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A panel study on patients with dominant cerebellar ataxia highlights the frequency of channelopathies

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Autosomal dominant cerebellar ataxias have a marked heterogeneous genetic background, with mutations in 34 genes identified so far. This large amount of implicated genes accounts for heterogeneous clinical presentations, making genotype–phenotype correlations a major challenge in the field. While polyglutamine ataxias, linked to CAG repeat expansions in genes such as *ATXN1*, *ATXN2*, *ATXN3*, *ATXN7*, *CACNA1A* and *TBP*, have been extensively characterized in large cohorts, there is a need for comprehensive assessment of frequency and phenotype of more 'conventional' ataxias. After exclusion of CAG/polyglutamine expansions in spinocerebellar ataxia genes in 412 index cases with dominantly inherited cerebellar ataxias, we aimed to establish the relative frequencies of mutations in other genes, with an approach combining panel sequencing and TaqMan® polymerase chain reaction assay. We found relevant genetic variants in 59 patients (14.3%). The most frequently mutated were channel genes [*CACNA1A* ($n = 16$), *KCND3* ($n = 4$), *KCNC3* ($n = 2$) and *KCNA1* ($n = 2$)]. Deletions in *ITPR1* ($n = 11$) were followed by biallelic variants in *SPG7* ($n = 9$). Variants in *AFG3L2* ($n = 7$) came next in frequency, and variants were rarely found in *STBN2* ($n = 2$), *ELOVL5*, *FGF14*, *STUB1* and *TTBK2* ($n = 1$ each). Interestingly, possible risk factor variants were detected in *SPG7* and *POLG*. Clinical comparisons showed that ataxias due to channelopathies had a significantly earlier age at onset with an average of 24.6 years, versus 40.9 years for polyglutamine expansion spinocerebellar ataxias and 37.8 years for *SPG7*-related forms ($P = 0.001$). In contrast, disease duration was significantly longer in the former (20.5 years versus 9.3 and 13.7, $P=0.001$), though for similar functional stages, indicating slower progression of the disease. Of interest, intellectual deficiency was more frequent in channel spinocerebellar ataxias, while cognitive impairment in adulthood was similar among the three groups. Similar differences were found among a single gene group, comparing 23 patients with *CACNA1A* expansions (spinocerebellar ataxia 6) to 22 patients with *CACNA1A* point mutations, which had lower average age at onset (25.2 versus 47.3 years) with longer disease duration (18.7 versus 10.9), but lower severity indexes (0.39 versus 0.44), indicating slower progression of the disease. In conclusion, we identified relevant genetic variations in up to 15% of cases after exclusion of polyglutamine expansion spinocerebellar ataxias, and confirmed *CACNA1A* and *SPG7* as major ataxia genes. We could delineate firm genotype–phenotype correlations that are important for genetic counselling and of possible prognostic value.

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Abbreviation: SCA = spinocerebellar ataxia

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Introduction

Hereditary cerebellar ataxias are a heterogeneous group of neurological diseases presenting as a cerebellar syndrome, with a combination of gait alteration, limb incoordination, dysarthria, and eye movement anomalies (Schols *et al.*, 2004). Additional neurological (e.g. pyramidal signs, seizures, cognitive impairment, extrapyramidal symptoms such as dystonia) or extraneurological (e.g. cardiomyopathy, diabetes, chorioretinal dystrophy) signs often complete the clinical picture along with evolution (Durr, 2010). Progressive cerebellar syndromes can be of various origins, but many forms are genetically inherited. They can be transmitted on all described inheritance modes, including autosomal dominant.

The genetic background of autosomal dominant cerebellar ataxias, so-called spinocerebellar ataxias (SCAs), is heterogeneous, with at least 34 genes identified. The most frequent forms are due to CAG trinucleotide repeat expansions in seven genes leading to polyglutamine stretch elongation in their respective proteins: *ATXN1* (MIM 601556)/SCA1 (MIM 164400) (Orr *et al.*, 1993), *ATXN2* (MIM 601517)/SCA2 (MIM 183090) (Pulst *et al.*, 1996), *ATXN3* (MIM 607047)/SCA3 – Machado-Joseph disease (MIM 109150) (Kawaguchi *et al.*, 1994), *CACNA1A* (MIM 601011)/SCA6 (MIM 183086) (Zhuchenko *et al.*, 1997), *ATXN7* (MIM 607640)/SCA7 (MIM 164500) (David *et al.*, 1997), *TBP* (MIM 600075)/SCA17 (MIM 607136) (Koide *et al.*, 1999), *ATN1* (MIM 607462)/dentatorubral-pallidoluysian atrophy (MIM 125370)

(Koide *et al.*, 1994). In SCA8, CTG/CAG trinucleotide repeat expansions were described in patients (Koob *et al.*, 1999), then also in controls (Stevanin *et al.*, 2000; Worth *et al.*, 2000), leading to controversy as to their pathogenic role. In transgenic mice, they induce a progressive neurological phenotype, and encode almost pure polyglutamine stretches from the *ATXN8* transcript, and an RNA with non-coding CUG expansion from the opposite strand transcript *ATXN8OS* (Moseley *et al.*, 2006).

Intronic expansions have also been described in SCA10 (Matsuura *et al.*, 2004) (MIM 603516) with ATTCT repeats in intron 9 of *ATXN10* (MIM 611150), SCA12 (Holmes *et al.*, 1999) (MIM 604326) with CAG repeats in the 5'UTR region of *PPP2R2B* (MIM 604325), SCA31 (Sato *et al.*, 2009) (MIM 117210) with TGGAA pentanucleotide repeats in a common intron of genes *BEAN* (MIM 612051) and *TK2* (MIM 188250), and SCA36 (Kobayashi *et al.*, 2011) (MIM 614153) with GGCCTG hexanucleotide repeats in the first intron of *NOP56* (MIM 614154).

Conventional mutations have been described in 22 genes so far (Supplementary Table 1): *SPTBN2*/SCA5 (Ikeda *et al.*, 2006), *TTBK2*/SCA11 (Houlden *et al.*, 2007), *KCNC3*/SCA13 (Waters *et al.*, 2006), *PRKCG*/SCA14 (Chen *et al.*, 2003), *ITPR1*/SCA15-16 (van de Leemput *et al.*, 2007), *KCND3*/SCA19-22 (Duarri *et al.*, 2012; Lee *et al.*, 2012), *TMEM240*/SCA21 (Delplanque *et al.*, 2014), *PDYN*/SCA23 (Bakalkin *et al.*, 2010), *EEF2*/SCA26 (Hekman *et al.*, 2012) (one family), *FGF14*/SCA27 (van Swieten *et al.*, 2003), *AFG3L2*/SCA28 (Di Bella *et al.*, 2010), *ELOVL4*/SCA34 (Cadieux-Dion *et al.*, 2014) (one family), *TGM6*/SCA35 (Wang *et al.*, 2010*a*), *ELOVL5*/SCA38 (Di Gregorio *et al.*, 2014), *CCDC88C*/SCA40 (Tsoi *et al.*, 2014) (one family), *TRPC3*/SCA41 (Fogel *et al.*, 2015) (one patient), *CACNA1A* (Yue *et al.*, 1997), *DNMT1* (Winkelmann *et al.*, 2012), *OPA1* (Amati-Bonneau *et al.*, 2008; Hudson *et al.*, 2008), *VAMP1*/SPAX1 (Bourassa *et al.*, 2012) (SPAstic ataXia type 1, one family), and *GRID2* (Coutelier *et al.*, 2015*b*). The involvement of *IFRD1* in SCA18 is debated (Brkanac *et al.*, 2009), as the only reported mutation is rather frequent in the ExAC database (www.exac.broadinstitute.org, 58 heterozygous patients/60 655); and as the affected amino acid is not conserved in distant mammals such as the elephant. Biallelic mutations in *POLG* are responsible for a broad clinical spectrum, including sensitive or cerebellar ataxia in some instances (Van Goethem *et al.*, 2001; Luoma *et al.*, 2005). If autosomal dominant transmission has been well documented in progressive external ophtalmoplegia (Van Goethem *et al.*, 2001; Lamantea *et al.*, 2002), it is still unclear whether heterozygous variant can cause cerebellar ataxia (Schulte *et al.*, 2009).

Compared to polyglutamine ataxias, these conventional forms are more slowly progressive (Durr, 2010), with a longer survival and a less severe disability stage (Monin *et al.*, 2015). The cerebellar component is more important than additional signs, which are prominent in polyglutamine SCAs, where intrafamilial presentation varies more (Marelli *et al.*, 2011a). Mutations in *DNMT1*, *OPA1* and *POLG* account for more complex phenotypes, with deafness and narcolepsy in the first case, and mitochondria-related symptoms in both others (optic atrophy, spasticity). *VAMP1* variants are responsible for a markedly spastic ataxia.

Until recently, the classical genetic work-up in dominant cerebellar ataxias was to genotype patients for CAG repeats in polyglutamine SCA genes. Mutations in other genes were only looked for in case of clinical suspicion, which was not frequent because phenotype–genotype correlations are non-univocal (Coutelier *et al.*, 2015*c*), and few specific symptoms exist for most conventional SCAs. Hence, many patients were never screened for most of these genes, and diagnostic yield remained low, with around 40% of individuals without diagnostic (Durr, 2010; Ruano *et al.*, 2014). We thus aimed to (i) assess the efficiency of a panel sequencing approach in dominant cases of cerebellar ataxias; and (ii) study the frequency and phenotype of a large cohort of index cases for mutations in conventional SCA genes.

Material and methods

Patient recruitment, clinical evaluation and initial genetic work-up

Four hundred and twelve index patients were recruited as part of the SPATAX cohort of patients with Spastic Paraplegia and Ataxia (https://spatax.wordpress.com/). They were examined by at least one member of the SPATAX network, and clinically assessed with a standardized evaluation form (https://spatax.files.wordpress.com/2013/09/fichecliniquespatax-eurospa-2011.pdf). Functional stages were evaluated as follows: 0, no functional handicap; 1, no functional handicap but signs at examination; 2, mild functional handicap, able to run; 3, moderate functional handicap, unable to run, limited walking without aid; 4, severe functional handicap, walking with one stick; 5, walking with two sticks; 6, unable to walk, requiring wheelchair; 7, confined to bed. Autosomal dominant inheritance was assumed based on positive first-degree familial history in more than 95% of patients. However, in many cases, DNA was only available for the index patient. Five patients were sporadic, three had unclear familial history due to premature death of one parent, and four had first or second degree relatives with another neurological disorder (Parkinson's disease, tremor, supranuclear palsy or uncategorized motor impairment). Patients gave informed consent, and blood samples were collected in accordance with local French regulations [Paris Necker ethics committee approval (RBM 01-29 and RBM 03-48) to A.B. and A.D.]. DNA was extracted using standard procedures. Polyglutamine expansions in *ATXN1*, *ATXN2*, *ATXN3*, *ATXN7*, *CACNA1A* and *TBP* were excluded in all

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patients. In previous research settings, several genes have been screened for in a subset of patients and positive cases therefore excluded (including four SCA5, one SCA11, one SCA12, six SCA13, 11 SCA14, one SCA22, three SCA23, nine SCA28, 12 SCA36). During the course of the study, causative mutations were nevertheless identified in six patients of the cohort (one *HTT*, one *FA2H*, one SCA1, two SCA2, one SCA3). *ITPR1* deletions, responsible for the SCA15 subtype of SCA, were looked for in all index cases, with a TaqMan® PCR assay designed by Obayashi *et al.* (2012), in parallel with panel sequencing. Results were analysed for probes 1 to 3 only, as probe 4 gave poorly reproducible results.

Gene list establishment

All genes implicated in ataxia by April 2014 were included: *AFG3L2*, *CACNA1A*, *DNMT1*, *EEF2*, *ELOVL5*, *FGF14*, *GRID2*, *IFRD1*, *ITPR1*, *KCNC3*, *KCND3*, *OPA1*, *PDYN*, *POLG*, *PRKCG*, *SPTBN2*, *TGM6*, *TTBK2*, and *VAMP1*, as mentioned above. Because point mutations in *CACNA1A* were first recognized to cause episodic ataxia (Ophoff *et al.*, 1996) (EA), then extended to progressive cerebellar ataxia (Yue *et al.*, 1997), and because interictal ataxia may occur in episodic ataxia, three genes involved in episodic ataxia were also included: *KCNA1* (EA1; Browne *et al.*, 1994), *CACNB4* (EA5; Escayg *et al.*, 2000), and *SLC1A3* (EA6; Jen *et al.*, 2005). A few other genes whose mutations are related to other core traits before ataxia were also added: *ITM2B*, linked to dementia (British-type and Danish-type) with ataxia (Vidal *et al.*, 1999); *NOL3*, whose mutation in one pedigree caused familial cortical myoclonus, later followed by ataxia (Russell *et al.*, 2012); *REEP1* and *SPG7*, whose variants are associated with prominent hereditary spastic paraplegia, with a frequent cerebellar component (Casari *et al.*, 1998; Zuchner *et al.*, 2006; Klebe *et al.*, 2012). If *SPG7* causative variants are classically biallelic in hereditary spastic paraplegia, autosomal dominant transmission has been established in optic neuropathy (Klebe *et al.*, 2012). Relatives of affected individuals that carried heterozygous variants were also shown to harbour frequent cerebellar atrophy, or slight late-onset cerebellar signs (Klebe *et al.*, 2012). *STUB1*, implicated in autosomal recessive cerebellar ataxia (Shi *et al.*, 2013), was also included to assess a potential implication in SCAs, since we otherwise found a heterozygous variant segregating in one family. Because they had not been described in SCAs by the time we designed the panel, *CCDC88C*, *ELOVL4*, *TMEM240* and *TRPC3* were not included.

The full list of known genes considered for sequencing is described in Supplementary Table 1.

Candidate genes were also included, which brought the number of genes to 65. Among them, *CACNA1G* was sequenced, which allowed confirmation of its implication in SCA (Coutelier *et al.*, 2015*a*).

Panel sequencing

Primers to amplify the 1150 amplicons covering the 65 genes selected were designed by Fluidigm Primer D3 Design Assay. DNA was amplified using the Fluidigm Access Array™ system, according to the manufacturer's protocol (Fluidigm). Briefly, 48 × 48 chips were loaded with, on one side, 50 ng of DNA and a PCR mix containing FastStart™ High Fidelity enzyme (Roche); and, on the other side, pools of up to 12 primer couples diluted in Access Array™ loading reagent. After chip loading, amplification, and chip harvesting, samples were barcoded with an index PCR on 100 × diluted harvested product. Indexed pools were purified with SPRIselect beads (Beckman Coulter), and then pooled equimolarily. Once purified, the final library was sequenced on a MiSeq Illumina Sequencer as 2 × 300 base pair reads using standard protocols (Illumina).

Bioinformatics processing and variant analysis

Fastq sequences were aligned to the human genome v19 reference sequence using BWA (Li and Durbin, 2009), then GATK (McKenna *et al.*, 2010) for local realignment and recalibration, as well as detection of variants. Variants were annotated with Annovar (Wang *et al.*, 2010*b*), then filtered with the following criteria: (i) quality (PASS GATK filter); (ii) effect on coding sequence (exonic non-synonymous or splice variant); (iii) frequency in public databases (< 1% in EVS); (iv) internal database frequency (< 5%); and (v) heterozygosity (allelic frequency between 0.3 and 0.7). Manual curation of all variants was performed, with a refinement on public frequency threshold made possible by the release of ExAC database (http://exac.broadinstitute.org; < 0.01% for heterozygous variants) and a correlation to the clinical picture. All possibly relevant variants were Sanger sequenced using classical procedures, as well as most of the variants of unknown significance. When available, segregation of the variant was assessed in family members (Table 1 and Supplementary Fig. 3).

Statistical analysis of clinical features of patients

Clinical features were compared between (i) patients with a molecular diagnosis after screening and patients without diagnosis; (ii) patients carrying a conventional mutation in a channel gene (*CACNA1A*, *CACNA1G*, *KCNA1*, *KCNC3*, *KCND3*), a conventional mutation in the *SPG7* pathway (heterozygous in *AFG3L2*, biallelic in *SPG7*), and patients with a CAG repeat expansion in *ATXN1*, *ATXN2* or *ATXN3*; and (iii) patients carrying a conventional mutation in *CACNA1A* and patients carrying an expansion in *CACNA1A* (SCA6). For all comparisons, relatives of index cases in which segregation of the variant had been confirmed were included. For comparisons (ii) and (iii),

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Table 1 Class 1, class 2 and class 3 variants

Patient ID	Gene	Status	Variant	Pathogenicity predictions	GERP++	Other	ExAC	Segregation	ACMG classification
AAD-636-3	*CACNA1A*	Class 1	NM_001127222:c.1745G > A:p.R582Q	Known variant	–	–	0	NA	Pathogenic
AAD-414-3	*CACNA1A*	Class 1	NM_001127222:c.2039_2040del:p.680_680del	Frameshift variant	–	–	0	NA	Pathogenic
AAD-899-13	*CACNA1A*	Class 1	NM_001127222:c.3787G > A:p.E1263K	Known variant	–	–	0	TRUE (2 affected)	Pathogenic
AAD-555-11	*CACNA1A*	Class 1	NM_001127222:c.4034G > A:p.R1345Q	Known variant	–	–	0	NA	Pathogenic
AAD-870-1	*CACNA1A*	Class 1	NM_001127222:c.4034G > A:p.R1345Q	Known variant	–	–	0	NA	Pathogenic
AAD-202-7	*CACNA1A*	Class 1	NM_001127222:c.4979G > A:p.R1660H	4/4	4.96	Close to known R1664Q, recurrent in another study	0	NA	Likely pathogenic
AAD-750-3	*CACNA1A*	Class 1	NM_001127222:c.4996C > T:p.R1666W	Known variant	–	–	0	NA	Pathogenic
14GX000698	*CACNA1A*	Class 1	NM_001127222:c.5200G > T:p.E1734X	Nonsense variant	–	–	0	NA	Pathogenic
AAD-82-4	*CACNA1A*	Class 1	NM_001127222:c.5248C > T:p.R1750W	Known variant	–	–	0	TRUE (6 affected)	Pathogenic
AAD-890-6	*CACNA1A*	Class 1	NM_001127222:c.6028C > T:p.Q2010X	Nonsense variant	–	–	0	NA	Pathogenic
AAD-883-1	*CACNA1A*	Class 2	NM_001127222:c.835C > T:p.R279C (+ NM_001127221:c.5575A > G:p.I1859V (non-canonical transcript))	4/4	4.24	Close to known C287Y	0/0	NA	Likely pathogenic
AAD-603-1	*CACNA1A*	Class 2	NM_001127222:c.880C > T:p.P294S	4/4	5.42	Close to known G293R	0	NA	Likely pathogenic
AAD-875-1	*CACNA1A*	Class 2	NM_001127222:c.2026G > A:p.G676R	4/4	4.58	Close to known E668K	0	NA	Likely pathogenic
AAD-263-11	*CACNA1A*	Class 3	NM_001127222:c.2056G > A:p.G686S	2/4	3.55	Close to known E668K	0	NA	Uncertain significance; no strict criteria for benign, divergent *in silico* predictions
AAD-747-11	*CACNA1A*	Class 3	NM_001127222:c.4448G > T:p.R1483L	3/4	5.13	Close to known F1491S	0	NA	Likely pathogenic
AAD-70-7	*CACNA1A*	Class 3	NM_001127221:c.5578_5579insAT:p.S1860fs (non-canonical transcript)	Frameshift variant	–	Not in canonical transcript	0	NA	Pathogenic
AAD-785-9	*SPG7*	Class 1	NM_003119:c.1529C > T:p.A510V; c.858dupT:p.A286fs	A510V + frameshift variant	–	–	306/0	NA	Pathogenic/pathogenic
AAD-248-12	*SPG7*	Class 1	NM_003119:c.958G > T:p.E320X; c.1047dupC:p.G349fs	Nonsense + frameshift variants	–	–	0/0	NA	Pathogenic/pathogenic
AAD-420-1	*SPG7*	Class 1	NM_003119:c.1529C > T:p.A510V;c.1519C > T:p.Q507X	A510V + nonsense variant	–	–	306/0	NA	Pathogenic/pathogenic
AAR-541-13	*SPG7*	Class 1	NM_003119:c.1529C > T:p.A510V;c.1519C > T:p.Q507X	A510V + nonsense variant; also detected by WES (Coutelier *et al.*, in preparation)	–	–	306/0	TRUE (3 affected)	Pathogenic/pathogenic
AAD-951-11	*SPG7*	Class 1	NM_003119:c.1529C > T:p.A510V x2	Homozygous A510V	–	–	306/306	NA	Pathogenic/pathogenic
AAD-847-18	*SPG7*	Class 1	NM_003119:c.1529C > T:p.A510V x2	Homozygous A510V	–	–	306/306	TRUE (3 affected)	Pathogenic/pathogenic
AAD-1033-1	*SPG7*	Class 1	NM_003119:c.1529C > T:p.A510V x2	Homozygous A510V	–	–	306/306	NA	Pathogenic/pathogenic
SAL-399-1026	*SPG7*	Class 1	NM_003119:c.1529C > T:p.A510V x2	Homozygous A510V	–	–	306/306	NA	Pathogenic/pathogenic
AAD-458-5	*SPG7*	Class 1	NM_003119:c.1529C > T:p.A510V;c.2249C > T:p.P750L	A510V + known variant	–	–	306/3/120860	NA	Pathogenic/pathogenic
AAD-886-8	*AFG3L2*	Class 1	NM_006796:c.1961C > T:p.T654I	Known variant	–	–	0	NA	Pathogenic
AAD-994-1	*AFG3L2*	Class 1	NM_006796:c.1996A > G:p.M666V	Known variant	–	–	0	NA	Pathogenic
AAD-315-17	*AFG3L2*	Class 1	NM_006796:c.2059A > G:p.K687E	Recurrent variant, 3/4	5.62	In proteolytic domain	0	NA	Likely pathogenic
AAD-512-11	*AFG3L2*	Class 1	NM_006796:c.2059A > G:p.K687E	Recurrent variant, 3/4	5.62	In proteolytic domain	0	NA	Likely pathogenic
AAD-541-19	*AFG3L2*	Class 2	NM_006796:c.1450G > C:p.A484P	4/4	6.03	In AAA domain	1/121360	NA	Uncertain significance; no criteria for benign
AAD-1009-1	*AFG3L2*	Class 2	NM_006796:c.2062C > G:p.P688A	4/4	5.62	In proteolytic domain, close to known Y689H	0	NA	Likely pathogenic
AAD-1005-1	*AFG3L2*	Class 3	NM_006796:c.1430C > T:p.P477L	2/4	6.03	In AAA domain	0	NA	Uncertain significance; no strict criteria for benign, divergent *in silico* predictions
AAD-722-1	*KCND3*	Class 1	NM_004980:c.1348C > T:p.L450F	Previously reported patient	–	–	14/90828	NA	Likely pathogenic
AAD-114-9	*KCND3*	Class 1	NM_004980:c.1897C > T:p.P633S	Previously reported patient	–	–	3/121388	NA	Uncertain significance; no strict criteria for benign, divergent *in silico* predictions
AAD-374-1	*KCND3*	Class 2	NM_004980:c.1086T > G:p.I362M	4/4	−8. but conserved amino acid across species	Close to known T352P and M373I	0	TRUE (3 affected)	Likely pathogenic

(continued)

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Table 1 Continued

Patient ID	Gene	Status	Variant	Pathogenicity predictions	GERP++	Other	ExAC	Segregation	ACMG classification
AAD-484-1	*KCND3*	Class 2	NM_004980:c.1094T > C:p.M365T	4/4	5.59	–	0	NA	Uncertain significance; no criteria for benign
AAD-350-9	*KCNA1*	Class 1	NM_000217:c.677C > T:p.T226M	Known variant	–	–	0	NA	Pathogenic
ENE-13-18	*KCNA1*	Class 2	NM_000217:c.508G > T:p.A170S	4/4	4.91	Heterozygous A510V in *SPG7*	0	TRUE (two affected)	Likely pathogenic
AAD-982-3	*KCNC3*	Class 1	NM_004977:c.1268G > A:p.R423H	Known variant	–	–	0	TRUE (two affected)	Pathogenic
AAD-449-3	*KCNC3*	Class 3	NM_004977:c.1420G > A:p.A474T	3/4	3.36	–	0	NA	Uncertain significance; no criteria for benign
AAD-479-5	*SPTBN2*	Class 2	NM_006946:c.1294C > A:p.R432S	4/4	3.7	In second spectrin repeat	0	NA	Likely pathogenic
AAD-483-1	*SPTBN2*	Class 3	NM_006946:c.1261G > A:p.E421K	3/4	4.62	In second spectrin repeat	0	NA	Likely pathogenic
AAD-285-3	*ELOVL5*	Class 1	NM_001242828:c.214C > G:p.L72V	Previously reported patient	–	–	0	NA	Likely pathogenic
AAD-958-1	*FGF14*	Class 2	NM_004115:c.351G > T:p.Q117H	4/4	5.87	–	0	NA	Uncertain significance; no criteria for benign
14GX000693	*STUB1*	Class 3	NM_005861:c.433A > C:p.K145Q;c.502C > T:p.L168F	3/4/4/4	4.59/4.59	Trans, close to W147C and L165F/concordant phenotype, mild cognitive impairment	82/116864/0	NA	Pathogenic/pathogenic
AAD-915-6	*TTBK2*	Class 1	NM_173500:c.1306_1307del:p.D436fs	Previously reported patient	–	–	0	NA	Pathogenic

List of variants of Classes 1, 2 and 3 (as described in main text) found by sequencing 412 index patients with dominantly inherited cerebellar ataxia. Pathogenicity predictions were assessed by Annovar annotation with SIFT, PolyPhen 2 HDIV, LRT and MutationTaster. WES = whole exome sequencing.

patients identified in a parallel study using whole exome sequencing in an independent autosomal recessive ataxia cohort (Coutelier *et al.*, submitted), were added. The full list of included patients, along with their mutations, is available in Supplementary Table 4. Statistical analyses were performed with the IBM SPSS Statistics version 21 software. Mean ages at onset, at examination and disease durations were compared with a parametric ANOVA. Clinical features frequencies were compared using non-parametric Fisher exact test.

Results

Panel sequencing allows satisfying coverage of about 90% of amplicons

Mean coverage per patient was 450×, with an average 90% of bases covered more than 30×. We found the difference between the mean and the median coverage to be a better indicator of sequencing quality for each individual than the mean coverage alone. Indeed, for five subjects, even though mean coverage was high (472× to 633μ), median coverage per base was much lower: some amplicons had been covered up to > 100 000× while most of them were not covered at all (Supplementary Fig. 1). We found this indicator to generally correlate with percentage of bases above 30×. The three most extreme patients had median coverage of 1×, 4×, and 14×; and percentage of bases above 30× of 19.4%, 46.6%, and 46.7%, respectively. Because of the poor quality of the results, all three were excluded from further analysis.

Coverage was satisfactory in ~90% of the 27 studied SCA gene amplicons (n = 344/382), i.e. average percentage of 10× coverage was above 90%. All amplicons were, however, not equally covered; Supplementary Fig. 2 shows the proportion of amplicons (y) with more than (x)% of coverage over both thresholds of 10 and 30×. A median of 301 amplicons (79%) was perfectly covered (100% at 30× coverage), while 24 (6%) were completely missed (0% at 30×) (Supplementary Fig. 2). Ninety per cent of amplified regions were covered above 10× for 18 genes (18/27, 67%); between 80 and 90% for an additional six (Supplementary Table 2).

Among all 65 genes, from 286 to 1428 (mean 357) SNPs, and from 51 to 80 (mean 67) indels were detected per patient; in known SCA genes, these numbers decreased to 107–652 (mean 152) SNPs and 12–33 (mean 25) indels.

Diagnostic rate reaches 7.2% of the naïve SCA cohort

After analysis, as described above, 0 to 4 variants were sequenced per patient, with a median number of 0 and an average of 0.51 in known genes; 87% of them were true

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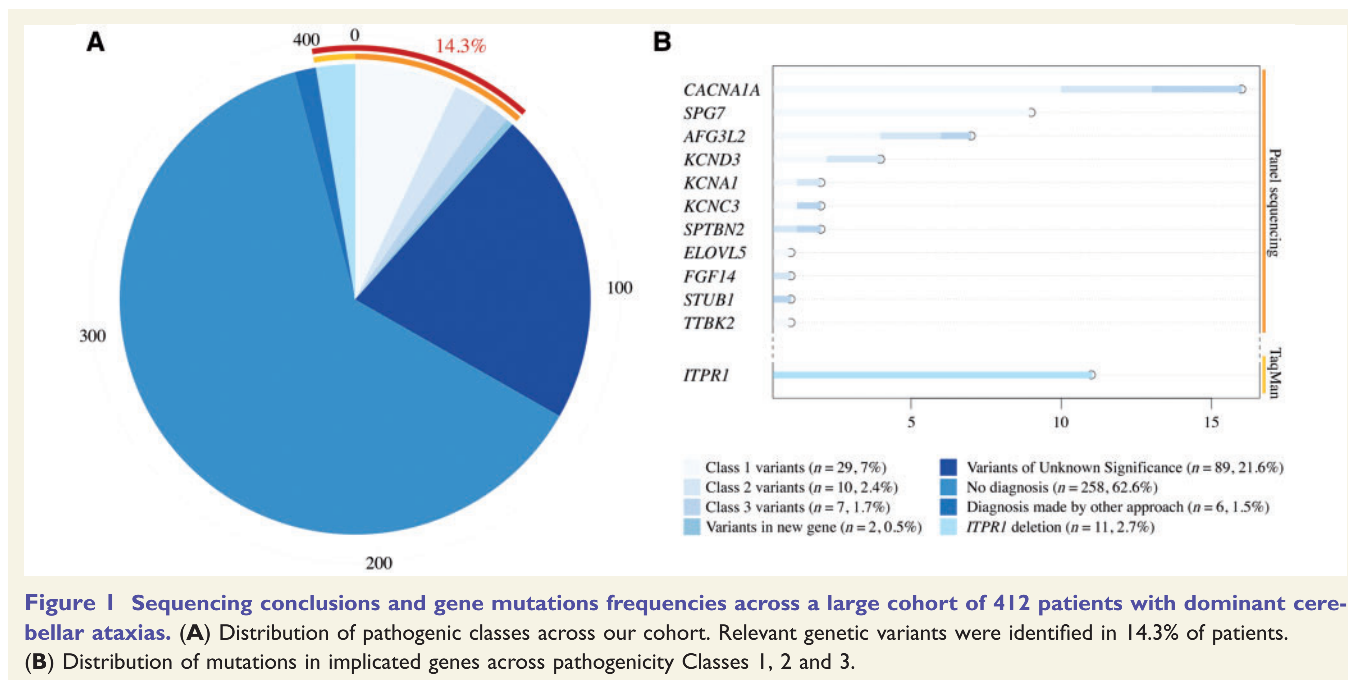

Figure 1 Sequencing conclusions and gene mutations frequencies across a large cohort of 412 patients with dominant cerebellar ataxias. (**A**) Distribution of pathogenic classes across our cohort. Relevant genetic variants were identified in 14.3% of patients. (**B**) Distribution of mutations in implicated genes across pathogenicity Classes 1, 2 and 3.

positives. As clinical presentations are rarely specific in dominant forms of cerebellar ataxia, we established a classification of variant pathogenicity based on stringent genetic criteria only. The variants fitting the characteristics used for filtering were distinguished into (Table 1 and Supplementary Table 5) (i) Class 1 variants: pathogenic variants (known or recurrent or null variant, or confirmed segregation in family; (ii) Class 2 variants: probably pathogenic variants (four concordant pathogenicity predictions by SIFT, PolyPhen HDIV, LRT and MutationTaster and GERP++ > 3); (iii) Class 3 variants: possibly causative variants (convincing genetic arguments but one of the above is missed); (iv) variants of unknown significance. All Class 1, 2, and 3 variants have been submitted to ClinVar. We chose not to strictly use American College of Medical Genetics and Genomics (ACMG) criteria for pathogenicity (Richards *et al.*, 2015), since we believe they are not perfectly suited for rare diseases. Variants sometimes fail to be classified as pathogenic due to the lack of fulfilled criteria linked to those disease group specificities, but not because they show evidence towards benign classification. Cohorts are indeed too small to reach statistical significance of variant frequency (criterion PS4). Furthermore, in cerebellar ataxias, *de novo* mutations (criteria PS2 and PM6) are not as classically expected as they are, for example, in autism spectrum disorders. Finally, the marked clinical and genetic heterogeneity of this disease group renders criterion PS1 (previous report of the variant) and PP4 (strict clinicogenetic concordance) difficult to achieve, while we believe that intra-study recurrence and clinical presumption should be taken into account. However, we assessed ACMG criteria for each Class 1, 2, and 3 variant, and they are summarized in Table 1 and detailed in Supplementary Table 3.

Altogether, 62.6% of patients had no convincing variants in known genes (either no variant at all, either benign polymorphism). The diagnostic yield (Class 1 + 2) was 9.5% (39 patients) (Fig. 1A), with variants across 11 genes. Another seven patients were classified as carrying possibly causative variants, and two were diagnosed with mutations in a new causative gene (Coutelier *et al.*, 2015*a*), which bring the percentage of patients with relevant genetic information to 11.7%. The most frequently mutated gene was *CACNA1A*, in 16 unrelated patients. Next in frequency were *SPG7* biallelic variants (nine patients), then monoallelic *AFG3L2* mutations (seven patients). Four patients harboured mutations in *KCND3*, including two that had been reported previously (Duarri *et al.*, 2012). Mutations in the seven other genes were classified in Classes 1, 2 or 3 for only one or two patients (Fig. 1B).

The TaqMan® PCR assay aiming at *ITPR1* allowed consistent detection of probe deletions in 11 patients (Supplementary Table 6), four of whom had been previously reported by other techniques (Marelli *et al.*, 2011*b*), validating the approach. The combined approach of panel sequencing and TaqMan® PCR assay allowed the identification of relevant genetic variants in 14.3% of patients (59 patients). Since polyglutamine expansions had previously been excluded in the cohort and are estimated to account for about half the patients with SCA, those patients would constitute 7.2% of the original cohort.

Possible risk factor variants are detected

Of interest were four specific variants, one in *SPG7* and three in *POLG*, which were recurrently found in our cohort. Their frequency in public databases is too high to

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consider them pathogenic alone; however, they were more frequent than expected in the SCA cohort. The *SPG7* p.A510V variant is found in 306/121348 chromosomes (0.25%) in the ExAC database; whereas in our study it was found in 20/824 chromosomes (2.43%). Even when considering only heterozygous patients, it represented 8/808 (0.99%). These results are statistically significant, with a Fisher's exact test *P*-value of 1.837×10^{-13} if patients with biallelic variants are considered, and 0.00129 if they are excluded.

Three *POLG* variants were also recurrent: 576/121362 (0.47%) chromosomes harbour the p.G517V variant in ExAC; 10/824 in our study (1.21%). The p.G268A variant is present in 422/121102 chromosomes (0.35%) in ExAC, and 7/824 (0.85%) in our cohort. Finally, 195/120460 ExAC chromosomes harbour the p.L392V change (0.16%), while it is the case for 4/824 chromosomes (0.49%) we sequenced. We calculated the significance of these results using a Fisher's test, at a 5% threshold of significance. All variants were shown to be statistically more frequent in our cohort (Table 2). No Dunn-Sidak correction was applied, since data are independent (in our cohort at least, no patient carries two of these four variants).

All four variants have slightly higher frequencies in the European American population of Exome Variant Server (Table 2). While considering those, the *SPG7* p.A510V is the only statistically significantly associated with ataxic presentation. However, the tendency for the others to be more frequent in our cohort remains true.

Of interest, we could identify the *POLG* p.G517V variant in a 5-plex family. Among the five patients, only three carried the variant, which establishes that it is not causative *per se*.

Diagnosed patients have earlier presentations than undiagnosed patients

Patients carrying Class 1 (known, recurrent, null or segregating) and Class 2 (unanimously predicted pathogenic and conserved) mutations were compared to patients without molecular diagnosis, either only harbouring variants of unknown significance, or no mutation at all in screened genes (Table 3). Age at onset was significantly earlier in diagnosed cases (32.6 versus 45.7), as was age at examination (48.5 versus 57.5), while disease duration was longer (16.8 versus 12). Severity index (disease stage divided by disease duration) was significantly lower in diagnosed patients (0.38 versus 0.58), which means that the disease progression was slower.

Conventional channelopathies have earlier onsets and more slowly progressive courses

Statistical comparison of clinical presentation of patients with SCA1, 2 or 3 (polyglutamine SCAs) versus patients with channelopathies or mAAA-related cerebellar ataxia (monoallelic *AFG3L2* and biallelic *SPG7* mutations) allowed highlighting of several differences (Table 4). Channelopathies had the earliest age at onset (average 24.6, versus 37.8 for mAAA-related forms and 40.9 for polyglutamine SCAs). Age of onset distribution was illustrative of the difference in tendency (Fig. 2). Disease duration was the longest for channelopathy SCAs (20.5 versus 9.3 and 13.7), for similar functional stages, indicating slower progression of the disease. Peripheral neuropathy, demonstrated by distal muscle wasting, and ophthalmoplegia were more frequent in mAAA-related ataxias, which is consistent with the mitochondrial role of proteins encoded by both *SPG7* and *AFG3L2*. Channelopathies coincided with more pure cerebellar presentations. However, intellectual deficiency was exclusively found in this group, while prevalence of cognitive impairment in adulthood was similar among all groups. Dysphagia, as with most other clinical associated signs, was more prominent in polyglutamien SCAs, frequently associating brainstem involvement, not showed in brain MRI collected for the most frequent punctual mutations found in this cohort (*CACNA1A*, *SPG7* and *AFG3L2*) (examples in Fig. 3). In each subgroup, brain MRI showed cerebellar atrophy. No associated signs were described, except for slight pons atrophy in SCA6. Cerebellar involvement was more prominent in patients

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Table 2 Potential risk factors identified

Gene	Variant	ExAC			ADCA cohort				EVS – European Americans			
		Wild-type count	Mutant count	Frequency	Wild-type count	Mutant count	Frequency	*P*	Wild-type count	Mutant count	Frequency	*P*
SPG7	p.A510V	121042	306	0.0025	804	20	0.0243	$\mathbf{1.84 \times 10^{-13}}$	8561	39	0.004555543	$\mathbf{5.66 \times 10^{-8}}$
POLG	p.G517V	120786	576	0.0047	814	10	0.0121	**0.007156**	8526	72	0.008444757	0.241075
POLG	p.G268A	120680	422	0.0035	817	7	0.0085	**0.02796**	8551	47	0.005496433	0.326859
POLG	p.L392V	120265	195	0.0016	820	4	0.0049	**0.04772**	8584	14	0.001630941	0.066154

Four recurrent rare variants were identified in our cohort, with higher frequencies than observed in the ExAC (http://exac.broadinstitute.org/) population. They were all significantly more present in our SCA cohort (bold font), as established with a Fischer test, at the threshold of 5%. Compared to the frequencies observed in the European American population of the Exome Variant Server (EVS, http://evs.gs.washington.edu) database, the difference is only statistically significant for the *SPG7* p.A510V variant.

Table 3 Clinical comparison between patients with dominant inherited cerebellar ataxias with or without mutations of diagnostic value after screening

	Diagnostic mutation	Non-diagnostic mutation/VUS	*P*
n = 410	63	347	
Females/males	22/41	174/173	**0.03**
Age at onset in years (range)	32.6 ± 20 (0–78)	45.7 ± 17 (0–69)	**0.001**
Age at examination in years (range)	48.5 ± 17 (3–79)	57.5 ± 13 (13–88)	**0.001**
Disease duration in years (range)	16.8 ± 14 (1–60)	12.0 ± 11 (1–70)	**0.005**
Functional stage (0–7)	2.8 ± 1.2	3.1 ± 1.3	0.09
Severity index (functional stage/ duration)	0.38 ± 0.53	0.58 ± 0.69	**0.04**
Cerebellar dysarthria	71%	69%	0.5
Extensor plantar reflex	29%	29%	1
Increased reflexes	13%	15%	0.08
Abolished reflexes	8%	13%	
Distal wasting	5%	11%	0.23
Decreased vibration sense at ankles	39%	46%	0.31
Dystonia	5%	5%	1
Parkinsonian syndrome	7%	11%	0.48
Ophthalmoplegia	7%	9%	0.71
Dysphagia	20%	29%	0.24
Urinary dysfunction	26%	31%	0.53
Late cognitive impairment	14%	20%	0.34
Early intellectual deficiency	14%	5%	**0.018**

Clinical comparison of natural evolution of the disease and main phenotypic traits in 63 patients with a diagnostic mutation (46 index cases, including 28 Class 1 variants, two causative *CACNA1G* variants, 10 Class 2 variants, and six *ITPR1* deletions; and 17 affected relatives) versus 347 index patients without molecular diagnosis [89 variant of unknown significance (VUS) and 258 without any variant after panel analysis].

carrying mutations in *CACNA1A* than in *SPG7* or *AFG3L2*, and further marked for the patient with point mutation than the patient with CAG expansion (Fig. 3).

Specific assessment of patients with *CACNA1A* expansions versus conventional mutations yielded similar observations. We compared 23 patients with *CACNA1A* expansions (SCA6) to 22 patients with *CACNA1A* point mutations identified in this study or in another independent whole exome sequencing study of sporadic and recessive cases (Coutelier *et al.*, submitted for publication). Clinical characteristics are summarized in Table 4. Individuals with conventional mutations had lower average age at onset (25.2 versus 47.3 years) and longer disease duration (18.7 versus 10.9). The severity index was higher for SCA6 patients (0.44 versus 0.39), indicating more rapid progression. The limited size of the reported groups might explain the lack of statistical power of the analysis, but this confirms the tendency observed between ataxias linked to channel gene point mutations and SCA1, 2 and 3. Pyramidal involvement is more frequent in SCA6 cases with expansions, whereas ophthalmoplegia is limited to patients with conventional mutations.

Clinical features of patients with *SPG7* mutations

Nine patients carried Class 1 *SPG7* mutations; four were homozygous for the p.A510V variant, four carried the p.A510V in compound heterozygosity with either a null variant (*n* = 3, two nonsense and one frameshift) or a missense mutation (*n* = 1), and one carried both a nonsense and a frameshift mutation. Average age at onset was 38 years, with a tendency for homozygous p.A510V carriers to have a later onset (42.5 years) than heterozygous carriers (33 years), even though the patient with two null mutations had an onset at 40 years. Seven of nine patients had brisk deep tendon reflexes, 6/9 had an extensor plantar reflex, and 3/9 were clearly spastic. On MRI, 7/9 patients presented cerebellar atrophy. Among the nine probands, five had a clear dominant history, with cerebellar signs reported in the mother (deceased or not examined, without DNA available for sequencing). Two pedigrees were multiplex one-generation families, with 3/10 and 3/6 affected probands, respectively; motor impairment in the grandfather and alcoholism in the father were reported in one; and dementia in the father in the ninth patient.

Discussion

In summary, we aimed to explore the nosology of dominantly inherited cerebellar ataxias linked to conventional mutations through assessment of their relative frequency in a large cohort, and genotype–phenotype correlations in the most numerous groups. We used an amplicon-based panel sequencing technique for 65 genes, including 27 known genes, on 412 index patients. We obtained good coverage of most amplicons (90% of the sequence of 90% of the amplicons was at least covered at 10 ×), with an acceptable rate of false positive variants detected (<15%). We combined the panel study with a TaqMan® PCR assay to detect deletions in *ITPR1*.

We yielded a very probable or definite diagnostic in 50 patients (12.1%), a possible diagnostic in seven, and confirmed the implication of a new gene in two, for a total of 14.3% (*n* = 59). Considering that about half the patients with dominantly inherited cerebellar ataxia bear $(CAG)_n$ repeat expansions, which had been previously excluded in this population, the diagnostic percentage amongst general population falls to 7.2%. This is rather low compared to other studies using panel sequencing or exome sequencing on undiagnosed cerebellar ataxia cohorts. Indeed, diagnostic rate in previous studies, on smaller cohorts though, ranged

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Table 4 Clinical comparison between patients with cerebellar ataxias due to distinct mutational subgroups

	SCAs polyQ[a]	Channelopathies[b]	*SPG7*-related[c]	*P*
n = 184 (index cases: *n* = 162)	109 (106)	37 (25)	38 (31)	
Females/males	53/56	14/23	11/27	0.088
Age at onset in years (range)	40.9 ± 12.1 (12–78)	24.6 ± 22 (0–78)	37.8 ± 12.5 (10–70)	**0.001**
Age at examination in years (range)	50.3 ± 12.9 (22–79)	44.7 ± 19.3 (3–79)	51.5 ± 10.1 (31–78)	0.073
Disease duration in years (range)	9.3 ± 6.8 (1–30)	20.5 ± 15.7 (1–60)	13.7 ± 11.4 (2–48)	**0.001**
Functional stage (0–7)	3.3 ± 1.5	3.1 ± 1.4	3.2 ± 1.1	0.602
Severity index (functional stage/duration)	0.55 ± 0.4	0.39 ± 0.66	0.43 ± 0.36	0.149
Cerebellar dysarthria	79%	70%	69%	0.4
Extensor plantar reflex	42%	27%	54%	0.08
Increased reflexes	20%	13%	32%	**0.001**
Abolished reflexes	31%	3%	3%	
Distal wasting	4%	6%	16%	**0.034**
Decreased vibration sense at ankles	55%	38%	49%	0.21
Dystonia	13%	6%	8%	0.52
Parkinsonian syndrome	2%	3%	8%	0.21
Ophthalmoplegia	21%	3%	25%	**0.03**
Dysphagia	42%	16%	29%	**0.017**
Urinary dysfunction	32%	18%	47%	**0.03**
Late cognitive impairment	10%	11%	14%	0.79
Early intellectual deficiency	0%	23%	0%	**0.005**

	CACNA1A polyglutamine expansions	*CACNA1A* point mutations	*P*
n = 45 (index cases *n* = 27)	23 (11)	22 (16)	
Females/males	11/12	10/12	1
Age at onset in years (range)	47.3 ± 11.4 (24–67)	25.2 ± 21.6 (0–65)	**<0.001**
Age at examination in years (range)	59.0 ± 14.3 (24–80)	43.9 ± 19.7 (3–70)	**0.005**
Disease duration in years (range)	10.9 ± 8.3 (0–31)	18.7 ± 16.2 (1–60)	0.054
Functional stage (0–7)	3.8 ± 1.9	3.1 ± 1.5	0.24
Severity index (functional stage/duration)	0.44 ± 0.27	0.39 ± 0.71	0.79
Cerebellar dysarthria	78%	55%	0.22
Extensor plantar reflex	23%	23%	0.18
Increased reflexes	35%	9%	**0.01**
Abolished reflexes	0%	14%	
Distal wasting	9%	4.5%	0.49
Extrapyramidal syndrome	13%	9%	0.1
Decreased vibration sense at ankles	52%	32%	0.34
Ophthalmoplegia	0%	4.5%	0.46
Cognitive impairment	9%	14%	0.87
Intellectual deficiency	4.30%	24%	0.09

Top: Clinical comparison between patients with cerebellar ataxias due to polyglutamine expansions (SCAs polyQ), mutations in channels genes (channelopathies) and mutations in *SPG7* linked genes (*SPG7*-related). *Bottom*: Clinical comparison between patients with cerebellar ataxias due to mutations in *CACNA1A*, either polyglutamine expansions or point mutations. Values with a significant *P*-value of <5% are highlighted in bold.

[a]SCA1 = 28; SCA2 = 24; SCA3 = 57.

[b]*CACNA1A* = 22; *CACNA1G* = 4; *KCNA1* = 3; *KCNC3* = 2; *KCND3* = 6.

between 18 and 46% (Nemeth *et al.*, 2013; Fogel *et al.*, 2014; Gomez and Das, 2014; Sawyer *et al.*, 2014; Pyle *et al.*, 2015). However, most of these studies aimed at patients with undiagnosed ataxias, regardless of the transmission mode or age at onset. Among adult patients, diagnostic rate was usually somehow lower, with the identification of the genetic background in 8.3% of 24 undiagnosed adult-onset progressive patients (capture sequencing; Nemeth *et al.*, 2013), 21% of 76 adult and sporadic cases (whole exome sequencing; Fogel *et al.*, 2014), and 33% of 12 sporadic patients (Keogh *et al.*, 2015). Moreover, many variants in the latter studies were described in genes whose mutations are transmitted on a recessive mode, which for the most part, were not included in our study.

This is low compared to diagnostic yield in other Mendelian diseases, which usually reaches about a third on average (Gomez and Das, 2014; Novarino *et al.*, 2014), and which could have several explanations. First, the most frequent mutations in SCAs remain polyglutamine expansions. Conventional mutations were estimated to

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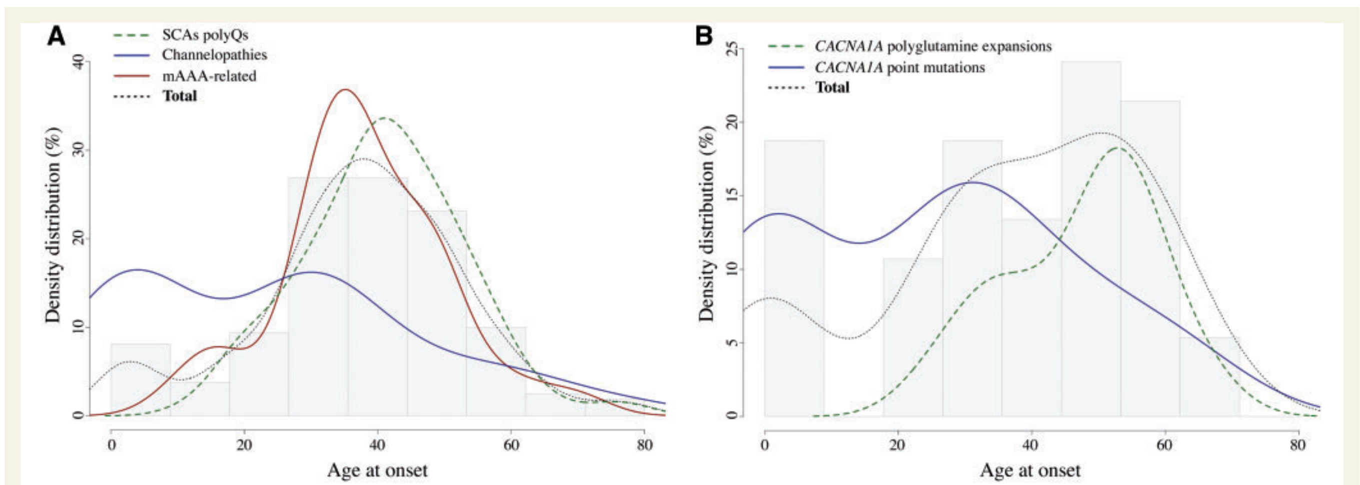

Figure 2 Age at onset density distribution for dominant cerebellar ataxias. (**A**) Age at onset density distribution for dominant cerebellar ataxias due to mutations in channel genes (*CACNA1A*, *CACNA1G*, *KCNC3*, *KCND3*, *KCNA1*) (solid blue), mAAA-related ataxias (biallelic *SPG7*, monoallelic *AFG3L2*) (solid red), or SCA1, 2 and 3 (dotted green). (**B**) Age at onset density distribution for dominant cerebellar ataxias due to *CACNA1A* polyglutamine expansions (dotted green) versus point mutations (solid blue). Light dotted dark lines and grey background histograms show the age at onset distribution for all considered patients as a whole.

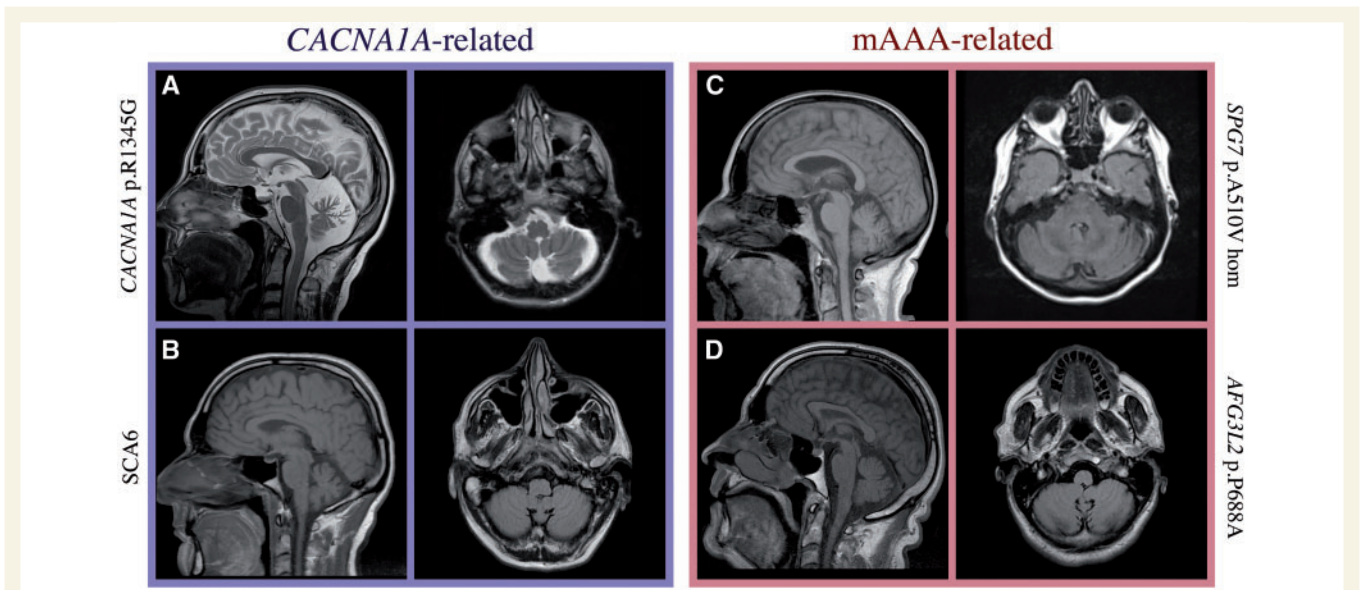

Figure 3 Brain MRI of patients carrying mutations in *CACNA1A* (point mutations and expansion), *SPG7* and *AFG3L2*, with similar severity indexes. (**A**) T_2-weighted sagittal and axial brain MRI showing marked cerebellar atrophy of Patient AAD-870, *CACNA1A* mutation p.R1345Q; age at onset: 28; age at examination: 52; severity index 0.13. (**B**) T_1-weighted sagittal and T_2 FLAIR axial brain MRI showing cerebellar and brainstem atrophy of Patient AAD-980, SCA6 [$(CAG)_n$ = 27]; age at onset: 45; age at examination: 47; severity index 0.5. (**C**) T_1-weighted sagittal and axial brain MRI showing mild cerebellar atrophy of Patient AAD-1033, *SPG7* homozygous mutation p.A510V; age at onset: 57; age at examination: 70; severity index 0.15. (**D**) T_1-weighted sagittal and axial brain MRI showing slight vermian atrophy of Patient AAD-1009; *AFG3L2* mutation p.P688A; age at onset: 47; age at examination: 57; severity index 0.1. Brainstem atrophy (arrow) is only noticed in the SCA6 patient, which is in line with the observed phenotypic differences. Cerebellar atrophy is more prominent in *CACNA1A*-related ataxias, and in particular in point mutation cases, than in mAAA-linked cases.

account for less than 10% of SCA cases (Durr, 2010). This, however, remained to be formally demonstrated, as, in many studies, only polyglutamine expansions were consistently looked for. We were thus able to confirm that estimate of ~10%, when reporting to the total number of cases with ataxia, including polyglutamine ataxias. Furthermore, we also report a relatively high number of variants of unknown significance (21.6%). This is not

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very surprising in cerebellar ataxias either, for which the phenotype is rarely pathognomonic, since most heterozygous rare variants within a known gene with some damaging effect predicted by *in silico* tools may often be considered. To further confirm pathogenicity of those variants, a convergence of evidences should be used, as described in the ACMG guidelines (Richards *et al.*, 2015). In the absence of biomarkers, and since functional studies cannot be performed for each variant, it is difficult to obtain higher confidence in pathogenicity. A major criterion, however, is the recurrence of (extremely) rare variants in patients with similar phenotypes, hence the importance of publicly sharing data. Finally, our cohort comes from a highly specialized university hospital, including a national reference centre for rare diseases, and some of the patients have already been variably screened for several genes and partly cleaned out through more than 20 years of genetic screening efforts. The study is thus biased in that the cohort is, for the most part, not naïve. This is confirmed by the identification of already known variants in some of the patients, which were found elsewhere during the study process. All in all, this low identification rate raises the attention of other genes, mutation types, and pathogenic mechanisms, which remain to be discovered.

Despite these facts, we could still identify the genetic origin of the ataxic phenotype in ~15% of the patients (~7.2% considering all ataxic patients before expansion screening). More importantly, among these patients, most carried variants in two genes, previously little recognized in progressive ataxia: *CACNA1A* and *SPG7*. Of note, this panel study also allowed us to describe patients with mutations in new SCA genes such as *CACNA1G*, which has been described elsewhere (Coutelier *et al.*, 2015*a*). Deletions in *ITPR1* are also relatively frequent, and should be looked for (Marelli *et al.*, 2011*b*).

CACNA1A point mutations were first recognized in episodic ataxia type 2 (EA2, loss-of-function variants) and familial hemiplegic migraine type 1 (gain-of-function variants) (Ophoff *et al.*, 1996). Small size polyglutamine expansions were then implicated in SCA6 (Zhuchenko *et al.*, 1997). They were shortly after described in EA2 (Jodice *et al.*, 1997), while point mutations were conversely implicated in SCA6 (Yue *et al.*, 1997). However, diagnostic work-up in progressive ataxia often fails to identify point mutations in *CACNA1A*, rather focusing on searching for polyglutamine expansions. We establish here that these are frequent causes of undiagnosed ataxia. Interestingly, no correlation seems to exist between specific variants and the phenotypic presentation, whether hemiplegic migraine, progressive, or episodic ataxia.

However, clinical presentations associated with point mutations differ from those linked to polyglutamine expansions, with an earlier age at onset for a similar functional disability score after a longer evolution. This observation was made while exclusively regarding mutations in *CACNA1A*, or considering wider SCA gene groups. The associated phenotype also differs. Channelopathies show a pure cerebellar syndrome, frequently combined with intellectual deficiency. This is in line with more general observations comparing the clinical presentation of patients with mutations in channel genes (*CACNA1A*, *CACNA1G*, *KCND3*, *KCNC3*), and patients with classical polyglutamine ataxias (SCA1, SCA2, SCA3). Those observations are of importance in the clinically heterogeneous group of cerebellar ataxias, where genetic counselling might prove difficult even amongst families. While phenotypic variability still lacks convincing genetic explanation, this is a first step in the delineation of firm genotype–phenotype correlation tendencies.

Though point mutations in *CACNA1A* are thus frequent in progressive ataxia, this does not seem to be the case for mutations in other episodic ataxia genes. Two causative or possibly causative variants were found in *KCNA1*, but the clinical picture of the patients included episodic ataxia. No variant was detected in *SLC1A3*. For *CACNB4*, the only variant we detected, p.R473H, is carried by a patient with a causative *CACNA1A* mutation. It is, however, conserved (GERP++ 5.26) and predicted to be pathogenic by all four software programs used, and might be a modifying factor, although the rarity of the observation makes it impossible to demonstrate. While we did not identify episodic symptoms in patients carrying *KCND3* or *KCNC3* variants, blurring of phenotypes seems to occur bidirectionally, and testing of SCA genes, especially channel genes, might prove to be successful in diagnosing episodic ataxias.

SPG7-linked phenotype has largely been remodelled since its first implication in autosomal recessive spastic paraplegia (Casari *et al.*, 1998), then optic neuropathy with a clear dominant pattern in one family (Klebe *et al.*, 2012). In the latter study, cerebellar ataxia co-occurred in 39% of patients (with cerebellar atrophy at MRI in almost all cases), and in some related heterozygous carriers. Biallelic mutations in *SPG7* were recently recognized as a major cause of undiagnosed ataxia (Pfeffer *et al.*, 2015). We confirm these results in our cohort, with nine patients identified as undoubtedly *SPG7*-mutated, carrying previously reported or loss-of-function biallelic mutations. All but one eventually developed pyramidal signs; 3/8 (37.5%) were clearly spastic. *SPG7* biallelic variants hence account for ~2% of our patients' cohort (9/412 = 2.2%). This is not as high as reported in Pfeffer's study (15/72 = 20.8%). Our cohort, however, predominantly includes patients with autosomal dominant transmission. This also sheds light on the fact that mutations in recessive cerebellar ataxia genes might be implicated in some of them, but missed because of the use of a panel strategy, which was noted in other studies (Fogel *et al.*, 2014; Keogh *et al.*, 2015). However, among those nine patients, at least five had a clear history of dominant transmission of cerebellar signs. This raises, again, the question of dominant inheritance of *SPG7* mutations, possibly with later-onset cerebellar signs in heterozygous carriers, as previously described in two individual patients carrying a heterozygous inframe deletion (Klebe *et al.*,

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2012). Unavailability of both DNA and patients for thorough examination keeps us from reaching more definite conclusions in our families.

Those examples of phenotype remodelling, linked to mutations in a given gene, raise the question of other overlapping genes, which we would not have included in our panel, and whose mutations may also give rise to cerebellar ataxia, even though not yet recognized. This is a main disadvantage of our choice of sequencing technique, and the next step in evaluating those patients would be to use whole exome or genome sequencing. However, amplicon-based panel sequencing has proven fast, still efficient, and markedly cost-effective.

Some variants of interest identified in our study may be risk factors in cerebellar ataxia; they are rare ($<1\%$), but too frequent to be considered pathogenic alone.

The pathogenicity of *SPG7* p.A510V variant has long been discussed; it is now recognized to be causative while homozygous, or in compound heterozygosity with a second pathogenic heterozygous variant (Elleuch *et al.*, 2006; Roxburgh *et al.*, 2013; Sanchez-Ferrero *et al.*, 2013). Among the nine patients we identified with SPG7 mutations, respectively, four were homozygous for that change, and four were compound heterozygous, with a loss-of-function or previously reported variant. The overall frequency of p.A510V variant in our cohort was significantly higher than in ExAC chromosomes, which was still the case when biallelic SPG7 patients were excluded. It might thus be a hypomorphic allele conferring a risk for the disease, even though not causative on its own. This is similar to what has been described for the p.S44L variant in *SPG4*; even though not pathogenic alone in most if not all cases, and quite frequent in general population, these variants are pathogenic at the homozygous state, and accentuate or induce a phenotype in combination with another pathogenic variant (Chinnery *et al.*, 2004).

The pathogenicity of *POLG* variant p.G517V has also been debated. It was first described in several pedigrees with dominant transmission (Horvath *et al.*, 2006; Burusnukul and de los Reyes, 2009; Hopkins *et al.*, 2010). Due to its occurrence in patients presenting variable ages of onset and clinical pictures, the mutant protein polymerase activity was assessed and shown to be similar to wild-type (Kasiviswanathan and Copeland, 2011). We demonstrated, in a 5-plex family that the variant does not segregate, being present in three patients only. This would argue against its being causative in SCA. *POLG* p.G268A variant is also reported in patients with diverse presentations (Graziewicz *et al.*, 2006). Mutant protein was shown to induce an altered growth phenotype in yeast, in a recessive pattern (Baruffini *et al.*, 2006). To the best of our knowledge, no functional study has been performed on variant p.L392V. Insufficient evidence has been gathered to consider *POLG* heterozygous variants causative in ataxia.

In some SCA genes, no variant of interest was identified (*SLC1A3*, *PDYN*, *ITM2B*); in several others, all (rare) identified changes were classified as variants of unknown significance (*EEF2*, *IFRD1*, *NOL3*). This sheds light on the fact that these genes are, at best, rarely implicated in ataxia, if at all. The case of *IFRD1* is enlightening. Only one variant has been described so far (Brkanac *et al.*, 2009). Its rarity and lack of conservation made its implication in dominant forms of cerebellar ataxia doubtful; the fact that we could not identify any other pathogenic variant in such a large cohort reinforces the presumption that the variant identified by Brkanac and colleagues (2009) may not be causative.

In conclusion, we sequenced a large cohort of index cases with dominantly inherited cerebellar ataxia, with an amplicon-based panel including 27 known genes. We reached a satisfactory coverage on most regions, though the obtained results will not allow direct implementation of the technique for diagnostic purposes. Combining the panel approach with a TaqMan® PCR assay aiming *ITPR1*, we identified (possibly) causative variations in up to 14.3% of cases, which is low compared to other diseases, but expected in SCAs. We confirmed the prominent implication of *CACNA1A* and *SPG7* point mutations in the disease, and underlined genotype–phenotype correlations in *CACNA1A*-linked progressive ataxias, with an earlier and purer phenotype for point mutations compared to polyglutamine expansions. This observation is more generally corroborated in ataxia-channelopathies.

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Supplementary material

Supplementary material is available at *Brain* online.

Appendix 1

SPATAX network collaborators

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