SPG30	KIF1A	Rare	Kinesin-like protein KIF1A	Pure to complex forms with cerebellar ataxia, psychomotor developmental delay

Loss of function and haploinsufficiency appear to best explain the pathology of the disease, as most pathogenic SPAST mutations affect spastin functional domains, cause the loss of large portions of the gene or preclude formation of functional spastin protein through mRNA nonsense-mediated decay due to large deletions or frameshift mutations.

In conclusion, HSP-SPG4 causal mutations lead to a degenerative process that predominantly involves UMNs, whereas reports of a few cases in which additional LMN degeneration was observed exemplifies the overlapping phenotypes of neurodegenerative diseases, such as the phenotype of juvenile ALS, which is sometimes related to several genes involved in both ataxia (for example, AOA1) and HSP (for example, SPG11).

4.2. HSPs with both UMN and LMN involvement

4.2.1. SPG11

Approximately 20% of ARHSPs arise following SPG11 mutations, making it the most frequently mutated gene in ARHSPs

[43,44]. The associated clinical phenotype comprises, in most cases, a combination of progressive spastic gait, accompanied by limb weakness, peripheral neuropathy and progressive cognitive decline (for a review, see Stevanin et al. [45]). Atrophy of the corpus callosum can be detected by MRI in 41–77% of patients carrying SPG11 mutations [43]. Cerebellar ataxia and retinopathy are also frequent [43,46]. Onset is usually during infancy or adolescence. The disease progresses rapidly, often leaving patients wheelchair-bound within one or two decades of onset [44].

LMN alterations are detectable on EMG, and provoke fibrillation and muscle-wasting in both the upper and lower limbs [43,47,48]. The crucial role played by spatacsin/SPG11 in UMNs and LMNs is further underlined by the fact that SPG11 mutations have been detected in patients affected by autosomal-recessive juvenile ALS [49] and autosomal-recessive Charcot-Marie-Tooth disease type 2 [50].

Located on chromosome 15 (15q21.1), the human SPG11 gene spans an 8-kb region containing 40 exons that encode spatacsin [43]. This protein is widely expressed in the central

nervous system, especially in cortical and spinal motor neurons [51]. Its interaction with both spastizin, encoded by SPG15, and AP5Z1/SPG48, a member of the fifth adaptor protein complex (AP-5), suggests a role in membrane trafficking [52]. This is in agreement with the still undetermined role of spatacsin in autophagy and lysosome turnover [53–55]. Spatacsin is required for normal neuronal development and functioning, as its absence leads to the impairment of axonal transport and affects spinal motor axon branching, as shown by human intermediate progenitor cells, and zebrafish and mouse knock-down models [56,57].

More than 100 pathogenic mutations have, to date, been linked to HSP-SPG11 and almost exclusively lead to loss of function [58,59].

4.2.2. BICD2

The bicaudal drosophila homolog 2 (BICD2) protein, encoded by the homonym gene, is a motor-adaptor protein that interacts with proteins involved in both retrograde and anterograde cargo transport [60]. BICD2 is composed of a C-terminal domain that binds to different cargoes, such as the G protein Rab6, as related to its role in vesicle trafficking between the Golgi apparatus and ER [61]. In synapses, BICD2 is involved in the recycling of synaptic vesicles following binding to clathrin heavy chains through its C-terminal [62]. Through its N-terminal portion, BICD2 interacts with the dynein/dynactin motor complex and the heavy chain of two kinesins belonging to family 5, type 5A (KIF5A/SPG10) and 5B (KIF5B) [63]. This N-terminal interaction is strongly increased following deletion of the C-terminal portion, impairing normal functioning of dynein/dynactin and leading, along with other effects, to Golgi fragmentation, diminished retrograde transport and axonal swelling, as observed in neurons of a BICD2 mouse model [64]. Mutations affecting BICD2 have been detected along its entire coding sequence and are mostly linked to the onset of autosomal-dominant, predominantly lower-extremity spinal muscular atrophy type 2 (SMALED2) [60]. SMA due to BICD2 mutations is characterized by an extremely early onset (before the first decade), predictable in some cases through the finding of fetal abnormalities (such as reduced fetal movement). The lower limbs are especially affected, with both proximal and distal muscle-wasting of varying severity. However, most patients maintain the capacity to walk without support.

HSP-BICD2 patients can exhibit impairment of predominantly LMNs or of both UMNs and LMNs [65]. Patients present with lower-limb muscle-wasting, contractures and weakness in addition to lower-limb spasticity and hyperreflexia. Onset starts in adulthood with minor symptom severity and a slower progression, thereby allowing the distinction of HSP-BICD2 from BICD2-related SMA [66].

5. Conclusion

Based on the fact that the motor neuron degeneration underlying HSPs can involve both UMN and LMN axons, the present attempt was made to classify each form of HSP based on the motor neurons affected. HSPs arise following the dying-back degeneration of UMN axons of the corticospinal tracts.

The combination of both UMN and LMN degeneration is detectable in some forms of HSP, leading to a phenotype that is rather more ALS-like, with muscular atrophy and muscle weakness as the main symptoms of LMN involvement.

However, the distinction between exclusively UMN involvement and impairment of both UMNs and LMNs can be difficult due to the presence of infraclinical signs. This clinical picture is further complicated by the fact that the neurodegenerative process affecting LMNs can first manifest through disturbances at the cell-body level, which further affects normal axon functioning or directly involves the axon, resulting in the dying-back motor neuropathy already observed in UMNs.

To clarify the nature of the neurodegenerative process, clinical examination should systematically be accompanied by electrophysiological investigations [EMG and electroneurography (ENG)], which are crucial for guiding clinicians to the correct clinical and genetic diagnoses.

Disclosure of interest

The authors declare that they have no competing interest.

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Hereditary ataxias and paraparesias: clinical and genetic update

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Purpose of review

This review aims at updating the clinical and genetic aspects of hereditary spastic paraplegias (HSPs) and hereditary cerebellar ataxias (HCAs), focusing on the concept of spastic-ataxia phenotypic spectrum and on newly identified clinical overlaps with other neurological and nonneurological diseases.

Recent findings

Next-generation sequencing (NGS) has allowed the discovery of new genes involved in HSPs and HCAs. They include new HCAs genes such as *GRM1* (SCA44), *FAT2* (SCA45), *PLD3* (SCA46), *SCYL1* (SCAR21), *UBA5* (SCAR24) and *XRCC1* (SCAR26) as well as *CAPN1* (SPG76) and *CPT1C* (SPG73) in HSPs. Furthermore, NGS allowed enriching known genes phenotype, reinforcing the overlap between HSPs and HCAs defining the spastic ataxia spectrum. Clear examples are the expanded phenotypes associated with mutations in *SPG7*, *PNPLA6*, *GBA2*, *KIF1C*, *CYP7B1*, *FA2H*, *ATP13A2* and many others. Moreover, other genes not previously linked to HCAs and HSPs have been implicated in spastic or ataxic phenotypes.

Summary

The increase of HSPs and HCAs-related phenotypes and the continuous discovery of genes complicate clinical diagnostic in practice but, at the same time, it helps highlighting common pathological pathways, therefore opening new ways to the development of common therapeutic approaches.

Keywords

cerebellar ataxia, spastic ataxia, spastic paraplegia

INTRODUCTION

The introduction in everyday genetic diagnostic process of innovative approaches, such as next-generation sequencing (NGS), including whole exome sequencing (WES), has recently revolutionized the clinicogenetic distinction between hereditary cerebellar ataxias (HCAs) and hereditary spastic paraplegias (HSPs). HCAs are characterized by the predominance of Purkinje cells and/or spinocerebellar tracts involvement, variably combined with atrophy of brainstem or of other regions of the nervous system. Pyramidal tract degeneration is the hallmark of HSPs, often accompanied by posterior cordal tracts impairment. To date, 72 (35 genes for SCAs and 22 for SCARs) and 79 loci (65 genes) are, respectively, known to be involved in HCAs and HSPs onset, while eight loci are responsible for spastic ataxia [1]. Disease-causing mutations are mostly inherited through autosomal dominant and recessive transmission, even if in both diseases all the known inheritance patterns are observed [2,3].

The recent discovery of a remarkable number of genes that when mutated produced hybrid phenotypes, ranging from a more pure ataxia to pure spastic paraparesis, led to the introduction of a new concept of spastic ataxia phenotypic spectrum, rather than referring to HCAs and HSPs as two separated diseases [4]. A clear example is given by the observation that SPG7 (spastic paraplegia type 7) is one of the most frequent form of late-onset spastic

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KEY POINTS

- Update of new genes causative for hereditary cerebellar ataxias and spastic paraplegias, supporting the concept of spastic-ataxia spectrum.
- To point out the clinical and genetic overlap between ataxias and paraplegias.
- Highlight recent and innovative therapeutic approaches.

ataxia [5,6]. Autosomal recessive ataxia of Charlevoix–Saguenay (SACS), late-onset Friedreich ataxia (FA, *FXN*), adult-onset Alexander disease (*GFAP*), cerebrotendinous xanthomatosis (*CTX*) [7] result in spastic ataxia, although they do not belong to HSP or HCA genetic classifications. Clinical and genetic diagnostic process is made more difficult by the presence of overlapping phenotypes and by the fact that, to date, genetic diagnosis is still missing for about 50% of HCAs or HSPs patients [8[•],9,10]. Polyglutamine expansions diseases (*ATXN1/SCA1*, *ATXN2/SCA2*, *ATXN3/SCA3*, *CACNA1A/SCA6*, *ATXN7/SCA7*, *TBP/SCA17*, *ATN1/DRPLA*) are the most frequent dominant HCAs. The onset of these diseases is between 20 and 40 years, with anticipation in successive generations. Major clinical features are progressive cerebellar ataxia associated, according to the genotype, with other neurological or extraneurological signs (pyramidal syndrome, parkinsonism, myoclonus, dystonia, dementia, seizure, retinal degeneration) [10]. Regarding HSPs, *SPAST/SPG4* is the most common mutated gene in both familial and sporadic cases. Typical age at onset is between the third and fourth decade manifesting with lower limbs spasticity variously associated with muscle wasting, hyperreflexia, decreased vibration sense at ankles. Variable penetrance can be observed with some *SPG4* carriers being asymptomatic for life [3].

The aim of the present review is to report on newly identified HSPs and HCAs genes and on the phenotypic expansion in genes belonging to both HSPs/HCAs networks or responsible of other neurological or nonneurological diseases, with a special focus on the spastic ataxia spectrum.

NEW GENES IN CEREbellAR ATAXIAS AND SPASTIC PARAPLEGIAS

In 2017, by WES, Nibbeling *et al.* [11^{••}] found five new genes causative for autosomal dominant cerebellar ataxias: *FAT2*, *PLD3*, *KIF26B*, *EP300* and *FAT1*. Mutations in *FAT2*, responsible for SCA45, were

reported in two families with a slow progressive cerebellar ataxia. The proband showed a pure cerebellar ataxia, without additional details about other family members. *PLD3* mutations were identified in a family already described in 1995 [12] with autosomal dominant inheritance and combination of cerebellar ataxia and sensory neuropathy. This gene has been associated with SCA46 and the phenotype includes cerebellar dysarthria, oculomotor abnormalities and mild cerebellar atrophy with a mean age at onset of 53.5 years. *KIF26B* mutation was found in a single family with a late age at onset (74–90 years) and spastic ataxia phenotype. Patients with *FAT1* mutations presented with variable ages at onset (10–70 years) with cerebellar ataxia variably associated with other neurological signs. In one additional case with late-onset cerebellar ataxia, a heterozygous mutation in *EP300* was detected.

Another gene involved in autosomal dominant cerebellar ataxias is *GRM1*, encoding the metabotropic glutamate receptor 1 (mGluR1) and causative for SCA44. Two distinct phenotypes were reported in three SCA44 families: cerebellar ataxia associated with pyramidal syndrome with cerebellar atrophy and cerebellar ataxia with intellectual deficiency without cerebellar atrophy. Disease onset varied from childhood up to 50 years. Glutamate is the most common excitatory neurotransmitter and the increased mGluR1 signalling could provoke excitotoxicity on Purkinje cells. The work of Watson *et al.* [13] showed the inhibitor effect, *in vitro*, of Nitazoxanide on mutant forms of mGluR1, suggesting it as a potential drug in SCA44. Another molecule used to modulate mGluR1 activity with some improvement in motor task was baclofen in SCA1 mice [14]. Channelopathies play an important role in cerebellar ataxia, particularly mutations in *CACNA1A* in dominant and recessive forms [6,15[•]]. Another recently identified gene belonging to the voltage-dependent calcium channels family is *CACNA1G* that produces a mildly progressive cerebellar ataxia with vermian atrophy, pyramidal signs and variable age at onset (9–78 years) [16].

Among recessively transmitted cerebellar ataxias, a newly identified gene is *CHP1* (Calcineurin Homologous Protein-1), found in two siblings of a consanguineous family with cerebellar ataxia and motor neuropathy associated with cognitive impairment, spastic paraparesis and abnormal ocular saccades. Brain MRI showed hypoplasia of cerebellar vermis [17[•]]. *SCYL1*, *UBA5* and *XRCC1* are causative of SCAR21 (hepatocerebellar neuropathy syndrome) [18], SCAR24 (cerebellar ataxia, cataract, childhood onset) [19] and SCAR26 (cerebellar ataxia, oculomotor apraxia, axonal neuropathy), respectively [20].

As in the case of HCAs, different new genes have recently been associated with HSP, leading to both pure and complicated diseases. Among pure and dominantly inherited HSPs, *CPT1C*/SPG73 is the newest added to the SPGs list. It encodes the neuronal isoform of the carnitine palmitoyl-transferase enzyme that localizes at the endoplasmic reticulum, wherein it was shown to interact with *ATL1*/SPG3A [21]. A new gene involved in recessive complicated HSP is *CAPN1*/SPG76 with variable age at onset ranging from congenital to adult: the related phenotype is characterized by spastic ataxia accompanied in some cases by mild cognitive decline and cerebellar atrophy [22–24]. A more complicated phenotype was observed in presence of pathogenic mutations impairing the correct functioning of kinesin light chain protein 4, encoded by *KLC4*. The related phenotype was complicated spastic paraplegia with progressive deafness and retinitis pigmentosa. Bilateral signal changes in the dentate nucleus of the cerebellum, in the posterior leg of the internal capsule and in the subcortical white matter were highlighted at brain MRIs [25]. Exome sequencing was useful also to identify SPG23 causative gene, *DSTYK*, coding for a kinase involved in cell death regulation. A homozygous deletion was identified segregating in unrelated affected patients presenting with early-onset spastic paraplegia accompanied by premature hair greying and skin depigmentation, typical SPG23 features [26]. Furthermore, spastic paraparesis and cerebellar ataxia were recently observed in association with *VPS13D* mutations. With onset in childhood, carrier patients developed spastic ataxia complicated by developmental delay, axial hypotonia and hyperkinetic movements as well as generalized dystonia. Brain MRI revealed bilateral symmetric T2 hyperintensities in the basal ganglia and brain stem leading to the addition of *VPS13D* to the group of spastic ataxia genes [27].

PHENOTYPIC EXTENSION AND CLINICAL VARIABILITY UPDATE

In addition to new genes discovery, NGS is constantly allowing the expansion of the clinical phenotype associated to mutations affecting genes already known to be causative of HSPs and HCAs (Table 1) [24,28–72]. Cognitive decline or intellectual disability, reported with different severity degrees, were the most frequent newly identified HSPs additional features. Cognitive deterioration was observed in presence of *CYP2U1*/SPG56 [28], *AP4S1*/SPG52 [29], *ZFYVE26*/SPG15 [30] and SPG7 [31] mutations. For *KIF1A*/SPG30 mutated patients, language impairment [32] coupled to intellectual

disability [33] have been reported. Poor intellect and undeveloped speech was noticed when examining *TFG*/SPG57 mutated patients [34].

Even the category of HSP complicated by epilepsy was enriched of several new entries. Epileptic seizures were observed in addition to HSP in patients carrying *KIF1A*/SPG30 [32] and *FARS2*/SPG77 [35] mutations. Febrile and focal seizures characterized patients with *AP4S1*/SPG52 mutations [29], while myoclonic seizures were presented by *KIF5A*/SPG10 carriers [36].

Cerebellar ataxia was an additional clinical feature in a great variety of genes increasing the group of spastic ataxia genes. The results of a study conducted in undiagnosed cerebellar ataxia patients ($n=412$), without dominant transmission, showed that the most frequent genes involved were SPG7, *SACS*, *SETX*, *SYNE1*, *CACNA1A* [6]. SPG7, initially described as a pure or complicated HSP with cerebellar atrophy, represents a frequent cause of undiagnosed cerebellar ataxia [5] and spastic ataxia [73]. This gene should be considered in patients with late-onset cerebellar ataxia, even in absence of pyramidal syndrome at disease onset [33]. Mild cerebellar atrophy is frequently linked to SPG7 mutations [15[¶]], as it happens in the case of SCA28 [74]. This association is not surprising, as paraplegin forms oligomeric mAAA protease complex with the homologous AFG3L2 protein, mutated in SCA28. Moreover, additional clinical features such as progressive external ophthalmoplegia, parkinsonism [75], as well as palatal tremor [31] and impaired emotional communication [76], have been reported in SPG7 carrier patients. SPG7 is known to be causative for autosomal recessive HSPs, but was frequently reported also in sporadic cases and more rarely in families with autosomal dominant transmission [15[¶],77]. However, it is not known whether genetic factors account for these clinical differences. Another phenotypic expansion is reported for the *FA2H* gene, with a recent effort to establish phenotype–genotype correlations [78]. *FA2H* gene is reported in childhood form of recessive HSP with dystonia, dysarthria, epilepsy and cognitive deficiency [79], but in the last years, leukoencephalopathy [80] and neurodegeneration with iron brain accumulation (NBIA) [81] have been added. SPG39 caused by mutations in *PNPLA6* [82], can also give rise to other related syndromes. Beyond spastic paraparesis, cerebellar ataxia is one of the most common feature often associated with hypogonadotropic hypogonadism (Gordon Holmes syndrome) [37] or with chorioretinal dystrophy (Boucher–Neuhäuser syndrome) [38]. Nevertheless, pure cerebellar ataxia phenotypes have been reported in a large Parsi family [39].

Table 1. New phenotypic manifestations associated with known hereditary spastic paraplegias and hereditary cerebellar ataxias causative genes

Disease	Gene	SPG/SCA	Mode of transmission	Additional associated symptoms	Age at onset	MRI	Reference
HSP	–	SPG7	AR	Pure cerebellar ataxia	–	–	[33]
	KIF5A	SPG10	AD	Cognitive decline, macrocephaly, palatal tremor	41 years	–	[31]
				Pseudobulbar palsy, fasciculations, ALS-like	66 years	Normal	[42]
				Myoclonic seizures, progressive leukoencephalopathy	Congenital	Dandy-Walker variant, TCC, WMH, ventricular dilatation, pons atrophy	[36]
				Ptois, optic nerve atrophy, oculogyric crises, dysphagia, apnoea	3 months– 5 years	Increased T2 signal in brainstem and pons	[43]
	–	SPG11	AR	ALS-like	29–68 years	–	[44]
	–	SPG11	AR	Subacute episodes of gait worsening (\approx MS)	\approx 20 years	WMH, TCC, C3-C4-T2 hypersignal with oedema and GE	[45]
	ZFYVE26	SPG15	AR	Pes cavus, cognitive decline, PNP	4 years	TCC	[30]
	–	SPG20	AR	Generalized muscle weakness, intentional tremor, dysarthria, ataxia, pseudobulbar palsy, pectus carinatum	5 years	–	[46]
	B4GALNT1	SPG26	AR	Febrile ataxia	5–8 years	–	[47]
HSP	DDHD1	SPG28	AR	Retinal dystrophy	40 years	NBIA	[48]
	KIF1A	SPG30	AD	Cognitive and language impairment, seizures, optic nerve atrophy, PNP, hypotonia	14 years	CA, TCC	[32]
	–	–	–	Intellectual disability	3 months–20 years	–	[33]
	REEP1	SPG31	AR	Severe congenital axonal neuropathy, respiratory distress, distal arthrogryposis, congenital PNP, SMA RD1-like	5 years	–	[49]
	NT5C2	SPG45	AR	Dysarthria, developmental delay	7–10 months	CC dysgenesis, cerebral atrophy	[50]
	AP4M1	SPG50	AR	NBIA	Childhood	–	[51]
	AP4S1	SPG52	AR	Cerebral palsy, febrile and focal seizures, microcephaly, facial dysmorphism, cognitive disability	5–15 years	Delayed myelination, TCC, WMH	[29]
	DDHD2	SPG54	AR	Gaze-evoked horizontal nystagmus, dysarthria, limbs ataxia and truncal ataxia, postural tremor in the head and upper extremities, dysphagia, urinary incontinence	45 years	Mild CA, TCC, WMH	[52]
	CYP2U1	SPG56	AR	Cognitive decline	6 years old	Spinal cord hydromyelia	[28]
	–	–	–	Activity-induced dystonia (face and upper/lower limbs)	5–33 years	Delayed myelination, TCC, WML, GPH, dorsal hydromyelia	[53]
HSP	TFG	SPG57	AR	Undeveloped speech, sleep disturbances, severe movement disability, poor intellect	4–5 years	WM myelination reduction, cerebral atrophy	[34]
	CAPN1	SPG76	AD	–	Congenital	–	[24]
	FARS2	SPG77	AD	Epilepsy	15 months	–	[35]
	BICD2	–	AD	Myopathic alteration	Congenital	LL fat infiltration	[54]
	SOX10	–	AD	Early onset sensorineural hearing loss, mild pigmentary abnormalities	2 months	–	[55]
	ATXN7	SCA7	AD	Severe infantile form	–	–	[56]
	TTBK2	SCA11	AD	–	Childhood	–	[57]
	ITPR1	SCA15	AD	–	–	Hydrocephalus (improvement with ventriculoperitoneal shunting)	[58]
	GRID2	SCAR18	AR	Semidominant transmission, congenital onset	Congenital–46 years	–	[59]
	ELOVL5	SCA38	AD	Pes cavus, hyposmia	–	–	[60]
HCA	PNPLA6	SPG39	AR	Hypogonadotropic hypogonadism	18–23 years	–	[37,38]
	–	–	–	Pure CA	–	–	[39]
	GBA2	SPG46	AR	–	–	–	[61]
	IFRD1	SMNA	AD	–	4–10 years	Normal	[62]
	KCNA1	EA1	AD	Hand tremor, malignant hyperthermia	–	–	[63]
	SLC52A2	BVVL52	AR	Severe hearing loss	8 – 9 years	Optic atrophy	[64]
	COG5	CDG	AR	Freidreich ataxia like	< 2 years	–	[65]
	DNMT1	HSN 1E	AD	Cognitive decline	–	–	[66]
	–	–	–	Scleroderma, endocrinopathy, immunodeficiency	8 years	–	[67]
	SLC25A46	PCHD1	AR	Myoclonic ataxia, optic atrophy, exotropia, PNP	15 years	CA	[68]
HCA	ITPR1	SCA15-16-19	AD	Gillespie syndrome	–	–	[69]
	SYNE1	SCAR8/ARCA1	AR	Motor neuron and brainstem dysfunction	–	–	[70]
	TSEN54	PCH2A	AR	Exaggerated startle response	Newborn	–	[71]
	KCNC1	MEAK	AD	Myoclonus, epilepsy	3–15 years	Prominent CC	[72]
	SCA3/MJD	MJD1	AD	Spastic ataxia, bilateral upper-eyelid contracture, genetic anticipation	15–30 years	Cervical spinal cord atrophy	[41]
	SCA1	SCA1	AD	SP as early presentation of SCA1	35 years	–	[40]

ALS, amyotrophic lateral sclerosis; CA, cerebellar ataxia; CC, corpus callosum; GE, gadolinium enhancement; GPH, globus pallidus hyperintensities; LL, lower limbs; MS, multiple sclerosis; NBIA, neurodegeneration with brain iron accumulation; PNP, polyneuropathy; SMARD1, spinal muscular atrophy with respiratory Distress 1; TCC, thin corpus callosum; WM, white matter; WMH, white matter hyperintensities; WML, white matter lesion.

The clinical overlap between cerebellar ataxias and HSPs was once again highlighted by the fact that mutations in SCAs genes produced, at least in some phases of the disease, a more prominent pyramidal syndrome than the ataxia. This was the case of SCA1 carrier patients that manifested neurological features typical of spastic paraplegia during the initial phases of the disease, which later evolved in cerebellar syndrome [40]. Spasticity also characterizes patients carrying *MJD1/SCA3* mutations [41], as reported before [10].

Clinical and genetic complexity is evident and the clinician might be confused when facing such complex and overlapping phenotypes. To tackle the issue, a tool has been created to find the diagnosis in autosomal recessive cerebellar ataxias (ARCAs). The Recessive Ataxias ranking differential DIagnosis ALgorithm (RADIAL) outperformed ataxia experts, supporting its use in clinical practice to guide the investigations [83]. Providing detailed clinical and paraclinical features of a patient with suspected recessive ataxia, the algorithm predicts different possible candidate genes with high sensitivity and specificity.

HEREDITARY SPASTIC PARAPLEGIA AND CEREBELLAR ATAXIAS AS A NEW MANIFESTATION OF OTHER NEUROLOGICAL DISORDERS

A further widening of the clinical phenotype presented by HSPs and cerebellar ataxias patients can be observed when considering genes causative of HSPs/cerebellar ataxias allelic diseases. The observation of HSP or cerebellar ataxia as a novel clinical manifestation allowed to broaden the phenotypical spectrum of other neurological diseases (Table 2) [84–100].

Spastic paraplegia, complicated by additional features, was present in patients carriers of causative mutations affecting genes previously associated with early-onset fatal encephalopathy and severe pulmonary hypertension [84], Charcot–Marie–Tooth disease [85], Parkinson’s disease [33] and leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation [86], among others. Finally, a dominant-negative mutation in *TUBB2A* was associated with a spastic ataxia phenotype [87] while previously involved in dominant cortical dysplasia (OMIM 615763). Phenotype–genotype correlations could help the diagnostic process but most of the time are lacking. One exception is the correlation found for *POLR3A*, gene involved in the spectrum of hypomyelinating leukodystrophies (HLDs) with the observation that patients carrying the c.1909 + 22G>A variant in a

compound heterozygous state present with a spastic ataxia phenotype rather than HLD [88].

Spastic ataxia was a complicating feature in presence of *CAV1* mutations responsible for a broad clinical phenotype composed by congenital, generalized lipodystrophy, partial lipodystrophy, congenital cataracts and neurodegeneration syndrome and primary pulmonary hypertension [89] as well as in presence of *GRN* mutations, causative of frontotemporal dementia [90].

The recent expansion of the overlap between HSPs and other neurological diseases allowed to consider common cellular mechanisms involved in HSPs. Impairment to the correct functioning of motor proteins (such as KIF1C, KIF5A, TUBB2A and so on) was known to be deleterious, especially at the neuronal level, leading to HSPs [101–103]. Impaired cargo transport due to *DYNC1H1* and *DNM2* mutations was already known to be associated with different neurodegenerative diseases such as spinal muscular atrophy with lower extremity dominance (SMA-LED) and Charcot–Marie–Tooth, and as recently observed, also to HSP [85,104].

NEW CHALLENGES IN DIAGNOSTIC AND THERAPEUTIC PROCESSES

The introduction of NGS techniques undoubtedly had, and is still having, a strong impact in unravelling the genetic causes underlying complex neurological diseases such as HSPs and HCAs. The number of new genes causing both HSPs and HCAs has rapidly increased, especially in the last decade, allowing to establish new overlaps with diverse neurological or extra neurological diseases. The first thing that clearly jumps out when considering phenotypic overlaps is the strong superposition between HSPs and HCAs. As already noticed, cerebellar ataxia can be the primary clinical feature of HSP genes [4,105,106], as well as spastic paraplegia can be the first clinical presentation in some HCA genes [40,41]. It is therefore more and more justified to consider HSPs and HCAs as part of a common spectrum, as sharing not only an increasing number of genes but also some pathological pathways [4]. Moreover, spastic ataxia was observed as a newly identified clinical entity associated with genes previously not related to HSPs and HCAs [7].

Another issue that furthermore complicates the diagnostic process is the absence of phenotype–genotype correlations, as well as the usual lack of specific biomarkers. Recently, the evidence of 25-hydroxycholesterol (25-OHC) and 27-hydroxycholesterol (27-OHC) as biomarkers in SPG5 [107] may simplify the genetic testing of HSP and has allowed to test molecules as atorvastatin and

Table 2. New allelic diseases to hereditary spastic paraplegias and cerebellar ataxia

Disease	Gene	Mode of transmission	New associated symptoms	Age at onset	MRI	Reference
Early-onset fatal encephalopathy, severe pulmonary hypertension	NFU1	AR	SP, mild cognitive impairment, progressive scoliosis, severe bladder dysfunctions, PDH deficiency	18 months	–	[59]
SMA-LED, MCD, CMT2O, ID with neuronal migration defects	DYNC1H1	AD	cHSP, bilateral cataracts, extremities ataxia, focal epilepsy	15 years	TCC	[104]
CMT2M, CMTDIB, ADCNM, LCCS5	DNM2	AD	HSP, bilateral pes cavus, LL wastings	10–37 years	–	[85]
Severe congenital disorder of glycosylation	PMM2	AR	Late-onset ataxia	<10 years	–	[33]
PD (PARK8)	LRRK2	AR	HSP	–	–	
Dominant optic atrophy syndrome	OPA1	AD	Neuropathy, cataract	20 years	–	
Early infantile epileptic encephalopathy – 32 (EIEE32)	KCNA2	AD	cSP, early-onset absence epilepsy	–	–	[91]
			SP, dysarthria	–	–	[92]
DYT4, H-ABC	TUBB4A	AD	SP, mild ID, cerebellar hypometabolism	Childhood	Iron deposition in the globi pallidi, mild CA	[93]
FTLD	GRN	AR	Spastic ataxia	20 years	CA	[90]
IBSL	DARS2	AR	Slowly progressive SP	20 years	Extensive WM lesions	[86]
MEGDEL syndrome	SERAC1	AD	cHSP, infantile-onset cognitive developmental delay, febrile seizures, slight impairment of fine motor skills, dystonia, dysphagia, speech reduction	2–7 years	–	[94]
KRS, NBIA, late-onset PD, NCL	ATP13A2/SPG78	AD	cHSP, cognitive deficits, supranuclear gaze palsy	32 years	Vermian and hemispheric CA, WM changes, TCC, 'ear of the lynx sign', T2 hyperintensities	[95]
HUPRAS	SARS2	AR	Severe spastic tetraparesis	7–8 months	T2 hyperintensities, CA, elevate thalamic lactate	[96]
			cHSP, UL dysmetria, pes cavus, hammer toes	12 years	–	[89]
IMMD, FTD, ALS, SMAJ	CHCHD10	AD	cHSP, UL dysmetria, deafness, global ophthalmoparesis with bilateral ptosis	25 years	–	[89]
COXPD24	NARS2	AR	cHSP, epilepsy, ID, scoliosis, muscular biopsy abnormalities, severe LL hypoesthesia, chronic kidney disease	7 years	–	

Table 2 (Continued)

Disease	Gene	Mode of transmission	New associated symptoms	Age at onset	MRI	Reference
HUPRAS	SARS2	AR	cHSP, UL dysmetria, pes cavus, hammertoes	12 years	–	
SPAX1	VAMP1	AD	Mild SP, UL dysmetria, cervical dystonia, slow saccades, supranuclear palsy	40 years	–	
SPAX4	MTPAP	AR	SP, oromandibular and cervical dystonia, supranuclear ventricular palsy	6 years	Optic atrophy	
CGL3, LCCNS, PPH3	CAV1	AD	spastic ataxia, aquileu clonus, pigmentary retinopathy, congenital cataracts	12 years	–	
INAD1, NBIA2B, PD (PARK14)	PLA2G6	AR	cH SP, cognitive impairment	3–5 years	Optic atrophy	[97]
MRT49	GPT2	AR	Dystonia, cognitive decline, Microcephaly, cognitive decline, dysmorphic feet, synophrys	4–7 years 12 months	Cerebellar atrophy, NBIA	[98]
TACH	POLR3A	AR	adolescent-onset spastic ataxia	2 months–51 years	Hyperintensities along the superior cerebellar peduncles, hypointense correlate in T1-weighted images, cervical cord atrophy, hypoplasia of the corpus callosum	[88]
COXPD26	TRMT5	AD	SP, muscle weakness, developmental delay, cerebral palsy	Childhood	Deep WM changes	[99]
FDFM	ADCY5	AD	SP, mild foot dystonic posture, right hand writer's cramp	Unclear	normal	[100]
CDCBM5	TUBB2A	AD	Spastic ataxia and sensory motor neuropathy, strabismus, pendular nystagmus, mild mental defect	4 years	Periventricular T2 hyperintense areas, bilateral optic atrophy, TCC, cerebellar vermis atrophy	[87]

ADCNM, autosomal dominant centronuclear myopathy; ALS, amyotrophic lateral sclerosis; CA, cerebellar ataxia; CDCBM5, complex cortical dysplasia with other brain malformations – 5; CGL3, lipodystrophy, congenital, generalized type 3; cHSP, complicated hereditary spastic paraplegia; CMT2O Charcot-Marie-Tooth disease type 2O; CMTDIB, Charcot-Marie-Tooth dominant intermediate; COXPD24, combined oxidative phosphorylation deficiency 24; COXPD26, combined oxidative phosphorylation deficiency 26; DYT4, dystonia type 4; FDFM, familial dyskinetic with facial myokymia; FTD, frontotemporal dementia; H-ABC, hypomyelination with atrophy of the basal ganglia and cerebellum; HUPRA syndrome, hypoleukemia, pulmonary hypertension, renal failure in infancy, and alkalosis; HUPRAS, hyperuricemia, pulmonary hypertension, renal failure, alkalosis; ID, intellectual disability; IMMD, autosomal dominant isolated mitochondrial myopathy; INAD1, infantile neuroaxonal dystrophy 1; KRS, Kufor-Rakeb syndrome; LBSL, leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation; LCCNS, partial lipodystrophy, congenital cataracts and neurodegeneration syndrome; LCCS5, lethal congenital contractures syndrome type 5; LL, lower limbs; MCD, malformations of cortical development; MEGDEL, 3-methylglutaconic aciduria, deafness, encephalopathy, Leigh-like syndrome; MFM7, myofibrillar myopathy 7; MRT49, autosomal recessive mental retardation-49; NBIA, neurodegeneration with brain iron accumulation; NBIA2B, neurodegeneration with brain iron accumulation 2B; NCL, neuronal ceroid lipofuscinosis; ODDD, oculodentodigital dysplasia; PD, Parkinson's disease; PDH, pyruvate dehydrogenase; PPH3, primary pulmonary hypertension type 3; SMAJ, Spinal muscular atrophy, Jokela type; SMA-LED, spinal muscular atrophy with lower extremity dominance; SP, spastic paraplegia; TCC, thin corpus callosum; UL, upper limbs; WM, white matter.

chenodeoxycholic acid, resulting in the restoration of bile acids levels [108]. Improving knowledge regarding gene functions and biological pathways shared by different genes might open the way to the test of new therapeutic possibilities. This approach led to different phase 1/2 therapeutic trials, sometimes with positive results, such as riluzole administration in SCAs or Friedreich Ataxia patients [109], docosahexaenoic acid in SCA38 patients [110], 4-aminopyridine in episodic ataxia 2 (EA2) [111], valproic acid in SCA3 patients [112], while in the case of other molecules, their effective benefits still remains unclear [113]. More promising and innovative therapeutic approaches might result from intracerebroventricular injections of antisense oligonucleotides (ASOs), already tested in SCA2 and SCA3 mice models. In SCA2 mice, ASOs led to a downregulation of ATXN2 mRNA with consequent decrease of mutant protein, restoring of normal firing frequency of Purkinje cells with delayed onset and improvement of motor skills [114]. In SCA3, ASOs showed a diffused delivery in mice brain and consequent decrease of mutant ATXN3 protein by more than 50% with a good tolerance profile [115]. The development of ASOs treatments in SCA patients is therefore very awaited as well as the identification of biomarkers of disease progression useful to define the potential efficacy of therapies in these slowly progressive disorders.

CONCLUSION

While the number of new HSPs and HCAs causative genes is still raising, their discovery rate has not increased, reaching a sort of plateau. Phenotypic expansion of already known disease-related genes complicated the diagnostic practice. In this regard, data sharing could be improved in order to solve the 'one gene, one family' concern. Expansion of spastic ataxia related phenotypes has a major impact in pointing out common pathological pathways.

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Conflicts of interest

The authors declare to have no conflict of interest.

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Spastic Paraplegia 4

Synonyms: *SPAST-HSP*, *SPG4*

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Summary

Clinical characteristics

Spastic paraplegia 4 (SPG4; also known as *SPAST-HSP*) is characterized by insidiously progressive bilateral lower-limb gait spasticity. More than 50% of affected individuals have some weakness in the legs and impaired vibration sense at the ankles. Sphincter disturbances are very common. Onset is insidious, mostly in young adulthood, although symptoms may start as early as age one year and as late as age 76 years. Intrafamilial variation is considerable.

Diagnosis/testing

The diagnosis of *SPAST-HSP* is established in a proband with characteristic clinical features and a heterozygous pathogenic variant in *SPAST* identified by molecular genetic testing.

Management

Treatment of manifestations: Antispastic drugs for leg spasticity; anticholinergic antispasmodic drugs for urinary urgency; regular physiotherapy to stretch spastic muscles and prevent contractures. Consideration of botulinum toxin and intrathecal baclofen when oral drugs are ineffective and spasticity is severe and disabling. Urodynamic evaluation in order to initiate treatment when sphincter disturbances become a problem.

Surveillance: Evaluation every 6-12 months to update medications and physical rehabilitation.

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Genetic counseling

SPAST-HSP is inherited in an autosomal dominant manner with age-related, nearly complete penetrance and is characterized by significant intrafamilial clinical variability. Most individuals diagnosed with *SPAST*-HSP have an affected parent. The proportion of cases caused by a *de novo* pathogenic variant is low. Each child of an individual with *SPAST*-HSP has a 50% chance of inheriting the pathogenic variant. Prenatal testing and preimplantation genetic diagnosis are possible if a pathogenic *SPAST* variant has been identified in an affected family member. Because of variable clinical expression, results of prenatal testing cannot be used to predict whether an individual will develop *SPAST*-HSP and, if so, what the age of onset, clinical course, and degree of disability will be.

Diagnosis

Suggestive Findings

Spastic paraplegia 4 (SPG4; also known as *SPAST*-HSP) **should be suspected** in individuals with the following:

- Characteristic clinical symptoms of insidiously progressive bilateral leg stiffness affecting gait with or without spasticity at rest and mild proximal weakness, often accompanied by urinary urgency
- Neurologic examination demonstrating corticospinal tract deficits affecting both legs (spastic weakness, hyperreflexia, and extensor plantar responses). Mildly impaired vibration sensation in the ankles is present in the majority of individuals.
- Family history consistent with autosomal dominant inheritance, or exclusion of other causes of spastic paraplegia in simplex cases (i.e., a single occurrence in a family)

Note: The presence of other signs/symptoms suggestive of complicated hereditary spastic paraplegia does not exclude *SPAST*-HSP, although it reduces its probability.

Brain and spinal cord MRI

- Often normal in individuals with *SPAST*-HSP
- Spinal cord atrophy can occur in *SPAST*-HSP, but is less pronounced than in other genetic causes of HSP.
- Mild vermis atrophy, a thin corpus callosum, subtle white matter changes, and/or cerebellar atrophy have been reported [Duning et al 2010, da Graça et al 2019].

Note: The MRI is useful in identifying anomalies of the brain, cerebro-medullary junction, and medulla that are characteristic of disorders discussed in Differential Diagnosis.

Electromyography (EMG) with nerve conduction velocities (NCV) is used to exclude peripheral nervous system involvement, which could raise the possibility of an alternative diagnosis as severe polyneuropathy is not a frequent symptom of *SPAST*-HSP. Karle et al performed neurophysiologic examinations of 128 individuals with HSP, including 35 individuals with *SPAST*-HSP, and showed that massively elongated central motor conduction time argued against *SPAST*-HSP; however, reduced amplitudes and prolonged latencies were reported, in particular in individuals with a *SPAST* pathogenic missense variant [Karle et al 2013].

Establishing the Diagnosis

The diagnosis of *SPAST*-HSP **is established** in a proband with Suggestive Findings by identification of a heterozygous pathogenic variant in *SPAST* by molecular genetic testing (see Table 1).

Note: (1) Failure to detect a pathogenic variant/deletion does not absolutely exclude the diagnosis. (2) Once non-genetic causes have been excluded, testing for *SPAST*-HSP should be considered in simplex cases (i.e.,

individuals with no family history of spasticity), as *SPAST* pathogenic variants can be identified in approximately 10%-20% of simplex cases [Erichsen et al 2009a, Shoukier et al 2009, Fei et al 2011].

Molecular genetic testing approaches can include a combination of **gene-targeted testing** (single-gene testing, multigene panel) and **comprehensive genomic testing** (exome sequencing, exome array, genome sequencing) depending on the phenotype.

Gene-targeted testing requires that the clinician determine which gene(s) are likely involved, whereas genomic testing does not. Because the phenotype of *SPAST*-HSP is broad, individuals with the distinctive findings described in Suggestive Findings are likely to be diagnosed using gene-targeted testing (see Option 1), whereas those with a phenotype indistinguishable from many other inherited disorders with spastic paraplegia are more likely to be diagnosed using genomic testing (see Option 2).

Option 1

When the phenotypic and laboratory findings suggest the diagnosis of *SPAST*-HSP, molecular genetic testing approaches can include **single-gene testing** or use of a **multigene panel**:

- **Single-gene testing.** Sequence analysis of *SPAST* detects missense, nonsense, and splice site variants, as well as small intragenic deletions/insertions. The combination of *in silico* predictive algorithms and information retrieved from population databases is essential to establish the pathogenic role of variants of unknown significance [Richards et al 2015]. If no pathogenic variant is found on sequence analysis, perform gene-targeted deletion/duplication analysis to detect intragenic deletions or duplications.
- **A multigene panel** that includes *SPAST* and other genes of interest (see Differential Diagnosis) is most likely to identify the genetic cause of the condition at the most reasonable cost while limiting identification of variants of uncertain significance and pathogenic variants in genes that do not explain the underlying phenotype. Note: (1) The genes included in the panel and the diagnostic sensitivity of the testing used for each gene vary by laboratory and are likely to change over time. (2) Some multigene panels may include genes not associated with the condition discussed in this *GeneReview*. (3) In some laboratories, panel options may include a custom laboratory-designed panel and/or custom phenotype-focused exome analysis that includes genes specified by the clinician. (4) Methods used in a panel may include sequence analysis, deletion/duplication analysis, and/or other non-sequencing-based tests. For this disorder, a multigene panel that also includes deletion/duplication analysis is recommended (see Table 1).

For an introduction to multigene panels click [here](#). More detailed information for clinicians ordering genetic tests can be found [here](#).

Option 2

When the phenotype is indistinguishable from many other inherited disorders characterized by spastic paraplegia, **comprehensive genomic testing** (which does not require the clinician to determine which gene[s] are likely involved) is the best option. **Exome sequencing** is most commonly used; **genome sequencing** is also possible.

If exome sequencing is not diagnostic – and particularly when evidence supports autosomal dominant inheritance – **exome array** (when clinically available) may be considered to detect (multi)exon deletions or duplications that cannot be detected by sequence analysis.

For an introduction to comprehensive genomic testing click [here](#). More detailed information for clinicians ordering genomic testing can be found [here](#).

Table 1. Molecular Genetic Testing Used in Spastic Paraplegia 4

Gene ¹	Method	Proportion of Probands with a Pathogenic Variant ² Detectable by Method
<i>SPAST</i>	Sequence analysis ³	75%-80% ⁴
	Gene-targeted deletion/duplication analysis ⁵	20%-25% ⁶

1. See Table A. Genes and Databases for chromosome locus and protein.

2. See Molecular Genetics for information on allelic variants detected in this gene.

3. Sequence analysis detects variants that are benign, likely benign, of uncertain significance, likely pathogenic, or pathogenic. Pathogenic variants may include small intragenic deletions/insertions and missense, nonsense, and splice site variants; typically, exon or whole-gene deletions/duplications are not detected. For issues to consider in interpretation of sequence analysis results, click [here](#).

4. Benign variants can affect the phenotype (see Genotype-Phenotype Correlations and Molecular Genetics).

5. Gene-targeted deletion/duplication analysis detects intragenic deletions or duplications. Methods used may include quantitative PCR, long-range PCR, multiplex ligation-dependent probe amplification (MLPA), and a gene-targeted microarray designed to detect single-exon deletions or duplications.

6. Exon and multiexon deletions and duplications account for approximately 20%-25% of *SPAST* pathogenic variants [Beetz et al 2006, Depienne et al 2007b].

Clinical Characteristics

Clinical Description

The cardinal clinical feature of spastic paraplegia 4 (*SPAST*-HSP) is insidiously progressive bilateral lower-limb spasticity associated with brisk reflexes, ankle clonus, and bilateral extensor plantar responses. Sphincter disturbances are very frequent (77%), in particular urinary urgency and incontinence. Increased reflexes in the upper limbs may also occur (65%), but other symptoms and findings in the upper limbs are rare. A frequent additional feature is decreased, but not abolished, vibration sense at the ankles, occurring in 60% of individuals [Parodi et al 2018]. Around 50% of affected individuals have proximal weakness in the lower limbs [Kanavin & Fjermestad 2018, Parodi et al 2018, Schneider et al 2019].

Age at onset of symptoms ranges from infancy to the eighth decade. Age at onset is variable even among family members with the same pathogenic variant. A recent study including more than 500 individuals with *SPAST*-HSP confirmed that the age at onset is characterized by a bimodal distribution, with a major first peak before the first decade, and a second, lower one between the third and fifth decades. Penetrance is not complete; an estimated 6% of individuals remain asymptomatic throughout life. Penetrance is reported to be lower in females than in males [Parodi et al 2018].

Disease severity generally worsens with the duration of the disease, although some individuals remain mildly affected all their lives. Disease severity is variable even among family members with the same pathogenic variant. After long disease duration (20 years), approximately 50% of individuals need assistance for walking, and approximately 10% require a wheelchair. Disease progression is more rapid in individuals with late onset (age >35 years) than in those with early onset [Loureiro et al 2013, Chrestian et al 2016, Polymeris et al 2016, Parodi et al 2018].

Comparing men and women, Parodi et al [2018] observed that symptomatic females more often had increased upper-limb reflexes than males, representing a more severe and diffused disorder in women.

Leg spasms are frequent and may also develop before the onset of spasticity. Spasms are more frequent after physical activity, and tend to disappear when spasticity becomes more severe.

Bladder dysfunction remains one of the most frequent problems for affected individuals and may be more frequent in individuals with *SPAST*-HSP than in all individuals with HSP; Schneider et al [2019] reported

urologic involvement in 91.2% of individuals with *SPAST*-HSP compared to 74.5% of individuals with HSP. The most frequent symptoms are urinary incontinence, hesitancy, increased frequency of micturition, and urgency. Incomplete bladder emptying may also occur [Braschinsky et al 2010]. The anal sphincter may also be affected, resulting in uncontrollable flatulence or fecal incontinence, affecting respectively 31.4% and 8.7% of individuals with HSP in one study [Kanavin & Fjermestad 2018]. In a study of urodynamic findings in 29 individuals with HSP, Fourtassi et al [2012] described signs of central neurogenic bladder in 82.7%, with detrusor overactivity in 52% and detrusor sphincter dyssynergia in 65.5%.

Pes cavus and mild spastic dysarthria may be observed.

Subtle cognitive impairment has been documented [Erichsen et al 2009b]; but its relation to the disease remains undetermined. Cognitive deficits appear late in the disease course and are not present in all affected members of a given family. When detected by neuropsychological testing, the impairment is often subtle, limited to executive dysfunction, and without noticeable effect on daily living. No definite correlation with the type of pathogenic variant in *SPAST* has been established.

Extensive neuropsychological assessment of nine adults with *SPAST*-HSP (including one asymptomatic individual) identified cognitive impairment fulfilling multidomain non-amnesic mild cognitive impairment criteria, with executive impairment and impaired social cognition [Chamard et al 2016] as suggested by Tallaksen et al [2003], where a familial aggregation of cognitive impairment suggested the implication of modifiers. In the large *SPAST*-HSP study by Parodi et al [2018], intellectual disability was described in 4.2%.

Other findings compatible with a complex form of HSP are uncommon in *SPAST*-HSP but do not exclude the diagnosis. Also, whether these additional findings are related to *SPAST*-HSP or coincidental remains to be clarified.

Neuropathy has been reported in individuals with *SPAST*-HSP, but without compelling evidence of a shared underlying pathologic mechanism. Kumar et al [2012] found peripheral abnormalities in nerve conduction studies in two of 11 individuals with *SPAST*-HSP.

Non-motor symptoms are more frequent than previously acknowledged. Servelhere et al [2016] studied 30 individuals and found that fatigue, pain, and depression were frequent and often severe manifestations in individuals with *SPAST*-HSP.

Restless legs syndrome has been associated with *SPAST*-HSP [Sperfeld et al 2007], but this remains to be confirmed.

Hand tremor was reported in 10% of a large cohort of Dutch individuals with *SPAST*-HSP [de Bot et al 2010].

Seizures, intellectual disability, and cerebellar ataxia are rare. A few individuals with severe dementia have been reported [Murphy et al 2009]. However, too few neuropathologic studies have been performed in persons with *SPAST*-HSP for a general picture of the distribution of cortical and medullar lesions in the disease to emerge.

Neuroimaging. Newer MRI studies using advanced neuroimaging techniques have shown widespread involvement of gray and white matter in individuals with *SPAST*-HSP [Lindig et al 2015, Rezende et al 2015, Liao et al 2018, Rucco et al 2019]. In a study of 11 individuals, fractional anisotropy was reduced in the corticospinal tracts, cingulate gyri, and splenium of the corpus callosum [Rezende et al 2015]. Resting-state fMRI studies in 12 individuals with *SPAST*-HSP showed abnormal functional activity in several brain areas [Liao et al 2018]. Rucco et al [2019] performed magnetoencephalography of ten individuals with *SPAST*-HSP and described global network rearrangements. Using diffusion tensor imaging and tract-based special statistics, Lindig et al [2015] found that imaging findings in the 15 included individuals correlated with disease duration and severity.

Genotype-Phenotype Correlations

Recently, after analyzing a cohort of more than 500 individuals with *SPAST*-HSP, Parodi et al [2018] showed that missense variants were associated with an earlier age of onset (by 10 years), when compared to truncating variants. This finding provides an explanation for the bimodal age of onset distribution typical of *SPAST*-HSP.

It is important to note that age at onset and clinical severity are highly variable for a given variant, even in the same family. The observed difference in age of onset between related individuals ranged from 27 years to 69 years [Parodi et al 2018]. Furthermore, two family members with the same variant can have in one case a pure spastic paraparesis and in the other a complex disease. For example, Orlacchio et al [2004] reported wide phenotypic variability with the p.Asn386Ser variant, with some individuals presenting with intellectual disability and others showing brain MRI abnormalities including thin corpus callosum or cerebellar atrophy.

The most plausible explanation for intra and interfamilial variability is the presence of genetic modifiers. Svenson et al [2004] reported two rare nonsynonymous *SPAST* variants, c.131C>T (p.Ser44Leu) and c.134C>A (p.Pro45Gln) acting as age-of-onset modifiers. In several analyzed families, the individuals who had a *SPAST* pathogenic variant on one allele and either a c.131C>T or c.134C>A variant on the other allele (*in trans*) had a very early onset, suggesting that these alleles could modify the HSP phenotype [Svenson et al 2004, McDermott et al 2006, personal communication]. The *SPAST* variant c.131C>T has a frequency of 0.4% in a control population, c.134C>A is even more rare in the [gnomAD Database](#) [Karczewski et al 2019]. In addition to the two *SPAST* variants, an *HSPD1* variant was proposed as a *SPAST*-HSP age-at-onset modifier [Svenstrup et al 2009], but its role remains under discussion.

Penetrance

Penetrance is age dependent and mostly complete in individuals with *SPAST*-HSP. It is estimated to be 85% by age 45 years [Fonknechten et al 2000] and complete at 70 years [Parodi et al 2018]. It should be emphasized that age dependence is explained partly by variability in age at onset and partly by the difficulty in determining the precise age of onset; thus, neurologic examination is important. Penetrance is greater if pyramidal signs as well as spastic gait are considered: approximately 6% of individuals who have a *SPAST* variant are completely asymptomatic on examination; approximately 20% have abnormal signs when examined, but no awareness of being affected.

Penetrance may be gender dependent. Parodi et al [2018] reported a higher penetrance in males (94%) than females (88%), and greater gender discordance in individuals with onset before the third decade (91% vs 70%).

Nomenclature

The gene in which mutation is responsible for spastic paraplegia at the SPG4 locus, *SPAST*, was previously known as *SPG4*.

SPAST-HSP may also be referred to as SPG4. Previously it was also known as hereditary spastic paraplegia, spastin type [Marras et al 2016].

Prevalence

The most recent epidemiologic study estimates a global prevalence of autosomal dominant-HSP (AD-HSP) of 1-5:100,000 [Ruano et al 2014].

Among AD-HSPs, *SPAST* is the most frequently associated gene in both familial and simplex cases [Lo Giudice et al 2014, Ruano et al 2014]. Reports from many European countries as well as the US, Canada, Japan, and China appear to indicate that *SPAST*-HSP accounts for 40% of inherited AD-HSPs and 20% of simplex HSPs [Erichsen et al 2009a, Takiyama et al 2010, Fei et al 2011, Lo Giudice et al 2014, Ruano et al 2014].

Geographic prevalence may vary; Meijer et al [2002] found fewer families with *SPAST*-HSP among North American families than expected from reports in European families.

Genetically Related (Allelic) Disorders

Some individuals with a *SPAST* pathogenic variant have lower motor neuron degeneration, leading to an ALS-like phenotype [Meyer et al 2005, Parodi et al 2017].

Differential Diagnosis

See [Hereditary Spastic Paraplegia Overview](#) for a review of the differential diagnosis.

SPAST-HSP is the most frequently occurring form of autosomal dominant hereditary spastic paraplegia, accounting for an estimated 40% of AD-HSP [Lo Giudice et al 2014]. Because *SPAST* is the most commonly involved gene in AD-HSP, it is the first and most relevant gene to be tested. The other main types of autosomal dominant pure spastic paraplegia to consider are SPG3A, SPG31, and SPG10.

With the exceptions of [SPG3A](#), SPG31, and SPG10, no significant differences have been established between SPG4 and other types of pure dominant spastic paraplegia.

Table 2. Other Types of Autosomal Dominant Pure Spastic Paraplegia (AD-HSP) to Consider in the Differential Diagnosis of *SPAST*-HSP

Gene(s)	Disorder	Clinical Features Distinguishing the Disorder from <i>SPAST</i> -HSP
<i>ATL1</i>	SPG3A	<ul style="list-style-type: none">• Earlier onset (often age <10 yrs)• More muscle wasting in lower limbs & scoliosis• Fewer sphincter disturbances• Less frequent impairment of vibration sense at the ankles & ↑ reflexes in upper limbs• 2nd most common type of AD-HSP
<i>KIF5A</i>	SPG10 (OMIM 604187)	More frequent peripheral neuropathy, amyotrophy, or parkinsonism
<i>REEP1</i>	SPG31 (OMIM 610250)	<ul style="list-style-type: none">• More frequent peripheral neuropathy or amyotrophy• 3rd most common type of AD-HSP

In simplex cases (i.e., spasticity in one individual in a family), all possible causes of spasticity in the legs must be considered because several non-genetic causes of spasticity are more common than *SPAST*-HSP.

Management

Evaluations Following Initial Diagnosis

To establish the extent of disease and needs in an individual diagnosed with spastic paraplegia 4 (*SPAST*-HSP), the evaluations summarized in this section (if not performed as part of the evaluation that led to the diagnosis) are recommended:

- Neuro-urologic examination is advised for individuals who have sphincter disturbances.
- Whether neuropsychological testing should be performed to assess the cognitive impairment frequently reported in individuals with *SPAST*-HSP remains unclear. So far, no consensus exists on the type of tests that should be performed, or on the timing or purpose of the tests. Considering that cognitive impairment is often absent or is detectable only by neuropsychological testing, one should be wary of increasing the burden of individuals with *SPAST*-HSP, and probably only recommend further testing when required by the affected individual.

- Electrophysiologic investigations may be advisable in case of pain and/or edema in the lower limbs to evaluate for associated neuropathy. Neuropathy, while not a feature of *SPAST*-HSP per se, may occur in individuals with *SPAST*-HSP for other reasons and should be investigated and adequately treated. Because of the underlying HSP, the neuropathy may remain undiagnosed if routine investigations are not conducted.
- Spinal MRI examination to exclude any additional degenerative disorder can be considered if unusual symptoms or pain are present.
- Consultation with a clinical geneticist and/or genetic counselor is appropriate.

Treatment of Manifestations

Treatment is symptomatic as there is still no curative or disease-modifying treatment for *SPAST*-HSP. Care by a multidisciplinary team that includes a general practitioner, neurologist, clinical geneticist, physiotherapist, physical therapist, social worker, and psychologist should be considered.

Symptomatic treatment includes use of the following:

- Antispastic drugs for leg spasticity
- Anticholinergic antispasmodic drugs for urinary urgency
- Regular physiotherapy for stretching of spastic muscles. Stretching should be done manually at all levels (hips, knees, ankles) and preceded by heat conditioning. Early regular physiotherapy can prevent contractures to a certain extent. Intensive and early physiotherapy delays the development of symptoms related to spasticity and prolongs the ability to walk [Author, personal observation]. To date, the effectiveness of physical therapy in individuals with HSP is only documented in a small number of case reports and uncontrolled studies.

Botulinum toxin and intrathecal baclofen can be proposed when oral drugs are ineffective and spasticity is severe and disabling. In children, orthopedic treatment and botulinum toxin injections may also contribute to better ambulatory function. A recent study of a mixed cohort of 33 individuals with HSP suggested that botulinum toxin-A injections provide some benefits, not only for spasticity, but also for fatigue [Servelhere et al 2018]. However, studies are scarce and more systematic studies are needed to confirm these observations.

Urodynamic evaluation should be performed early in all affected individuals complaining of urgency or other problems, such as voiding difficulties, urine retention, and/or frequent urinary infections. Such symptoms should be monitored and treated according to individual needs and disease evolution. Follow up of the sphincter disturbances is important to prevent bladder dysfunction. Treatment options include anticholinergic drugs and intravesical botulinum-toxin injections [Joussain et al 2019].

Surveillance

Specialized outpatient evaluations are suggested every six to 12 months to update medications and physical rehabilitation.

Evaluation of Relatives at Risk

See Genetic Counseling for issues related to testing of at-risk relatives for genetic counseling purposes.

Therapies Under Investigation

Search [ClinicalTrials.gov](https://clinicaltrials.gov) in the US and [EU Clinical Trials Register](https://clinicaltrialsregister.eu) in Europe for access to information on clinical studies for a wide range of diseases and conditions.

Other

A double-blind crossover trial with gabapentin did not show improvement of spasticity in persons with SPAST-HSP [Scheuer et al 2007].

Genetic Counseling

Genetic counseling is the process of providing individuals and families with information on the nature, inheritance, and implications of genetic disorders to help them make informed medical and personal decisions. The following section deals with genetic risk assessment and the use of family history and genetic testing to clarify genetic status for family members. This section is not meant to address all personal, cultural, or ethical issues that individuals may face or to substitute for consultation with a genetics professional. —ED.

Mode of Inheritance

Spastic paraplegia 4 (SPAST-HSP) is inherited in an autosomal dominant manner.

Risk to Family Members

Parents of a proband

- Individuals diagnosed with SPAST-HSP usually have a symptomatic parent who has the SPAST pathogenic variant; however, a parent with the SPAST pathogenic variant may have no symptoms.
- An individual with SPAST-HSP may, more rarely, have the disorder as the result of a *de novo* pathogenic variant [Parodi et al 2018].
- Recommendations for the evaluation of parents of a proband with an apparent *de novo* pathogenic variant include neurologic examination for evidence of spasticity and molecular genetic testing if a SPAST pathogenic variant has been identified in a family member.
- If the pathogenic variant found in the proband cannot be detected in the leukocyte DNA of either parent, possible explanations include a *de novo* pathogenic variant in the proband or germline mosaicism in a parent. Though theoretically possible, no instances of a proband inheriting a pathogenic variant from a parent with germline mosaicism have been reported.
- The family history of some individuals diagnosed with SPAST-HSP may appear to be negative because of failure to recognize the disorder in family members, reduced (age-related) penetrance, or early death of the parent before the onset of symptoms. Therefore, an apparently negative family history cannot be confirmed unless molecular genetic testing has been performed on the parents of the proband.

Sibs of a proband. The risk to the sibs of the proband depends on the clinical/genetic status of the proband's parents:

- If a parent of the proband is affected or has the pathogenic variant, the risk to the sibs of inheriting the variant is 50%. Note: Significant intrafamilial variability in age of onset and clinical severity is observed in SPAST-HSP.
- If the proband has a known SPAST pathogenic variant that cannot be detected in the leukocyte DNA of either parent, the recurrence risk to sibs is estimated to be 1% because of the theoretic possibility of parental germline mosaicism [Rahbari et al 2016].
- If the parents of a proband are clinically unaffected but have not undergone molecular genetic testing, sibs of the proband are still presumed to be at increased risk for SPAST-HSP because of the possibility of reduced penetrance in a parent or the theoretic possibility of parental germline mosaicism.

Offspring of a proband. Each child of an individual with SPAST-HSP has a 50% chance of inheriting the pathogenic variant.

Other family members. The risk to other family members depends on the status of the proband's parents: if a parent is affected or has the *SPAST* variant present in the affected family member, his or her family members are at risk.

Related Genetic Counseling Issues

Considerations in families with an apparent *de novo* pathogenic variant. When neither parent of a proband with an autosomal dominant condition has the pathogenic variant identified in the proband or clinical evidence of the disorder, the pathogenic variant is likely *de novo*. However, non-medical explanations including alternate paternity or maternity (e.g., with assisted reproduction) and undisclosed adoption could also be explored.

Predictive testing (i.e., testing of asymptomatic at-risk individuals)

- Predictive testing for at-risk relatives is possible once a *SPAST* pathogenic variant has been identified in an affected family member.
- Potential consequences of such testing (including, but not limited to, socioeconomic changes and the need for long-term follow up and evaluation arrangements for individuals with a positive test result) as well as the capabilities and limitations of predictive testing should be discussed in the context of formal genetic counseling prior to testing.

Predictive testing in minors (i.e., testing of at-risk individuals age <18 years)

- For asymptomatic minors at risk for typically adult-onset conditions for which early treatment would have no beneficial effect on disease morbidity and mortality, predictive genetic testing is considered inappropriate, primarily because it negates the autonomy of the child with no compelling benefit. Further, concern exists regarding the potential unhealthy adverse effects that such information may have on family dynamics, the risk of discrimination and stigmatization in the future, and the anxiety that such information may cause.
- For more information, see the National Society of Genetic Counselors [position statement](#) on genetic testing of minors for adult-onset conditions and the American Academy of Pediatrics and American College of Medical Genetics and Genomics [policy statement](#); ethical and policy issues in genetic testing and screening of children.
- In a family with an established diagnosis of *SPAST*-HSP, it is appropriate to consider testing of symptomatic individuals regardless of age.

Family planning

- The optimal time for determination of genetic risk and discussion of the availability of prenatal testing is before pregnancy.
- It is appropriate to offer genetic counseling (including discussion of potential risks to offspring and reproductive options) to young adults who are affected or at risk.

DNA banking is the storage of DNA (typically extracted from white blood cells) for possible future use. Because it is likely that testing methodology and our understanding of genes, allelic variants, and diseases will improve in the future, consideration should be given to banking DNA of affected individuals.

Prenatal Testing and Preimplantation Genetic Diagnosis

Once a *SPAST* pathogenic variant has been identified in an affected family member, prenatal testing for a pregnancy at increased risk and preimplantation genetic diagnosis are possible. Because of variable clinical expression, the results of prenatal testing cannot be used to predict whether an individual will develop *SPAST*-HSP and, if so, what the age of onset, clinical course, or degree of disability will be.

Differences in perspective may exist among medical professionals and within families regarding the use of prenatal testing, particularly if the testing is being considered for the purpose of pregnancy termination. While most centers would consider decisions regarding prenatal testing to be the choice of the parents, discussion of these issues is appropriate.

Resources

GeneReviews staff has selected the following disease-specific and/or umbrella support organizations and/or registries for the benefit of individuals with this disorder and their families. GeneReviews is not responsible for the information provided by other organizations. For information on selection criteria, click [here](#).

- **EURO HSP**

Plateforme Maladies Rares

99 Rue Didot

Paris 75014

France

Phone: 33 1 56 53 52 61

Email: president@eurohsp.eu

www.eurohsp.eu

- **HSP Research Foundation**

P.O. Box 4064

Warrimoo New South Wales NSW 2774

Australia

www.hspersunite.org.au

- **Spastic Paraplegia Foundation, Inc.**

7700 Leesburg Pike

Ste 123

Falls Church VA 22043

Phone: 877-773-4483 (toll-free)

Email: information@sp-foundation.org

sp-foundation.org

- **Tom Wahlig-Foundation**

Tom Wahlig Stiftung

Büro Veghestrasse 22

Germany

Phone: 49 (0) 251 - 20 07 91 20

Email: nfo@hsp-info.de

www.hsp-info.de/en/foundation.htm

- **A.I. Vi.P.S.**

Associazione Italiana Vivere la Paraparesi Spastica Onlus

Via Tevere, 7
20020 Lainate (MI)
Italy

Phone: 39 392 9825622

Email: nfo@vipsonlus.it

www.vipsonlus.it

- **National Institute of Neurological Disorders and Stroke (NINDS)**

PO Box 5801

Bethesda MD 20824

Phone: 800-352-9424 (toll-free); 301-496-5751; 301-468-5981 (TTY)

[Hereditary Spastic Paraplegia Information Page](#)

- **Norsk forening for Arvelig-Spastisk Paraparese / Ataksi (NASPA)**

The Norwegian association for individuals with hereditary spastic paraplegia and ataxia

PO Box 9217

Oslo 0134

Norway

Phone: 47 24 10 24 00

Fax: 47 24 10 24 99

Email: naspa@nhf.no

www.naspa.no

Molecular Genetics

Information in the Molecular Genetics and OMIM tables may differ from that elsewhere in the GeneReview: tables may contain more recent information. —ED.

Table A. Spastic Paraplegia 4: Genes and Databases

Gene	Chromosome Locus	Protein	Locus-Specific Databases	HGMD	ClinVar
<i>SPAST</i>	2p22.3	Spastin	SPAST database Human Variation Database - SPAST	SPAST	SPAST

Data are compiled from the following standard references: gene from [HGNC](#); chromosome locus from [OMIM](#); protein from [UniProt](#). For a description of databases (Locus Specific, HGMD, ClinVar) to which links are provided, click [here](#).

Table B. OMIM Entries for Spastic Paraplegia 4 ([View All in OMIM](#))

182601	SPASTIC PARAPLEGIA 4, AUTOSOMAL DOMINANT; SPG4
604277	SPASTIN; SPAST

Molecular Pathogenesis

Introduction. *SPAST* encodes a 616-amino acid protein named spastin, a putative nuclear member of the AAA (ATPases associated with diverse cellular activities). Claudiani et al [2005] have shown that two spastin isoforms – M1 and M87, of 68 kd and 60 kd, respectively – were synthesized from the *SPAST* mRNA through usage of two

different translational start sites. Spastin-M87 was detected in both spinal cord and cerebral cortex, whereas appreciable levels of spastin-M1 were observed only in spinal cord [Solowska et al 2010].

Spastin is widely expressed in the neurons of the central nervous system, including the cortex and striatum. Distal degeneration of long tracts in the spinal cord is associated with a microglial reaction [Wharton et al 2003]. In addition, the presence of a large number of thin axons in the pyramidal tracts may suggest the existence of a regeneration process [Parodi et al 2018]. Tau-pathology outside the motor system may also be observed [Wharton et al 2003] but is not common to all the analyzed cases [Parodi et al 2018].

Within the cells, spastin acts as a microtubule-severing protein and is responsible for different aspects of microtubule dynamics, such as their length, number, and mobility [Errico et al 2002]. Four proteic domains allow spastin to accomplish its enzymatic function, as well as to interact with other proteins.

- Spastin N-terminal domain is involved in both lipid metabolism [Papadopoulos et al 2015] and endoplasmic reticulum morphogenesis, after interacting with ATL1/SPG3A and REEP1/SPG31 [Park et al 2010].
- The microtubule-interacting and trafficking domain (MIT) allows spastin to interact with CHIMP1 and IST1, two proteins belonging to the endosomal-sorting complex required for transport (ESCRT-III), being therefore involved in both cytokinesis and endosomal-tubule recycling [Reid et al 2005, Connell et al 2009, Allison et al 2013].
- The microtubule-binding domain (MTBD) and the AAA ATPase cassette are responsible for the microtubule binding and ATP hydrolysis [White et al 2007].

Mechanism of disease causation. Spastin loss of function, and consequent haploinsufficiency, has been proposed as the mechanism of disease causation: the majority of *SPAST* pathogenic variants affect the AAA cassette domain and almost 20% of affected individuals were found to have large deletions [Depienne et al 2007a, Parodi et al 2018]. In addition, reduced spastin mRNA was observed in individuals with premature protein termination [Bürger et al 2000].

An alternative to the loss-of-function model, the finding that *SPAST* pathogenic variants in the AAA domain led to constitutive binding to microtubules, could suggest a dominant-negative effect [Errico et al 2002]. The abnormal spastin-microtubule interaction was observed leading to organelle transport impairments, possibly underlying degeneration of the corticospinal axons [McDermott et al 2003].

An additional alternative hypothesis was recently proposed by Solowska et al [2010] and Solowska et al [2014]. After observing that some of the *SPAST* pathogenic variants located outside the AAA cassette may act through another pathogenic mechanism, they generated a mouse model overexpressing human spastin and carrying a *SPAST* pathogenic variant. Adult homozygous mice presented with spastic-like tremors and gait impairments as well as decreased microtubule stability, leading the researchers to conclude in favor of a gain (rather than loss) - of-function mechanism [Qiang et al 2019].

In conclusion, the debate concerning *SPAST*-HSP pathogenic mechanism remains open. It must be emphasized that the *SPAST* mutation spectrum, which mostly includes pathogenic variants introducing premature termination codons and therefore leading to degradation of the mRNA by nonsense-mediated decay, argues in favor of haploinsufficiency (i.e., disease occurs once the level of functional spastin falls below a critical level), rather than a dominant-negative effect [Patrono et al 2002, Schickel et al 2007]. The recent observation that missense pathogenic variants are associated with earlier onset [Parodi et al 2018] may suggest, in specific cases, the existence of pathogenic mechanisms other than haploinsufficiency.

***SPAST*-specific laboratory considerations.** *SPAST* undergoes alternate splicing with variable inclusion of exon 4. No pathogenic variants have been reported in exon 4, however, suggesting that the isoform lacking exon 4 is

the predominant functional form of spastin in the adult nervous system. This transcript variant is [NM_199436.1](#) (see Table A, **Gene**).

Both exon/multiexon deletions and duplications may be pathogenic *SPAST* variants.

Table 3. Notable *SPAST* Variants

Reference Sequences	DNA Nucleotide Change	Predicted Protein Change	Comment [Reference]
NM_014946.3 NP_055761.2	c.131C>T	p.Ser44Leu	Earlier disorder onset, more severe phenotype [Svenson et al 2004, McDermott et al 2006]
	c.134C>A	p.Pro45Gln	Earlier disorder onset, more severe phenotype [Svenson et al 2004, McDermott et al 2006]

Variants listed in the table have been provided by the authors. *GeneReviews* staff have not independently verified the classification of variants.

GeneReviews follows the standard naming conventions of the Human Genome Variation Society (varnomen.hgvs.org). See [Quick Reference](#) for an explanation of nomenclature.

References

Published Guidelines / Consensus Statements

Committee on Bioethics, Committee on Genetics, and American College of Medical Genetics and Genomics Social, Ethical, Legal Issues Committee. Ethical and policy issues in genetic testing and screening of children. Available [online](#). 2013. Accessed 6-6-19.

National Society of Genetic Counselors. Position statement on genetic testing of minors for adult-onset conditions. Available [online](#). 2018. Accessed 6-6-19.

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