

RESEARCH ARTICLE

Dissecting the Phenotype and Genotype of *PLA2G6*-Related Parkinsonism

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ABSTRACT: Background: Complex parkinsonism is the commonest phenotype in late-onset *PLA2G6*-associated neurodegeneration.

Objectives: The aim of this study was to deeply characterize phenogenotypically *PLA2G6*-related parkinsonism in the largest cohort ever reported.

Methods: We report 14 new cases of *PLA2G6*-related parkinsonism and perform a systematic literature review.

Results: *PLA2G6*-related parkinsonism shows a fairly distinct phenotype based on 86 cases from 68 pedigrees. Young onset (median age, 23.0 years) with parkinsonism/dystonia, gait/balance, and/or psychiatric/cognitive symptoms were common presenting features. Dystonia occurred in 69.4%, pyramidal signs in 77.2%, myoclonus

in 65.2%, and cerebellar signs in 44.6% of cases. Early bladder overactivity was present in 71.9% of cases. Cognitive impairment affected 76.1% of cases and psychiatric features 87.1%, the latter being an isolated presenting feature in 20.1%. Parkinsonism was levodopa responsive but complicated by early, often severe dyskinesias. Five patients benefited from deep brain stimulation. Brain magnetic resonance imaging findings included cerebral (49.3%) and/or cerebellar (43.2%) atrophy, but mineralization was evident in only 28.1%. Presynaptic dopaminergic terminal imaging was abnormal in all where performed. Fifty-four *PLA2G6* mutations have hitherto been associated with parkinsonism, including four new variants reported in this article. These are mainly nontruncating,

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which may explain the phenotypic heterogeneity of childhood- and late-onset *PLA2G6*-associated neurodegeneration. In five deceased patients, median disease duration was 13.0 years. Brain pathology in three cases showed mixed Lewy and tau pathology.

Conclusions: Biallelic *PLA2G6* mutations cause early-onset parkinsonism associated with dystonia, pyramidal and cerebellar signs, myoclonus, and cognitive impairment. Early psychiatric manifestations and bladder

overactivity are common. Cerebro/cerebellar atrophy are frequent magnetic resonance imaging features, whereas brain iron deposition is not. Early, severe dyskinesias are a tell-tale sign. © 2021 The Authors. *Movement Disorders* published by Wiley Periodicals LLC on behalf of International Parkinson and Movement Disorder Society

Key Words: NBIA; parkinsonism; *PLA2G6*; PLAN; systematic review

PLA2G6 encodes the calcium-independent phospholipase A₂β (iPLA₂β), which hydrolyzes membrane phospholipids and lysophospholipids, thereby regulating membrane homeostasis and generating lipid second messengers involved in cell proliferation, Ca²⁺ signaling, mitochondrial dynamics, and apoptosis.^{1,2}

Biallelic *PLA2G6* mutations were initially associated with infantile (INAD) and atypical neuroaxonal dystrophies (ANADs).^{3,4} INAD presents with psychomotor regression/delay between 6 and 36 months, and its clinic picture includes early axial hypotonia progressing to spastic tetraparesis, intellectual disability, strabismus, optic atrophy, and axonal sensorimotor neuropathy, with death occurring by age 10 years because of bulbar dysfunction.^{3,5,6} ANAD usually manifests between 1.5 and 6.5 years with prominent language difficulty and autistic-like traits, along with cerebellar, pyramidal, and dystonic features. ANAD shows fairly slow progression during early childhood and rapid deterioration at the turn of the first decade of life.⁴ Neuroradiological findings in INAD/ANAD encompass cerebellar atrophy, cerebellar cortical magnetic resonance imaging (MRI)-T2 hyperintensity, iron deposition in the globus pallidus (GP) and/or substantia nigra (SN), white matter abnormalities, vertically oriented splenium of the corpus callosum, claval hypertrophy, and thinning of the optic pathway.⁷ Axonal degeneration with distended axons (spheroid bodies) throughout the central and peripheral nervous systems is the pathological hallmark of INAD/ANAD.^{3,4,6}

In 2009, *PLA2G6* was linked to dystonia-parkinsonism with onset in the second to third decades of life.⁸ Since then, additional phenotypes manifesting later than INAD/ANAD have been described, including parkinsonism either isolated or combined with other neurological/psychiatric features,^{9,10} ataxia,^{11,12} and spastic paraplegia.^{13,14}

Growing evidence suggests that *PLA2G6*-associated neurodegeneration (PLAN) is a phenotypic continuum.^{12,15} For instance, childhood-onset phenotypes and *PLA2G6*-related parkinsonism share Lewy and tau pathology.¹⁶ Equally, there are unsolved questions about PLAN, particularly late-onset phenotypes. Controversies remain regarding why *PLA2G6* mutations cause such a wide phenotypic spectrum, and why the same mutation leads to different phenotypes, even in the same pedigree.

Little is known about late-onset PLAN progression because ongoing natural history studies mainly focus on INAD.^{17,18} Finally, treatments with disease-modifying potential have not hitherto been explored in late-onset PLAN.¹⁹ Some PLAN cases show brain iron deposition, thus raising the option of chelation therapy, as in pantothenate kinase-associated neurodegeneration.²⁰ More promisingly, small molecule therapies are under investigation in cell and murine models, and a viral vector-based gene therapy has been tested in the *PLA2G6*-INAD mouse with encouraging results and is approaching completion of preclinical studies,^{15,21} as reviewed elsewhere.¹⁹ Promptly recognizing *PLA2G6*-related phenotypes, in particular *PLA2G6* parkinsonism among early-onset parkinsonism from different etiologies, may therefore have considerable therapeutic implications in the not-too-distant future.

We report 14 new cases of *PLA2G6*-associated parkinsonism carrying 13 different mutations, 4 of which are novel. By merging data from this series and a systematic literature review, we deeply characterize phenotypically and genotypically *PLA2G6*-related parkinsonism, highlight clinicoradiological hints for diagnosis, outline its natural history, and discuss poorly understood issues in late-onset PLAN.

Subjects and Methods

We identified new cases of *PLA2G6*-associated parkinsonism from six centers and systematically searched PubMed (14/03/2021) for parkinsonism in genetically confirmed PLAN published since 2006 (discovery paper). The search strategy was “*PLA2G6*” AND “parkins*,” with no language restriction. Additional references from relevant articles were identified and reviewed. According to the search strategy, the systematic review was driven by the presence of parkinsonism in genetically confirmed PLAN cases; thus, subsequent results were not restricted to any age at symptom onset. Only cases carrying biallelic *PLA2G6* mutations with individual information were included. Predefined categories for data extraction were sex; ethnicity; age and symptom(s) at onset; age at last assessment; different neurological and psychiatric symptoms/signs; findings

from laboratory, neuroimaging, and other investigations; family history; genotype; and treatment response. Neuropathology specimens available at Queen Square Brain Bank and videos of published cases were reviewed. Phenotypic features were recorded as non-missing if explicitly stated to be present/absent. *PLA2G6* variants were (re-)annotated in reference to transcript NM_003560.4. Combined Annotation Dependent Depletion, PolyPhen-2, Sorting Intolerant From Tolerant, MutationTaster, and Protein Variation Effect Analyzer were used to predict the impact of missense mutations on the protein structure and function. We assessed sequence conservation across species using Genomic Evolutionary Rate Profiling and/or visual multiple sequence alignment (Clustal Omega).²² gnomAD and ClinVar were retrieved on August 11, 2021. Mutations were finally classified according to American College of Medical Genetics and Genomics guidelines.²³ Descriptive statistics were performed using IBM SPSS Statistics. Results are provided as valid percentages (ie, counts divided by the total number of nonmissing observations) for dichotomous variables and median with interquartile range (IQR; weighted average) for continuous variables.

Results

Clinicogenetic features of 14 new cases of *PLA2G6* parkinsonism from 12 families are summarized in Table 1^{8,12,24} and detailed in Supporting Information Files 1, 2, and 3. Progression of clinical manifestations over time in the new cases is outlined in Supporting Information File 4. They carried 13 *PLA2G6* mutations, including 4 of which are novel (Table 2).^{3,7,8,12,14,16,24-39} We screened for eligibility 162 references from PubMed search results ($n = 136$) and their reference lists (Supporting Information File 5). We found 40 references reporting 72 additional cases from 57 pedigrees and carrying 46 different *PLA2G6* mutations. Predefined data were extracted (Supporting Information File 3).^{7-10,12-14,16,24,25,29-32,35,39-63} Overall, 86 cases from 68 kindreds contributed to the phenotypic description of *PLA2G6*-related parkinsonism.

Phenotype

The cohort included 45 female patients (52.3%; Fig. 1A). Ethnicity was traceable in 84/86 (97.7%; Fig. 1B). Developmental milestones were unremarkable in 42/45 (93.3%) patients, whereas one case experienced long-term toe-walking,¹⁶ one slower development,⁴¹ and one unspecified developmental delay stabilized by therapy.⁴⁴ Convergent strabismus was noticed in case 10 since age 2 years.

Median age of symptom onset calculated for 81/86 (94.2%) cases was 23.0 years (IQR, 11.0; Fig. 1C), with 70/83 (84.3%) patients having onset before age

31 years. Median age at last assessment determined in 67/86 (77.9%) cases was 31.0 years (IQR, 12.0). Median disease duration at last assessment in 68/86 (79.1%) cases was 7.0 years (IQR, 11.75). Presenting symptoms are summarized in Fig. 1D. Extrapyramidal features (parkinsonism/dystonia; 38/79, 48.1%), gait/balance problems (29/79, 36.7%), and psychiatric/cognitive issues (25/79, 31.6%), either alone or combined, were the most common manifestations at onset. Psychiatric manifestations (eg, severe anxiety, major depression, psychosis) were isolated presenting symptoms in 16/79 (20.2%) patients,^{10,16,25,29,44,57,59,60} foot dragging was the sole initial complaint in 8/79 (10.1%),^{8,9,24,40,45,46,63} and urinary incontinence was a major symptom at onset in 2/79 (2.5%).^{16,50}

Parkinsonism, either isolated or combined with other neurological/psychiatric features (Fig. 1E), started at a median age of 25.0 years (IQR, 9.25) and was present at onset or within 1 year in 45/68 (66.2%) patients. In the remaining 23 cases, the median time interval between symptom onset and onset of parkinsonism was 3.0 years (IQR, 9.00). Hyposmia/anosmia was not reported as a premotor symptom in any of our cases and was mentioned only once in the literature.⁵⁵ Parkinsonism presented as an akinetic-rigid syndrome in nearly half of the cases, whereas 36/67 (53.7%) patients had rest tremor mostly affecting the upper limbs and/or rarely the lower limbs.⁵⁶⁻⁵⁸ Parkinsonian signs were asymmetric at onset in 40/50 (80.0%) cases. Hallucinations were reported in 16/30 (53.3%) cases, either visual (9), auditory (2), or both (4). Delusions were present in 11/23 (47.8%). Sleep disturbances, including sleep fragmentation and acting out dreams, were mentioned in 10/16 (62.5%) patients. Polysomnography was available in cases 1 and 14, showing prolonged rapid eye movement phase and sleep apnea, respectively.

Dystonia was present in 50/72 cases (69.4%; Fig. 1E). When specified, dystonia was reported to affect the limbs only (17 cases) or to be generalized (8). Trunk dystonia was frequent, with overt opisthotonus in four cases. Cranial dystonia was reported in 11 patients (facial grimacing in 5, oromandibular dystonia in 4, blepharospasm in 3). Myoclonus, spontaneous and/or stimulus sensitive, was reported in 15/23 (65.2%) cases at any point in the disease course but never at onset (Fig. 1E). In case 2, we documented the cortical origin of myoclonus on neurophysiology (electromyography bursts duration < 50 ms, electroencephalographic discharges time-locked to individual myoclonic jerks detected with jerk-locked back averaging). Pyramidal signs were detected in 44/57 subjects (77.2%; Fig. 1E). Reduced muscle strength was mentioned in only one case.²⁹ Cerebellar signs were observed in 29/65 cases (44.6%; Fig. 1E), including mild dysmetria, gait ataxia, or gaze-evoked nystagmus. Postural and/or action tremor was mentioned in 10 cases.

TABLE 1 Clinical, neuroradiological, and genetic features of new cases of PLA2G6-related parkinsonism

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9	Case 10	Case 11	Case 12	Case 13	Case 14
Sex/current age (y)	F/43	F/36	F/25	M/22	M/20	M/33	F/28	M/24	F/24	F/Deceased age 36	F/33	M/24	M/24	M/36
Ethnicity	White British	White British	Indian	Indian	Indian	Indian	Indian	Indian	Indian	Pakistani	Pakistani	German	Indian	Pakistani
Parental consanguinity	No	No	No	No	Yes	Yes	No	No	No	Yes	Yes	No	Yes	Yes
Family history	Unremarkable	Unremarkable	Unremarkable	Brother similarly affected (no details available)	Sister similarly affected (no details available)	Unremarkable	Unremarkable	Sister affected (case 9)	Brother affected (case 8)	Sister affected (case 11)	Sister affected (case 10)	No	Brother similarly affected (no details available)	Two siblings affected ^{8,12}
Age at onset (y)	27	29	21	15	16	29	25	17	22	23	21	22	21	31
Symptom at onset	Dystonia right arm	Parkinsonism and executive dysfunction	Parkinsonism and psychiatric features	Psychiatric features (aggressiveness, disinhibition, episodes of fear, emotional lability)	Psychiatric features	Parkinsonism	Parkinsonism	Parkinsonism	Parkinsonism, dystonia, and behavioral issues	Psychiatric features (anxiety, unusual emotional states)	Psychiatric features (anxiety, depression)	Balance difficulty, bradykinesia	Psychiatric features (behavioral changes, stubbornness)	Gait and balance difficulties
Motor features	Parkinsonism (bradykinesia, rigidity, rest tremor) Dystonia Pyramidal signs (hyperreflexia, ankle clonus) Cerebellar signs Myoclonus	Parkinsonism (bradykinesia, rigidity) Dystonia Cerebellar signs (limb dysmetria) Dysphagia	Parkinsonism (bradykinesia, rigidity) Dystonia Cerebellar signs (limb dysmetria) Dysphagia	Parkinsonism (bradykinesia, rigidity) Dystonia Pyramidal signs (hyperreflexia) Myoclonus	Parkinsonism (bradykinesia, rigidity) Dystonia Pyramidal signs (hyperreflexia, spasticity LL, ankle clonus) Myoclonus	Parkinsonism \ (bradykinesia, rigidity) Dystonia Pyramidal signs (hyperreflexia, spasticity LL, ankle clonus) Myoclonus	Parkinsonism (bradykinesia, rigidity, rest tremor) Dystonia Pyramidal signs (hyperreflexia, spasticity, Babinski sign) Dysarthria Myoclonus	Parkinsonism (bradykinesia, rigidity, rest tremor) Dystonia Pyramidal signs (hyperreflexia, spasticity, Babinski sign) Dysarthria Myoclonus	Parkinsonism (bradykinesia, rigidity) Dystonia Pyramidal signs (hyperreflexia, Hoffman sign) Dysarthria Myoclonus	Parkinsonism (bradykinesia, rigidity) Dystonia Cerebellar signs (gait ataxia) Pyramidal signs (hyperreflexia, crossed adductor response) Dysarthria Myoclonus	Parkinsonism (bradykinesia, rigidity, rest tremor) Pyramidal signs (Babinski sign) Myoclonus	Parkinsonism (bradykinesia, rigidity, rest tremor) Pyramidal signs (hyperreflexia, ankle clonus) Cerebellar signs (limb dysmetria) Dysarthria	Parkinsonism (bradykinesia, rigidity, rest tremor) Blepharospasm Pyramidal signs (hyperreflexia) Cerebellar signs (gaze-evoked nystagmus) Dysarthria	Parkinsonism (bradykinesia, rigidity) Dystonia Pyramidal signs (hyperreflexia, spasticity LL, ankle clonus) Cerebellar signs (mild gait ataxia, limb dysmetria, terminal tremor) Myoclonus
Nonmotor features	Postural instability Cognitive impairment Anxiety Depression	Postural instability Cognitive impairment Anxiety Depression Apathy Urinary issues (urgency, nocturia, incontinence)	Postural instability Cognitive impairment Anxiety Depression Urinary issues (urgency, urge incontinence)	Postural instability Cognitive impairment Aggressive behaviors Urinary issues (frequency, urgency, incontinence) Constipation	Postural instability Cognitive impairment Aggressive and abusive behaviors Urinary incontinence	Postural instability Cognitive impairment Anxiety Depression Apathy Emotional lability Urinary incontinence	Postural instability Cognitive impairment Apathy Emotional lability Urinary issues Visual hallucinations Constipation	Postural instability Cognitive impairment Apathy Emotional lability Urinary issues Constipation	Postural instability Cognitive impairment Apathy Abusive behaviors Emotional lability	Postural instability Cognitive impairment Anxiety Depression Emotional lability Urinary issues (urgency, incontinence)	Postural instability Cognitive impairment Anxiety Depression Emotional lability	Postural instability Cognitive impairment Dysphagia	Postural instability Cognitive impairment Behavioral issues	Postural instability Cognitive impairment Postural instability Cognitive impairment
Response to L-dopa	Initial good response, then worsening of dystonia Early L-dopa-induced dyskinesias	Initial good response Early L-dopa-induced dyskinesias after 8 months of treatment	Initial optimal response L-dopa-induced dyskinesias after 8 months of treatment	Modest response Early L-dopa-induced dyskinesias	Good response L-dopa-induced dyskinesias after 3 months of treatment	Good response L-dopa-induced dyskinesias after 5 months of treatment	Good response L-dopa-induced dyskinesias after 2 months of treatment	Good response Early L-dopa-induced dyskinesias	Good response Apathy Abusive behaviors Emotional lability	Good response L-dopa-induced dyskinesias after a few months of treatment	Very good response L-dopa-induced dyskinesias after a few months of treatment	Optimal response	Good response	Good response Early L-dopa-induced dyskinesias (mainly affecting his jaw)

(Continues)

TABLE 1 *Continued*

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9	Case 10	Case 11	Case 12	Case 13	Case 14
Brain MRI	Unremarkable (no mineralization on SWI); cerebellar atrophy on follow-up MRI	Iron deposition on SWI; cerebellar atrophy on follow-up MRI	Cerebral atrophy (FT)	Cerebellar atrophy on T2*	Cerebellar atrophy No mineralization on T2*	Cerebellar atrophy No mineralization on T2*	Cerebellar atrophy No mineralization on T2*	Cerebellar atrophy Iron deposition on SWI (SN, putamen, GP)	Cerebellar atrophy Iron deposition on SWI (SN, caudate, putamen)	Mild cerebral atrophy (biparietal)	Mild cerebral atrophy on T2*	Mild generalized cerebral atrophy	Cerebral atrophy (FT)	Mild generalized cerebral atrophy
Presynaptic dopaminergic terminal imaging	DaTscan: reduced uptake of tracer throughout the striata bilaterally (> putamen)	N.A.	^{99m} Tc-TRODAT-1 single photon emission computed tomography SPECT/CT: reduced tracer uptake in the striatum bilaterally	¹⁸ F-DOPA PET: asymmetrical decrease in tracer uptake in the BG	¹⁸ F-DOPA PET: asymmetrical decrease in tracer uptake in the BG	¹⁸ F-DOPA PET: severe decrease in tracer uptake in bilateral putamen more than caudate	¹⁸ F-DOPA PET: asymmetrical decrease in tracer uptake	N.A.	N.A.	N.A.	N.A.	DaTscan: reduced tracer uptake in the striatum bilaterally	N.A.	DaTscan: reduced tracer uptake in the putamen and striatum bilaterally
Negative genetic tests before definitive diagnosis	<i>TOR1A, LRRK2, PRKN, PINK1, POLG, mtDNA, DJ-1, SNCA, VPS35</i>	<i>HTT, FTL, PANK2, WDR45</i>	SCAs (<i>ATXN1, ATXN2, ATXN3, CACNA1A, ATXN7, ATXN10, PPP2R2B</i>)	None	None	None	None	None	None	<i>HTT, DRPLA, PRNP, TBP, POLG, common mtDNA variants, PRKN</i>	None	None	None	None
Genetics	<i>PLA2G6</i> (NM_003560.4) c.956C>T p.(Thr319Met); c.1061T>C p.(Leu345Pro)	WGS Compound heterozygote c.238G>A (p.Ala80Thr); c.1924A>G (p.Thr642Ala)	NGS gene panel Compound heterozygote c.673C>T (p.His225Tyr); c.2311G>A (p.Asp771Asn)	WES Homozygote c.2222G>A (p.Arg741Gln)	WES Homozygote c.2222G>A (p.Arg741Gln)	NGS gene panel Homozygote c.1937C>T (p.Pro646Leu)	WES Compound heterozygote c.2370T>G (p.Tyr790*); c.1511C>T (p.Ser504Leu)	WES Homozygote c.2222G>A (p.Arg741Gln)	WES Homozygote c.2222G>A (p.Arg741Gln)	WES Homozygote c.2222G>A (p.Arg741Gln)	Sanger sequencing Homozygote c.2222G>A (p.Arg741Gln)	NGS gene panel Compound heterozygote c.1021G>A (p.Ala341Thr); c.1898C>T (p.Ala633Val)	WES Homozygote c.2222G>A (p.Arg741Gln)	Sanger sequencing Homozygote c.2222G>T (p.Arg741Trp)

M, male; F, female; LL, lower limbs; L-dopa, levodopa; MRI, magnetic resonance imaging; SWI, susceptibility-weighted MRI sequence; FT, frontotemporal; T2*, T2*-weighted gradient echo MRI sequence; BG, basal ganglia; N.A., not available; SPECT, single photon emission computed tomography; CT, computed tomography; PET, positron emission tomography; SCA, spinocerebellar ataxia; mtDNA, mitochondrial DNA; WGS, whole-genome sequencing; NGS, next-generation sequencing; WES, whole-exome sequencing; SN, substantia nigra; GP, globus pallidus.

TABLE 2 Analysis of genetic variants in the PLA2G6 gene detected in the new 14 cases with parkinsonism

	Case 1		Case 2		Case 3		Cases 4, 5, 8–11, 13		Case 6	Case 7	Case 12		Case 14
	PLA2G6 variant (NM_003560.4)	Mutation type	c.238G>A p.Ala80Thr	Miscense	c.2311G>A p.Asp771Asn	Miscense	c.2222G>A p.Arg741Gln	Miscense	c.1937C>T p.Pro646Leu	c.2370T>G p.Tyr790 ^a	c.1511C>T p.Ser504Leu	c.1021G>A p. Ala341Thr	c.1898C>T p. Ala633Val
CADD score	23.2	29.8	23.0	2.41	25.5	27.8	25.7	29.6	29.6	35.0	23.0	26.4	28.7
GERP Phred score	2.48	2.48	2.41	2.41	2.62	2.24	2.10	6.43	6.43	−4.68	6.51	2.48	2.31
PolyPhen-2 (HumDiv score)	Probably damaging (0.997)	Probably damaging (1.000)	Possibly damaging (0.855)	Possibly damaging (0.971)	Possibly damaging (0.632)	Probably damaging (0.990)	Probably damaging (1.000)	Probably damaging (0.999)	Probably damaging (0.999)	N.A.	Benign (0.011)	Probably damaging (0.999)	Probably damaging (0.987)
SIFT (score)	Tolerated (0.072)	Damaging (0.001)	Tolerated (0.069)	Damaging (0.007)	Damaging (0.035)	Tolerated (0.147)	Tolerated (0.064)	Damaging (0.004)	Damaging (0.004)	N.A.	Tolerated (0.358)	Damaging (0.003)	Tolerated (0.145)
PROVEAN (score)	Deleterious (−4.18)	Deleterious (−6.64)	Deleterious (−2.63)	Deleterious (−3.34)	Deleterious (−3.37)	Deleterious (−2.62)	Neutral (−2.06)	Deleterious (−4.63)	Deleterious (−4.63)	N.A.	Neutral (−0.04)	Deleterious (−3.26)	Deleterious (−2.61)
MutationTaster	Disease causing	Disease causing	Disease causing	Disease causing	Disease causing	Disease causing	Disease causing	Disease causing	Disease causing	Disease causing	Disease causing	Disease causing	Disease causing
ClinVar	Uncertain significance (criteria provided, multiple submitters, no conflicts)	Not reported	Conflicting interpretations of pathogenicity: pathogenic/uncertain significance (criteria provided, conflicting interpretations)	Not reported	Pathogenic/likely pathogenic (criteria provided, multiple submitters, no conflicts)	Not reported	Pathogenic/likely pathogenic (criteria provided, multiple submitters, no conflicts)	Not reported	Not reported	Conflicting interpretations of pathogenicity: likely pathogenic/likely pathogenic/uncertain significance (criteria provided, conflicting interpretations)	Not reported	Not reported	Not reported
gnomAD v3.1	46/0;	1/0;	2/0;	1/0;	Not found	Not found	Not found	Not found	Not found	9/0;	2/0;	1/0;	2/0;
Total allele count/total homozygotes; aggregated total AF [population-specific AF] ^a	AF = 0.0003021 [European non-Finnish AF: 0.0002058]	AF = 0.000006568 [European non-Finnish AF: 0.00001470]	AF = 0.00001316 [European non-Finnish AF: 0.0001470]	AF = 0.000006578 [European non-Finnish AF: 0.00001471]	Not found	Not found	Not found	Not found	Not found	AF = 0.00005915 [South Asian AF: 0.000]	AF = 0.00001314 [South Asian AF: 0.000]	AF = 0.000006569 [European non-Finnish AF: 0.00001470]	AF = 0.00001315 [European non-Finnish AF: 0.00001473]
Classification (ACMG)	Uncertain significance	Likely pathogenic	Uncertain significance	Likely pathogenic	Likely pathogenic	Uncertain significance	Pathogenic	Likely pathogenic	Likely pathogenic	Pathogenic	Uncertain significance	Likely pathogenic	Likely pathogenic
References	–	3, 16	3, 25, 26	–	27, 28	–	8, 24, 29–32	–	–	3, 7, 12, 33, 34	35	3, 36, 37	38
													8, 12, 14, 29, 36, 39

^aPopulation-specific allele frequency relevant to the case is reported in square brackets (cases 1, 2, and 12: European non-Finnish; cases 3–11, 13, and 14: South Asian). ClinVar and gnomAD v3.1 were retrieved on August 11, 2021. CADD, Combined Annotation Dependent Depletion; GERP, Genomic Evolutionary Rate Profiling; N.A., not available; SIFT, Sorting Intolerant From Tolerant; PROVEAN, Protein Variation Effect Analyzer; AF, allele frequency; ACMG, American College of Medical Genetics and Genomics.

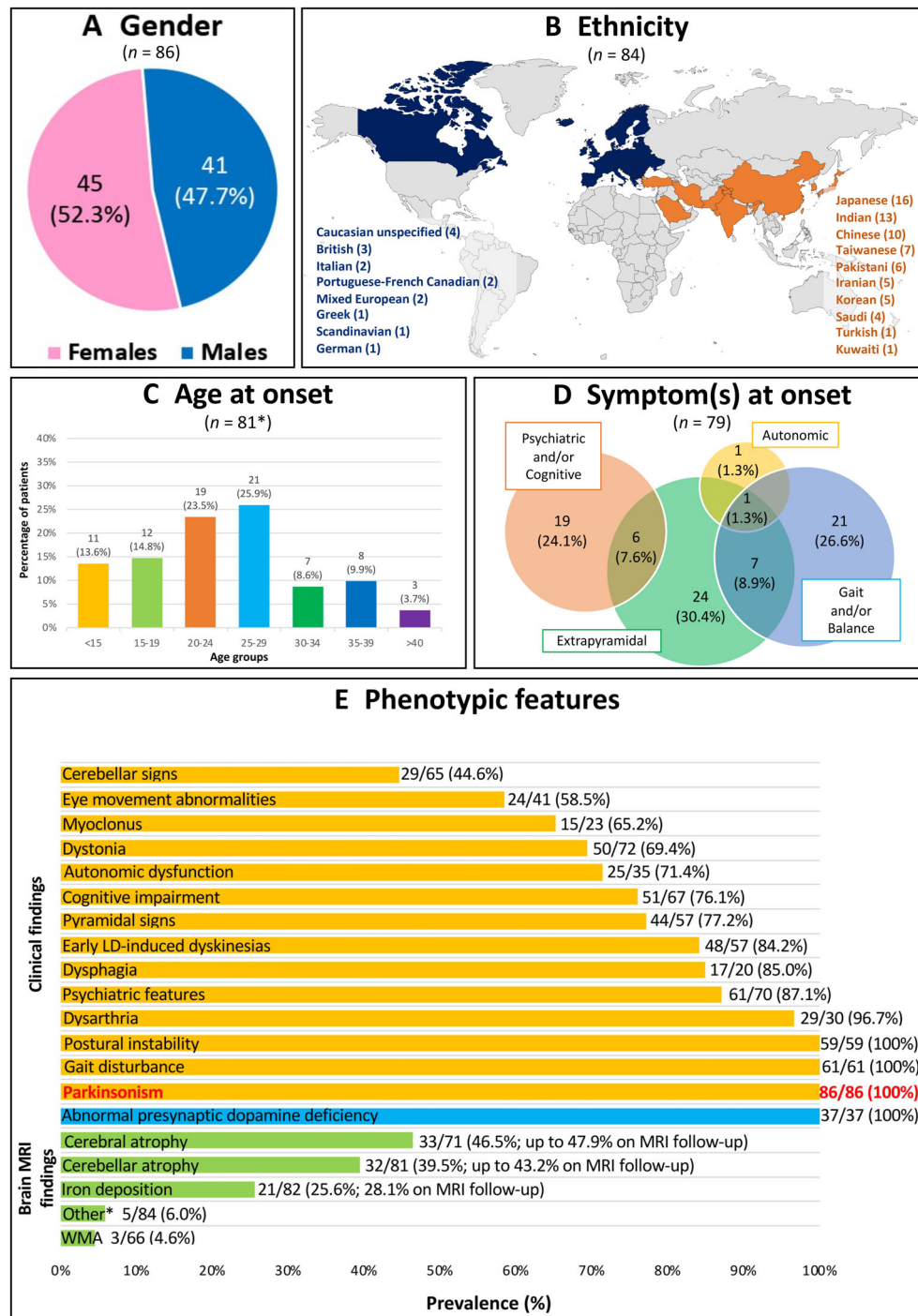


FIG. 1. Descriptive statistics of the whole cohort with PLA2G6-related parkinsonism, including new and previously published cases. The number of cases for which the information was available (nonmissing observations) is indicated by *n* in (A)–(D) and by fractions' denominator in (E). (A) Sex distribution. (B) Ethnicity: number of cases according to ethnicity are reported in brackets. (C) Age at symptom onset according to age group. *Two additional cases with unspecified age of onset of parkinsonism before the age of 31 years.⁴¹ (D) Symptom(s) at onset according to major descriptive categories. (E) Phenotypic spectrum of PLA2G6-associated parkinsonism with clinical features (yellow bars), brain magnetic resonance imaging (MRI) findings (green bars), and findings on presynaptic dopaminergic terminal imaging (blue bar). LD, levodopa; WMA, white matter abnormalities. [Color figure can be viewed at wileyonlinelibrary.com]

Gait abnormalities were constantly present early in the disease course (Fig. 1E). When details were available (39 cases), gait was described as having parkinsonian (46.2%), ataxic (30.8%), pyramidal (23.1%),

dystonic (17.9%) features, either alone or in combination. Freezing of gait was mentioned in three patients. Postural impairment was also constantly reported (Fig. 1E), causing loss of walking independence with a

median time interval of 3.0 years (IQR, 3.0) after symptom onset.

Dysautonomia was present in 25/35 (71.4%) patients (Fig. 1E), with symptoms of bladder overactivity and/or urinary incontinence reported in 71.9% of cases and constipation in 50%. Orthostatic hypotension was mentioned in two cases.^{42,43}

Cognitive impairment was documented early in the disease course in 51/67 (76.1%) patients (Fig. 1E). Psychiatric comorbidity was present in 61/70 (87.1%) cases (Fig. 1E), including depression (80.0%), anxiety (79.2%), and signs of frontal lobe impairment (87.0%), encompassing, among others, apathy, paranoid thoughts, aggressive behaviors, and emotional lability.

Eye movement abnormalities were reported in 24/41 (58.5%; Fig. 1E) cases, with fragmented pursuit and/or reduced gaze range in the vertical plan being the most frequent. Eyelid-opening apraxia was reported in six cases.^{8,24,25,55} Dysarthria was described in 29/30 (96.7%; Fig. 1E) cases. Swallowing difficulties were reported in 17/20 (85.0%) cases (Fig. 1E) and, in 6 cases with details available,^{7,8,12,16,24,30,51} overt dysphagia and/or the need of percutaneous endoscopic gastrostomy occurred a median interval of 10 years (IQR, 7.0) after symptom onset. Sensory signs were reported in one case.¹⁶ Generalized seizures occurred in five patients some years into the disease course.^{8,16,24,31,39} Oculogyric crises were reported in four cases.^{10,30,58}

Parkinsonism responded to levodopa in 71/73 (97.3%) cases. Levodopa-induced dyskinesias were reported in 46/57 (80.7%) cases and appeared within the first year of treatment in most cases, even with low levodopa doses (≤ 300 mg/day). In some cases, levodopa caused behavioral changes with psychotic manifestations, or dystonic reactions mainly affecting the oromandibular or cervical region. In 17/18 cases, dopamine agonists had a beneficial response; however, some cases experienced negative side effects, including psychosis and hypersexuality. Response to other pharmacological treatments is detailed in Supporting Information File 3. Four patients underwent bilateral deep brain stimulation (DBS) of the subthalamic nucleus (STN) with excellent outcome,⁵⁷ and one patient underwent bilateral DBS of the GP internus (GPi; case 1) with improvement of trunk dystonia and control of levodopa-induced dyskinesias. One patient underwent unilateral pallidotomy with transient relief of motor fluctuations.^{52,53}

Five patients died at a median age of 36.0 years (IQR, 13.0), showing a median disease duration of 13.0 years (IQR, 14.5).

Brain MRI was available in 82/86 (95.3%) patients. Cerebral atrophy was reported in 34/71 cases (47.9%), being described as mild to moderate in severity and generalized or mainly involving the frontotemporal lobes (Fig. 1E; Supporting Information File 2B,F,G,L,O). Mild to marked cerebellar atrophy affecting the vermis and/or the hemispheres was observed in 32/81

(39.5%; Fig. 1E; Supporting Information File 2B,G,L,N) cases. In 21/82 (25.6%) cases, there was evidence of iron deposition on MRI-T2/T2*/SWI sequences (Fig. 1E; Supporting Information File 2D–I). Interestingly, iron deposition was not detected in 15 cases in which T2*/SWI sequences were performed (Fig. 1E; Supporting Information File 2A–P). White matter T2 hyperintensities were reported in three cases,^{8,24,40,56} mainly in the frontal lobes. Additional findings on brain MRI (Fig. 1E) were claval hypertrophy in four patients,^{7,12,14,31,56} vertically oriented corpus callosum in two,^{14,31} and the swallow tail sign in two.⁶¹ Follow-up brain MRI scans were available in seven patients, showing progression of cerebral and/or cerebellar atrophy in three cases,¹⁶ as well as appearance of iron deposition in two.^{8,24,51} In three patients, CT scan excluded MRI-T2/SWI hypointensity corresponding to basal ganglia calcifications. Spine MRI, available in eight cases, showed no signal abnormalities from the spinal cord.

Dopamine imaging with presynaptic tracers was available in 37/86 cases (43.0%) and invariably abnormal (Fig. 1E; Supporting Information File 2C,E,H,M,Q). In one patient, ¹¹C-raclopride (RAC)-positron emission tomography for postsynaptic receptor function revealed increased RAC uptake in the putamen more than in the caudate.²⁵ ¹⁸F-fluorodeoxyglucose-positron emission tomography, performed in seven cases, showed global hypometabolism of cerebral cortex and cerebellum in one case,⁴⁷ hypometabolism in the frontoparietal regions in three,²⁹ and hypometabolism in the temporoparietal regions or parieto-occipital lobes in one case each.^{10,39}

EEG was abnormal in four of eight cases where reported, with diffuse slowing and multifocal epileptiform abnormalities a few years into the disease course.^{16,24,31,59} Nerve conduction studies were performed in seven patients, showing signs of distal sensory neuropathy in two.^{7,16} Four patients with pyramidal signs underwent motor-evoked potentials (MEPs), which showed delayed central motor conduction time in two cases, including case 1.⁵⁰ In case 12, motor-evoked potential was abnormal despite the absence of clinically detectable pyramidal signs.

Retinopathy was excluded by ophthalmological assessment in five cases.^{8,24,25,40,63} Visual-evoked potentials were normal in three cases.^{8,24,29}

CSF analysis revealed decreased homovanillic acid in 4/13 (30.8%) cases,^{8,24,29} 2 of which had subsequent normal phenylalanine loading test.^{8,24}

Genotype

Parental consanguinity was reported in 35/64 (54.7%) cases. Overall, 46/86 (53.5%) patients carried homozygous variants in *PLA2G6*, whereas segregation

analysis revealed that 40/86 (46.5%) were compound heterozygotes.

The new cases carried 13 distinct *PLA2G6* mutations, including four novel missense variants (c.956C>T, c.1924A>G, c.2311G>A, and c.1937>T), four missense variants previously reported in only childhood-onset phenotypes (c.1061T>C, c.673C>T, c.1021G>A, and c.1898C>T), and one nonsense (c.2370T>G) and three missense variants (c.238G>A,

c.2222G>A, and c.2239C>T) described in both childhood- and late-onset phenotypes (Table 2). The novel variants were either absent (n = 2) or exceedingly rare (minor allele frequency, MAF < 0.1%; n = 2) in gnomAD, highly conserved across species, and predicted pathogenic by at least four predetermined prediction tools (Table 2). Among variants previously reported in only childhood-onset phenotypes, the variant c.1061T>C was in compound heterozygosity with

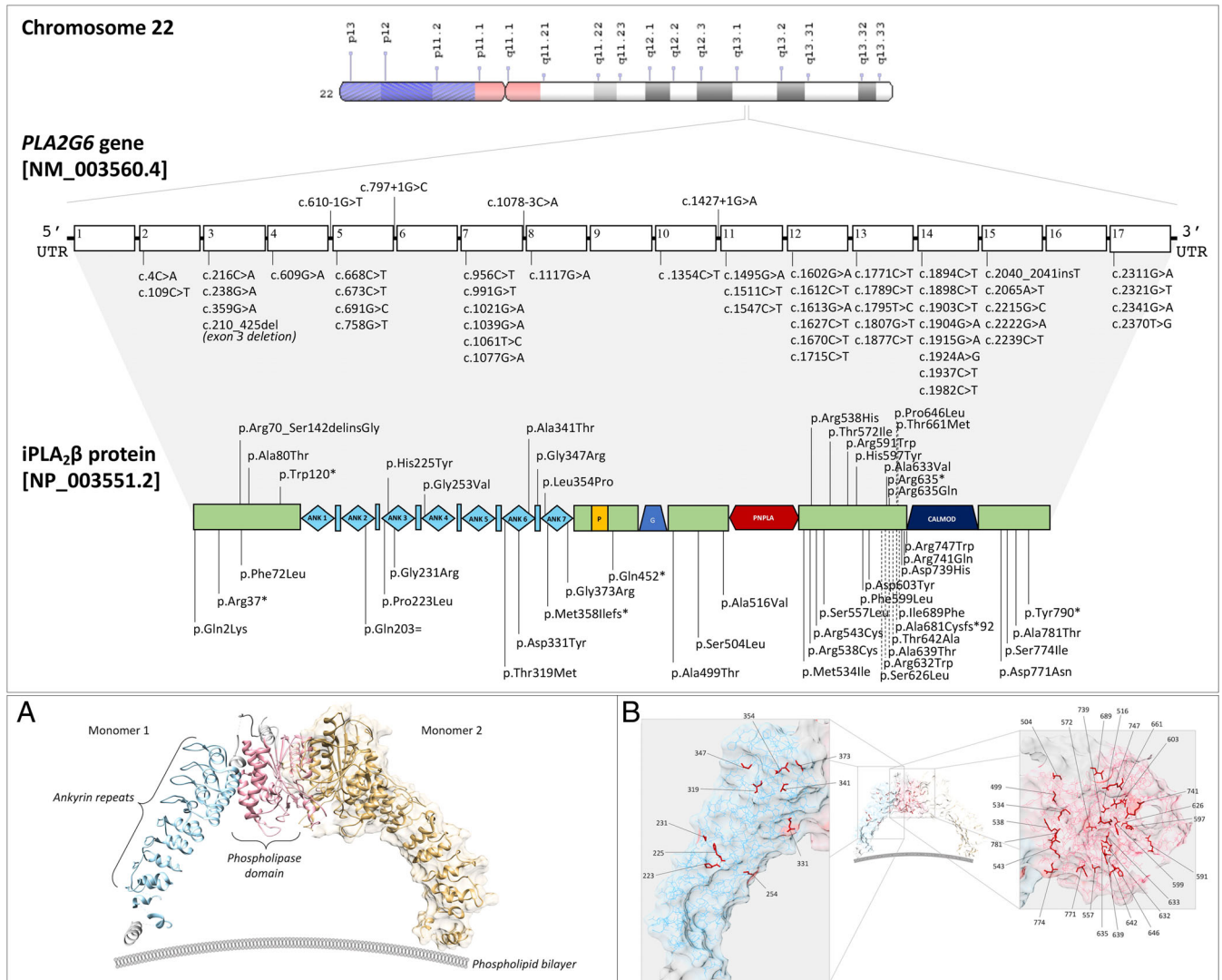


FIG. 2. Overview of genetic variants and protein changes associated with PLA2G6-related parkinsonism. Large box: schematic of the *PLA2G6* gene and its product with genetic variants and corresponding protein changes associated with parkinsonism. Upper part: ideogram of chromosome 22 showing the localization of the *PLA2G6* gene. Middle part: schematic of the *PLA2G6* gene with 54 mutations linked to parkinsonism. Lower part: schematic of the *PLA2G6* protein product (iPLA₂β) with predicted protein changes. The protein structure encompasses seven ankyrin repeats (light blue), a proline-rich motif (yellow), a glycine-rich nucleotide binding motif (light blue), a lipase motif (pink), and a proposed C-terminal Ca²⁺-dependent calmodulin binding domain (dark blue). Small boxes: structural distribution of coding mutations in *PLA2G6*. **(A)** Crystal structure of the dimeric iPLA₂β complex, displayed in association with the phospholipid bilayer cell membrane. The dimer consists of catalytic domains, which firmly interact through an extensive interface, and ankyrin domains, which are oriented outward from the catalytic core and anchor the protein to the membrane in its inactive state.¹ The active site of each subunit is proposed to adopt an open conformation for phospholipids to access the catalytic regions in the absence of membrane interaction. Monomer 1 shows domain organization, with the ankyrin repeats colored light blue and the phospholipase domain colored pink. **(B)** Distribution of coding mutations in *PLA2G6* across the functional domains of iPLA₂β, showing localization with the ankyrin (left-hand inset, blue) and phospholipase domains (right-hand inset, pink). Protein coordinates are derived from Malley et al.,¹ protein database file 6AUN. Images are generated using UCSF Chimera.⁷¹ UTR, untranslated region. [Color figure can be viewed at wileyonlinelibrary.com]

another missense variant in a case of neurodegeneration with brain iron accumulation (NBIA)³ and with nonsense and frameshift variants in two INAD cases.^{3,16} The variant c.238G>A was found in compound heterozygosity with a nonsense variant in an NBIA case³ and

in homozygosity in two cases (onset 8 and 14 years, respectively).^{25,26} The variant c.1021G>A was previously found in compound heterozygosity with a missense variant in two siblings with INAD³ and in homozygosity in another INAD case,³⁶ whereas the

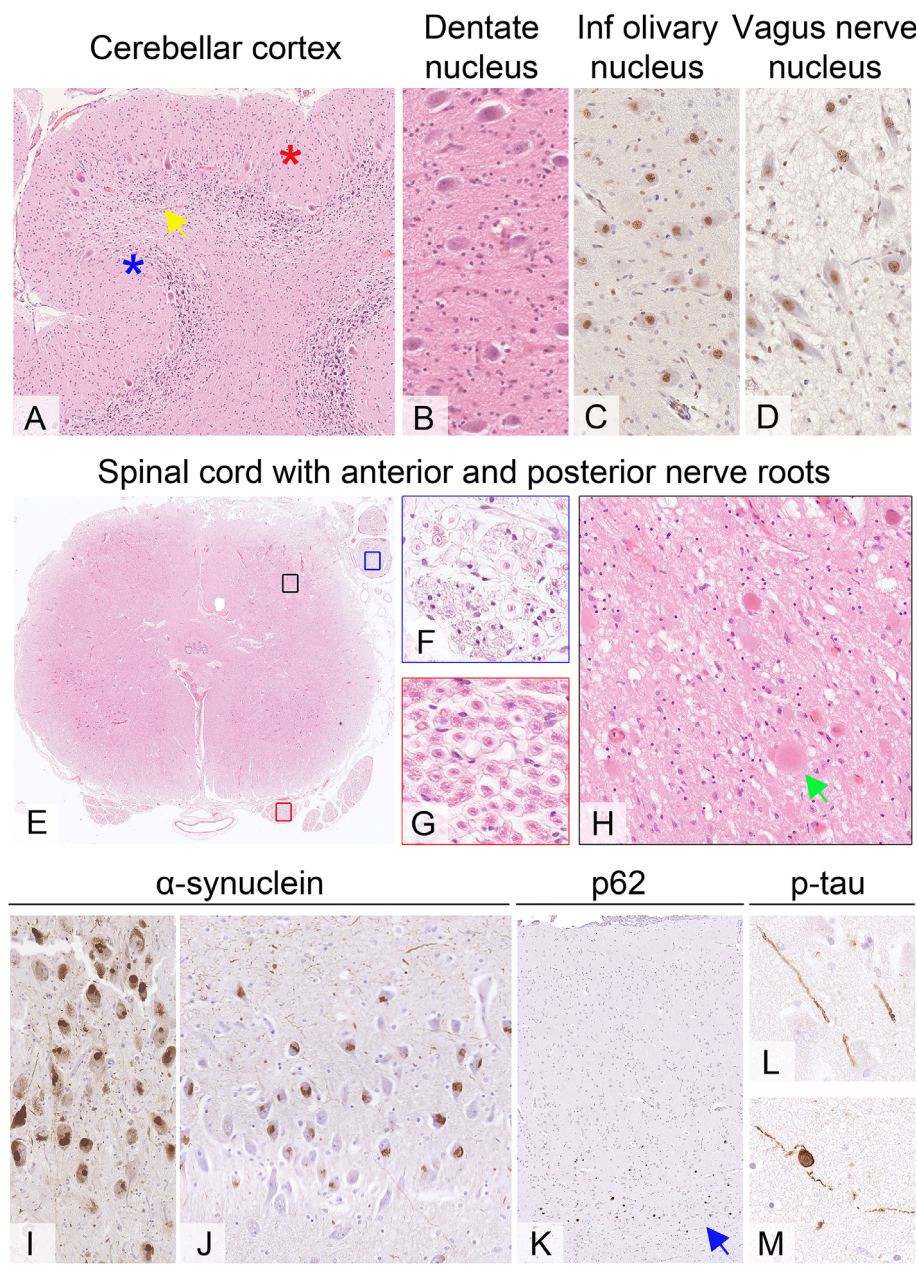


FIG. 3. Postmortem findings in the brain and spinal cord of a patient carrying c.109C>T and c.1078-3C>A mutations in *PLA2G6* (previously published).¹⁶ (A) In the cerebellar cortex, there is a severe depletion of granule cells (yellow arrow), prominent gliosis in the molecular layer (red asterisk), and to a lesser extent depletion of the Purkinje cells (blue asterisk), shown on hematoxylin and eosin (H&E)-stained section. (B) The dentate nucleus in the cerebellum, demonstrated on H&E-stained section. (C) The inferior olivary nucleus in the medulla (preparation immunostained for nonphosphorylated TDP43) shows only mild gliosis and no significant neuronal loss. (D) The dorsal vagus nerve nucleus, although containing Lewy bodies (not shown), does not demonstrate any severe neuronal depletion. (E) Transverse H&E-stained section of the spinal cord at the lumbar level shows (F) that the posterior nerve roots are much more prominently depleted of myelinated fibers (G) compared with the anterior nerve roots. (H) In the posterior horns, there are frequent, variably large axonal spheroids (green arrow, H&E-stained section), but no apparent Lewy body or tau pathology (not shown). (I) Lewy bodies are particularly numerous in the less atrophic medial part of the substantia nigra, (J) in the CA2 region of the hippocampus, and (K) across the deep layers of all the neocortical regions (I, J, immunostained for α -synuclein; K, immunostained for p62; positive inclusions in K are highlighted with blue arrow). (L, M) Occasional isolated neuropil threads (L) and rare tangles (M) are seen in the medial temporal lobe. Scale bars: 300 μ m (A); 100 μ m (B–D, F–J, L, M); 2.5 mm (E); 550 μ m (K). [Color figure can be viewed at wileyonlinelibrary.com]

variant c.673C>T was detected in the homozygous state in two kindreds with childhood-onset cases.²⁷ Among the variants reported in both childhood- and late-onset phenotypes, the nonsense variant c.2370T>G was in compound heterozygosity with a missense variant in case 7 (age at onset: 25 years) and found in the homozygous state in an INAD case³ and in compound heterozygosity with another nonsense variant (c.1674del) in two sisters with INAD.³³ Overall, these observations suggest a gradient in the age of onset and phenotype severity reflecting variants' impact on the transcript.

The number of *PLA2G6* mutations associated with parkinsonism increases to 54 (Table 2, Fig. 2). These included 46 nontruncating (44 missense and 2 in-frame deletions) and 8 truncating (4 splicing, 2 nonsense, and 2 frameshift) changes; therefore, missense variants were predominant in *PLA2G6*-related parkinsonism (Fig. 2; Supporting Information File 3). Most *PLA2G6* mutations were present in only one pedigree, whereas 12 occurred in more than one family. The most frequent variants were c.991G>T (17 pedigrees, mainly Chinese and Taiwanese),^{9,10,35,41,43,45-47,59,61,62,64} c.2222G>A (12 Indian, Pakistani and Saudi families),^{8,24,29-32,63} and c.1904G>A (10 pedigrees),^{13,42,43,54} with the latter being hitherto detected in only Japanese kindreds, which suggests a founder effect.

Enzymatic activity of the mutant iPLA2 β was documented in only one included report.⁴⁵ Transfection of cDNA encoding *PLA2G6* carrying the homozygous variant c.991G>T into HEK293T cells revealed about 30% of residual protein activity compared with the wild-type protein.⁴⁵

Pathology

Brain pathology has been reported in three cases.^{16,48,52,53} In one case, brain autopsy revealed cerebellar cortical atrophy with severe loss of granule cells, gliosis in the molecular layer, and to a lesser extent, Purkinje cell depletion. Cytoarchitecture of the dentate and inferior olivary nuclei was comparably well preserved. There were occasional neuroaxonal spheroids in the GP, STN, thalamus, and ambiguous nucleus, and frequent ones in the gracile and cuneate nuclei and posterior horns of the spinal cord. There was severe atrophy of the ventrolateral parts of the SN with better pigmented neuron preservation medially. The locus ceruleus showed mild depletion, and the vagus nerve nucleus in the medulla showed no evidence of severe neuronal loss. Nevertheless, Lewy bodies were present in the brainstem nuclei in the tegmentum and frequently in the less atrophic medial part of the SN, in the Meynert nucleus, medial temporal lobe, and occasionally in the putamen. Lewy pathology was particularly widespread across the deep layers of the neocortex affecting all, including occipital lobes (Braak stage 6). There was also very mild tau pathology in the medial

temporal lobe with rare neurofibrillary tangles and occasional neuropil threads (Fig. 3).¹⁶ One patient underwent brain biopsy of the frontal cortex, which showed severe Lewy pathology and moderate tau pathology (neuropil threads).¹⁶ Postmortem evaluation in another case showed widespread cortical and limbic structure atrophy, Lewy bodies in the SN and locus ceruleus, Alzheimer's disease-like pathology, mainly in the temporal lobe structures, abundant gliosis detected by glial fibrillary acid protein, and excessive iron accumulation in the reticularis portion of the SN, but also the GP and ventral forebrain.^{52,53} No cases hitherto reported had nerve or rectal biopsy available. Muscle biopsy was unremarkable in three cases and showed neurogenic changes and reduced cytochrome oxidase activity (complex IV) in one case.^{7,12,16} Skin biopsy, which, however, did not search for α -synuclein, was unremarkable in three cases.^{8,24} Bone marrow aspiration was normal in two cases.^{8,24}

Discussion

We provided in-depth phenotypic and genotypic characterization of the largest cohort of *PLA2G6*-related parkinsonism hitherto reported. Distinct aspects from this phenogenotypic overview are discussed later.

Although the age of symptom onset ranged between the first and seventh decades of life, most cases manifested between late second and third decades of life. Patients with earlier onset^{7,12,56} showed overlapping clinoradiological features with ANAD cases but lacked the typical rapid deterioration in late childhood,⁴ and instead had slower symptom progression with parkinsonism from the second decade of life, which supports the notion of PLAN phenotypes as a phenotypic continuum.^{12,15} Psychiatric features were frequently observed early in the disease course, often preceding extrapyramidal manifestations, and were the only presenting symptoms in one-fifth of the cohort.^{10,16,25,29,30,44,59,60} This evidence should warn clinicians against misdiagnosis of psychiatric disorders and initiation of potentially detrimental treatments (ie, antipsychotics) in the context of (at least preclinical) extrapyramidal involvement.²⁹ Interestingly, onset or rapid deterioration of symptoms in *PLA2G6*-related parkinsonism occurred during pregnancy/postpartum (cases 2, 3, and 11)³⁰ or in vitro fertilization (case 1) in five cases. This is in keeping with likely hormone-related symptom deterioration during pregnancy and, most often, the perimenopausal and postmenopausal period previously reported in idiopathic Parkinson's disease (PD).⁶⁵ These observations, along with the age of onset and high prevalence of psychiatric manifestations, suggest that female individuals with *PLA2G6* parkinsonism can be at risk to be misdiagnosed as

having pregnancy- or postpartum-related psychiatric morbidity, autoimmune encephalitis, or functional motor disorders in the early disease stages.

Parkinsonism frequently showed dramatic, albeit unsustained, response to levodopa. Levodopa efficacy was most often limited by the occurrence of severe levodopa-induced dyskinesias, as well as exacerbation of psychiatric symptoms a few weeks to a few years after treatment initiation. We suggest that early-onset levodopa-induced dyskinesias are a tell-tale sign and, along with other clinicoradiological red flags discussed in this article, essentially limit the differential diagnosis to Kufor-Rakeb syndrome. Five patients underwent bilateral DBS (STN or GPi) with good to excellent outcome,^{54,55,57} thus making DBS a potential option in early disease stages with intractable treatment fluctuations. Dystonia was the second most frequent motor feature in *PLA2G6*-related parkinsonism. Isolated foot dragging was the presenting feature in ~10% of cases,^{8,24,40,45} thus initially suggesting *PRKN*-PD.⁶⁶ We noticed a high prevalence of extensor truncal dystonia in our cases and previous literature, thus confirming dystonic opisthotonus as a hint to suspect NBIA syndromes. Oculogyric crises were also observed,^{10,30,58} thus expanding the spectrum of disorders possibly manifesting with these paroxysmal dystonic manifestations.^{67,68} Finally, oromandibular dystonia seemed less prevalent than in pantothenate kinase-associated neurodegeneration. Although pyramidal features are not particularly diriment in early-onset parkinsonian, pallidopyramidal, or NBIA syndromes, (even subtle) cerebellar signs could point toward *PLA2G6*. Finally, myoclonus was frequently reported a few years into the disease course, and we first proved its cortical origin on neurophysiology in case 2.

Cerebellar atrophy was detected on MRI in more than 40% of cases. Genetic *PLA2G6* ablation in mice causes cerebellar atrophy, with loss of Purkinje cells, reactive astrogliosis, microglia activation, and up-regulation of proinflammatory cytokines. Less frequent and (in most cases) severe degree of cerebellar atrophy in late-onset PLAN might reflect higher residual iPLA₂β activity. It could also correlate with disease duration because we documented cerebellar atrophy on follow-up MRI in three cases where it was not initially detected. Iron deposition was found in ~25% of cases. Interestingly, it was often not documented on dedicated MRI-T2*/SWI sequences. When available, dopamine imaging invariably documented signs of nigrostriatal degeneration. Notably, in one patient, RAC for post-synaptic receptor function revealed increased RAC uptake in the putamen more than in the caudate, thus indicating a largely presynaptic dopaminergic abnormality as seen in idiopathic PD.²⁵

Pathogenic *PLA2G6* mutations causing different PLAN phenotypes are scattered throughout protein

domains and may therefore impair iPLA₂β function through a variety of loss-of-function mechanisms, affecting either its enzymatic activity, regulation, or interactions at the macromolecular level. No mutations hitherto linked to *PLA2G6* parkinsonism affect the primary structure of iPLA₂β domains responsible for its enzymatic activities. Most of them are nontruncating and might determine less detrimental effects than truncating variants, which are found more frequently in INAD/ANAD. As previously suggested, different mutation sites in iPLA₂β domains can lead, directly or indirectly, to different changes in its enzymatic activity, which might be a critical factor in the phenotypic heterogeneity of PLAN.⁶⁹ This is supported by the observation that all individuals with two null alleles manifest with INAD, while most patients with ANAD or late-onset phenotypes carry two missense *PLA2G6* mutations.¹⁵ Furthermore, it is consistent with the few biochemical and enzymatic studies available. Engel et al.⁶⁹ demonstrated in vitro that mutations associated with different *PLA2G6* phenotypes have different impact on its catalytic activity. Mutations associated with INAD/NBIA cause loss of enzyme activity, with mutant proteins exhibiting less than 20% of wild-type enzymatic activity in both lysophospholipase and phospholipase assays. In contrast, three mutations associated with dystonia-parkinsonism (c.1894C>T, c.2222G>A, and c.2239C>T) do not impair phospholipase or lysophospholipase catalytic activity. Shi et al.⁴⁵ documented that the *PLA2G6* variant c.991G>T is associated with approximately 30% residual protein activity compared with the wild-type iPLA₂β. Finally, Zhou et al.⁷⁰ found defective activation of endogenous store-operated Ca²⁺ and iPLA₂β activation in cells from a patient with familial PD carrying the c.2239C>T mutation in *PLA2G6*. Functional studies of the tertiary/quaternary structure of mutant iPLA₂β and its residual enzymatic activity/regulation are needed to further explore phenotype-genotype correlations.

Limitations of this study are mainly attributable to its retrospective nature. Frequencies of clinicoradiological features were calculated as valid percentages, and this, or any possible alternative imputation, is not exempt from risk for underestimation or overestimation. Furthermore, considerations on the natural disease history were limited by the lack of precise information on symptom/sign onset and follow-up in most published cases. Nevertheless, we are confident this is the most comprehensive analysis of the largest population with *PLA2G6*-related parkinsonism hitherto reported. Finally, we acknowledge that the search strategy did not allow to compare *PLA2G6* cases with and without parkinsonism in perspective of gene-specific therapies. However, we believe that clinicoradiological clues highlighted by this study might increase the clinical suspicion and prompt genetic testing for *PLA2G6*

parkinsonism among different etiologies of early-onset parkinsonism, thus favoring precision medicine in the not-too-distant future.

In conclusion, biallelic *PLA2G6* mutations cause early-onset parkinsonism with dystonia, pyramidal and cerebellar signs, myoclonus, and cognitive impairment. Early psychiatric manifestations and bladder overactivity are common. Early, severe dyskinesias are a tell-tale sign. Against this background, irrespective of whether iron deposition is present on brain MRI, the detection of cerebellar atrophy points toward *PLA2G6* among other genetic causes of early-onset parkinsonian, pallidopyramidal, and NBIA syndromes. These clinico-radiological features should prompt *PLA2G6* mutation analysis, which might have considerable therapeutic implications in the near future, given promising preclinical disease-modifying strategy development.¹⁹ ■

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DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary material as well as on request from the corresponding authors.

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(2) Statistical Analysis: A. Design, B. Execution, C. Review and Critique;
(3) Manuscript: A. Writing of the first draft, B. Review and Critique.

F.M.: 1A, 1B, 1C, 2A, 2B, 3A

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