

Genome Sequencing Uncovers Phenocopies in Primary Progressive Multiple Sclerosis

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Objective: Primary progressive multiple sclerosis (PPMS) causes accumulation of neurological disability from disease onset without clinical attacks typical of relapsing multiple sclerosis (RMS). However, whether genetic variation influences the disease course remains unclear. We aimed to determine whether mutations causative of neurological disorders that share features with multiple sclerosis (MS) contribute to risk for developing PPMS.

Methods: We examined whole-genome sequencing (WGS) data from 38 PPMS and 81 healthy subjects of European ancestry. We selected pathogenic variants exclusively found in PPMS patients that cause monogenic neurological disorders and performed two rounds of replication genotyping in 746 PPMS, 3,049 RMS, and 1,000 healthy subjects. To refine our findings, we examined the burden of rare, potentially pathogenic mutations in 41 genes that cause hereditary spastic paraplegias (HSPs) in PPMS (n = 314), secondary progressive multiple sclerosis (SPMS; n = 587), RMS (n = 2,248), and healthy subjects (n = 987) genotyped using the MS replication chip.

Results: WGS and replication studies identified three pathogenic variants in PPMS patients that cause neurological disorders sharing features with MS: *KIF5A* p.Ala361Val in spastic paraplegia 10; *MLC1* p.Pro92Ser in megalencephalic leukodystrophy with subcortical cysts, and *REEP1* c.606 + 43G>T in Spastic Paraplegia 31. Moreover, we detected a significant enrichment of HSP-related mutations in PPMS patients compared to controls (risk ratio [RR] = 1.95; 95% confidence interval [CI], 1.27–2.98; *p* = 0.002), as well as in SPMS patients compared to controls (RR = 1.57; 95% CI, 1.18–2.10; *p* = 0.002). Importantly, this enrichment was not detected in RMS.

Interpretation: This study provides evidence to support the hypothesis that rare Mendelian genetic variants contribute to the risk for developing progressive forms of MS.

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Primary progressive multiple sclerosis (PPMS) is a rare form of multiple sclerosis (MS) characterized by progressive accumulation of disability from disease onset

without the attacks typically observed in the relapsing form of the disease (RMS).¹ PPMS represents an unmet need in the care of neurological patients because of its

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Additional supporting information can be found in the online version of this article.

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TABLE 1. Demographic Data in Study Cohorts Used to Identify MS Phenocopy Mutations

	Discovery Phase		Phase 1 Replication			Phase 2 Replication			
Platform	Complete Genomics Inc.		Illumina OpenArray			Targeted Genotyping			
Variants	15.2 million		15			4			
Cohort source	UCSF	1KG ^a (64), CGI ^b (11), UCSF (6)	Germany	UCSF		Italy		UCSF	
Phenotype	PPMS ^c	Controls	PPMS	PPMS	RMS	PPMS	RMS	RMS	Controls
Sample size	38	81	142	269	460	335	340	2,249	1,000
Age at onset									
Mean ± SD	42.9 ± 9.9	NA	—	40.1 ± 11.1	31.2 ± 9.2	40.7 ± 9.5	29.5 ± 9.4	31.9 ± 9.5	NA
Median (range)	47 (25–54)	NA	—	40 (5–66)	31 (4–61)	40 (18–66)	28 (10–64)	31 (5–69)	NA
Disease duration									
Mean ± SD	11.3 ± 8.1	NA	—	19.2 ± 11.3	22.6 ± 9.9	11.1 ± 7.3	9.0 ± 5.3	13.8 ± 10.6	NA
Median (range)	10 (0–37)	NA	—	18 (1–56)	20 (10–62)	10 (1–45)	8 (1–39)	12 (0–66)	NA
Female sex (%)	22 (58)	39 (48)	93 (65)	155 (58)	475 (60)	171 (50.6)	179 (52.6)	1,886 (84)	516 (52)

^a1000 Genomes Project, Complete Genomics Data.
^bComplete Genomics Inc. Public Genomes.
^cPatients meet 2010 International Panel Criteria for PPMS.
MS = multiple sclerosis; SD = standard deviation; UCSF = University of California San Francisco; PPMS = primary progressive multiple sclerosis; NA = not applicable; HSP = hereditary spastic paraplegia; PPMS = primary progressive multiple sclerosis; RMS = relapsing multiple sclerosis.

poor response to MS disease-modifying therapies and its relentless clinical course that resembles neurodegenerative disorders.² Compared to RMS, PPMS patients are older at onset, men and women are equally affected, and the most common clinical presentation is a progressive spastic paraparesis.^{3,4} Moreover, some family studies demonstrate a higher concordance in MS disease course (PPMS versus RMS) within affected siblings than expected by chance.^{5–7} These observations suggest that unique genetic and environmental susceptibility factors may, in part, influence risk for PPMS.

To date, genetic studies have not shown a difference between PPMS and RMS susceptibility,⁸ either because of a strong shared genetic susceptibility or because of a lack of power given the lower prevalence of primary progressive disease and consequent under-representation in genome-wide association studies (GWAS) and other screens. Furthermore, the role of Mendelian genes has not been systematically studied in MS, despite the observation that there is widespread comorbidity among

Mendelian and complex diseases.⁹ PPMS shares clinical features with specific Mendelian neurological disorders (ie, potential MS phenocopies) that cause progressive neurological disability attributed to injury to the central nervous system (CNS). Examples of genetic disorders that resemble PPMS include hereditary spastic paraplegias (HSPs), inherited leukodystrophies, and mitochondrial disorders.^{3,10,11}

This study aims to determine whether mutations causative of genetic disorders that share features with MS contribute to disability in PPMS. Furthermore, we hypothesized that genomes from subjects with PPMS are enriched for mutations in genes involved in monogenic disorders that share clinicopathological features of MS. To this end, we performed whole-genome sequencing (WGS) in a well-characterized PPMS cohort and validated identified variants in multiple independent PPMS cohorts. We next examined whether PPMS patients carried mutations in specific classes of MS phenocopy disorders. Specifically, we hypothesized that PPMS patients

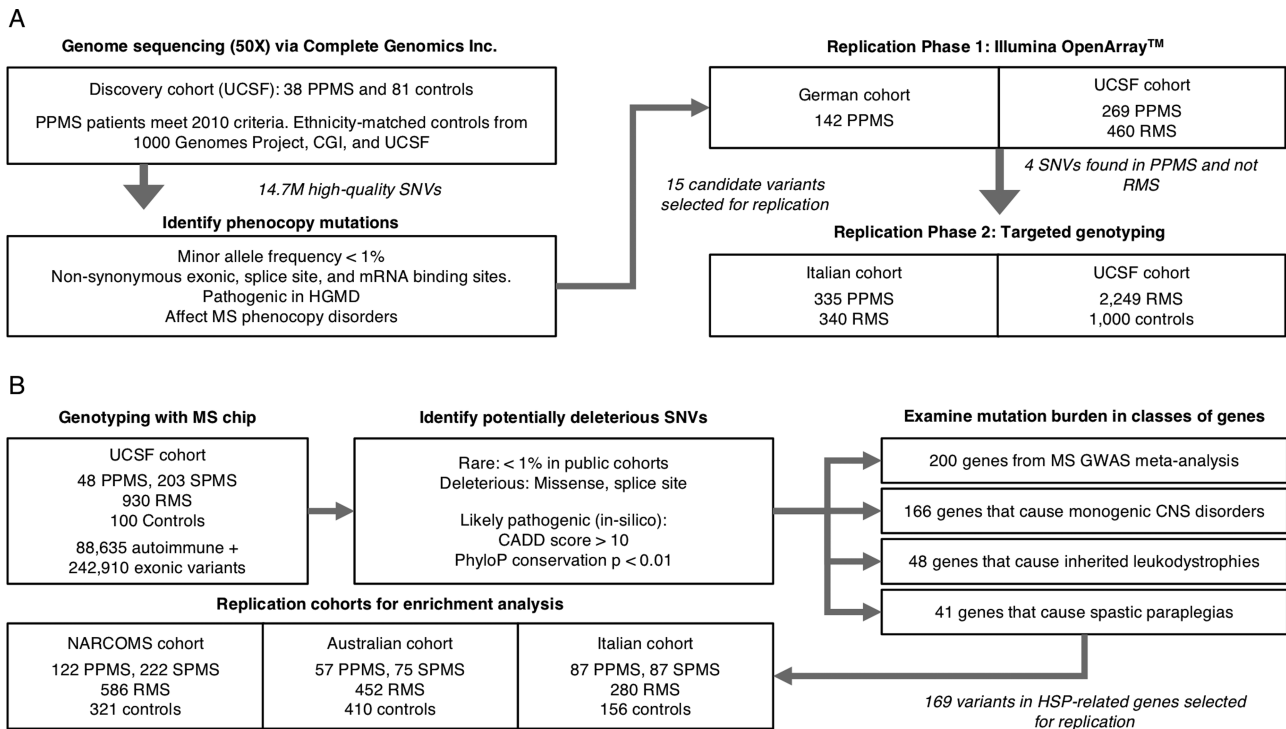


FIGURE 1: Summary of study cohorts, genotyping platforms, and variant selection. (A) Schematic of study design used for identifying MS phenocopy variants. The WGS discovery cohort included 38 PPMS patients (who met 2010 International Panel Criteria) and 81 ethnicity-matched controls sequenced using the Complete Genomics Inc. (CGI) platform. Fifteen candidate variants were selected for Phase 1 replication genotyping in 411 PPMS and 460 RMS patients using OpenArray. Four top candidate variants exclusively found in PPMS and not RMS patients were selected for Phase 2 replication in 335 PPMS and 340 RMS patients from an Italian cohort and in 2,249 RMS and 1,000 controls from UCSF. (B) Schematic of study design used for determining the burden of HSP-related mutations in PPMS. The discovery cohort comprised of 48 PPMS patients (who met 2010 International Panel Criteria) and 100 controls genotyped on the MS replication chip. Replication patients included an additional 266 PPMS, 1,702 RMS, and 887 control subjects from three additional cohorts (NARCOMS, Australian, and Italian) genotyped on the same platform. All subjects examined were of European ancestry. CADD = Combined Annotation Dependent Depletion; CNS = central nervous system; GWAS = genome-wide association studies; HGMD = Human Gene Mutation Database; HSP = hereditary spastic paraplegia; MS = multiple sclerosis; NARCOMS = North America Research Committee on Multiple Sclerosis; PhyloP = phylogenetic conservation p value; PPMS = primary progressive multiple sclerosis; RMS = relapsing multiple sclerosis; SNVs = single-nucleotide variants; SPMS = secondary progressive multiple sclerosis; UCSF = University of California San Francisco; WGS = whole-genome sequencing.

were enriched for mutations in genes that caused hereditary spastic paraplegias, a rare group of conditions that cause progressive leg weakness and spasticity resembling PPMS.¹² Last, we performed similar analyses in RMS and secondary progressive multiple sclerosis (SPMS) patients to determine whether MS phenocopy mutations identified in this study were unique to PPMS.

Patients and Methods

Cohorts

All human studies were approved by each respective institutional ethics review committee, and all participants provided written informed consent. To investigate the role of MS phenocopy mutations in PPMS pathogenesis, we examined WGS data in a discovery cohort of 38 PPMS patients of European descent and 81 ethnicity-matched controls. PPMS patients in this group were recruited at or referred to the University of California San Francisco (UCSF) between 1996 and 2013 and

satisfied 2010 International Panel Criteria¹³ (Table S1). WGS data for controls were obtained from the 1000 Genomes Project,¹⁴ Complete Genomics Inc. (CGI) Public Genomes,¹⁵ and healthy individuals recruited at UCSF. We subsequently performed replication genotyping in 142 PPMS patients from Germany and in 269 PPMS and 460 RMS patients recruited at UCSF (Phase 1 replication). We performed a second round of replication in 335 PPMS and 340 RMS patients from Italy and in 2,249 RMS patients and 1,000 healthy controls recruited at UCSF (Phase 2 replication). In total, the discovery and replication cohorts included 784 PPMS and 3,049 RMS patients and 1,081 controls (Table 1; Fig 1A).

To test the hypothesis that PPMS patients may be enriched for mutations in genes that cause spastic paraplegias, we examined a cohort of 48 PPMS patients of European descent recruited at UCSF and 100 ethnicity-matched controls who were genotyped using the MS replication chip.¹⁶ These PPMS patients met 2010 International Panel Criteria and included 25 patients from our WGS cohort and 23 patients

TABLE 2. Discovery and Replication Cohorts Used to Examine Burden of Spastic Paraplegia Mutations

	Discovery Cohort				Replication Cohort 1				Replication Cohort 2				Replication Cohort 3			
Platform	MS Replication Chip				MS Replication Chip				MS Replication Chip				MS Replication Chip			
Variants	331,536				169 ^a				169 ^a				169 ^a			
Cohort	UCSF				NARCOMS ^b				Australia				Italy			
Diagnosis ^d	CTL	RMS	SPMS	PPMS	CTL	RMS	SPMS	PPMS	CTL	RMS	SPMS	PPMS	CTL	RMS	SPMS	PPMS
Samples	100	930	203	48 ^c	321	586	222	122	410	452	75	57 ^c	156	280	87	87
Age at onset																
Mean ± SD	NA	32.8 ± 9.4	31.3 ± 9.3	42.3 ± 11.2	NA	32.6 ± 9.8	31.0 ± 9.2	40.7 ± 11.1	NA	36 ± 9.8	35 ± 9.4	42 ± 10.8	NA	28.2 ± 8.9	29.1 ± 9.4	39.9 ± 10.1
Median, range	NA	32 (5–64)	31 (11–58)	42 (22–66)	NA	33 (4–61)	30 (10–65)	41 (12–66)	NA	36 (14–64)	33 (15–59)	41 (20–72)	NA	27 (7–52)	27 (15–59)	40 (19–60)
Disease duration																
Mean ± SD	NA	12.6 ± 9.5	23.0 ± 10.0	11.2 ± 7.4	NA	21.9 ± 11.3	28 ± 10.9	22.6 ± 10.4	NA	8 ± 6.5	21 ± 10.2	13 ± 9.9	NA	8.1 ± 7.0	17.3 ± 7.9	10.8 ± 8.2
Median range	NA	12 (0–54)	22 (3–48)	10 (0–37)	NA	20 (1–66)	28 (0–58)	20 (2–56)	NA	6 (0–41)	22 (3–44)	10 (1–43)	NA	6 (0–37)	17 (3–41)	9 (1–45)
Female sex	22%	74%	68%	40%	47%	81%	81%	61%	77%	77%	76%	61%	38%	66%	70%	57%

^aOne hundred sixty-nine HSP-related variants were examined in these replication cohorts.

^bNorth American Research Committee on Multiple Sclerosis.

^cPatients met 2010 International Panel Criteria for PPMS.

^dCTL = control subjects; RMS = relapsing MS patients excluding those with known secondary progression; SPMS = secondary progressive MS; PPMS = primary progressive MS.

SD = standard deviation; UCSF = University of California San Francisco; NA = not applicable; HSP = hereditary spastic paraplegia.

who were exclusively genotyped on the MS chip (Table S1). For replication, we examined three additional cohorts of European ancestry genotyped on the same platform. These included the North America Research Committee on Multiple Sclerosis (NARCOMS; 122 PPMS and 321 controls), an Australian cohort (57 PPMS and 410 controls), and an Italian cohort (87 PPMS and 156 controls). Last, we examined RMS and SPMS patients from these cohorts to investigate whether MS phenocopy mutations are unique to patients with PPMS. In total, we performed enrichment analysis in 314 PPMS, 587 SPMS, 2,248 RMS, and 987 healthy subjects (Table 2; Fig 1B). A description of all cohorts and genotyping methods is provided in Tables 1 and 2 and Figure 1.

WGS and Replication Genotyping

For each sample selected for genome sequencing, we derived lymphoblastoid cell lines (LCLs) from whole blood samples¹⁷ and extracted 15 µg of DNA from each of these cell lines for sequencing using the CGI platform.¹⁵ The LCL is a convenient research tool for obtaining virtually unlimited amounts of biological material from an individual, and there is high

concordance for single-nucleotide variant (SNV) calls obtained from WGS using LCL and whole blood.¹⁸ CGI performed DNA read mapping to the human genome (reference hg19) and provided variant calls using CGI proprietary software.¹⁹ We performed additional quality control by removing low-quality calls (heterozygous calls with VarScoreVAF <40 and homozygous calls with VarScoreVAF <20) and variants with less than 95% call rate. We confirmed European ancestry using Identity-By-Descent analysis of WGS variants.²⁰ We annotated WGS variants with curated data from the Human Gene Mutation Database^{21,22} (HGMD) and selected SNVs that were functionally deleterious (nonsynonymous exonic, splice site, or mRNA binding site), rare (<1% in public cohorts), classified as pathogenic, affected monogenic neurological disorders, and were exclusively found in PPMS patients. We used an allele frequency cutoff of 1% to identify potentially pathogenic mutations, which, in Mendelian disorders, have allele frequencies below 0.1%.²³ Candidate MS phenocopy variants were then selected for replication genotyping using the Illumina OpenArray system (Illumina, San Diego, CA) in Phase 1 replication and using targeted individual genotyping in Phase 2 replication.

Mutation Enrichment Analysis

To determine whether PPMS patients are enriched for mutations in genes that cause spastic paraplegias, we examined cases and controls who were genotyped using the custom MS replication chip, which includes 88,635 autoimmune markers and 242,910 exonic variants from the Illumina HumanExome Bead-Chip v1.1. Quality control included the following SNV-level exclusion criteria: (1) missingness > 0.05 ; (2) Hardy-Weinberg equilibrium p value $< 10^{-6}$; and (3) differential missingness in cases and controls p value < 0.001 . We annotated variants using Ingenuity Variant Analysis²⁴ and extracted all rare (minor allele frequency [MAF] $< 1\%$ in public data sets), functionally deleterious (missense and splice site), and potentially pathogenic (Combined Annotation Dependent Depletion [CADD] score > 10 ; Phylogenetic conservation p value [PhyloP] < 0.01) variants within 41 genes that cause spastic paraplegias (Table S4).^{12,25–29} We performed logistic regression on the number of potentially pathogenic variants per individual adjusted for subject sex and used $p = 0.05$ as the threshold for significance. To understand the impact of variant selection on enrichment results, we performed sensitivity analysis using different in silico predicted pathogenicity scores including CADD, PhyloP, Sorting Intolerant From Tolerant (SIFT), and Polymorphism Genotyping v2 [PolyPhen-2]; Table S5). To evaluate the likelihood of finding an enrichment of spastic paraplegia variants in PPMS patients by chance, we randomly permuted PPMS and healthy control status in our discovery cohort 10,000 times and determined the permutation p value as the likelihood of observing an enrichment in HSP-related genes greater than or equal to that found in our discovery cohort.

To replicate our findings from mutation enrichment analysis, we examined the same spastic paraplegia variants in three additional PPMS cohorts genotyped on the MS replication chip. We performed meta-analysis across these four (discovery plus three replication) cohorts using a random-effects model examining the mean number of mutations per individual in PPMS compared to controls. To determine whether the enrichment of HSP-related mutations is unique to PPMS, we also examined RMS and SPMS patients genotyped on the MS replication chip from these four cohorts. We calculated the average number of spastic paraplegia variants per individual in each phenotype and used a t test to determine whether the average burden of HSP-related variants differed between MS disease-course subtypes.

To investigate the relationship between spastic paraplegia variants and the burden of common MS susceptibility variants, we calculated the MS Genetic Burden (MSGB) using MS replication chip genotypes. The MSGB is obtained by summing the number of independently associated MS risk alleles weighted by their beta coefficients, obtained from a large GWAS meta-analysis, at 177 (of 200) non-MHC (major histocompatibility complex) loci and 18 (of 32) MHC variants, which includes the HLA-DRB1*15:01-tagging single-nucleotide polymorphism (SNP) rs3135388.¹⁶ Subsequently, we examined whether the average MSGB differed between PPMS ($n = 170$), SPMS ($n = 425$), RMS ($n = 1516$), and healthy subjects ($n = 421$) in the UCSF and NARCOMS cohorts using pair-wise t tests

between groups. Finally, we examined whether the mean MSGB differed in PPMS and SPMS patients who carried a HSP-related variant compared to those who did not carry any such variants.

Results

Genome sequencing in a well-characterized cohort of 38 PPMS patients and 81 ethnicity-matched controls using CGI yielded on average greater than $50 \times$ depth of coverage and identified more than 3 million SNVs, 5,000 insertion deletions, 1,500 structural variants, and 250 copy number variants (CNVs) per sample. After performing quality control, we found 14,709,637 high-quality SNV calls in the autosomal and sex chromosomes across these 119 genomes that comprised our discovery cohort (Fig 1).

We searched for candidate MS phenocopy variants, by annotating the 14.7 million SNVs identified in the 119 genomes, and found 1,287 pathogenic variants directly involved in 714 Mendelian disorders. Of these, 691 variants involved in 474 disorders were identified in PPMS patients, and 1,029 variants involved in 609 disorders were identified in controls. This included 52 variants on the sex chromosomes; however, none of these affected neurological disorders that share features with MS. Fifteen of the 691 variants found in PPMS patients were rare, functionally deleterious, affected neurological disorders and were absent in controls. We attempted an independent replication to validate the WGS calls in the discovery cohort by genotyping these 15 variants in an additional 411 PPMS and 460 RMS patients using the Illumina OpenArray platform (Phase 1 replication). Twelve of the 15 candidate variants were confirmed, and four were exclusively found in PPMS (and not in RMS) patients in the combined discovery and initial replication cohort. To further assess whether these four pathogenic variants were PPMS specific, we selectively genotyped them in an additional set of PPMS ($n = 335$) and RMS ($n = 2,589$) patients, in addition to healthy subjects ($n = 1,000$; Phase 2 replication). The variants selected for replication genotyping are summarized in Table 3.

The four variants selected for Phase 2 replication were observed at a higher frequency in PPMS patients ($n = 784$) compared to over 36,000 European individuals from the Exome Aggregation Consortium (ExAC-EUR).³⁰ Of note, three of these variants had been previously reported in disorders that potentially mimic MS: *KIF5A* p.Ala361Val (risk ratio [RR] = 23), a dominant variant for spastic paraplegia 10 (SPG10 [MIM: 604187])²⁷; *MLC1* p.Pro92Ser (RR = 1.8), a recessive variant for megalencephalic leukodystrophy with subcortical cysts (MLC [MIM: 604004])³¹; and *REEP1* c.606 + 43G>T (RR = 1.6), a dominant variant affecting mRNA binding at the 3' untranslated region (UTR) of

TABLE 3. MS Phenocopy Variants Selected for Phase 1 and Phase 2 Replication

Genetic Variant			Number of Individuals Carrying a Heterozygous Mutation							Minor Allele Frequency (%) ^a			Risk vs ExAC EUR	
			Discovery Cohort (WGS)		Phase 1 Replication (OpenArray)		Phase 2 Replication (targeted typing)			All	All	All	ExAC EUR	RR for PPMS
Gene	Variant ^b	Disorder (inheritance) ^c	38 PPMS	81 CTRLS	411 PPMS	460 RMS	335 PPMS	2589 RMS	1000 CTRLS	PPMS	RMS	CTRLS	EUR	PPMS
<i>KIF5A</i>	p.A361V	SPG10 (AD)	1	0	0	0	0	1	0	0.064	0.016	0.000	0.003	23.3
<i>TSC2</i>	p.E75K	TS ^e (AD)	1	0	0	0	0	0	0	0.064	0.000	0.000	0.019	3.3
<i>MLC1</i>	p.P92S	MLC (AR)	1	0	0	0	0	4	0	0.064	0.066	0.000	0.034	1.9
<i>REEP1</i>	c.606 +43G>T	SPG31 (AD)	1	0	2	0	0	2	2	0.191	0.033	0.093	0.115	1.7
<i>SCN9A</i>	p.W1538R	PE (AD)	1	0	6	3	Not selected for Phase 2:			0.67	0.33	0.00	0.173	3.9
<i>HPD</i>	p.I335M	TYRSN3 (AR)	1	0	3	3	Variant was found in at least one RMS patient in Phase 1.			0.33	0.33	0.00	0.158	2.1
<i>CACNA1A</i>	p.P897R	EA2 (AD)	1	0	1	2				0.22	0.22	0.00	0.141	1.6
<i>DCTN1</i>	p.T1249I	ALS (AD)	1	0	3	4				0.22	0.43	0.00	0.416	0.5
<i>D2HGDH</i>	p.A426T	D-2-HGA (AR)	3	0	7	3				0.89	0.33	0.00	1.101	0.8
<i>ADAR</i>	p.P193A	AGS (AR, AD)	1	0	1	1				0.22	0.11	0.00	0.281	0.8
<i>NOTCH3</i>	p.S497L	CADASIL (AD)	2	0	7	4				0.67	0.43	0.00	1.395	0.5
<i>SLC6A5</i>	p.T690T	HKPX3 ^e (AD)	1	0	0	4				0.11	0.43	0.00	0.271	0.4
<i>NF1</i>	p.D176E	NF1 (AD)	1 ^c	0	2	5	Not selected for Phase 2:			0.24	0.54	0.00	0.512	NA
<i>TSC2</i>	p.L1423L	TS ^e AD	1 ^c	0	0	0	CGI genotypes not validated on OA			0.00	0.00	0.00	0.423	
<i>LRRK2</i>	p.E334K	PD (AD)	1 ^c	0	0	0				0.00	0.00	0.00	0.484	

^aMinor allele frequencies (MAFs) are calculated for 784 PPMS, 3,049 RMS, and 1,081 control subjects for the top four candidate variants. For all other variants, MAF is calculated for 449 PPMS, 460 RMS, and 81 control subjects. The ExAC European (including Finnish) cohort was used as the reference to calculate relative risk for candidate variants.

^b“c” denotes coding DNA sequence position; “p” denotes protein amino acid position. Only the variant corresponding to the primary transcript according to ExAC is provided.

^cPhenocopy disorder abbreviations: SPG = spastic paraplegia; TS = tuberous sclerosis; MLC = megalencephalic leukodystrophy with subcortical cysts; PE = primary erythromelalgia; TYRSN3 = tyrosinemia type 3; EA2 = episodic ataxia 2; ALS = amyotrophic lateral sclerosis; D-2-HGA = D-2-hydroxyglutaric aciduria; AGS = Aicardi-Goutières syndrome; CADASIL = cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy; HKPX3 = hyperekplexia 3; NF1 = neurofibromatosis type 1; PD = Parkinson's disease.

^dThese variants were not validated during Phase 1 replication genotyping.

^eThese variants have not yet been reported to be pathogenic in this disorder.

REEP1 causing spastic paraplegia 31 (SPG31 [MIM: 610250]).²⁵ The last variant, *TSC2* p.Glu75Lys (*RR* = 3.3), is a variant of unknown significance for tuberous sclerosis (*TSC2* [MIM: 613254]).

REEP1 c.606 + 43G>T was found in a PPMS patient (65-0008) with spastic paraparesis from our WGS discovery cohort and in 2 additional PPMS patients (52-0139 and 52-1859) with a progressive spinal cord syndrome in our Phase 1 replication genotyping. This variant was also found in 2 RMS patients (21-

0003, MSGENE02-528) and 2 controls (9961-50050, 9961-51000901) in our Phase 2 replication genotyping at a frequency comparable to the ExAC European cohort. *KIF5A* c.C1082T p.Ala361Val was found in a PPMS patient (02-0069) with spastic paraparesis from our discovery cohort and was also found in 1 RMS patient (MSGENE02-539) in our Phase 2 replication genotyping at a lower frequency compared to that observed in PPMS patients. *MLC1* c.274C>T p.Pro92Ser was found in a PPMS patient (04-1225) with brain-predominant disease

TABLE 4. PPMS Patients Who Carry a Reported Phenocopy Variant

ID	Sex	Age at Onset	Progressive course	2010 International Panel Criteria ^a					Genetic Variant				
				Positive CSF (elevated IgG index, OCBs)	Brain MRI	Spine MRI	Gad +	Meets criteria	Gene	Disorder (inheritance)	Reported Variant ^b	Type ^c	Platform ^d
020069	F	37	+	ND	+	+	-	+	<i>KIF5A</i>	SPG10 (AD)	p.A361V	mis	CGI
041225	F	51	+	+(+,+)	+	-	-	+	<i>MLC1</i>	MLC (AR)	p.P92S	mis	CGI
650008	F	50	+	+(+,+)	+	+	-	+	<i>REEP1</i>	SPG31 (AD)	c.606 + 43G>T	3'	CGI
650084	F	48	+	+(-,+)	+	+	-	+	<i>SPG7</i>	SPG7 (AR,AD)	c.1552 + 1G>T	ss	MS chip
700019	F	61	+	+(+,+)	+	+	+	+	<i>SPG7</i>	SPG7 (AR,AD)	p.A510V	mis	MS chip
520139 ^e	F	26	+	+	-	+	-	+	<i>REEP1</i>	SPG31 (AD)	c.606 + 43G>T	3'	OA
521859 ^e	M	57	+	ND	+	ND	-	-	<i>REEP1</i>	SPG31 (AD)	c.606 + 43G>T	3'	OA

^a2010 International Panel Criteria includes (1) progression since onset without relapses and (2) two of the following three criteria: positive CSF (elevated IgG index or oligoclonal bands), brain lesions consistent with MS, and spinal cord lesions consistent with MS. “+” denotes satisfies criteria, “-” denotes does not satisfy criteria, “ND” denotes test was not done. GAD denotes gadolinium enhancement on at least one magnetic resonance imaging brain or spinal cord.

^b“c” denotes coding DNA sequence position; “p” denotes protein amino acid position. Only the variant corresponding to the primary transcript according to ExAC is provided.

^cVariant types: mis = missense; ss = splice site; 3' = 3'UTR.

^dGenotyping platforms: CGI = WGS via Complete Genomics Inc.; MS chip = MS replication chip; OA = OpenArray.

^ePPMS patients from Phase 1 replication cohort. CSF for patient 520139, performed at the NIH in 1977, was reported to be consistent with MS; however, results were not available.

from our discovery cohort. Of note, the *MLC1* variant was also found in 4 RMS patients (52-1463, 05-0032, 60-0354, and 60-0362) with the same frequency as that observed in PPMS patients, and in 0 controls, in our Phase 2 replication genotyping. Last, *TSC2* c.G223A p.Glu75Lys was found in a PPMS patient (60-0385) with a mild disease course from our discovery cohort. These results are summarized in Table 3, and detailed clinical information is provided in Table 4 and Tables S2 and S3.

Given that two of the top four genes identified by WGS (*REEP1* and *KIF5A*) are associated with progressive spinal cord injury, we hypothesized that PPMS patients may be generally enriched for deleterious mutations in genes that associate with the hereditary spastic paraplegias. Spastic paraplegias are a rare group of conditions that cause degeneration of motor axons in the corticospinal tract resulting in progressive leg weakness and spasticity,¹² providing a plausible basis for phenotypic mimicry

(ie, phenocopy) with MS. To test this hypothesis, we examined 48 PPMS patients of European ancestry who satisfied 2010 International Panel Criteria and 100 matched controls genotyped using the custom MS (ie, replication) chip, which includes more than 240,000 exonic variants. We extracted 169 rare, functionally deleterious, and potentially pathogenic variants within the 41 genes known to cause spastic paraplegias^{12,25–29} (Table S4). Interestingly, PPMS patients harbored, on average, significantly more variants (0.29 per individual) in these genes than did controls (0.11), and the risk for PPMS increased with the number of potentially pathogenic variants ($RR = 2.65$; 95% confidence interval [CI], 1.18–5.96; likelihood ratio [LR], $p = 0.028$).

To evaluate the likelihood of finding an enrichment of spastic paraplegia variants in PPMS patients by chance, we randomly permuted PPMS and healthy control status in our discovery cohort 10,000 times and

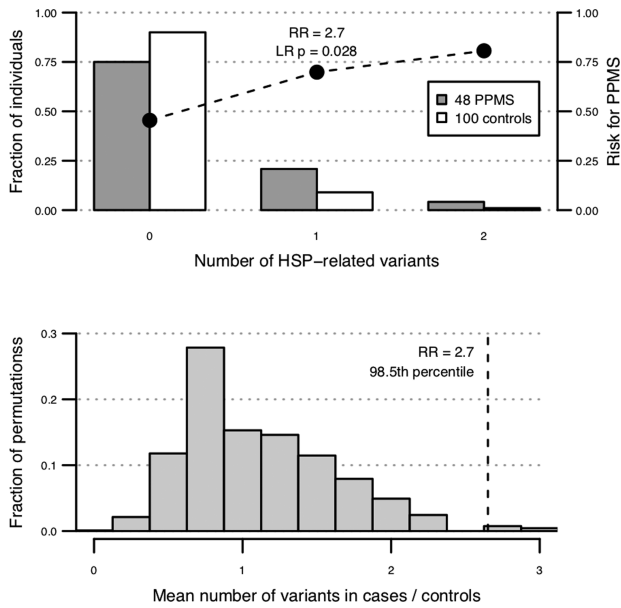


FIGURE 2: Burden of spastic paraplegia mutations in PPMS. (A) Forty-eight PPMS patients genotyped on the MS replication chip are enriched for rare (MAF < 1% in public data sets), functionally deleterious (missense and splice site), and potentially pathogenic (CADD score, > 10; PhyloP conservation, $p < 0.01$) variants in 41 genes known to cause spastic paraplegias, and the relative risk for PPMS increases with the number of HSP-related variants carried by an individual (cases mean = 0.29; controls mean = 0.11; $RR = 2.7$; logistic regression, $p = 0.028$). (B) Random permutation of PPMS case and control status shows that the 2.7-fold enrichment of pathogenic variants in 41 HSP-related genes is greater than in 98.5% of 10,000 permutations ($p = 0.015$). HSP = hereditary spastic paraplegia; LR = likelihood ratio; MAF = minor allele frequency; MS = multiple sclerosis; PhyloP = phylogenetic conservation p value; PPMS = primary progressive multiple sclerosis; RR = risk ratio.

calculated the enrichment of HSP-related variants determined using the same criteria. Strikingly, the 2.7-fold enrichment of spastic paraplegia variants in PPMS was greater than in 98.5% of case-control permutations ($p = 0.015$), suggesting that the observed enrichment was unlikely attributed to chance (Fig 2). Sensitivity analysis demonstrated a persistent enrichment of HSP variants in PPMS after applying various in silico predicted pathogenicity criteria (Table S5). No significant enrichment was detected in 48 genes affecting inherited leukodystrophies,¹¹ 166 genes affecting other Mendelian disorders that involve the CNS,³² or in the 200 genes associated with MS in a recent GWAS meta-analysis.¹⁶ Whereas we observed a trend toward enrichment of variants in 48 genes affecting inherited leukodystrophies, this was not significant ($RR = 1.9$; 95% CI, 0.9–4.2; LR, $p = 0.091$). A summary of discovery cohort PPMS patients who carry a reported MS phenocopy variant is shown in Table 4.

To replicate our finding that PPMS patients are enriched for mutations in genes that cause spastic paraplegias, we examined three additional cohorts of European ancestry genotyped on the MS replication chip. Using the same variant selection criteria as described previously, we detected an enrichment of HSP-related mutations in 122 PPMS patients (0.3 variants per individual) recruited through NARCOMS compared to 321 controls (0.12; $RR = 2.56$; 95% CI, 1.64–4.01; LR, $p = 6.0 \times 10^{-4}$), as well as in an Australian cohort of 57 PPMS patients (0.26) compared to 410 controls (0.14; $RR = 1.83$; 95% CI, 1.00–3.35; LR, $p = 0.049$). No enrichment was observed in an Italian cohort of 87 PPMS patients (0.08) compared to 156 controls (0.1; $RR = 0.84$; 95% CI, 0.35–2.02; $p = 0.8$). Meta-analysis of the discovery and three replication cohorts confirmed a significant enrichment of potentially pathogenic HSP mutations in 315 PPMS patients compared to 987 controls ($RR = 1.95$; 95% CI, 1.27–2.98; random-effects model, $p = 0.002$; Fig 3).

To determine whether the enrichment of variants observed in spastic paraplegia genes was unique to PPMS patients or whether this also contributes to the risk for SPMS, we examined genotypes from SPMS ($n = 587$) and RMS ($n = 2,248$) patients of European ancestry from the four cohorts in our meta-analysis. On average, PPMS ($n = 315$) patients harbored a significantly higher number of spastic paraplegia variants (0.23 per individual) compared to RMS patients (0.14; $n = 2,248$; mean difference [MD] = 0.10; 95% CI, 0.06–0.14; t test,

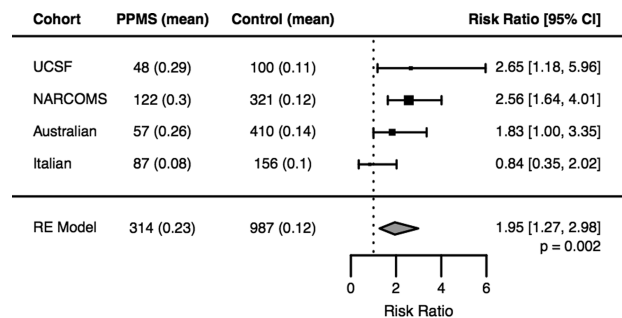


FIGURE 3: Meta-analysis of HSP-related mutations across multiple cohorts. Examination of 314 PPMS patients and 987 controls genotyped on the MS Replication Chip across four cohorts (UCSF, NARCOMS, Australian, and Italian) confirmed the observation that PPMS patients harbor significantly more potentially pathogenic HSP mutations compared to controls ($RR = 1.95$; random effects model, $p = 0.002$). HSP = hereditary spastic paraplegia; MS = multiple sclerosis; NARCOMS = North America Research Committee on Multiple Sclerosis; PPMS = primary progressive multiple sclerosis; RR = risk ratio; UCSF = University of California San Francisco; RE = random effects.

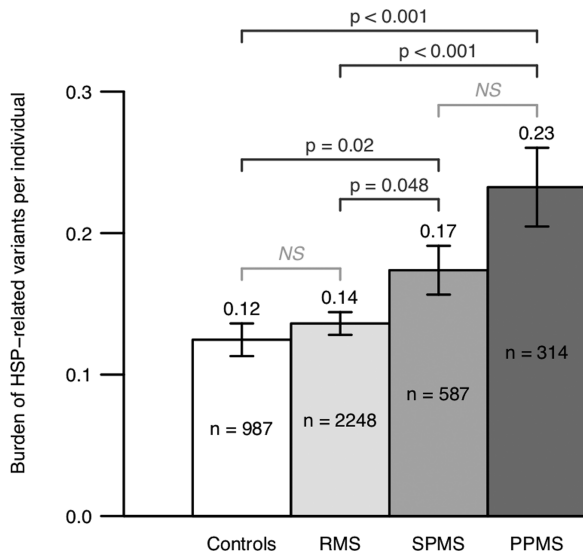


FIGURE 4: HSP-related mutation burden in PPMS, SPMS, RMS, and controls. Examination of 314 PPMS, 2,248 RMS, 587 SPMS, and 987 control subjects from four cohorts (UCSF, NARCOMS, Australian, and Italian) showed that PPMS patients (0.23 variants per individual) on average harbored a significantly higher number of potentially pathogenic HSP-related mutations compared to RMS (0.14) and controls (0.12; t test, $p = 3.8 \times 10^{-4}$ for PPMS vs controls; $p = 9.6 \times 10^{-4}$ for PPMS vs RMS). Moreover, SPMS patients (0.17), on average, also harbored a higher number of HSP-related mutations compared to RMS (0.14) and controls (0.12; $p = 0.018$ for SPMS vs controls, $p = 0.048$ for SPMS vs RMS). Importantly, no significant enrichment was detected in RMS patients compared to healthy controls ($p = 0.4$). HSP = hereditary spastic paraplegia; NS = not significant; PPMS = primary progressive multiple sclerosis; RMS = relapsing multiple sclerosis; SPMS = secondary progressive multiple sclerosis.

$p = 9.6 \times 10^{-4}$) and compared to controls (0.12; $n = 987$; MD = 0.11; 95% CI, 0.05–0.17; $p = 3.8 \times 10^{-4}$). Interestingly, SPMS patients ($n = 587$) also harbored a higher number of spastic paraplegia variants (0.17 per individual) compared to RMS patients (0.14; $n = 2,248$; MD = 0.04; 95% CI, 0.00–0.07; $p = 0.048$) and compared to controls (0.12; $n = 987$; MD = 0.05; 95% CI, 0.01–0.09; $p = 0.018$). By contrast, no significant enrichment was found in RMS patients compared to controls (MD = 0.01; 95% CI, –0.02 to 0.04; $p = 0.4$; Fig 4). Whereas we observed a trend toward enrichment of HSP-related variants in PPMS patients (0.23) compared to SPMS patients (0.17), this was not significant (MD = 0.06; 95% CI, –0.01 to 0.12; $p = 0.07$). These results suggest that the enrichment of spastic paraplegia variants is unique to patients with a progressive disease course and is not present in all forms of MS.

We subsequently hypothesized that the risk for developing a progressive form of MS is related to the accumulation of rare deleterious variants that directly

affect degenerative neurological disorders, and this risk is independent of the genetic burden that confers susceptibility for MS. To test this hypothesis, we calculated the MS Genetic Burden (MSGB) in PPMS ($n = 170$), SPMS ($n = 425$), RMS ($n = 1516$), and healthy subjects ($n = 421$) from our UCSF and NARCOMS MS replication chip cohorts. We found that, on average, MS patients ($n = 2,111$) have a higher MSGB (mean = 22.7) compared to healthy controls (21.7; (MD = 1.0; 95% CI, 0.86–1.13; $p = 2.2 \times 10^{-42}$). We did not detect a significant difference in MSGB between PPMS (22.9), SPMS (22.8), and RMS (22.7) patients (PPMS versus RMS, MD = 0.15; 95% CI, –0.06 to 0.37; $p = 0.17$; SPMS versus RMS, MD = 0.07; 95% CI, –0.07 to 0.21; $p = 0.34$; PPMS versus SPMS, MD = 0.8; 95% CI, –0.16 to 0.33; $p = 0.49$). Importantly, we observed no significant difference between PPMS patients who carry a HSP variant (22.9; $n = 44$) and those who do not (22.9; $n = 126$; MD = 0.02; 95% CI, –0.55 to 0.48; $p = 0.95$). Likewise, we detected no difference between SPMS patients who carry a HSP variant (23.0; $n = 62$) and those who do not (22.7; $n = 363$; MD = 0.22; 95% CI, –0.14 to 0.58; $p = 0.23$). These results suggest that rare HSP-related variants modulate the risk for developing a progressive disease course independent of the overall genetic burden that confers risk for developing MS.

Discussion

Although many disorders resemble MS clinically and radiographically, we are unaware of previous reports associating Mendelian disorder genes in PPMS. Systematic review of clinical records indicates that patients who carried a MS phenocopy-related mutation were not misdiagnosed with PPMS, but rather carry clinical characteristics of both PPMS and the phenocopy disorder. Specifically, the PPMS patients with pathogenic mutations in *REEP1* and *KIF5A* have demyelinating-appearing lesions on magnetic resonance imaging. Cerebrospinal fluid (CSF) was obtained in 2 of 3 *REEP1* carriers and both had elevated intrathecal gammaglobulin synthesis. CSF was not obtained in the *KIF5A* pA361V carrier. Carriers of these phenocopy mutations experienced progressive spastic paraparesis typical of both PPMS and HSP. Additional studies are needed to better understand the impact of these MS phenocopy mutations on disease severity in PPMS.

Furthermore, to our knowledge, this is the first study to report that PPMS patients are enriched for mutations in genes that cause spastic paraplegias. We show that the enrichment of HSP-related variants in PPMS is significantly higher than expected by chance, is validated in meta-analysis across multiple cohorts, and is not present in RMS patients. Importantly, SPMS patients

also harbor a detectable enrichment of spastic paraplegia mutations, suggesting that there might be a shared genetic susceptibility to progressive forms of MS, and that carrying such variants might increase the risk for developing secondary progression after an earlier relapsing-onset course. Last, we observe no significant difference in the burden of common MS susceptibility variants in PPMS and SPMS patients who carry a spastic paraplegia variant compared to those who do not. These findings suggest that rare mutations in genes that cause degenerative neurological disorders contribute to a progressive disease course, and this effect is independent of the burden of common MS susceptibility variants that influences the risk for developing MS.

We acknowledge a number of limitations. First, although we comprehensively examined pathogenic SNVs, which are better documented in literature, we did not analyze CNVs, structural variants, or intergenic regulatory mutations that might also be pathogenic. Second, the size of the discovery WGS cohort was limited by the high cost of genome sequencing. After performing WGS in the pilot PPMS cohort, we subsequently devoted resources to independent replication rather than additional sequencing. Third, we did not identify pathogenic SNVs on the sex chromosomes, and thus our results do not explain the difference in gender distribution between PPMS (equally affects males and females) and RMS (female predominant). Fourth, although we used an expert-curated and experimentally validated list of spastic paraplegia genes, a more comprehensive gene list will emerge as additional HSP-related loci are discovered. Therefore, our analysis might underestimate the prevalence of such mutations in progressive forms of MS. Fifth, the enrichment of spastic paraplegia mutations in PPMS patients was not detected in the Italian cohort. The genetic variation of Italian Europeans can be distinguished from that of other European populations,⁸ and known common pathogenic spastic paraplegia mutations in this population^{33–35} were not captured on the MS replication chip. Last, whereas clinical genetic testing may be useful in refining a diagnosis when it is in question, the utility of genetic testing in PPMS patients is currently limited given the lack of disease-modifying treatments for the Mendelian genetic variants that might contribute to a progressive disease course.

Proposed mechanisms for PPMS pathogenesis include compartmentalized leptomeningeal inflammation behind a relatively intact blood–brain barrier, oxidative stress driving mitochondrial injury, chronic microglial activation causing oligodendrocyte dysfunction and axonal injury, and age-related iron accumulation.^{36–40} By examining MS phenocopy mutations, this study identified genes encoding

neuroaxonal proteins (*KIF5A*), mitochondrial function (*REEP1*, *SPG7*), and astrocyte osmoregulation (*MLC1*), supporting the hypothesis that genetic variation contributes to progressive neuronal and glial dysfunction in PPMS. However, our approach does not comprehensively assess all proposed mechanisms for PPMS, including complex local autoimmune and glial-mediated pathways that do not manifest as monogenic disorders.

KIF5A c.C1082T p.Ala361Val (a reported dominant mutation for SPG10²⁷) was found in a PPMS patient (02-0069) from our WGS discovery cohort. We considered the presence of this variant to be responsible for a progressive myelopathy characteristic of SPG10, highlighting a potential MS phenocopy. This variant was previously reported in a SPG10 patient with adult-onset (age 35) spastic paraparesis from an affected family spanning 4 generations.²⁷ *KIF5A* encodes an axonal motor protein responsible for anterograde transport. Reduced expression of *KIF5A* has been observed in MS white matter lesions,⁴¹ and some SPG10 patients have demyelinating-appearing lesions in the spinal cord.²⁶ Whereas *KIF5A* is located in the MS susceptibility locus *CYP28B1-OS9*, the top SNP in this region (rs701006) does not influence *KIF5A* expression,⁴² and there is no clear linkage between this common MS susceptibility variant and the rare *KIF5A* variant (rs121434444).¹⁴ We hypothesize that disruption to axonal transport may be, in part, responsible for neurodegeneration and spinal cord injury in progressive forms of MS; however, additional studies are needed to confirm this association and to understand its functional impact.

REEP1 c.606 + 43G>T (a reported dominant variant for SPG31²⁵) was found in a PPMS patient (65-0008) from our discovery cohort and 2 PPMS patients (52-0139, 52-1859) from our replication cohorts. Reported SPG31 patients who carried this variant had heterogeneous clinical characteristics, ranging from mild paraparesis to severe tetraparesis with bulbar dysfunction. Functional studies show that *REEP1* facilitates mitochondrial-endoplasmic reticulum (ER) interactions, and altered ER-mitochondrial contacts cause intracellular Ca²⁺ overload resulting in axonal injury. We found additional evidence for the role of mitochondrial dysfunction in PPMS by identifying reported pathogenic mutations for spastic paraplegia 7 (SPG7 [MIM: 607259])^{12,43–46} in two PPMS patients (70-0019, 65-0084) from our MS chip discovery cohort (Table 4). *SPG7* encodes the mitochondrial protein paraplegin, and mutations in this gene cause complex spastic paraplegia attributed to complex I deficiency and increased sensitivity to oxidative stress.⁴⁷ These findings provide evidence for a pathogenic role of *REEP1* and *SPG7* in PPMS, and supports the hypothesis

that mitochondrial dysfunction and diminished tolerance to oxidative stress may contribute to progressive myelopathy in PPMS.

The *MLC1* c.274C>T p.Pro92Ser variant (a reported recessive and isolated heterozygous mutation in MLC,³¹ a leukodystrophy characterized by myelin swelling and cystic changes arising from dysfunction of MLC1 cell junction proteins on astrocytic foot processes) was found in a PPMS patient (04-1225) from our WGS discovery cohort. Of note, this variant was also found in 4 RMS patients (52-1463, 05-0032, 60-0354, and 60-0362) with the same frequency as that observed in PPMS patients, and in 0 controls, in our Phase 2 replication genotyping. This variant is rare (MAF 0.03%) and, to our knowledge, has not been examined through GWAS. Neuropathology studies show that active MS lesions have reduced staining for perivascular astrocytic MLC1, whereas chronic lesions demonstrate upregulation of MLC1 attributed to astrogliosis.^{48,49} Astrocytes are hypothesized to play a role in MS disease progression through participation in the innate immune system, production of cytotoxic factors, and inhibition of remyelination by forming glial scar.⁵⁰ Despite these observations, the exact role of MLC1 in MS pathogenesis remains unclear. Given that *MLC1* p.Pro92Ser has been reported in leukodystrophy patients and is well characterized in functional studies, we hypothesize that this *MLC1* variant affects white matter injury through astrocyte-mediated osmoregulatory dysfunction in both PPMS and RMS patients, albeit with incomplete penetrance and variable expressivity.

TSC2 p.Glu75Lys was found in a PPMS patient (60-0385) from our WGS discovery cohort. White matter lesions in tuberous sclerosis are often caused by abnormal cortical development or neuronal migration rather than demyelination.⁵¹ Because of the lack of reported pathogenic cases, we consider *TSC2* p.Glu75Lys a variant of uncertain significance without a clear role in PPMS pathogenesis.

Understanding the role of phenocopies in PPMS has important clinical implications. The finding that PPMS patients are enriched for HSP-related mutations that cause progressive axonal injury is consistent with the observation that the most common clinical presentation in PPMS is a progressive spastic paraparesis^{3,52,53} and might help explain why these patients respond poorly to immunomodulatory therapies. Moreover, carrying a pathogenic mutation for a MS phenocopy disorder does not cause MS, but rather modulates the disease course through mechanisms independent of immune-mediated pathways implicated by reported MS susceptibility loci. Larger studies are needed to identify additional phenocopy disorders that might contribute to a

progressive disease course in MS. Longitudinal studies are needed to examine the predictive value of rare phenocopy variants on transition to secondary progression in patients with relapsing-onset disease. Last, translational studies are required to develop disease-modifying therapies for spastic paraplegias and other Mendelian disorders that share clinical features with MS. These efforts are instrumental for developing effective treatments that slow and prevent disability in patients with progressive forms of MS.

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Author Contributions

S.E.B., J.R.O., B.A.C.C., X.J., and S.L.H. were responsible for conception and design of the study. All authors were responsible for data acquisition and analysis. X.J., L.M. and S.E.B. were responsible for drafting the manuscript. All authors reviewed and accepted the final draft of the manuscript.

Potential Conflicts of Interest

Nothing to report.

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