Genetics of Hereditary Spastic Paraplegia

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Table of contents

List of Abbreviations

AAO	Age at onset
ACMG	American College of Medical Genetics and Genomics
AD	Autosomal dominant
AF	Allele frequency
AFO	Ankle foot orthosis
AR	Autosomal recessive
ANNOVAR	Annotate Variation
ATPase	Adenosine triphosphatase
BH4	Tetrahydrobiopterin
CIS	Clinically isolated syndrome
CK	Creatine kinase
CNV	Copy number variation
CP	Cerebral palsy
CS	Cervical stenosis
DRD	Dopa-responsive dystonia
EMG	Electromyogram
ER	Endoplasmic reticulum
GATK	Genome Analysis ToolKit
GCH1	Guanosine triphosphate cyclohydrolase I
GO	Gene Ontology
GTP	Guanosine triphosphate

HPABH4B	Tetrahydrobiopterin (BH4)-deficient hyperphenylalaninemia B
HSP	Hereditary Spastic Paraplegia
IAHSP	Infantile-onset ascending spastic paraplegia
LoF	Loss of function
MLPA	Multiplex ligation-dependent probe amplification
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NCV	Nerve conduction velocity
NGS	Next generation sequencing
NR	Not reported
PD	Parkinson's disease
SPRS	Spastic Paraplegia Rating Scale
SPG4	Spastic paraplegia type 4
UNK	Unknown
WES	Whole exome sequencing

Abstract

Background: Hereditary spastic paraplegias (HSPs) are a heterogeneous group of rare neurodegenerative disorders characterized by lower limb spasticity and weakness, with over 80 causative genes and loci described. The most common type of HSP, caused by heterozygous *SPAST* mutations, is spastic paraplegia type 4 (SPG4), with highly heterogeneous clinical manifestations.

A genetic diagnosis cannot be made for over half of HSP cases, even with the use of whole exome sequencing (WES). There are some potential explanations for these undiagnosed cases: WES cannot detect some genetic alterations such as copy number variations (CNVs); variants may be in new genes or genes associated with other conditions, such as *GCH1*, which has been described in dopa-responsive dystonia and Parkinson's disease.

Objectives: The general objective of this study was to better understand HSP and its novel features and improve its genetic diagnostic yield. **Aim 1)** Studying the genotype-phenotype correlation and clarifying novel clinical and genetic aspects of SPG4. **Aim 2)** Identifying the genetic diagnosis of three genetically unsolved HSP cases and introducing possible treatment options.

Methods: As a part of CanHSP, a Canadian consortium for the study of HSP, 696 HSP patients from 431 families were recruited and assessed. HSP-gene panel sequencing was performed on 379 cases, and 400 patients from 291 families underwent WES. To analyze the WES data, a list of HSP-related genes or genes associated with similar neurological disorders was used. The suspicious mutations were validated by Sanger

sequencing. For detection of CNVs, Multiplex ligation-dependent probe amplification was used.

Results: In the SPG4 study, 157 SPG4 patients from 65 families were identified to carry 41 different *SPAST* mutations, 6 of which were never reported before, as well as 6 CNVs. We reported three *de novo* cases, a family with probable compound heterozygous mutation, a case with homozygous mutation, three cases with pathogenic synonymous mutations, and novel or rarely reported signs and symptoms seen in SPG4 patients.

Among undiagnosed cases, we identified three patients with heterozygous *GCH1* mutations: monozygotic twins carrying a novel, in-frame deletion, p.(Ser77_Leu82del); and a case with a p.(Val205Glu) variant. The variants were predicted to be likely pathogenic and pathogenic respectively. All patients presented with childhood-onset lower limb spasticity, abnormal plantar responses, and hyperreflexia. The monozygotic twins presented with different manifestations, and both responded well to levodopa treatment. Structural analysis of the variants indicated a disruptive effect, and pathway enrichment analysis suggested that GCH1 shares processes and pathways with other HSP-associated genes.

Conclusion: As a heterogeneous type of HSP, SPG4 could present with diverse clinical manifestations and genetic features. In some cases, CNVs, *de novo* mutation, pathogenic synonymous mutations, and biallelic inheritance should be considered.

We suggest considering mutation in genes associated with other disorders, such as *GCH1*, in the diagnosis process of patients presenting with HSP symptoms, as well as levodopa trials in their treatment. The clinical differences seen between the monozygotic

twins could suggest the role of environmental factors, epigenetics, and stochasticity in the presentation of HSP.

Résumé

Contexte : Les paraplégies spastiques héréditaires (PSH) forment un groupe hétérogène de maladies neurodégénératives rares caractérisées par une spasticité et une faiblesse des membres inférieurs, plus de 80 gènes et loci causatifs ont été rapportés. Le type le plus courant de PSH est associé à des mutations hétérozygotes de *SPAST* qui entrainent la paraplégie spastique de type 4 (SPG4), avec des manifestations cliniques très hétérogènes.

Un diagnostic génétique ne peut être posé pour plus de la moitié des cas PSH malgré l'utilisation du séquençage de l'exome entier (WES). Il y a quelques explications potentielles pour cette fraction de cas non diagnostiqués : l'approche WES ne peut détecter certaines altérations génétiques telles que les variations du nombre de copies (VNC) ; des variations peuvent se trouver dans de nouveaux gènes ou des gènes associés à d'autres affections, telles que *GCH1* qui a été lié à la dystonie dopa-sensible et la maladie de Parkinson.

Objectifs : L'objectif général de cette étude était de mieux comprendre la PSH et ses nouvelles caractéristiques, et d'améliorer le diagnostic génétique. **Objectif 1)** Étudier la corrélation génotype-phénotype et clarifier les nouveaux aspects cliniques et génétiques de SPG4. **Objectif 2)** Identifier le diagnostic génétique de trois patients PSH génétiquement non résolus et introduire les options de traitement possibles.

Méthodes : Dans le cadre de CanHSP, un consortium canadien pour l'étude des PSH, 696 patients PSH issus de 431 familles ont été recrutés et évalués. Le séquençage du panel de gènes PSH a été réalisé sur 379 cas, et 400 patients de 291 familles au total ont été examiné par WES. Pour analyser les données WES, une liste de gènes liés aux

PSH ou associés à des troubles similaires a été utilisée. Les mutations suspectes ont été validées par séquençage de Sanger. Pour la détection des VNC, une amplification de sonde dépendante de la ligature multiplex a été utilisée.

Résultats : Dans l'étude SPG4, 157 patients SPG4 de 65 familles ont été identifiés qui portaient 41 mutations *SPAST* différentes, dont 6 étaient nouvelles, ainsi que 6 VNC. Nous avons rapporté trois patients avec variations *de novo*, une famille avec une mutation hétérozygote composée probable, un cas avec une mutation homozygote, trois cas avec des mutations délétères synonymes, et des signes et symptômes nouveaux ou rarement rapportés chez les patients SPG4.

Parmi les cas non diagnostiqués, nous avons identifié trois patients présentant des mutations *GCH1* hétérozygotes : des jumeaux monozygotes porteurs d'une nouvelle délétion dans le cadre, p.(Ser77_Leu82del); et un cas avec une variation p.(Val205Glu). Les deux variations sont respectivement prédites comme étant probablement pathogènes et pathogènes. Tous les patients présentaient une spasticité des membres inférieurs apparue dans l'enfance, des réponses plantaires anormales et une hyperréflexie. Les jumeaux monozygotes présentaient des manifestations différentes et tous deux ont bien répondu au traitement à la lévodopa. L'analyse structurelle des variations a révélé un effet perturbateur, et l'analyse d'enrichissement des voies a suggéré que GCH1 partage des processus et des voies avec d'autres gènes associés aux PSH.

Conclusion : En tant que type hétérogène de PSH, SPG4 pourrait présenter diverses manifestations cliniques et caractéristiques génétiques. Dans certains cas, les VNC, les

mutations *de novo*, les mutations pathogènes synonymes et l'hérédité biallélique doivent être prises en compte.

Nous suggérons de considérer la présence de variations dans des gènes associés à d'autres troubles, tels que le *GCH1* dans le processus de diagnostic des patients présentant des symptômes PSH, ainsi que des essais à la lévodopa pour leur traitement. Les différences cliniques observées entre les jumeaux monozygotes pourraient suggérer la contribution de facteurs environnementaux, de l'épigénétique et de la stochasticité dans la présentation de la PSH.

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Preface

A major goal of the projects constituting this Master thesis was to better understand the rare, heterogenous disorder, hereditary spastic paraplegia (HSP). This thesis complies with the Graduate and Postdoctoral Studies' guidelines and general requirements of a manuscript- based (article-based) Master's theses at McGill University. This thesis consists of two manuscripts (one already published, and another submitted to the MedRxiv) that address important research topics related to HSP patients.

This thesis consists of **six chapters**:

Chapter 1 provides a comprehensive literature review on HSP, and more specifically on its most common sub-type, SPG4, as well as the gene we suggest to be associated with HSP, *GCH1*;

Chapter 2 introduces the thesis rationale, hypothesis, and objectives of the two projects;

Chapters 3 to 5 include the two manuscripts, which constitute my thesis. Chapter 3 is the project on SPG4 which clarifies new genetic and clinical aspects of this HSP sub-type. Chapter 4 is the bridging chapter between the two manuscripts, which highlights the importance of considering genes associated with other neurological diseases in the genetic diagnosis of unsolved HSP patients. Chapter 5 is a description of three HSP patients with mutations in *GCH1*, a gene classically known to be associated with other neurological conditions;

Chapter 6 summarizes and discusses the overall findings and provides the final conclusions;

Chapter 7 provides the complete reference list; and

Chapter 8 contains supplementary material.

Contribution of authors

Parizad Varghaei wrote and prepared the thesis, and Patrick A Dion was involved in constructive feedback, constant support, managing the laboratory, thesis review and critique.

Chapter 3. Genetic, structural and clinical analysis of spastic paraplegia 4

Parizad Varghaei contributed to the original concept of the project, reviewed the patients' files and consulted the CanHSP database for signs, symptoms, family histories and pedigrees, imaging findings, and genetic analysis and diagnosis. Collected missing data by contacting physicians and/or genetic counselors. Was involved in data analysis. Interpreted the results. Entered each individual's characteristics in SPSS file and carried out statistical analysis. Performed literature review. Consulted publicly available online databases (such as VarSome, InterPro, gnomAD, UCSC, Ensembl, etc.) for determining variant characteristics such as pathogenicity, minor allele frequency, protein domains. Performed laboratory experiments such as polymerase chain reaction (PCR) and gel electrophoresis. Wrote the manuscript and reviewed it as it progressed.

Mehrdad A Estiar was involved in research project conception, organization, and execution, as well as in manuscript review and critique.

Setareh Ashtiani, Etienne Leveille, Marie-France Rioux, Grace Yoon, Mark Tarnopolsky, Kym M. Boycott, Nicolas Dupre, and Oksana Suchowersky were involved in data collection and patient recruitment.

Kheireddin Mufti assisted in manuscript preparation.

Simon Veyron and Jean-François Trempe carried out the protein structural analysis.

Dan Spiegelman and Eric Yu contributed through data analysis.

Guy A Rouleau led the project and was involved in data and sample collection and the CanHSP establishment; conception, organization, and overseeing the progression of the research project, as well as manuscript review and critique.

Ziv Gan-Or contributed to all stages of the research, from data and sample collection and establishment of the CanHSP database to the conception, design, and organization of the research projects and statistical analysis, to manuscript review and critique.

Chapter 5. *GCH1* mutations in hereditary spastic paraplegia

Parizad Varghaei collected the patients' information by going through their files and the CanHSP, and contacted physicians or genetic counselors for additional data. Was involved in conception of the research and data analysis. Carried out literature review, and interpretation of the results. Wrote the manuscript and reviewed it as it progressed.

Grace Yoon and Nicolas Dupre were involved in data collection and patient recruitment.

Mehrdad A Estiar contributed to data analysis and writing the first draft of the manuscript.

Simon Veyron and Jean-François Trempe carried out protein structural analysis.

Etienne Leveille assisted in data analysis.

Guy A Rouleau led the project and was involved in patient recruitment, as well as in conception, organization, and overseeing the progression of the research project, and manuscript review and critique.

Ziv Gan-Or was involved in conception, design, and organization of the research projects, as well as manuscript preparation, review and critique.

CHAPTER 1: INTRODUCTION

1. Background

1.1. Hereditary Spastic Paraplegia (HSP)

1.1.1. Definition and clinical manifestations

HSP is in fact the umbrella term for a rare group of heterogeneous rare neurodegenerative disorders resulting from genetic alterations and characterized by progressive bilateral lower limb spasticity and weakness. The first description of HSP dates to 1880 when Adolph Strümpell, a German neurologist, reported two brothers suffering from spastic paraplegia. Their father was described as “a little lame”, suggesting the autosomal dominant (AD) form of inheritance [1]. The condition was explained further in 1888 by Maurice Lorrain, A French physician [2]. HSP is therefore sometimes called Strümpell-Lorrain disease. Several cases of HSP were reported following the original descriptions, and Pratt, having considered additional manifestations incompatible with the first definition, named many of them as “HSP plus syndromes” [3]. In 1981, upon his observation of 22 HSP families, Harding defined criteria to classify HSP as pure or complicated [4], the classification which persists to date.

Apart from lower extremity spasticity and weakness, the pure (uncomplicated) form of HSP could present with corticospinal tract signs (such as hyperreflexia, upgoing plantar reflexes, clonus), decreased proprioception and vibration sense in the lower extremities, and hypertonic urinary disturbances. Some references also suggest that pes cavus, absence of ankle jerks, and mild wasting of distal muscles could also be seen in pure

HSP [4-7]. What distinguished pure from complicated (complex) HSP is the presence of additional neurological and non-neurological features in the latter form. These additional features include extrapyramidal signs, amyotrophy, ataxia, intellectual disability, cognitive decline, language development delay, dysarthria, visual dysfunction, optic atrophy, retinopathy, cataracts, epilepsy, ichthyosis, deafness, peripheral neuropathy, and dystonia [8-10].

Age at onset (AAO) of HSP ranges from infancy to eighth decade [4] and similar to other clinical presentations of HSP, could vary even among patients with the same causative mutations [11-13].

Uncomplicated HSP does not affect life expectancy; however, patients mostly suffer from progressive walking disturbances [11]. On the one hand, in most cases, HSP with early onset (<35 years) progresses more slowly, with only a small percentage of patients becoming wheelchair dependent. Patients with a later onset on the other hand, generally lose the independent walking ability in seventh to eighth decade of life [4, 6, 11, 14, 15].

The prevalence of HSP has been reported to range from 0.9 to 10 in 100,000, depending on the geographical area and the mode of inheritance [16-21], with the overall global prevalence of approximately 4.26/100,000 [21].

1.1.2. Pathophysiology or molecular mechanisms

Distal degeneration of descending corticospinal and sensory tracts is the main pathologic finding of HSP [22]; however, there is a lack of detailed pathological knowledge so far. Similarly, little is known about the exact molecular mechanism of HSPs. Identification of

the involved genes and their functions though, suggests that intracellular trafficking disturbances could be the common involved pathway. Disturbances of endolysosomal system activities, axonal transport, lipid metabolism, and organelle shaping, as well as mitochondrial functions, myelination, and axonal guidance are among the most commonly suggested underlying mechanisms [21]. The majority of HSP-related genes are involved in more than one of these mostly interconnected pathways, making it challenging to assign a definite dysfunction as the cause of neurodegeneration for a specific gene [9, 21, 23].

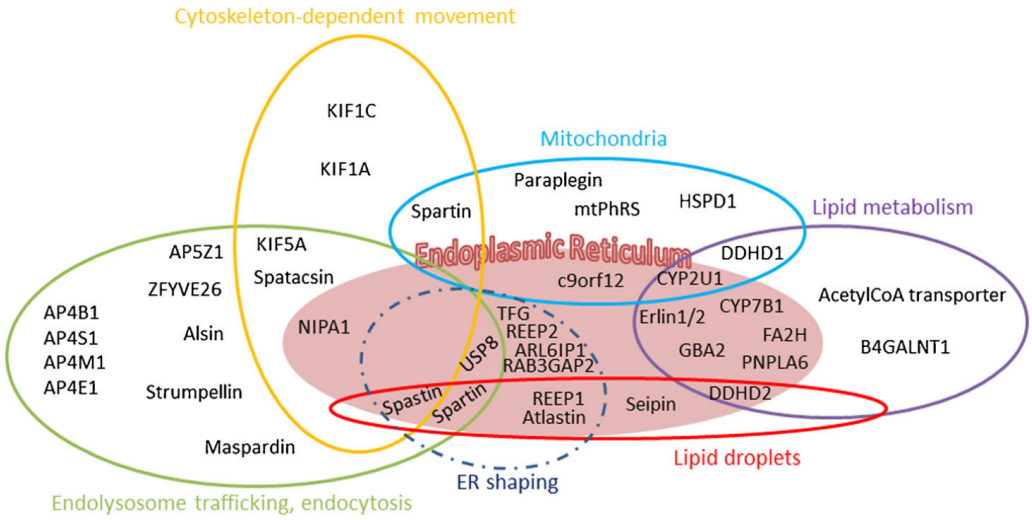

Figure 1. Overview of the pathways involved in hereditary spastic paraplegia [21].

1.1.3. Genetics

The genetic basis of HSP is even more complex. The introduction of next generation sequencing (NGS) revolutionized HSPs' genetic diagnosis with over 80 causative genes

and loci described to date, numbered in the order of discovery (SPG1-SPG83) (https://omim.org/phenotypicSeries/PS303350).

The genes could be inherited in all patterns of inheritance, including autosomal recessive (AR), autosomal dominant (AD), X-linked, and maternal transmission of mitochondrial DNA [23]. Recently, non-Mendelian mode of inheritance has also been described in HSP [24, 25]. Interestingly, some HSP genes can be transmitted with more than one form of inheritance [26].

On the one hand, accounting for 43% to 80% of the cases, AD form is the most common inheritance mode in HSP, [4, 19, 20, 27, 28] and causes pure type of the disease in 70-80% of the cases [9]. SPG4*,* accounting for approximately one third of all HSP cases and 17-79% of AD cases, is the most common HSP subtype [21]. Other common subtypes of HSP inherited in AD form are SPG3A (*ATL1*), SPG31 (*REEP1*), *and* SPG10(*KIF5A*). On the other hand, AR cases present with complicated HSP more frequently. The most common AR forms of HSP are SPG11 (*KIAA1840*), SPG5A(*CYP7B1*), SPG7(*SPG7*) and SPG15/*ZFYVE26* [8]. It is worth noting that the disease-causing genes frequencies vary largely depending on the geographical regions [29, 30]. In Canada, *SPAST* (SPG4, 48%), *ATL1* (SPG3A, 16%), and *KIAA0196* (SPG8, 5%) are the most common causes of autosomal dominant HSP. Mutations in the *SPG11* (8%) and *SPG7* (7%) genes are described as the most frequent causes for the autosomal recessive form [12].

1.2. Spastic paraplegia type 4 (SPG4)

Known as the most common type of both sporadic and familial HSP and caused by heterozygous mutations in *SPAST*, SPG4 (OMIM#182601) usually presents with a pure

type of the disease. Phenotype is mostly limited to pyramidal signs in the lower limbs, sometimes accompanied by sphincter disturbances and/ or deep sensory loss [31]. Symptoms could also include dysarthria and pes cavus in some cases [9, 13, 32-34]. AAO in SPG4 could range from birth to the eighth decade of life and is known to be variable even in patients of the same family [35, 36]. Two *SPAST* polymorphisms, p.(Ser44Leu) and p.(Pro45Glu), are among factors which could affect AAO, and more generally, could modify the disease, when carried by a patient with a pathogenic *SPAST* variant [37, 38]. A bimodal distribution of AAO has been described, first peak in the first decade, and second peak, which is smaller than the first one, in the third to fifth decade of life [35, 36]. In a recent study on the largest cohort of SPG4 patient, this bimodal distribution was related to the underlying mutation, and patients with missense mutations were suggested to show HSP symptoms earlier than those with loss of function (LoF) mutations [36]. Disease penetrance is up to 90%, and depends on age and sex [39].

SPAST encodes spastin, a protein from the adenosine triphosphatase (ATPase) associated with various cellular activities (AAA) family [40, 41]. Spastin has three main domains: an AAA domain which is responsible for the microtubule cleaving activity, a microtubule-interacting and endosomal trafficking domain, and a microtubule interacting domain [41]. It has two known isoforms: M1 and M87, which are respectively associated with endoplasmic reticulum (ER) and cytoplasm [42, 43].

Spastin hydrolyses ATP to sever microtubules (an essential step of axonal transport [44]), and regulates microtubule number, length, motility, remodeling, and disassembly [44-46]. In *Drosophila*, both the neural knockdown and neural overexpression of *SPAST* homolog were associated with neurodegeneration and locomotor dysfunction. Additionally, in the

subcellular scale, an excessive microtubule stabilization at neuromuscular synapses was seen [47]. Treatment with vinblastine, a microtubule targeting drug, was reported to revert these phenotypes [47]. Similarly, Knockdown of SPG4 homolog in mice resulted in impairment of axonal transport, causing axonal swelling and degeneration [48, 49]. Further experiments on cultures of neurons of the spastin-truncated mice suggested that impaired microtubule dynamics causes the axonal swelling [50]. Administration of microtubule targeting drugs was reported to rescue the abnormal phenotype of these neurons [50]. Furthermore, spastin, in association with REEP1 (*SPG31*) and atlastin-1 (*SPG3*), has been suggested to control the morphology of ER by linking microtubules to this organelle [51]. Both fly and worm models of SPG4 have shown ER stress signs, and administration of compounds that modulate ER stress resulted in a partial rescue of the locomotor abnormalities [52].

More than 200 *SPAST* mutations have been found to date [39, 40, 53-55]. Missense mutations occur mostly in the AAA domain, while other types of mutations are seen more evenly [39]. In one fifth of the cases with no detected point mutations, exon deletions are the causative genetic alteration [56].

While *SPAST* is an example of a well-known HSP-related gene, other genes which are classically known to cause other neurological diseases have been reported to be associated with HSP. *GCH1* is an example of such genes.

1.3. The guanosine triphosphate cyclohydrolase I (GCH1)

1.3.1. GCH1 protein

GCH1 encodes guanosine triphosphate (GTP) cyclohydrolase 1 (GCH1), an enzyme responsible for catalyzing tetrahydrobiopterin (BH4) biosynthesis [57]. BH4 is a known cofactor of tryptophan and tyrosine hydroxylase, which are respectively involved in the synthesis of serotonin and dopamine [58-60]. Impairment in the function of GCH1 would hence result in a deficit of these neurotransmitter and cause several neurological diseases, as listed below:

1.3.2 Dopa-responsive dystonia (DRD)

DRD belongs to the heterogeneous group of monogenic combined dystonias, and the most common causes of its AD form are the monoallelic mutations in *GCH1* [60]. Disease onset could range from 0 to 68 years of age, but is mostly in childhood. Symptoms usually begin in the foot, followed by insidious cranial progression that leads to generalized dystonia in teenage years. Most cases present additional features such as mild parkinsonian symptoms (tremor, bradykinesia, and rigidity). Patients mostly show diurnal changes in the symptoms likely due to circadian fluctuations in dopamine levels [61]. Penetrance is approximately 50% [62], and females are more likely to show symptoms of the disease [63]. In many cases, intrafamilial variability of symptoms is seen [64]. Patients respond dramatically to L-dopa treatment, while untreated cases develop significant disability due to motor symptoms [60].

A rare AR type of DRD resulting from biallelic mutations in *GCH1* has also been described, which manifests with a more severe phenotype. In addition to dystonia, patients could present with motor and mental development disorders, oculogyric crises, and hypotonia [65].

1.3.3 Tetrahydrobiopterin (BH4)-deficient hyperphenylalaninemia B (HPABH4B)

HPABH4B is another disorder caused by rare biallelic *GCH1* mutations. This rare condition could present with muscular hypotonia, developmental delay, seizures, hypertonia of the extremities, and other neurological features [57, 66, 67].

1.3.4 Parkinson's disease (PD)

Idiopathic PD could be seen in *GCH1* mutation carriers, with a later AAO [68-71]. These patients mostly show a mild form of PD, and need lower L-dopa doses for symptom control [69, 72]. Some cases however, have been reported to show a presentation similar to that of neurodegenerative PD, with manifestations such as dyskinesia induced by L-dopa, treatment fluctuations, and neurodegeneration signs [68, 73].

1.3.5 HSP

Two previous reports have suggested *GCH1* mutations in HSP. In the first report, a female was described with toe-walking that started at the age of 6. She had first been diagnosed as a case of cerebral palsy, and later, an HSP diagnosis was made. She was dependent on crutches by age 18 and later, developed symptoms such as dysarthria, functional impairment in the hands, and diurnal fluctuation of symptoms. On examination, she had spastic gait, brisk deep tendon reflexes, and extended big toes at rest while other toes

were flexed tonically. Whole exome sequencing (WES) showed a *GCH1* p.(Arg216Ter) variant, and she was started on treatment with carbidopa/levodopa, to which she responded well [74].

In the next report, four patients from three families were described. The first two cases, mother and daughter, had a disease onset in early childhood, and presented with spasticity in the lower limbs, spastic gait, abnormal plantar reflexes, and diurnal fluctuations. The mother, but not the daughter, also had bladder symptoms, dystonia, and parkinsonism. WES analysis revealed a *GCH1* c.454-2A>G.

The next case was a female with AAO of 14, who presented with spasticity of the lower limbs, spastic gait, hyperreflexia, abnormal plantar responses, bladder symptoms, as well as dystonia, parkinsonism, and diurnal fluctuations. *GCH1* p.(Glu65fs) variant was found by WES to cause the symptoms.

Finally, a male patient was described who had an AAO of 7, and suffered from spasticity of the lower extremities, spastic gait, hyperreflexia, abnormal plantar responses, parkinsonism, and diurnal fluctuation. WES revealed a *GCH1* p.(Met211fs) variant. All patients responded well to levodopa/carbidopa treatment [75].

Chapter 2: RATIONALE, HYPOTHESIS, AND OBJECTIVES

2.1. Rationale

Numerous aspects of HSP are still unknown. Although WES has revolutionized the genetic diagnosis of HSP and helped increase the number of the associated genetic regions from 20 loci and 9 genes in 2003 [76] to over 80 to date, it is not still possible to decipher the underlying cause in a significant percentage of cases. In the general clinical setting, the diagnostic yield of WES ranges from 25-50% for HSP [77, 78], and the gap is even higher in sporadic cases [8]. There are several potential explanations for the large fraction of unsolved cases. While structural variants as well as synonymous substitutions have been reported as disease-causing in different types of HSP and similar disorders, WES cannot identify certain changes such as copy number variations (CNV), and interpretation of synonymous variants is challenging. Furthermore, variants may occur in new genes or genes associated with non-HSP conditions. For instance, mutations in *ATP13A2*, which are known to cause Kufor-Rakeb syndrome (an atypical parkinsonism-dystonia disorder), have been reported in HSP patients [79, 80], and *ALS2* variants, which have been associated with juvenile amyotrophic lateral sclerosis [81-83] and juvenile primary lateral sclerosis [84, 85], are seen in infantile-onset ascending HSP [86-88]. A further example is *GCH1*, which has been classically described in PD [68, 70, 71] and DRD [89-91], but recent reports have suggested it could be associated with HSP as well [74, 75].

In addition, while HSP is classically considered to be caused by monogenic Mendelian mechanisms, this oversimplified view is not necessarily true. For instance, oligogenic inheritance [25] and modifier alleles [37, 38] have been suggested to play a role in HSP.

Interpretation of mode of inheritance could be even more complicated since some patients are asymptomatic due to the age- and sex-dependent penetrance seen in HSP [4, 8, 36].

Another challenge in the diagnosis of patients is that manifestations could vary among carriers of different mutations of a specific HSP gene or even among members of a family with the same mutation [12, 13]. This variety may well indicate the role of further genetic, environmental, and epigenetics factors which remain to be clarified [11]. What makes the puzzle of HSP even more complicated is that symptoms could overlap with other conditions which could sometimes lead to a misdiagnosis [92].

All the complexities mentioned above could make it challenging for clinicians to correlate clinical features of HSP with each specific genetic subtypes [93].

2.2. Hypothesis

SPG4, as the most common type of HSP, is a highly heterogeneous disorder, and has various novel features which need to be clarified. The underlying cause of HSP presentations in patients without a genetic diagnosis could be the structural or synonymous variants, *de novo* mutations, and inheritance forms other than those already known. Patients who remain genetically undiagnosed after being screened with the common gene panels could have mutations in genes that are not known to be HSP-associated.

2.3. Objectives

2.3.1. General Objective

The general objective of this study is to better understand HSP and its unknown aspects and to fill the genetic diagnostic gap for targeted therapies.

2.3.2. SPG4 Project Specific Objectives

To clarify the novel aspects, and the basis of the heterogeneity seen in SPG4, one of the most heterogeneous sub-types of HSPs, by studying a large cohort of patients who have been clinically assessed and followed up.

2.3.3. GCH1 Project Specific Objective

To identify the genetic diagnosis of undiagnosed cases by screening for genes not associated with HSP, and introducing possible treatment options.

CHAPTER 3: MANUSCRIPT 1

Genetic, structural and clinical analysis of spastic paraplegia 4

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Abstract

Background: Spastic paraplegia type 4 (SPG4), resulting from heterozygous mutations in the *SPAST* gene, is the most common form among the heterogeneous group of hereditary spastic paraplegias (HSPs).

Objective: To study genetic and clinical characteristics of SPG4 across Canada.

Methods: The *SPAST* gene was analyzed in a total of 696 HSP patients from 431 families by either HSP-gene panel sequencing or whole exome sequencing (WES). We used Multiplex ligation-dependent probe amplification to analyze copy number variations (CNVs), and performed *in silico* structural analysis of selected mutations. Clinical characteristics of patients were assessed, and long-term follow-up was done to study genotype-phenotype correlations.

Results: We identified 157 SPG4 patients from 65 families who carried 41 different *SPAST* mutations, six of which are novel and six are CNVs. We report novel aspects of mutations occurring in Arg499, a case with homozygous mutation, a family with probable compound heterozygous mutations, three patients with *de novo* mutations, three cases with pathogenic synonymous mutation, co-occurrence of SPG4 and multiple sclerosis, and novel or rarely reported signs and symptoms seen in SPG4 patients.

Conclusion: Our study demonstrates that SPG4 is a heterogeneous type of HSP, with diverse genetic features and clinical manifestations. In rare cases, biallelic inheritance, *de novo* mutation, pathogenic synonymous mutations and CNVs should be considered.

Introduction

Spastic paraplegia type 4 (SPG4, OMIM #182601) is the most frequent form of either sporadic or familial hereditary spastic paraplegias (HSPs), caused by heterozygous mutations in the *SPAST* gene.[1-3] With more than 80 potentially causative loci or genes reported to date,[4] HSPs are known to affect 1-10/100,000 of the population,[5] and autosomal dominant (AD) HSPs comprise 43%-80% of them.[5-11] Among all AD-HSPs, 70-80% are categorized as "pure"[12] with a phenotype limited to pyramidal signs in the lower limbs, with or without deep sensory loss and sphincter disturbances.[12-15] Of all pure AD-HSPs, about 40% are caused by *SPAST* mutations.[12]

SPAST encodes spastin, which is a protein from the AAA (ATPase associated with various cellular activities) family of ATPases.[16, 17] Spastin controls different aspects of microtubule dynamics (e.g. microtubule number, motility, length, disassembly and remodeling), and hydrolyses ATP to cleave microtubules,[18, 19] a necessary step in axonal transport.[20] Its mechanism involves binding to the C-terminus of tubulin and severing tubulin subunits from the microtubule in an ATP hydrolysis–dependent manner.[19] The structure of human spastin residues 323-610 was solved by cryoelectron microscopy.[21] The structure reveals an AAA+ ATPase homohexamer (Figure 1A), with ATPase active sites located at the interface between every adjacent subunit (Figure 1B).[22, 23] A tubulin peptide runs through the channel, suggesting a hand-over-hand mechanism of substrate translocation, with five subunits interacting with the peptide forming a spiral staircase and one displaced from the peptide/substrate (Figure 1A).[21]

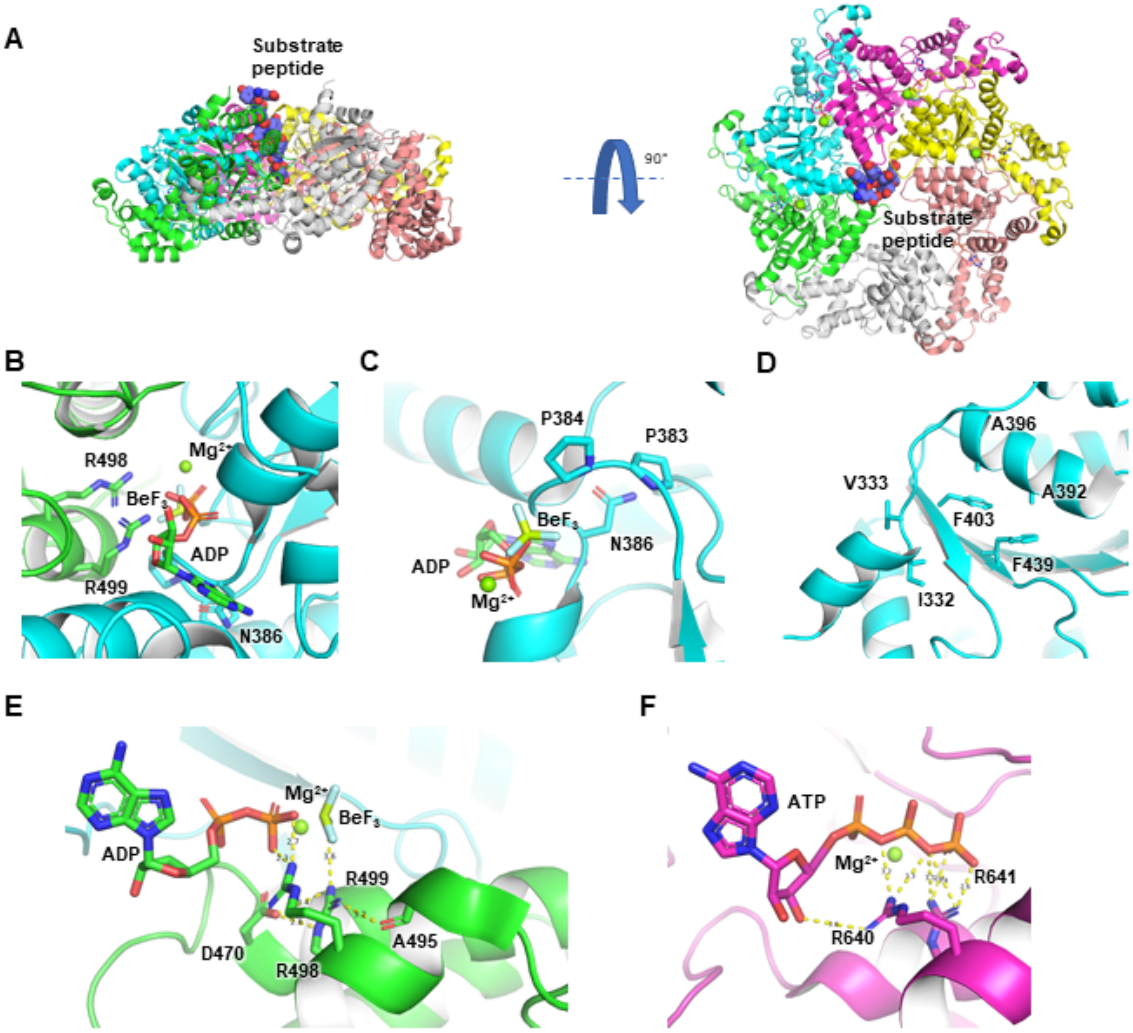

Figure 1. Structural analysis of human SPG4. A: Structure of SPG4 (PDB: 6PEN) forming a homohexameric assembly. The substrate peptide is shown as violet spheres. B: The interface between subunit A (green) and B (cyan) forms an active site. C: $Loop_{382-389}$ is part of the nucleotide binding pocket. D: β-$strand_{402-406}$ stabilizes $helix_{387-399}$ and the C-terminal residues of this structure. E: Arg498 and Arg499 interact with ADP, BrF_3, Asp470 and Ala495's main chain, stabilizing the nucleotide and the active site. Polar

interactions are shown as yellow dotted lines. F: *D. melanogaster* Spastin structure (PDB: 6P07) was solved with an ATP molecule in the active site. Arg640 and Arg641, homologous to Arg498 and Arg499 in human Spastin, are coordinating ATP in the active site.

To date, more than 200 *SPAST* mutations have been found.[3, 16, 24-26] Deletion/insertions, nonsense, and splice site mutations are distributed throughout the gene, while missense mutations are mostly clustered in the AAA domain.[3] Exon deletions may account for 20% of cases in whom point mutations are not detected.[27, 28]

The penetrance of SPG4 is up to 80-90% and is age-dependent,[3] with age at onset (AAO) that may range from infancy to the eighth decade of life.[1, 29] Most cases present as juvenile or adult-onset pure spastic paraplegia with urinary sphincter disturbances, pes cavus, and dysarthria. However, *SPAST* mutations are known to cause clinically heterogeneous manifestations, and a high variety of signs and symptoms is reported among carriers of different *SPAST* mutations. This clinical heterogeneity occurs even among patients harboring the same mutations,[30] suggesting that other factors affect the clinical presentation of SPG4.

Although previous studies have deciphered some aspects of this variability, the genotype-phenotype correlations and some rare features of SPG4 are not fully understood. In this study, we analyzed a large cohort of SPG4 patients from Canada to better clarify the genetic and clinical spectrum of the disease.

Methods

Population

A total of 696 HSP patients from 431 families were recruited in eight medical centers across Canada (Montreal, Quebec, Ottawa, Toronto, Hamilton, Calgary, Edmonton, and Vancouver) as part of CanHSP, a Canadian consortium for the study of HSP. Details about the diagnosis and recruitment process has been previously reported.[31] Clinical assessments were done including family history, demographic data, developmental history, AAO, and HSP core symptoms (lower extremity weakness and spasticity, extensor plantar responses, hyperreflexia, and bladder dysfunction). Other neurological, as well as non-neurological clinical presentations of HSP, were also assessed. For a group of the patients, the Spastic Paraplegia Rating Scale (SPRS), which is an indicator of severity in HSP, was measured.[32, 33] For another subset of patients, brain and/or spine MRI were performed. The disease was deemed pure unless the patient had at least one sign not attributable to the lateral corticospinal tract or pyramidal tract, including ataxia, intellectual disability, cognitive decline, language development delay, extrapyramidal signs, visual dysfunction, epilepsy, deafness, dysarthria, optic atrophy, peripheral neuropathy, and dystonia; in which case the disease was classified as complex.

All the data is stored in a central database at McGill University. All patients signed informed consent forms and the institutional review board approved the study protocol.

Genetic and data analysis

DNA was extracted from peripheral blood using a standard procedure.[34] Initially, HSP-gene panel sequencing was performed on 379 patients. Then, 194 genetically undiagnosed patients, and additional 206 patients who were not analyzed with panel sequencing (400 patients in total from 291 families), went through whole exome sequencing (WES).

For WES, the Agilent SureSelect Human All Exon v4 kit for capture and targeted enrichment of the exome was used. To analyze the WES data, we used a list of 785 HSP-related genes or genes associated with similar neurological disorders which cause spasticity (Supplementary Table 1). Illumina HiSeq 2000/2500/4000 system was used for sequencing captured samples. Using Burrows-Wheeler Aligner (BWA), the sequence reads were then aligned against the human genome (GRCh37 assembly).[35] We used the Genome Analysis ToolKit (GATK)[36] and Annotate Variation (ANNOVAR)[37] for variant calling and annotation, respectively. We excluded variant calls with a genotype quality less than 97 and less than 30x depth of coverage. Integrated Genomics Viewer was used to visually inspect the detected variants, and suspicious variants were validated by Sanger Sequencing. Sanger Sequencing was also used for assessing sporadic patients' parents, to determine if the proband had a *de novo* mutation.

SPAST variants (NM_014946) were initially selected based on identifying missense and LoF alleles, including frame-shift, splice-site, nonsense, and copy number variations (CNVs) with a minor allele frequency less than 0.01 in gnomAD.[38] The variants' pathogenicity has been determined using VarSome,[39] according to the American College of Medical Genetics and Genomics (ACMG) guideline. Variants classified as "Benign" and "Likely Benign", as well as intronic splice site variants higher than ±3 were excluded from

the analysis. To detect CNVs, ExomeDepth[40] was used on WES data, followed by 48 selected samples that went through Multiplex ligation-dependent probe amplification (MLPA) testing (MRC Holland, Amsterdam, The Netherlands) to confirm or exclude suspected *SPAST* CNVs. InterPro[41] was applied to identify domains and corresponding sites in the protein.

Statistical analysis

To determine the association between two categorical variables, one categorical variable with one continuous variable, and two continuous variables, Pearson chi-squared test, Mann-Whitney U test, and Spearman's rank correlation coefficient were used, respectively. *P*-value was set at <0.05 and Bonferroni correction for multiple comparisons was applied when necessary. SPSS was used to perform all statistical analyses.

***In silico* structural analysis**

The atomic coordinates of human spastin bound to a glutamate-rich peptide, ADP, BeF_3 and Mg^{2+} and D. melanogaster spastin bound to a glutamate-rich peptide, ADP, ATP and Mg^{2+}were downloaded from the Protein Data Bank (ID 6PEN and 6P07). The effect induced by each mutation was evaluated using the "mutagenesis" toolbox in The PyMOL Molecular Graphics System, Version 2.4.0 Schrödinger, LLC. and the DynaMut server http://biosig.unimelb.edu.au/dynamut/.[42]

Results

Cohort characteristics

We identified 65 families (15.1% of the families in HSP cohort), and a total of 157 patients (22.5% of HSP patients) with SPG4. Mean AAO was 22 years (0-67, SD: 19.89), and it followed a bimodal distribution; the first peak in the first 5 years of life, and the second peak from 35 to 44 years of age (Supplementary Figure 1). Mean age at examination was 43.4, and mean disease duration was 21 years. Patients with longer disease duration presented with higher SPRS scores (Spearman's correlation coefficient; p = 0.048). In contrast, age at onset was not associated with the severity of the disease (Spearman's correlation coefficient; p = 0.934). Complex HSP was seen in 24/65 (36.9%) of the probands, and 37/157 (23.6%) of all the patients. No significant differences were seen between male and female patients in AAO and other clinical manifestations. Table 1 details the clinical presentation of the patients.

Table 1. Frequency of signs and symptoms among different categories of gender, mutation type and protein domain.

Clinical presentation	Frequency	Gender			Type of mutation			Protein Domain (Missense mutations)					
		Male (n=72, 45.9%)	Female (n=85, 54.1%)	p value	LoF (n=21/41)	Missense(n=20/41)	p value	MIT[a]	AAA_ATPase[b]	AAA_lid_3[c]	Vps4_C[d]	Other domains	p Value
Mean Age at onset ±SD	-	25.9± 19.6	19± 20	.040	24± 20	27.4± 20.2	.465	38	22.9±19.2	32.5±21.7	29.3±23.3	30.5±19.1	.626
Mean SPRS score ±SD	-	23.1± 12.5	22± 14.7	.853	22± 8.7	22.8± 15.6	.696	28	20.1±14.3	6.0±2.8	3	34.6±6.6	.066
Lower extremity weakness	53/102 (52%)	25/44 (56.8%)	28/58 (48.3%)	.392	10 /24 (41.6%)	35 /50 (70%)	.019	-	24/32 (75%)	3/10 (30%)	1/1 (100%)	7/7 (100%)	.010
Lower extremity spasticity	92/109 (84.4%)	44/48 (91.7%)	48/61 (78.7%)	.064	25 /28 (89.3%)	45/49 (91.8%)	.708	-	32/32 (100%)	7/10 (70%)	-	6/7 (85.7%)	.008
Lower extremity hyperreflexia	102/111 (91.9%)	46/49 (93.9%)	56/62 (90.3%)	.496	25 /25 (100%)	51/53 (96.2%)	.325	-	32/33 (97%)	13/13 (100%)	-	6/7 (85.7%)	.261
Extensor plantar response	82/105 (78.1%)	38/44 (86.4%)	44/61 (72.1%)	.082	19 /24 (79.2%)	46/50 (92%)	.114	-	30/31 (96.8%)	10/12 (83.3%)	-	6/7 (85.7%)	.278
Abnormal bladder function	52/100 (52%)	25/43 (58.1%)	27/57 (47.4%)	.286	15 /22 (68.2%)	28/51 (54.9%)	.290	-	15/32 (46.9%)	6/11 (54.5%)	1/1 (100%)	6/7 (85.7%)	.227
Ankle clonus	59/97 (60.8%)	25/40 (62.5%)	34/57 (59.6%)	.777	14 /22 (63.6%)	34/47 (72.3%)	.464	-	19/28 (67.9%)	11/12 (91.7%)	-	4/7 (57.1%)	.189
Motor delay	9/50 (18%)	3/24 (12.5%)	6/26 (23.1%)	.331	0 /10 (0%)	7/34 (20.6%)	.118	-	6/22 (27.3%)	0/6 (0%)	0/1 (0%)	1/5 (20%)	.490
Speech delay or abnormality	8/47 (17%)	3/24 (12.5%)	5/23 (21.7%)	.400	0 /11 (0%)	8/31 (25.8%)	.061	-	6/20 (30%)	1/6 (16.7%)	0/1 (0%)	1/4 (25%)	.851
Learning disability	10/51 (19.6%)	3/25 (12 %)	7/26 (26.9%)	.180	0 /11 (0%)	8/34 (23.5%)	.076	-	7/22 (31.8%)	0/6	0/1 (0%)	1/5 (20%)	.387
Progressive cognitive deficiency	6/73 (8.2%)	5/36 (13.9%)	1/37 (2.7%)	.082	1 /19 (5.3%)	3/42 (7.1%)	.784	-	3/28 (10.7%)	0/6 (0%)	0/1 (0%)	0/7 (0%)	.656
Retinopathy or optic atrophy	1/71 (1.4%)	0/34 (0%)	1/38 (2.6%)	.348	-	-	-	-	-	-	-	-	-

Ocular movement abnormalities	1/72 (1.4%)	0/33 (0%)	1/38 (2.6%)	.341	1 /19 (5.3%)	0/41 (0%)	.139	-	-	-	-	-	-
Deafness	4/71 (5.6%)	3/34 (8.8%)	1/37 (2.7%)	.264	0 /19 (0%)	4/41 (9.8%)	.159	-	2/27 (7.4%)	0/6 (0%)	1/1 (100%)	1/7 (14.3%)	.017
Swallowing difficulty	4/72 (5.6%)	1/34 (2.9 %)	3/38 (7.9%)	.360	0 /20 (0%)	4/40 (10%)	.143	-	3/27 (11.1%)	0/6 (0%)	0/1 (0%)	1/6 (16.7%)	.774
Dysarthria	12/73 (16.4%)	5/35 (14.3%)	7/38 (18.4%)	.634	3 /20 (15%)	8/41 (19.5%)	.667	-	6/27 (22.2%)	1/6 (16.7%)	0/1 (0%)	1/7 (14.3%)	.914
Upper extremity weakness	5/72 (6.9%)	2/34 (5.9 %)	3/38 (7.7%)	.737	0 /19 (0%)	4/41 (9.8%)	.159	-	3/27 (11.1%)	0/6 (0%)	0/1 (0%)	1/7 (14.3%)	.807
Upper extremity hyperreflexia	31/77 (40.3%)	15/36 (41.7%)	16/41 (39%)	.814	11 /21 (52.4%)	15/44 (34.1%)	.159	-	8/27 (29.6%)	3/9 (33.3%)	1/1 (100%)	3/7 (42.9%)	.491
Sensory abnormalities	33/73 (45.2%)	15/35 (42.9%)	18/38 (47.4%)	.699	10 /21 (47.6%)	19/40 (47.5%)	.993	-	12/26 (46.2%)	3/6 (50%)	1/1 (100%)	3/7 (42.9%)	.753
Peripheral neuropathy	10/70 (14.3%)	4/35 (11.4%)	6/35 (17.1%)	.495	2 /20 (10%)	7/39 (17.9%)	.421	-	5/24 (20.8%)	1/8 (12.5%)	0/1 (0%)	1/6 (16.7%)	.914
Pes cavus	16/73 (21.9%)	9/35 (25.7%)	7/38 (18.4%)	.452	6 /19 (31.6%)	5/41 (12.2%)	.071	-	4/27 (14.8%)	0/6 (0%)	0/1 (0%)	1/7 (14.3%)	.759
Upper extremity ataxia	4/72 (5.6%)	1/35 (2.8%)	3/37 (8.1%)	.331	0 /19 (0%)	4/42 (9.5%)	.162	-	2/27 (7.4%)	1/7 (14.3%)	0/1 (0%)	1/7 (14.3%)	.893
Upper extremity Intent tremor	2/72 (2.8%)	0/35 (0%)	2/37 (5.4%)	.227	0 /19 (0%)	2/42 (4.8%)	.490	-	1/27 (3.7%)	0/7 (0%)	0/1 (0%)	1/7 (14.3%)	.331
Seizures	2/71 (2.8%)	0/34 (0%)	2/37 (5.4%)	.334	0 /19 (0%)	1/41 (2.4%)	.492	-	1/27 (3.7%)	0/6 (0%)	0/1 (0%)	0/7 (0%)	.912
Skeletal abnormality	4/69 (5.8%)	2/33 (6.1%)	2/36 (5.5%)	.929	0 /19 (0%)	3/40 (7.5%)	.220	-	2/27 (7.4%)	0/6 (0%)	-	1/7 (14.3%)	.621

Amyotrophy or lower motor neuron features	6/71 (8.5%)	5/35 (14.3%)	1/36 (2.8%)	.081	1/12 (8.3%)	3/19 (15.8%)	.542	-	3/33 (9.1%)	0/6 (0%)	-	1/8 (12.5%)	.823
Abnormal brain MRI	6/48 (12.5%)	4/28 (14.3%)	2/20 (10%)	.658	3 /13 (23.1%)	3/32 (9.4%)	.220	-	2/20 (10%)	0/5 (0%)	-	1/7 (14.3%)	.696
Abnormal spine MRI	8/53 (15.1%)	4/30 (13.3%)	4/23 (17.4%)	.683	2 /15 (13.3%)	6/32 (18.8%)	.645	-	4/17 (23.5%)	1/9 (11.1%)	-	1/6 (16.7%)	.735

Bonferroni corrected p value: 0.0017.

SD, standard deviation; SPRS, Spastic Paraplegia Rating Scale; MRI, magnetic resonance imaging; n, number; LoF, loss of function, MIT, microtubule interacting and trafficking; AAA, ATPase associated with various cellular activities; ATP, adenosine triphosphate.

[a] InterPro (IPR) number: IPR036181; [b] IPR003593; [c] IPR041569; [d] IPR015415

We detected 41 different *SPAST* mutations in our cohort (Table 2). Most mutations (22/34, 64.7%) occurred between amino acids 374 and 567, in the AAA cassette. Among the missense mutations, 15/20 (75%) were clustered in AAA cassette, while LoF mutations were more evenly distributed across gene (Figure 2). Presentation of the disease did not differ significantly between the two types of mutation (Table 1).

Table 2. Mutations identified in the current study and their characteristics.

Nucleotide Change	Amino acid Change	Number of Families	Number of Patients	AAO (Mean)	AF in gnomAD	Pathogenicity (VarSome)
c.127G>T	p.(Glu43Ter)	1	1	UNK	NR	Pathogenic
c.153C>G	p.(Tyr51Ter)	1	1	1	NR	Pathogenic
c.231G>A	p.(Trp77Ter)	1	1	0	NR	Pathogenic
c.562delG	p.(Ala188Profs Ter8)	1	1	38	NR	Pathogenic
c.687delT	p.(Ser229Argfs Ter11)	1	1	37	NR	Pathogenic
c.869A>G	p.(Lys290Arg)	1	1	55	NR	Likely Pathogenic
c.1098delG	p.(Glu366Aspfs Ter28)	1	1	42	NR	Pathogenic
c.1111C>T	p.(Leu371Phe)	2	2	29	NR	Pathogenic
c.1112T>C	p.(Leu371Pro)	1	1	30	NR	Pathogenic
c.1139T>C	p.(Leu380Pro)	1	1	28	NR	Likely Pathogenic
c.1144_1145	p.(Gly382_Pro383	1	2	15	NR	-

insGTC	insArg)					
c.1158T>G	p.(Asn386Lys)	1	1	40	NR	Pathogenic
c.1196C>T	p.(Ser399Leu)	3	8	33	NR	Pathogenic
c.1209C>A	p.(Phe403Leu)	2	5	35	NR	Likely Pathogenic
c.1212_1216del	p.(Asn405Lysfs Ter36)	1	1	2	NR	-
c.1220G>A	p.(Ser407Asn)	1	1	1	NR	Pathogenic
c.1242A>G	p.(Lys414Lys)	2	3	9	NR	Uncertain significance
c.1276C>G	p.(Leu426Val)	1	1	13	NR	Pathogenic
c.1300C>T	p.(Gln434Ter)	1	1	40	NR	Pathogenic
c.1321+1G>A		1	2	UNK	NR	Pathogenic
c.1356_1357 insGGG	p.(Gly452dup)	1	1	38	NR	-
c.1378C>T	p.(Arg460Cys)	1	1	40	NR	Pathogenic
c.1385A>G	p.(Lys462Arg)	1	1	14	NR	Pathogenic
c.1466C>T	p.(Pro489Leu)	1	4	19	NR	Likely Pathogenic
c.1492_1493 del	p.(Arg498Alafs Ter30)	1	1	50	NR	Pathogenic
c.1493+2T>A		1	1	39	NR	Pathogenic
c.1495C>T	p.(Arg499Cys)	2	4	6	NR	Pathogenic
c.1496G>A	p.(Arg499His)	4	4	2	NR	Pathogenic
c.1507C>T	p.(Arg503Trp)	3	4	34	4.82E-06	Pathogenic
c.1537-2A>G		1	7	17	NR	Pathogenic
c.1610T>G	p.(Leu537Arg)	1	1	UNK	NR	Pathogenic

c.1676G>A	p.(Gly559Asp)	8	15	33	NR	Pathogenic
c.1684C>T	p.(Arg562Ter)	1	2	10	NR	Pathogenic
c.1685G>A	p.(Arg562Gln)	1	1	65	NR	Pathogenic
c.1729-1G>C		2	2	27	NR	Pathogenic
c.1741C>T	p.(Arg581Ter)	2	2	42	4.81E-06	Pathogenic
c.1790delG	p.(Ser597Thrfs Ter3)	1	3	4	NR	Pathogenic
c.1844C>T	p.(Thr615Ile)	1	1	30	NR	Likely Pathogenic
del exon 1		3	3	42	NR	Pathogenic
del exons 5-7		1	2	UNK	NR	Pathogenic
del exons 16-17		1	1	UNK	NR	Pathogenic

AAO, age at onset; UNK, unknown; NR, not reported; AF, allele frequency.

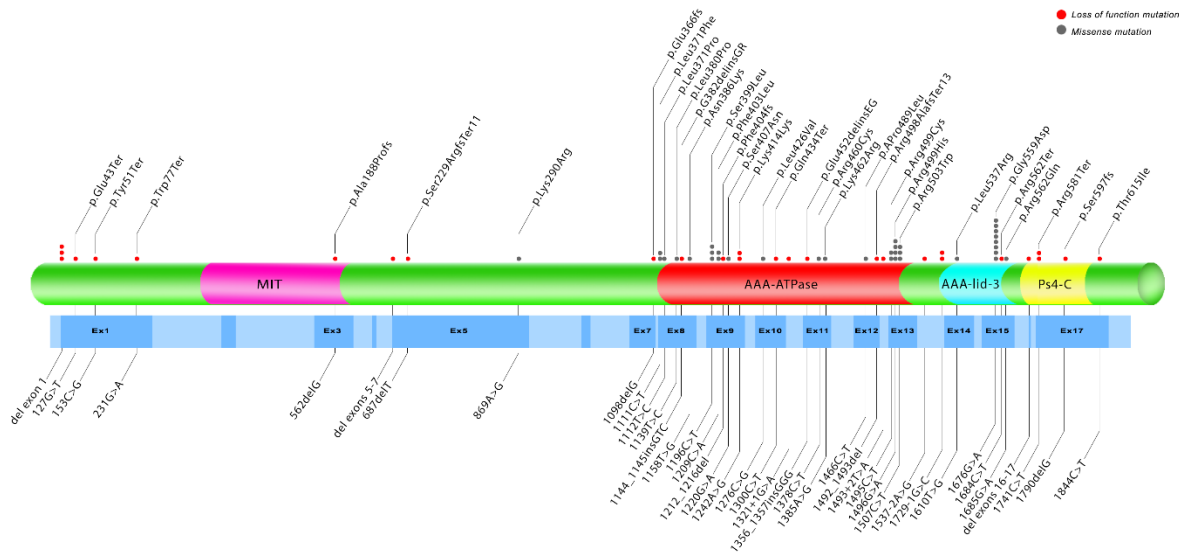

Figure 2. Schematic figure of the location of *SPG4* mutations in the current study. The schematic on top represents the spastin protein. Functional domains are demonstrated in different colors, including MIT (violet), AAA-ATPase (red), AAA-lid-3 (cyan), and Ps4-C (yellow). Mutations resulting in loss of function and missense mutations are indicated with red and grey circles respectively. The number of circles in a column demonstrate the number of families that carried each mutation. The bottom schematic represents the cDNA of *SPG4*. Exons (indicated with Ex) are represented by dark blue and introns by light blue.

Novel *SPAST* mutations

Of the 41 different *SPAST* mutations in our cohort, six were novel, including p.(Trp77Ter), p.(Glu366AspfsTer28), p.(Gly382_Pro383insArg), p.(Phe403Leu), p.(Arg498AlafsTer30), and p.(Ser597ThrfsTer3). We performed *in silico* analysis to investigate the impact of these mutations on the structure and activity of spastin, with

exception of p.(Trp77Ter), as Trp77 is not visible in any structure of spastin and could not be investigated.

Glu366 is located in the $loop_{365-377}$ and the frameshift mutation p.(Glu366AspfsTer28) changes all the residue subsequent to residue 365 and inserts a stop codon at position 394. This mutation truncates residues 394 to 616 which includes residues involved in oligomerization and ATP-binding, which will abrogate its enzymatic activity. Gly382 and Pro383 are located in the nucleotide-binding $loop_{382-389}$.[43] Residues Gly385 and Asn386 interact directly with the ADP, in a conformation that is shaped by the Pro383 and Pro384 (Figure 1C). The mutation p.(Gly382_Pro383insArg) lengthens this loop and likely destabilizes the interaction between the protein and the nucleotide. Phe403 forms a pi-stacking interaction with Phe439 and hydrophobic interactions with neighbouring residues (Figure 1D). Thus, mutation p.(Phe403Leu) would destabilize the domain and impair the ATPase activity.

In this structure, Arg498 and Arg499 are both involved in coordinating ADP. Arg498 is making direct interaction with the β phosphate of the nucleotide located in the active site, while Arg499 stabilizes $helix_{455-470}$ and coordinates BeF_3, a phosphate structural analog (Figure 1E). By homology with *D. melanogaster* spastin structure (PDB ID: 6P07), those residues seem to be also involved in ATP β and γ phosphate coordination (Figure 1F). As a result, we can hypothesize that the BeF_3 in the active site is mimicking the γ phosphate on an ATP. Mutation p.(Arg498AlafsTer30) causes residues 498 and 499 to be serine and valine residues, which would prevent ATP stabilization. In addition, this frame shift terminates the protein after residue 528, further disrupting the ATP-binding domain. This

mutation will therefore abolish the ATPase activity. Finally, mutation p.(Ser597ThrfsTer3) will induce a frame shift at residue 597 and truncate the protein at residue 600. The missing C-terminal residues form a helix involved in intersubunit interactions. This mutation would therefore destabilize the hexameric assembly, which would disrupt the ATPase activity.

Founder French-Canadian mutations and known CNVs

The most frequent *SPAST* mutation in our cohort, p.(Gly559Asp), which has previously been suggested to be a founder mutation in French-Canadian population[44], was carried by 8 families (12.3%) and 15 patients in total (9.5%). Seven out of 24 French-Canadian probands (29%) harbored this mutation, while among all the patients with other or unknown ancestral backgrounds, this mutation was present in only one out of 29 probands (3.4%, p=0.011). Moreover, the mutation p.(Phe403Leu) was only detected in patients with French-Canadian ancestral background, in 5 patients from 2 families, suggesting it may be a founder mutation. Seven patients (4.5%) from six families (9.2%) carried a CNV (Supplementary Table 2), all of which have been previously reported.[27]

Earlier age at onset and specific clinical features in patients with mutations in *SPAST* p.Arg499

The range of AAO in all 4 patients from 4 different families, two of which have previously been reported[45] with the p.(Arg499His) mutation was 1-3 years (Mann-Whitney U test; p = 0.003). Two patients were one year old when they started presenting with symptoms, one patient was two years old, and one patient was three years old. Patients with this mutation in our cohort were more likely to present with motor delay, speech delay,

dysarthria, learning disability, progressive cognitive deficits, and upper extremity weakness (Supplementary Table3).

Although statistically insignificant, cases who carried another mutation affecting the same amino acid residue, p.(Arg499Cys), also showed symptoms at a younger age (2, 3, 4, and 15 years of age, $p = 0.111$). When combined together, the two mutations occurred in Arg499 locus were associated with a younger AAO (Mann-Whitney U test; $p = 0.004$). *In silico* analysis of Arg499 is discussed above, along with Arg498. Furthermore, as Arg499 stabilizes the γ phosphate, mutations p.(Arg499Cys) or p.(Arg499His) would also impede ATP binding and catalysis.

Possible biallelic inheritance in SPG4

The *SPAST* p.(Ser44Leu) variant has been previously suggested to play modifier role in SPG4.[1, 46, 47] This variant was detected in one out of the 36 families that had undergone WES. From the three affected siblings, two had undergone WES, and both were heterozygous for the novel pathogenic mutation p.(Ser597ThrfsTer3), as well as for the p.(Ser44Leu) variant. The three siblings had complex HSP with disease onset in early childhood (clinical presentation of these patients is detailed in Supplementary Table 4). The unaffected father only carried the p.(Ser44Leu) polymorphism, and the unaffected mother was heterozygous for the pathogenic p.(Ser597ThrfsTer3) mutation and wild-type for p.(Ser44Leu) (Supplementary Figure 2). The last physical examination of the mother at the age of 56 revealed completely normal findings, including normal reflexes, gait, and motor exam, suggesting that the p.(Ser44Leu) variant may be a modifier of the disease,

or that extreme anticipation with undetected clinical effects in the mother exist in this family (Supplementary Figure 3, A).

Another patient with a Pakistani ancestral background and with consanguineous parents, carried a homozygous mutation, *SPAST* p.(Tyr51Ter). She had started to show symptoms at age one, including core HSP symptoms, motor and speech delay, swallowing difficulty, dysarthria, upper extremity weakness, pes cavus, and skeletal abnormalities. Her brain and total spine MRI were normal and her SPRS score was 40 when examined at 18 years of age. The patient's parents were both asymptomatic heterozygous carriers (Supplementary Figure 3, B).

Patients with novel or rarely reported clinical manifestations

In our cohort, four patients (Supplementary Table 5) presented with deafness, one of whom had a *de novo SPAST* p.(Arg499His) mutation, and the remaining three had familial SPG4 (Supplementary Figure 3, C, D). We also report a patient with ocular movement abnormality, in whom extraocular movements showed slightly hypometric saccades but pursuit was smooth, and a mild horizontal gaze evoked rotatory nystagmus. The disease-causing mutation in this French-Canadian patient was p.(Gln434Ter) (Supplementary Figure 3, E).

One of the signs less reported to date in SPG4 is upper extremity intention tremor, seen in two of the patients in the current study. The causative mutations in these two patients were p.(Arg503Trp) and p.(Leu371Pro). Their respective AAO was 50 and 30, and both presented with a severe form of the disease (respective SPRS scores: 34 and 39). The

first patient also had swallowing difficulty and peripheral neuropathy (Supplementary Figure 3, F), and both had upper extremity ataxia.

Another clinical feature seen in two of the patients in our cohort was seizures, which is quite rare in SPG4. One of the patients of French-Canadian ancestry who carried the mutation p.(Pro489Leu), had an AAO of 23 years, and she suffered from generalized seizures starting at the age of 42 (Supplementary Figure 3, G). The second patient harbored the splice-site mutation c.1321+1G>A, with AAO of 2 years, had episodes of atypical seizures at 58 (Supplementary Figure 3, H).

***De novo* cases**

The parents of the three sporadic cases in our cohort did not carry the causative *SPAST* mutations. The first patient, with *SPAST* p.(Ser407Asn), had an AAO of one year. She presented with lower extremity weakness and spasticity, upper and lower limb hyperreflexia, extensor plantar response, ankle clonus, and sensory abnormalities. She also showed signs of upper extremity weakness, motor and speech delay, dysarthria, learning disability, and swallowing difficulty. Her brain MRI was normal. The second patient had *de novo SPAST* p.(Arg499His). This Mexican-Salvadorian patient started to show symptoms at the age of one year, and her symptoms included lower extremity weakness, spasticity and hyperreflexia, extensor plantar responses, and ankle clonus. Furthermore, she had motor and speech delay, dysarthria, and learning disability. She had a normal brain MRI, and her SPRS score was 37. The last *de novo* patient, also with a p.(Arg499His) mutation, had an AAO of two years and SPRS score of 28, and apart

from core symptoms of HSP, had motor and speech delay, deafness, and dysarthria. The two latter patients have been previously reported.[45]

Cases with synonymous mutation and co-occurrence of multiple sclerosis and SPG4

We report three patients from two unrelated families who carried the synonymous mutation *SPAST* p.(Lys414Lys), previously reported to be pathogenic.[48] The first patient, with French-Canadian ancestral background, had an AAO of nine years, and presented with lower extremity spasticity, hyperreflexia, and extensor plantar responses. The second family had two affected individuals, father and daughter. The father started to have difficulty walking at the age of 65 which led to using a cane, had marked spasticity and brisk reflexes in the lower limbs along with ankle clonus, upgoing plantar responses, urge incontinency, and mild decrease of vibration sensation in the ankles and toes. His daughter noticed difficulty walking around 24 years of age, which progressed slowly. She had upper and lower limb weakness, bilateral Babinski sign and ankle clonus, and positive Hoffman sign. At the age of 34, lumbar puncture carried out due to diplopia was positive for oligoclonal bands and suggested a diagnosis of clinically isolated syndrome (CIS), which is considered as the first clinical episode in multiple sclerosis (MS).[49] She was treated with methylprednisone, and after one month, the diplopia resolved. (Supplementary Figure 3, I,J). In addition to this patient who is a case of co-occurrence of SPG4 and MS, from the 10 pedigrees indicated in Supplementary Figure 3, two demonstrate unaffected family members who have a definite or probable diagnosis of MS (Supplementary Figure 3, D and C).

Discussion

In this large-scale analysis of SPG4 from CanHSP, we report novel *SPAST* mutations, the possibility of a founder mutations in the French-Canadian population, novel characteristics of mutations occurring in Arg499, and potential biallelic inheritance. We also report patients with rare or novel clinical manifestations, and co-occurrence of SPG4 and MS.

Using *in silico* analysis, we predict that the novel mutations reported in this study cause a loss-of-function of spastin, either by substantially shortening the protein, affecting its ATPase activity, or disturbing the formation of a ternary and quaternary structure necessary for catalysis. Spastin, a microtubule-cleaving enzyme involved in the cytoskeletal rearrangement,[50-52] also has a role in intracellular trafficking, cytokinesis regulation and resealing of nuclear membrane.[53-55] In neurons, it is involved in the regeneration of axons and axonal transport.[51, 56-58] Mutations in *SPAST* could result in a disruption of normal organelle trafficking and distribution, and thus micro-organelle accumulation in axons and swelling of axons[59-63] which could result in HSP phenotype. We looked further into the three patients identified as sporadic and found out that all carried *de novo* mutations. Consistent with previous reports of more severe manifestations in patients with *de novo* mutations,[64] all patients showed a severe form of the disease. However, it is important to note that the mutation in two of the *de novo* patients (Arg499His), which is also carried by 33% of *de novo* patients,[64] is associated with a younger AAO, and infantile-onset ascending spastic paraplegia (IAHSP);

regardless of the mode of inheritance.[65, 66] Patients harboring this mutation in our cohort did not show all criteria to be diagnosed as IAHSP (spastic paraplegia progressing to tetraplegia, ocular gaze paralysis, pseudobulbar palsy[67]), however, they showed a higher probability of suffering from upper extremity weakness, dysarthria, speech and motor delay, learning disabilities and cognitive delay. It has been suggested that amino acid 499 is located in one of the highly conserved regions of the *SPAST* gene,[18] and mutations that occur in this region could have a significant effect on the hydrophobicity of the protein and its ability to sever microtubules.[45]

The mutation in our homozygous patient has been reported previously in a heterozygous patient;[68] however, the presence of an additional mutation or CNVs could not have been determined in that study, as only *SPAST* had been sequenced. Our patient with the homozygous mutation *SPAST* p.(Tyr51Ter) showed a severe form of the disease. Previously, there have been two reports of homozygous variants in *SPAST*. One, with the nonsense mutation p.(Ser545Ter), which also reports a complex and severe form of the disease,[69] and another, with the mutation p.(Leu534Pro), who showed a pure spastic paraparesis at the age of 39.[70] The patient from our cohort and the previously reported patient with p.(Ser545Ter) presented with a more severe form of the disease, probably because they carried nonsense mutations, while the latter patient had a missense mutation. Furthermore, in the latter study, the parents of the proband were asymptomatic carriers of the mutation.

The mode of inheritance observed in the patient with homozygous mutation and the family carrying the potential modifier mutation *SPAST* p.(Ser44Leu), may be explained by incomplete penetrance and/or anticipation[71-73] rather than biallelic inheritance, which

could be considered in prenatal diagnosis and genetic counselling. The synonymous mutation *SPAST* p.(Lys414Lys) we report in three patients has been previously reported to underlie the disease, and minigene assay suggested that this variant leads to an aberrant splicing effect.[48] Although we did not find other synonymous *SPAST* mutations in additional HSP patients, it is important to not exclude synonymous variants when analyzing HSP specifically and other diseases in general.

The AAO distribution in our cohort followed the same trend as previously reported,[1, 68] however, our results did not replicate previous findings which suggested that the underlying mutation and the patient sex can modify the disease, and that patients with later onset of the disease can have faster progression.[1, 24] In our data, we did not find an association between mutation type, sex, or late onset, with the disease course. Table 3 compares the frequency of symptoms not attributable to pure form of the disease in our cohort with previous studies. As shown, we report deafness in SPG4 for the first time. The co-occurrence of SPG4 and MS has been reported before;[74, 75] however, the results of studies assessing an association between HSP-related mutations and MS are controversial[76, 77] and further studies are required to verify such a relation.

Table 3. Frequency of symptoms not attributable to the pure form of hereditary spastic paraplegia in the present study compared to previous studies.

Sign/ Symptom	**Frequency in the current study**	**Previous studies**
Learning disabilities	19.6%	4.2% intellectual impairment [1] 1.5% Intellectual disability [68]
Speech delay or abnormality	17%	4/62 (6.5%) [68]
Dysarthria	16.4%	3% [68]
Cognitive deficiency	8.2%	Most frequent additional manifestation [78-82]
Upper extremity intention tremor	2.8%	Mild arm tremor in 10% of patients [68]
Seizure	2.8%	Case reports [69, 75, 83, 84]
Ocular movement abnormality	1.4%	Case reports [85]
Swallowing difficulty	5.6%	3% [68]
Upper limb weak ness	6.9%	Case reports [80]
Upper limb ataxia	5.6%	Case reports [80]
Peripheral neuropathy	14.3%	Case reports [86]
Deafness	5.6%	-

Our study has several limitations. For instance, in spite of being one of the largest HSP cohorts, the number of SPG4 patients was limited, especially for a genotype-phenotype correlation study. Furthermore, the families interested in participating in this study were probably those who had remained undiagnosed after the primary *SPAST* testing; therefore, the proportion of SPG4 patients in this study may be underestimated. In addition, clinical signs and symptoms for some of the patients were missing.

In conclusion, we report one of the largest SPG4 cohorts, with 41 different mutations including 6 novel mutations. We suggest that modes of inheritance other than autosomal dominant could be involved in SPG4. Our study sheds light on some rarely reported or unreported aspects of the disease and helps improve genetic counseling and clinical trials conducted on SPG4. Towards this goal, larger efforts and international collaborations are required.

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Author's Roles

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M.A.E.: Research project conception, organization and execution; manuscript review and critique.

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K.M.: Manuscript preparation.

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CHAPTER 4: BRIDGING CHAPTER "AUTOSOMAL DOMINANT PURE HSP BEYOND SPG4"

We aimed to establish the genetic diagnosis for as many patients as possible. Of the whole 696 subjects enrolled in our cohort, 379 underwent HSP-associated gene panel sequencing, and the genetic diagnosis of 185 cases was made using this cost-effective method. The remaining 194 cases, along with 206 patients on whom panel sequencing had not been performed (a total of 400 patients), were analyzed by WES. A total of 157 patients, from the 585 subjects that had been analyzed either with panel sequencing or WES, carried the *SPAST* gene alterations responsible for the HSP phenotype. This number included patients who carried rare pathogenic synonymous mutations and CNVs which were not detected in the initial analysis. To reach the highest rate of genetic diagnosis, we also analyzed non-HSP-associated genes that were previously described in conditions that have overlapping symptoms with HSP, including the *GCH1* gene. Three patients carried pathogenic mutations in this gene which is classically known to be involved in DRD and PD. These three patients, like the majority of cases with AD mode of inheritance including SPG4 patients, had pure HSP, and showed childhood-onset lower limb spasticity, hyperreflexia, abnormal plantar responses, and a spastic gait. In the next chapter, a more detailed description of these patients, the process of their diagnosis and treatment is reported.

CHAPTER 5: MANUSCRIPT 2

GCH1 mutations in hereditary spastic paraplegia

Running title: GCH1 in hereditary spastic paraplegia

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Conflict of interests

The authors declare no conflict of interests.

Data availability statement

Data available on request from the authors.

Abstract

GCH1 mutations have been associated with dopa-responsive dystonia (DRD), Parkinson's disease (PD) and tetrahydrobiopterin (BH_4)-deficient hyperphenylalaninemia B. Recently, *GCH1* mutations have been reported in five patients with hereditary spastic paraplegia (HSP). Here, we analyzed a total of 400 HSP patients (291 families) from different centers across Canada by whole exome sequencing (WES). Three patients with heterozygous *GCH1* variants were identified: monozygotic twins with a p.(Ser77_Leu82del) variant, and a patient with a p.(Val205Glu) variant. The former variant is predicted to be likely pathogenic and the latter is pathogenic. The three patients presented with childhood-onset lower limb spasticity, hyperreflexia and abnormal plantar responses. One of the patients had diurnal fluctuations, and none had parkinsonism or dystonia. Phenotypic differences between the monozygotic twins were observed, who responded well to levodopa treatment. Pathway enrichment analysis suggested that GCH1 shares processes and pathways with other HSP-associated genes, and structural analysis of the variants indicated a disruptive effect. In conclusion, *GCH1* mutations may cause HSP; therefore, we suggest a levodopa trial in HSP patients and including *GCH1* in the screening panels of HSP genes. Clinical differences between monozygotic twins suggest that environmental factors, epigenetics, and stochasticity could play a role in the clinical presentation.

Keywords: HSP, GCH1, Dystonia, Spastic paraplegia.

Introduction

The guanosine triphosphate cyclohydrolase I gene (*GCH1*, [OMIM] *600225*)* encodes GTP cyclohydrolase 1, a biosynthetic enzyme which catalyzes the synthesis of tetrahydrobiopterin (BH_4). BH_4 is a co-factor of tyrosine hydroxylase, an enzyme involved in the synthesis of the neurotransmitters dopamine and monoamines.[1,2] Due to its importance in the synthesis of these neurotransmitters, several neurological disorders may result due to GTP cyclohydrolase 1 deficiency.

Rare bi-allelic *GCH1* mutations may cause tetrahydrobiopterin (BH_4)-deficient hyperphenylalaninemia B (HPABH4B, OMIM #233910),[3] a rare disease which may include severe developmental delay, muscular hypotonia of the trunk, hypertonia of the extremities, seizures and other neurological symptoms.[4-6] Rare heterozygous mutations in *GCH1* are known to cause autosomal dominant dopa-responsive dystonia (DRD), which typically presents with dystonia of the lower limbs in childhood, diurnal fluctuations and a substantial response to levodopa treatment.[7-10] In recent years it has been shown that rare heterozygous *GCH1* mutations may also cause Parkinson's disease (PD),[11-14] and that common variants in the *GCH1* locus are associated with risk of PD.[14,15]

Two previous reports have implicated *GCH1* mutations in hereditary spastic paraplegia (HSP) patients,[16,17] yet their role in HSP is still not conclusive. HSP is a general term for a group of inherited neurodegenerative disorders characterized by lower limb spasticity and weakness, with or without additional neurological symptoms.[18] Herein, we report three additional HSP patients from two different families with heterozygous *GCH1* mutations, their phenotype and long-term follow-up.

Materials and Methods

Population

HSP patients (n=696) from 431 families were enrolled to CanHSP, a Canadian consortium for the study of HSP.[19] The patients were recruited from eight major medical centers across Canada (Montreal, Toronto, Quebec, Calgary, Edmonton, Ottawa, Hamilton, and Vancouver). Data regarding diagnosis, recruitment, and the cohort have been previously described.[19] All patients signed informed consent forms at enrollment and the study protocol was approved by the institutional review board.

Genetic analysis

Genomic DNA has been extracted from peripheral blood, according to standard procedures.[20] After diagnosis of a portion of the patients with panel sequencing, WES was performed on 400 patients from 291 families, using the Agilent SureSelect Human All Exon v4 kit for capture and targeted enrichment of the exome. Captured samples were sequenced in Illumina HiSeq 2000/2500/4000 system. Then, the sequence reads were aligned against the human genome (GRCh37 assembly) using Burrows-Wheeler Aligner (BWA).[21] Variant calling and annotation were done using Genome Analysis ToolKit (GATK)[22] and Annotate Variation (ANNOVAR).[23]

In order to rule out other genes known to be involved in HSP or in similar neurogenetic disorders in which spasticity is among the features, we analyzed the data using a list of 785 such genes (Supplementary Table 1). The nomenclature of *GCH1* variants is based

on its full transcript NM_000161. We used MutationTaster, FATHMM, EIGEN, SIFT, CADD and REVEL for assessing the pathogenicity of the variants.[24] Variants were classified according to the guidelines of American College of Medical Genetics and Genomics (ACMG) using VarSome.[25] Domain prediction was performed using Interpro,[26] and the reported mutations in PD, DRD and HSP were divided in two groups: within the main domain of the GCH1 protein (GTP_Cyclohydrolase_I_domain [IPR020602]) or outside of the main domain. Pearson Chi-square test was applied to determine whether an association between these two categorical variables (disease type and mutation location) exists. Gene Ontology (GO) enrichment analysis of *GCH1* with a list of known HSP associated genes (Supplementary table 2) was carried out using the g:Profiler,[27] with Benjamini-Hochberg adjusted *p*-values for statistical significance set at < 0.05.

In silico structural analysis

The atomic coordinates of human GCH1 bound to Zn^{2+} were downloaded from the Protein Data Bank (ID 1FB1). The effect induced by each mutation was evaluated using the "mutagenesis" toolbox in PyMol v. 2.4.0.

Results

Identification of *GCH1* mutations in HSP patients

Heterozygous *GCH1* mutations were identified in 3 HSP patients (representing 0.75% of HSP cases and 0.69% of HSP families with available WES data), including monozygotic twins from Family 1 and a single patient from Family 2. In family 1, following filtration, a novel heterozygous in-frame deletion in *GCH1*, c.229_246del: p.(Ser77_Leu82del), remained the only potential explanation for the phenotype of the twins. This variant was predicted to be likely pathogenic (Supplementary table 3) and was not reported in gnomAD.[28] The affected twins were adopted shortly after birth, and family history or DNA from genetic relatives are not available. In family 2, another *GCH1* variant was identified, c.614T>A: p.(Val205Glu), which is not reported in gnomAD, and has been analyzed in two functional studies. These studies have suggested a significant decrease in the activity[29] and a strong effect on the function of the protein.[30] In VarSome, this variant has been classified as likely pathogenic, however, when taking into account the PS3 criterion (which concerns functional studies), the verdict changes to pathogenic.

Sanger sequencing of DNA samples from the proband's parents indicated that the father carried the heterozygous mutation, while the mother had wild-type alleles. According to the proband, the father had no symptoms, but he was not seen by a neurologist. The variant is predicted to be deleterious by MutationTaster, FATHMM, EIGEN, SIFT, CADD (score of 26, within the top 0.5% of variants predicted to be most deleterious) and REVEL. Both variants occurred in the GTP_Cyclohydrolase_I_domain (IPR020602) which plays

an important role in catalyzing the biosynthesis of formic acid and dihydroneopterin triphosphate from GTP.[31] All variants were validated by Sanger sequencing.

Structural analysis of GCH1 pathogenic mutations

The structure of human GCH1 was solved by X-ray crystallography by Auerbach and colleagues.[32] The structure reveals a toroid-shaped D5-symmetric homodecameric assembly, with the catalytic sites located at the interface of three adjacent subunits (Figure 1A). By homology with its *E. coli* ortholog, the catalytic site is defined as Cys141, His210 and Cys212 from one subunit, Ser166 from a second subunit and $helix_{92\text{-}104}$ from a third subunit[33,34] (Figure 1B). To investigate the impact of the degenerative mutations on the structure, we performed *in silico* mutagenesis and evaluated the effect on the surrounding residues.

Val205 is located on a β-sheet ($strand_{202\text{-}209}$) and its sidechain points toward the core of the toroid, facing the C-terminal helices of two adjacent subunits. Its sidechain form hydrophobic interactions with Phe244, Val226 and Ile248 from the same subunit, and Val247 from the adjacent subunit, causing a leucine-zipper network of interactions, as valine, isoleucine and leucine have the same coupling energy[35] (Figure 1C). The HSP-associated mutation of Val205 to the polar residue glutamate would therefore strongly disrupt these interactions. Therefore, the Val205Glu mutation will likely destabilize the entire β-sheet and possibly the entire domain, hence preventing its oligomerization and, *in fine*, its activity. By contrast, the DRD-associated mutation of Val205 into Gly likely results in a milder destabilization of this zipper, weakening the stabilization of the C-terminal helix on the inside of the toroid on one hand, and destabilizing the interaction

between adjacent subunits. In addition, the Val205Glu would also induce more flexibility in the protein backbone as the absence of sidechain in glycine induce another degree of flexibility compared to others amino acids. Finally, several other mutations on the same β-sheet or on the facing helix have been reported to be causing DRD, supporting the hypothesis that disturbing this area is pathogenic.[36]

Residues Ser77 to Leu82 are located on a helix located on the outside of the toroid. This helix is involved in a homotypic interaction between two subunits on different halves of the toroid, through hydrophobic residues Leu71, Ala74, Ile78 and Leu82 from both helices (Figure 1D). As the two helices are anti-parallel, the Ser77_Leu82 deletion will abolish the interaction. As a result, this mutation will likely prevent the assembly of the 2 halves, either leading to homopentamers or to unstable monomers. In addition, $helix_{60-82}$ interacts with $helix_{92-104}$ near the catalytic site through the backbone OH of Asn64 and Gln103 sidechain and hydrophobic residues Leu68, Leu71, Ala72, Leu79, Leu91, Ala99 (Figure 1E). The Ser77_Leu82 deletion will shorten $helix_{60-82}$, shifting its residues and likely disturbing its interaction with $helix_{92-104}$ and disrupting its positioning as part of the catalytic site. Either way, this deletion will diminish the probability of formation of the active site. DRD-associated mutations Leu71Gln, Ala74Val and Leu79Pro have also been reported in this helix, supporting the hypothesis that disrupting the interaction between those helices is pathogenic, and that the degree of disruption of GCH1 leads to different pathologies.

In conclusion, the mutations Val205Glu and Ser77_Leu82del are probably affecting the proper homodecamerization of human GCH1, preventing the assembly of the three subunit-formed active sites. If Val205Glu seems to destabilize the proper folding of each

monomer, Ser77_Leu82del probably prevents the polymerization of the homodecamer. Overall, those mutations appear to be “loss-of-function” but it would be interesting to investigate whether helix$_{92\text{-}104}$ and the “inside of the toroid” have solely architectural roles, or function as well in catalysis.

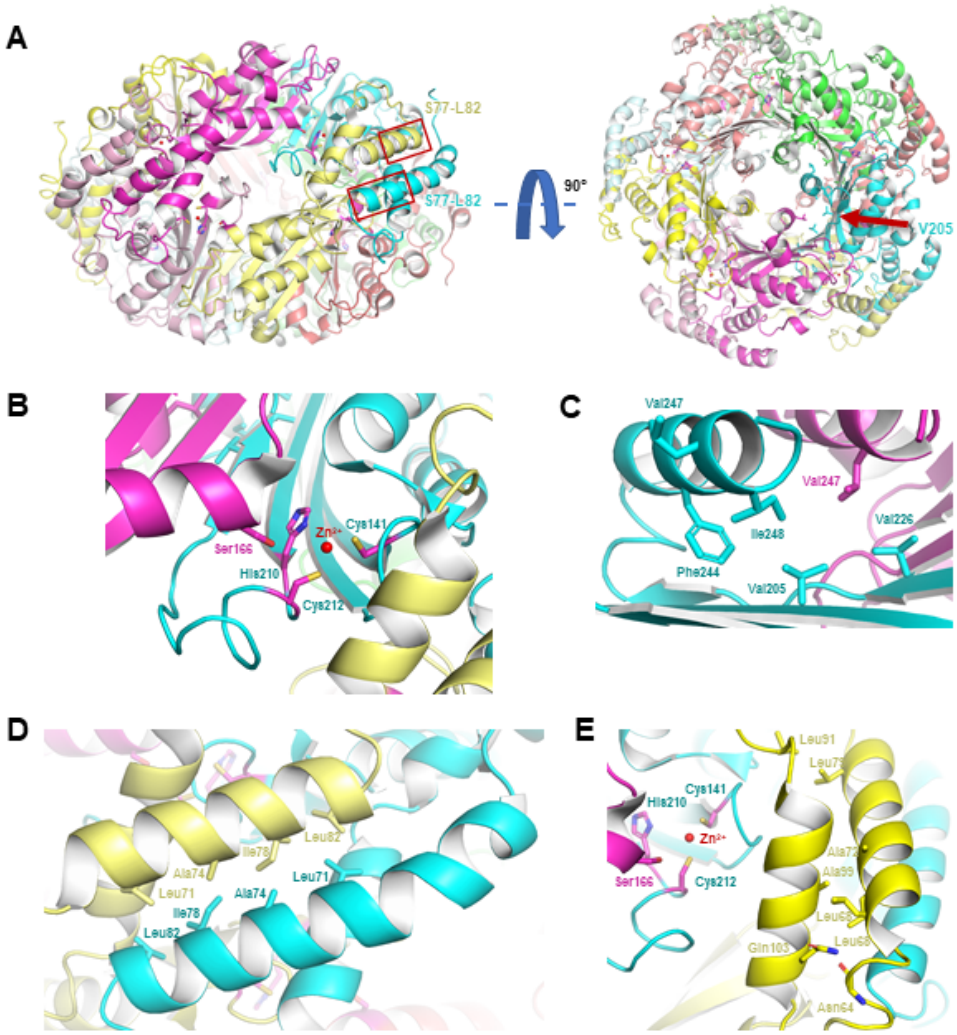

Figure 1. Structural analysis of GCH1 (protein database bank (PDB) code: 1FB1). A: Human GCH1 forms an homodecameric assembly. The Ser77-Leu82 region of subunit B (cyan) and D (pale yellow) are squared (left panel) and Val205 of subunit B is indicated with an arrow (right panel). B: The interface between subunit A (pink), B and D form an active site. The catalytic Cys141, Ser166, His210 and Cys212 are colored in magenta. C: Subunit B Val205 interacts with Phe244, Val226 and Ile248 of the same subunit, and Val247 of subunit A. D: Leu71, Ala74, Ile78 and Leu82 from subunit B and D form a hydrophobic-type interaction. E: Subunit D helix60-82 stabilize helix92-104 through the backbone OH of Asn64 and Gln103 sidechain and hydrophobic residues Leu68, Leu71, Ala72, Leu79, Leu91, Ala99.

Pathway enrichment analysis

Pathway enrichment analysis suggests that GCH1 shares similar processes and pathways with other HSP-associated genes, including: hydrolase activity, nucleoside-triphosphatase activity, small molecule binding, catalytic activity, pyrophosphatase activity, organic acid biosynthetic process, carboxylic acid biosynthetic process, oxoacid metabolic process, regulation of anatomical structure size and small molecule biosynthetic process (Supplementary Table 4).

Characteristics of HSP patients with *GCH1* mutations

Family 1

Cases A and B are male monozygotic twins, currently 18 years of age. The twins were conceived naturally to a healthy 17-year-old mother, and delivery was by cesarean section at 31.5 weeks. Both twins were adopted shortly after birth and live with their adoptive father and mother. Family history was reportedly negative for genetic or metabolic disorders, congenital malformations, neurological disorders, children with spasticity, seizure or mental challenges, history of other psychiatric disorders or autism.

Twin A clinical presentation: During the first years of life, twin A achieved developmental milestones at the appropriate time, and there was no early history of speech delay, vision or hearing problems. At the age of four years, twin A started to toe-walk and had an in-toed gait. His symptoms progressed slowly over the years, and he had multiple episodes of falling every week, and he later required a walker or occasionally a wheelchair. He could sit normally, had normal bladder control and attended a regular class with grades above average. On physical exam at 11 years, he had significant toe-walking, bilateral spasticity of the lower limbs, in-turned feet, history of frequent falls, and use of wheelchair most of the time. His neurological exam showed spasticity of lower limbs with hyperreflexia with brisk reflexes of 3+ at the knees, absent clonus, extensor plantar responses, bilateral pes planus, normal sensory exam, and a markedly spastic gait. No dystonic posture and bradykinesia were noted. Upper limb exam was normal.

Twin B clinical presentation: Twin B had normal development until the age of 9 years when he presented with toe walking, which progressed to lower limb spasticity with increasingly frequent falls. At age 11 years twin B required ankle foot orthosis (AFO) and received occupational therapy and physical therapy on a regular basis. He could carry out most activities of daily living independently, but needed to use a walker for ambulation

due to frequent falls. He had normal sitting posture, normal bladder function, and was in a regular classroom with grades above average. On physical exam at age 11 years, twin B presented with mild scoliosis, mild muscle atrophy spastic tone of lower limbs, hyperreflexia of 3+ at the knees, contractures at the ankles, power was 5/5 proximal, 4-/5 distal with pes cavus of the feet. Plantar responses were equivocal bilaterally. Dystonic posturing and bradykinesia were not detected. Sensory exams including vibration and proprioception was within normal limits. Upper limb exam was normal.

Treatment: Both brothers were treated with Sinemet 100/25 (carbidopa/levodopa) at age of 12 years, and within 6 weeks of starting therapy they showed a remarkable improvement. Formal neurological exam at the age of 13 revealed nearly normal muscle tone and strength of the lower extremities, with very mild weakness at the ankles. They were able to ambulate using ankle-foot orthoses and no longer required a walker or wheelchair. Due to the initial success of the levodopa trial, we did not perform lumbar puncture to measure catecholamines. Currently at age 18 years their neurological examination, including deep tendon reflexes and plantar responses, is completely normal, and the patients are able to run and jump. Both patients had normal creatine kinase (CK) levels, and magnetic resonance imaging (MRI) of the brain and spine revealed no specific abnormalities. Electromyogram (EMG) and nerve conduction velocity (NCV), microarray and metabolic testing were normal.

Family 2

Patient C from family 2 is a male of French-Canadian ancestry, born after a normal pregnancy and delivery without any complications, with normal language and motor skill

development until the age of 6 years, when he started to have difficulty walking. At 8, a possible diagnosis of cerebral palsy was made. At 9 ½ of age, a diffuse hyperreflexia was noted in lower limbs which was more significant on the right side. In addition, there was a retraction of the right Achilles tendon. When examined at the age of 10, his parents reported that he walks with toes turned inward. He had to stop frequently while walking due to muscle fatigue, and was unable to run. School performance was normal. Symptoms were reported to worsen toward the end of the day or with exercise. Family history was unremarkable. In his physical exam, muscle strength was 5/5 in the upper limbs, 5-/5 in the right leg (more noticeable in proximal muscles). Reflexes were 2+ in upper limbs as well as in the left patella, and 3+ in the right patella. Ankle jerks were brisk bilaterally. He had up-going toes in his right foot associated with 3 beats of clonus. When walking, he tended to drag his right leg slightly. Neither dystonia nor bradykinesia were noted. Over the years, the patient was lost to follow-up, and treatment was not initiated. Following our findings, we were able to locate the patient and suggest that they see a neurologist to consider a trial of levodopa.

Comparison to previously reported HSP patients with *GCH1* mutations

Table 1 details the main characteristics of the current and previous cases of HSP patients with *GCH1* mutations. Dystonia, the main manifestation of DRD, was not seen in our patients. Also, daily fluctuation, a prominent feature in DRD, which is also one of its diagnostic criteria[37] was absent in two of our patients. On the other hand, spasticity, which is not usually seen in the autosomal dominant form of DRD (as opposed to autosomal recessive form),[38] was seen in all three of our patients. Three of the five previously

reported HSP patients with *GCH1* mutations also had parkinsonism, whereas our patients did not show signs of bradykinesia, rigidity or tremor.

Table 1. Comparison of characteristics of patients in the current study with previously reported HSP patients with GCH1 mutations.

Patient	Sex	Age at onset (y)	Age at diagnosis (y)	First clinical diagnosis	Spastic gait	Spasticity Legs (L/R)	Hyperreflexia (L/R)	Plantar responses (L/R)	Bladder symptoms	Ankle clonus (L/R)	Motor delay	Speech delay/abnormality	Dystonia	Diurnal fluctuations	Parkinsonism	MRI
0	F	6	36	CP	+	+/+	+/+	NA	-	-	-	+ (dysarthria)	-	+	-	Normal
1.1	F	2	56	CP	+	+/+	+/+	↑ / ↑	+	-/-	-	-	+	+	+/-	NA
1.2	F	3	25	HSP	+	+/+	+/+	↑ / ↓	_	-/+	NA	NA	_	++	_	Normal
2	F	14	49	CS HSP	+	+/+	+/+	↓ / ↑	+	-	NA	NA	++	+	+	Normal
3	M	7	50	HSP	+	+/+	+/+	↑ / =	_	NA	NA	NA	_	+	++	NA
A*	M	4	11	HSP	+	+/+	+/+	↑ / ↑	-	-	-	-	_	_	_	Normal
B*	M	9	11	HSP	+	+/+	+/+	=/=	-	-	-	-	_	_	_	Normal
C*	M	6	10	CP	+	+/+	-/+	↑ / ↑	+	+	+	-	_	+	_	Normal

Abbreviations: y, year; F, female; M, male; L, left; R, right; MRI, Magnetic Resonance Imaging; CP, cerebral palsy; NA, not available; HSP, hereditary spastic paraplegia; CS, cervical stenosis; +, present; -, absent; Plantar responses: ↑, extensor; ↓, flexor; =, equivocal.

* Patients in the current study.

When examining the location of *GCH1* mutations in the current and previously reported cases of HSP, DRD and PD (Supplementary Table 5), there was no significant difference in the location of *GCH1* mutations comparing DRD, HSP and PD. (P=0.740, Pearson Chi-Square).

Discussion

We describe three HSP patients with likely pathogenic and pathogenic *GCH1* mutations, responsible for ~0.4% of all HSP patients and families in our cohort with 696 patients from 431 families. Patients A and B are monozygotic twins, who had bilateral spasticity of the lower limbs, spastic gait and hyperreflexia at the knees. In patient C, the main findings were spasticity of the lower limbs, spastic gait, hyperreflexia in the right lower limb, and diurnal fluctuation. Our patients had neither dystonia nor parkinsonism. These findings expand the genotype and phenotype knowledge about *GCH1* mutations in HSP.

The mutation in family 1, p.(Ser77_Leu82del), has not been reported before. However, the mutation in family 2, p.(Val205Glu), has been reported in three studies, two reporting families with typical DRD[29,30] and one reporting a patient with atypical DRD with isolated resting leg tremor.[39] The two former studies also reported family members who were asymptomatic carriers of the mutation, suggesting an incomplete penetrance, which is also supported by the reported lack of symptoms in the father of the proband who also carried the p.(Val205Glu) mutation.[29,30]

To date, *GCH1* mutations in HSP have been described in two reports,[16,17] including four female and one male patients with the age of onset between 2 and 14 years of age. Our patients, all male, were 4 to 9 years at diagnosis. Two of the patients in the previous

report had dystonia, three had parkinsonism, and all five had diurnal fluctuations. These symptoms were absent in our patients, except for diurnal fluctuation, which was reported in one patient. On the other hand, anatomical features (e.g. pes cavus, pes planus, scoliosis) as well as lower extremity weakness were seen in our patients, but have not been previously reported. The differences in phenotypes of the monozygotic twins in the current study (e.g. age at onset, presence of weakness, scoliosis, plantar response, muscle atrophy) may suggest that environment, epigenetics, and stochasticity [40-42] could play a role in the presentation of patients with *GCH1* mutations.

Interestingly, *GCH1* mutations which cause DRD, PD and HSP are found in overlapping regions of the protein (figure 2), which decreases the possibility that the location of the mutations alone affects the phenotype. Therefore, other genetic or environmental modifying factors may play a role in disease presentation and the mutation. Other genes have been reported with similarly overlapping phenotypes. For instance, mutations in *ATP13A2* cause Kufor-Rakeb syndrome,[43] which is an atypical parkinsonism-dystonia disorder. More recently, *ATP13A2* has been reported to also be associated with HSP.[44,45] *SPG7* is another example of a gene associated with HSP as well as parkinsonism.[46,47] In addition, *SPG11* mutations have been reported in patients with parkinsonism[48] and DRD[49] in addition to HSP.[50] These reports suggest a clinical-genetic overlap between these disorders, and that screening for mutations in these genes is indicated when the most common genetic causes have been ruled out.

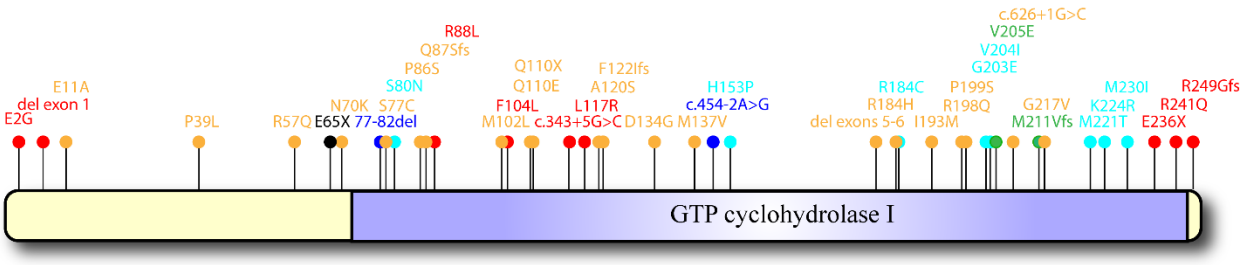

Figure 2. Schematic representation of the location of *GCH1* mutations in hereditary spastic paraplegia (HSP), dopa-responsive dystonia (DRD) and Parkinson's disease (PD) patients reported so far. The rectangle represents GCH1 protein, and the functional domain, GTP_CycloHydrolase_I, is indicated with vertical lines. Mutations associated with PD, DRD, and HSP are indicated in yellow, red, and dark blue respectively. Circles in green indicate mutations associated with both HSP and DRD, those in light blue with both PD and DRD, and those in black with PD, DRD and HSP.

To conclude, our results provide additional support for the involvement of *GCH1* in HSP and expand the clinical phenotype of *GCH1*-related disorders. We recommend trial of levodopa in HSP patients as well as adding *GCH1* to the sequencing panels of HSP genes to increase their genetic diagnostic yield.

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CHAPTER 6: SUMMARY OF THE FINDINGS, DISCUSSION, AND FINAL CONCLUSIONS

The major goal of this Master's thesis was to better understand HSPs, shed light on some novel aspects of this heterogeneous disease, and fill a part of the gap that exists in the genetic diagnosis of HSP.

In the first study, one of the largest SPG4 cohorts with 157 patients from 65 families was analyzed, and new genetic as well as clinical features were described. We suggested the probability of biallelic inheritance in SPG4 based on our findings in two families, one with homozygous mutation in a patient who had asymptomatic parents, and another family with a pathogenic mutation accompanied by a modifier variant. In the latter family, two male siblings with early-onset complex HSP were heterozygous for the known SPG4 modifier, *SPAST* p.(Ser44Leu), as well as for the pathogenic *SPAST* p.(Ser597ThrfsTer3). Their asymptomatic mother and father were heterozygous carriers of p.(Ser597ThrfsTer3) and p.(Ser44Leu) respectively. Although we suggest that biallelic inheritance could be a potential explanation, anticipation and reduced penetrance should also be taken into account. Particularly, since a previous study has suggested that SPG4 penetrance is lower in females compared to males [36].

We identified that synonymous, structural, and *de novo* variants could be the underlying genetic cause in a proportion of the genetically undiagnosed cases. We found synonymous mutations to be the underlying genetic cause of three SPG4 patients. We suggest that rare synonymous variants should not be excluded during WES data analysis. Of 157 SPG4 patients, seven (4.5%) carried CNVs. This finding emphasized the limitation

of WES in the detection of genetic causes in Mendelian disorders. By describing three patients with *de novo SPAST* mutations, we also repeated the findings of a review paper on *de novo* SPG4 patients published recently [94] which suggested a complex and relatively severe phenotype in carriers of *de novo* mutations. Additionally, in this study, p.(Arg499His) variant was reported to be the causative mutation in nine out of the 27 (33%) *de novo* SPG4 cases, and in our cohort, this mutation was seen in two out of three *de novo* patients. The reason why this specific domain is more prone to being mutated and cause *de novo* disease should be studied in the future. Another significant finding regarding the p.(Arg499His) in our study was the association of this variant with specific manifestations (such as motor delay and learning disability), regardless of inheritance mode.

Apart from deafness, which we reported for the first time in SPG4, other clinical manifestations are more or less similar to previous reports [36, 95]. A point to be noted is that although SPG4 is classically known to cause a pure form of HSP, complex form of the disease was seen in close to 37% of our cases, which is higher compared to the previous findings in which approximately 35%[36], 24%[96], 22%[95] and 12%[97] of SPG4 patients reported to show complex phenotypes. This could imply the necessity of including *SPAST* in the genetic screening of all patients with HSP presentation, including those who show the complex form.

In our cohort, analyzing *SPAST* provided diagnosis of 157 patients, most of whom presented with pure HSP phenotype and had an AD form of inheritance. The puzzle of three other patients, also with AD pure HSP, was unraveled by analyzing a gene not associated with HSP, the *GCH1* gene, which has been described in DRD and PD. In our

study, which is the third one reporting *GCH1* mutations in HSP, we described three patients, two monozygotic twins who presented with a typical pure HSP, and a third patient who had diurnal fluctuations of symptoms in addition to the manifestations of pure HSP. Parkinsonism and dystonia were completely absent in our patients, while from the five previously described HSP cases with *GCH1* mutations, three had parkinsonism, two had dystonia, and all five experienced diurnal fluctuations of symptoms [74, 75]. Another feature that distinguishes our patients from the previously reported ones is decreased lower limb muscle strength detected in the physical examination of our patients.

All these patients responded well to levodopa treatment. This finding is particularly important, as it indicates the importance of genetic diagnosis. There is no curative treatment for HSP, and a correct genetic diagnosis could help in controlling symptoms and thus improving the quality of life in some cases, such as these HSP patients in whom dopamine production was impaired due to *GCH1* mutations.

Although to the best of our knowledge the role of factors such as environment, epigenetics, and stochasticity has not been studied in HSP, the differences seen in clinical features of monozygotic twins who share 100% of their genome, show the probable role of such factors in the disease. These differences also highlight the importance of twin studies in clarifying contributions of further genetic and non-genetic factors.

Reports of HSP phenotypes caused by mutations in *GCH1* and some other genes classically known to be involved in neurological diseases other than HSP, underlie the importance of analyzing such genes to solve undiagnosed cases. Thus, in the process of genetic diagnosis of some patients, it is crucial to include all genes associated with the

diseases existing on the spectrum of neurodegenerative disorders. Our findings also support the hypothesis that neurodegenerations are a continuum of disorders and that neurodegenerative disorders share common pathways [92].

In conclusion, our studies expand the clinical and genetic knowledge of HSP. The possibility of biallelic inheritance and reduced penetrance could be taken into account in genetic counselling of SPG4 cases. We highly recommend not excluding genetic tests on patients based solely on the mode of inheritance or clinical symptoms. We suggest considering CNVs, synonymous variants, *de novo* mutations, and mutations in genes associated with other neurological diseases for genetic diagnosis of a proportion of unsolved HSP patients. Reaching an accurate diagnosis is essential for the genetic counseling of HSP families, and proper genetic counselling, which may influence the childbearing decisions of the affected individuals, could reduce the socio-economic burden of HSP. A levodopa trial could be recommended in HSP patients. For designing novel targeted therapies and clarifying the unknown aspects of a rare heterogeneous disorder like HSP, large genotype-phenotype correlation analysis through international collaborations and multicenter studies is required.

CHAPTER 7: REFERENCES

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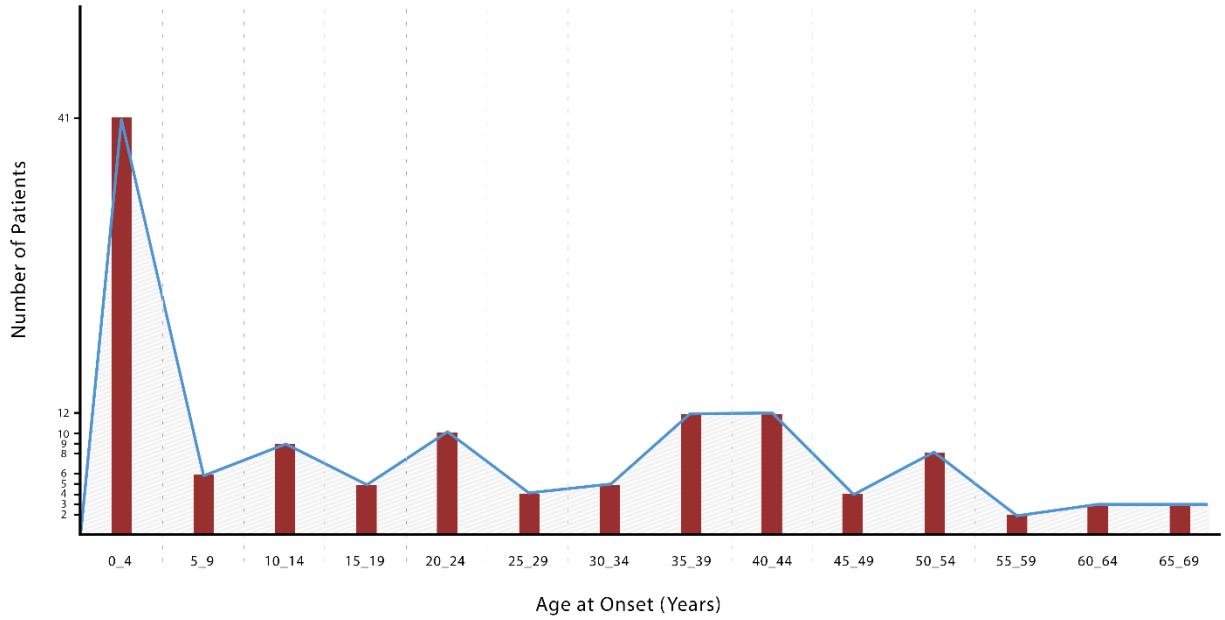
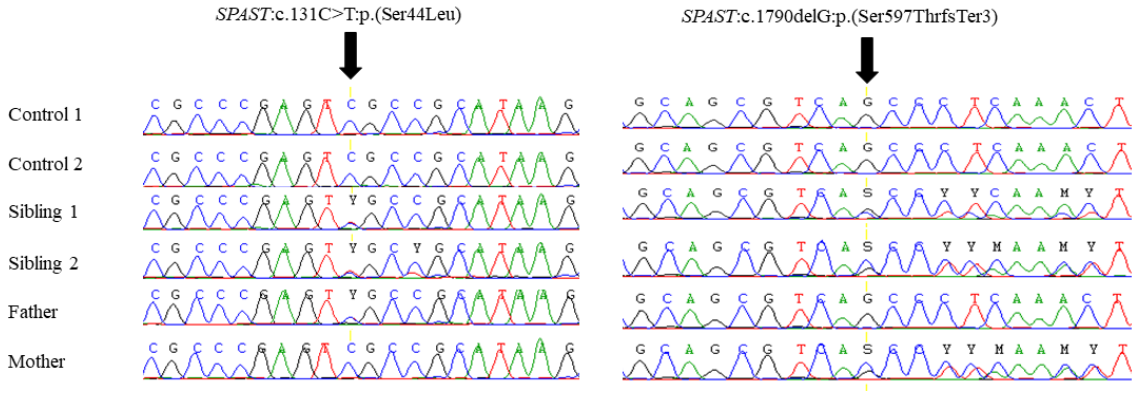
CHAPTER 8: APPENDICES

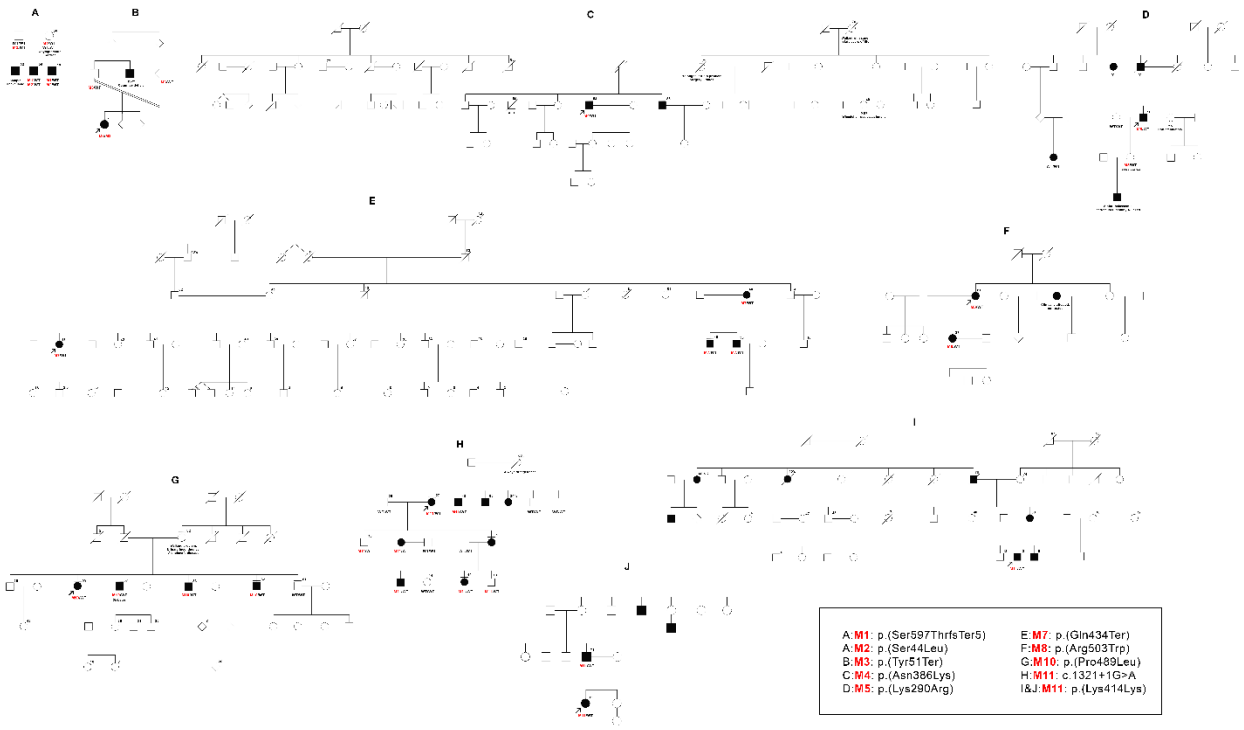
8.1. SPG4 project supplementary data



8.1.1. Supplementary Figure 1. Distribution of age at onset in the cohort. The onset of SPG4 mostly occurs in the first five years of life, and between 35-44 years of age.



8.1.2. Supplementary Figure 2. Sanger sequencing results of family with modifier mutation. Mother and father carry the p.(Ser597ThrfsTer3) and p.(Ser44Leu) mutations respectively. Siblings are heterozygote for both mutations.



8.1.3. Supplementary Figure 3. Pedigrees of patients described in the manuscript. Circles and squares represent females and males, respectively. Black filled symbols indicate individuals with symptomatic hereditary spastic paraplegia. Arrows and diagonal lines through the symbols represent the patient described in the manuscript and deceased individuals, respectively. Genotypes for the culprit mutations are labeled as wild-type (WT/WT), heterozygous ($M_{\text{Mutation number}}$/WT), or homozygous ($M_{\text{Mutation number}}$/$M_{\text{Mutation number}}$). Mutation numbers are explained in the box in lower right part of the figure. **A.** Family carrying modifier mutation, **B.** Patient with homozygous mutation, **C** and **D.** Patients with deafness, **E.** Patient with ocular movement abnormality, **F.** Patient with upper extremity tremor, **G.** Patient with generalized seizures, **H.** Patient with atypical seizures, **I** and **J.** Patients carrying pathogenic synonymous mutations.

8.1.4. Supplementary Table 1. Genes that are known or suspected to be involved in HSP or other similar disorders. (Material available online: https://onlinelibrary.wiley.com/doi/full/10.1111/cge.13955)

8.1.5. Supplementary table 2. Copy number variations (CNVs) in the current study.

CNV	**Number of families**	**Number of patients**	**Clinical presentation**
Deletion of exon 1	3	3	AAO:40 Pure HSP
			AAO:45 HSP complicated with amyotrophy/lower motor neuron features, and peripheral neuropathy
			AAO:29 Pure HSP
Deletion of exons 5-7	1	2	AAO: 46 Pure HSP
Deletion of exons 16-17	1	1	AAO: 39 Pure HSP
Deletion of exon 17	1	1	AAO:38 Pure HSP

AAO, age at onset; HSP, hereditary spastic paraplegia.

8.1.6. Supplementary table 3. Signs and symptoms seen more frequently in patients harboring the p.(Arg499His) mutation.

Sign/symptom	**Patients with Arg499His**	**Patients with other mutations**	**p value**
Motor delay	4/4 (100%)	3/38 (7.9%)	0.000003
Speech delay or abnormality	4/4 (100%)	4/37 (10.8%)	0.000019
Learning disability	3/4 (75%)	5/39 (12.8%)	0.00233
Progressive cognitive deficit	2/4 (50%)	2/56 (3.6%)	0.00032
Upper extremity weakness	2/4 (50%)	2/55 (3.6%)	0.00036
Dysarthria	4/4 (100%)	7/56 (12.5%)	0.000012

Bonferroni corrected p value: 0.00238.

8.1.7. Supplementary Table 4. Clinical manifestations of the family with probable biallelic inheritance. Patients 1 and 2 had undergone whole exome sequencing and were compound heterozygous carriers of the novel pathogenic mutation *SPAST* p.(Ser597ThrfsTer3), and the *SPAST* p.(Ser44Leu) variant. Sample from patient 3 was not available.

Pt	AAO	Spasticity	Delayed Motor Milestones	Speech Delay	Dysarthria	Learning difficulty	Dysphagia	UE weakness	LE Weakness	HR	Hoffman (R/L)	Babinski sign (R/L)
1	4 years	+ (LE>>UE)	-	-	-	-	-	+	+	+ (LE>UE)	-/-	+/+
2	Early childhood	+	+	+	Mild	-	-	-	+	+ (LE>UE)	+/+	+/+
3	Early childhood	+ (LE>>UE)	+	+	-	+	Mild	+	+	+ (LE>UE)	+/+	+/+

Pt, patient; AAO, age at onset; LE, lower extremity; UE, upper extremity; HR, hyperreflexia; R, right; L, left.

8.1.8. Supplementary table 5. Characteristics of SPG4 patients with deafness.

Mutation	**Age at onset**	**SPRS score**	**Other signs/symptoms attributed to complex HSP**	**Pedigree**
p.Asn386Lys	40	Not available	-	C
p.Lys290Arg	55	42	-	D
p.Arg499His	2	28	Motor delay, speech delay, dysarthria	- (*De novo*)
p.Arg581Ter	34	3	-	Not available

8.2. GCH1 project supplementary data

8.2.1 Supplementary Table 1. Genes that are known or suspected to be involved in HSP or other similar disorders. (Material available online: https://onlinelibrary.wiley.com/doi/full/10.1111/cge.13955)

8.2.2. Supplementary Table 2. Spastic paraplegia associated genes and loci. (Material available online : https://onlinelibrary.wiley.com/doi/full/10.1111/cge.13955)

8.2.3. Supplementary Table 3. Detailed ACMG criteria.

Mutation	**ACMG criteria**						
	PS3 †	**PM1** ‡	**PM2**§	**PM4** ¶	**PP2** *	**PP3** **	**PP5** ***
p.(Val205Glu)	Strong	Moderate	Moderate	NA	Supporting	Supporting	Moderate
p.(Ser77_Leu82del)	NA	Moderate	Moderate	Moderate	NA	Supporting	NA

Abbreviations: ACMG, American College of Medical Genetics; NA, not applicable.

† Well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product.

‡ Located in a mutational hot spot and/or critical and well-established functional domain (e.g., active site of an enzyme) without benign variation.

§ Absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium.

¶ Protein length changes as a result of in-frame deletions/insertions in a non-repeat region or stop-loss variants.

* Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease.

** Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.)

*** Reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent evaluation.

8.2.4. Supplementary Table 4. Gene Ontology enrichment analysis using g:Profiler (Benjamini-Hochberg adjusted p-value set at <0.05) (Material available online: https://onlinelibrary.wiley.com/doi/full/10.1111/cge.13955)

Supplementary Table 5 Reported mutations in *GCH1.*

Reference	Phenotype	Variant	Location (amino acid 73-248)†
[1]	DRD	Arg249Gly fs	No
[2]	DRD	Arg241Gln	Yes
[3]	DRD	Glu236X	Yes
[2]	PD, DRD	Met230Ile	Yes
[4]	PD,DRD	Lys224Arg	Yes
[4]	PD, DRD	Met221Thr	Yes
[2]	PD	Gly217Val	Yes
[5]	DRD,HSP	Met211Val fsX38	Yes
[6-8]	DRD, HSP	Val205Glu	Yes
[2]	PD, DRD	Val204Ile	Yes
[1]	PD,DRD	Gly203Glu	Yes
[9]	PD	Pro199Ser	Yes
[1]	PD	Arg198Gln	Yes
[1]	PD	Ile193Met	Yes
[4]	PD, DRD	Arg184Cys	Yes
[10]	PD	Arg184His	Yes
[1]	PD,DRD	His153Pro	Yes
[1]	PD	Met137Val	Yes
[2]	PD	Asp134Gly	Yes
[1]	PD	Phe122fs	Yes
[2]	PD	Ala120Ser	Yes
[11]	DRD	Leu117Arg	Yes
[2]	PD, DRD	Gln110X	Yes
[2]	PD	Gln110Glu	Yes
[2]	DRD	Phe104Leu	Yes
[1]	PD	Met102Leu	Yes
[12]	DRD	Arg88Leu	Yes
[1]	PD	Gln87Serfs	Yes
[1]	PD	Pro86Ser	Yes
[1]	PD,DRD	Ser80Asn	Yes
[1]	PD	Ser77Cys	Yes
[1]	PD	Asn70Lys	No
[1]	PD,DRD,HSP	Glu65X	No
[1]	PD	Arg57Gln	No
[1]	PD	Pro39Leu	No
[1]	PD	Glu11Ala	No
[13]	DRD	Glu2Gly	No
[2]	PD	c.626 + 1G>C	Yes
[14]	HSP	c.454-2A>G	Yes
[2]	DRD	c.343 + 5G>C	Yes
Current paper	HSP	c.229_246del:p.77_82del	Yes
[15]	PD	Deletion of exon 5 and 6	Yes

16	DRD	Deletion of exon 1	Yes
17	PD	Complete deletion of GCH1	Yes

Abbreviations: HSP, Hereditary spastic paraplegia; DRD, Dopa-responsive dystonia; PD, Parkinson's disease.
† Loss of function mutations were considered to be affecting this domain. (NM_000161.3)

Supplementary Table 5 References

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