

Efficacy of Exome-Targeted Capture Sequencing to Detect Mutations in Known Cerebellar Ataxia Genes

Marie Coutelier, MD, PhD; Monia B. Hammer, PhD; Giovanni Stevanin, PhD; Marie-Lorraine Monin, MD; Claire-Sophie Davoine, MS; Fanny Mochel, MD, PhD; Pierre Labauge, MD, PhD; Claire Ewencyk, MD, PhD; Jinhui Ding, PhD; J. Raphael Gibbs, PhD; Didier Hannequin, MD, PhD; Judith Melki, MD, PhD; Annick Toutain, MD, PhD; Vincent Laugel, MD, PhD; Sylvie Forlani, PhD; Perrine Charles, MD, PhD; Emmanuel Broussolle, MD, PhD; Stéphane Thobois, MD, PhD; Alexandra Afenjar, MD; Mathieu Anheim, MD, PhD; Patrick Calvas, MD, PhD; Giovanni Castelnovo, MD; Thomas de Broucker, MD; Marie Vidailhet, MD; Antoine Moulignier, MD; Robert T. Ghassia, MD; Chantal Tallaksen, MD, PhD; Cyril Mignot, MD, PhD; Cyril Goizet, MD, PhD; Isabelle Le Ber, MD, PhD; Elisabeth Ollagnon-Roman, MD; Jean Pouget, MD, PhD; Alexis Brice, MD; Andrew Singleton, PhD; Alexandra Durr, MD, PhD; for the Spastic Paraplegia and Ataxia Network

Supplemental content

IMPORTANCE Molecular diagnosis is difficult to achieve in disease groups with a highly heterogeneous genetic background, such as cerebellar ataxia (CA). In many patients, candidate gene sequencing or focused resequencing arrays do not allow investigators to reach a genetic conclusion.

OBJECTIVES To assess the efficacy of exome-targeted capture sequencing to detect mutations in genes broadly linked to CA in a large cohort of undiagnosed patients and to investigate their prevalence.

DESIGN, SETTING, AND PARTICIPANTS Three hundred nineteen index patients with CA and without a history of dominant transmission were included in the this cohort study by the Spastic Paraplegia and Ataxia Network. Centralized storage was in the DNA and cell bank of the Brain and Spine Institute, Salpêtrière Hospital, Paris, France. Patients were classified into 6 clinical groups, with the largest being those with spastic ataxia (ie, CA with pyramidal signs [$n = 100$]). Sequencing was performed from January 1, 2014, through December 31, 2016. Detected variants were classified as very probably or definitely causative, possibly causative, or of unknown significance based on genetic evidence and genotype-phenotype considerations.

MAIN OUTCOMES AND MEASURES Identification of variants in genes broadly linked to CA, classified in pathogenicity groups.

RESULTS The 319 included patients had equal sex distribution (160 female [50.2%] and 159 male patients [49.8%]; mean [SD] age at onset, 27.9 [18.6] years). The age at onset was younger than 25 years for 131 of 298 patients (44.0%) with complete clinical information. Consanguinity was present in 101 of 298 (33.9%). Very probable or definite diagnoses were achieved for 72 patients (22.6%), with an additional 19 (6.0%) harboring possibly pathogenic variants. The most frequently mutated genes were *SPG7* ($n = 14$), *SACS* ($n = 8$), *SETX* ($n = 7$), *SYNE1* ($n = 6$), and *CACNA1A* ($n = 6$). The highest diagnostic rate was obtained for patients with an autosomal recessive CA with oculomotor apraxia–like phenotype (6 of 17 [35.3%]) or spastic ataxia (35 of 100 [35.0%]) and patients with onset before 25 years of age (41 of 131 [31.3%]). Peculiar phenotypes were reported for patients carrying *KCND3* or *ERCC5* variants.

CONCLUSIONS AND RELEVANCE Exome capture followed by targeted analysis allows the molecular diagnosis in patients with highly heterogeneous mendelian disorders, such as CA, without prior assumption of the inheritance mode or causative gene. Being commonly available without specific design need, this procedure allows testing of a broader range of genes, consequently describing less classic phenotype-genotype correlations, and post hoc reanalysis of data as new genes are implicated in the disease.

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Author Affiliations: Author affiliations are listed at the end of this article.

Group Information: Members of the Spastic Paraplegia and Ataxia network are listed at the end of this article.

Corresponding Author: Alexandra Durr, MD, PhD, Institut du Cerveau et de la Moelle Épineuse, Centre de Référence de Neurogénétique, Hôpital de la Pitié-Salpêtrière, Assistance Publique–Hôpitaux de Paris, 47 boulevard de l'Hôpital, 75651 Paris, CEDEX 13, France (alexandra.durr@icm-institute.org).

Hereditary cerebellar ataxias (CAs) are clinically and genetically heterogeneous. All transmission modes have been described. The cardinal cerebellar syndrome is frequently part of a complex clinical spectrum with additional neurologic and/or extraneurologic symptoms.¹

The most frequent autosomal recessive (AR) CAs are Friedreich ataxia, caused by GAA repeat expansions in intron 1 of *FXN* (OMIM 606829),² and ataxia-telangiectasia, caused by loss-of-function mutations in *ATM* (OMIM 607585).³ Nonclassical clinical pictures have been described for both.^{4,5} Autosomal dominant (AD) CAs are most often caused by CAG repeat expansions in *ATXN1* (OMIM 601556), *ATXN2* (OMIM 601517), *ATXN3* (OMIM 607047), *CACNA1A* (OMIM 601011), *ATXN7* (OMIM 607640), *TBP* (OMIM 600075), or *ATNI* (OMIM 607462).⁶ Overall, mutations in more than 140 genes have been described, involving pathways such as mitochondrial functions, DNA repair, transcription and translation, ciliary structure, and lipid biosynthesis.^{1,7}

Sanger sequencing of candidate genes or panel approaches⁸ often fails to lead to a diagnosis.¹ We aimed to evaluate the efficacy of exome-capture sequencing followed by targeted analysis of genes broadly linked to CA⁹⁻¹² to identify mutations in 319 patients with undiagnosed CA and without an AD transmission history. To our knowledge, this CA cohort is the largest studied.

Methods

Patient Recruitment and Clinical Evaluation

Three hundred nineteen index cases of CA were recruited by the Spastic Paraplegia and Ataxia (SPATAX) network (<https://spatax.wordpress.com/>) and examined using a standardized form (<https://spatax.files.wordpress.com/2013/09/fichecliniquespatax-eurospa-2011.pdf>). The sexes were equally distributed. For the 210 patients with available information, the national origin was mostly France (112 [53.3%]) and continental Europe (39 [18.6%]). We classified the 298 cases with full phenotypic information into the following 6 clinical groups: (1) pure ataxic phenotype (n = 62), (2) additional spastic component (spastic ataxia) (n = 100), (3) complex late-onset clinical picture with extrapyramidal signs (n = 30), (4) metabolic presentation with mitochondrial features (sensorineural hearing loss, ptosis, ophthalmoplegia, optic atrophy, axonal neuropathy, cardiomyopathy, short stature, and/or diabetes type 1) or white matter changes (n = 70), (5) sensory ataxia (n = 19), or (6) a CA with oculomotor apraxia (AOA)-like presentation (n = 17) (Table). The remaining 21 patients were unclassified. The patients' familial anamneses included no evidence of AD transmission. Recessive inheritance was considered to be plausible based on consanguinity (101 of 298 [33.9%]) or family history consisting of at least 2 affected siblings (59 of 298 [19.8%]) with no case in other generations. According to a classic nonsystematic clinical approach, expansions of *FXN* (80 patients) or ADCA genes (132 patients, including *ATXN1* in 129, *ATXN2* in 129, *ATXN3* in 130, *CACNA1A* in 126, *ATXN7* in 110, *TBP* in 47, and *ATNI* in 102) and mutations in various genes (eg, *APTX* [OMIM 606350], *SETX* [OMIM

Key Points

Questions Is exome-targeted capture sequencing able to detect mutations in genes broadly linked to ataxia, and what is the prevalence of such mutations in a large cohort of undiagnosed patients with various phenotypic presentations?

Findings This cohort study of 319 patients with undiagnosed cerebellar ataxia used a sequencing approach that allowed the identification of genetically relevant variants in known genes in 91 (28.5%). This approach had the highest success rate for patients with spastic ataxia or a cerebellar ataxia with oculomotor apraxia-like phenotype; *SPG7*, *SACS*, *SETX*, *SYNE1*, and *CACNA1A* were the most frequently mutated genes.

Meaning Mutations were identified in a broad range of genes implicated in ataxia and related neurologic diseases, even in cohorts that underwent previous extensive screening.

608465], *SPG7* [OMIM 602783], *SACS* [OMIM 604490], and *POLG* [OMIM 174763]) were ruled out when the phenotype was suggested by means of clinical presumption or biological workup (ie, measurement of albumin, cholesterol, or α-fetoprotein level). This study was approved by the Paris Necker ethics committee. Written informed consent was obtained in accordance with French ethics regulations.

Sequencing and Bioinformatics

Exome-enriched libraries were prepared with the rapid-capture expanded exome kit (Nextera; Illumina) and sequenced on the manufacturer's system (HiSeq 2000; Illumina) (2 × 100-base pair paired-end reads). Sequence alignment and variant calling were performed following the Broad Institute's Genome Analysis Toolkit (GATK)¹³⁻¹⁵ version 3 best practices. Sequence reads were aligned against the reference human genome (UCSC hg19) using the Burrows-Wheeler Alignment tool.¹⁶ The Picard software toolset (<http://broadinstitute.github.io/picard/>) was used to calculate summary alignment metrics and mark duplicate reads. Local realignments near indels, base-score recalibration, variant calling, joint genotyping, and variant quality score recalibration were performed using GATK.

Variants were annotated with Annovar¹⁷ (<http://annovar.openbioinformatics.org/>) and sorted with SnpSift¹⁸ (<http://snpeff.sourceforge.net/SnpSift.html>) based on (1) quality (GATK filter pass, quality >30), (2) effect on the coding sequence of a protein-coding gene (exonic nonsynonymous single-nucleotide variant or splice variant), (3) frequency in public databases of no greater than 1% (Exome Variant Server [<http://evs.gs.washington.edu/EVS/>]) and Exome Aggregation Consortium [<http://exac.broadinstitute.org/>, <https://www.biorxiv.org/content/early/2016/05/10/030338>]), and (4) internal frequency (allele count ≤30; ie, ≤4.7%). IntersectBed (<http://bedtools.readthedocs.io/en/latest/>) allowed filtering variants affecting known CA or hereditary spastic paraplegia genes, because both overlap clinically, based on curation of PubMed performed in February 2015 and in-house lists (209 genes) (eTable 1 in the Supplement). Variants in other genes were not examined.

Table. Clinical Subgroups Among the Cohort

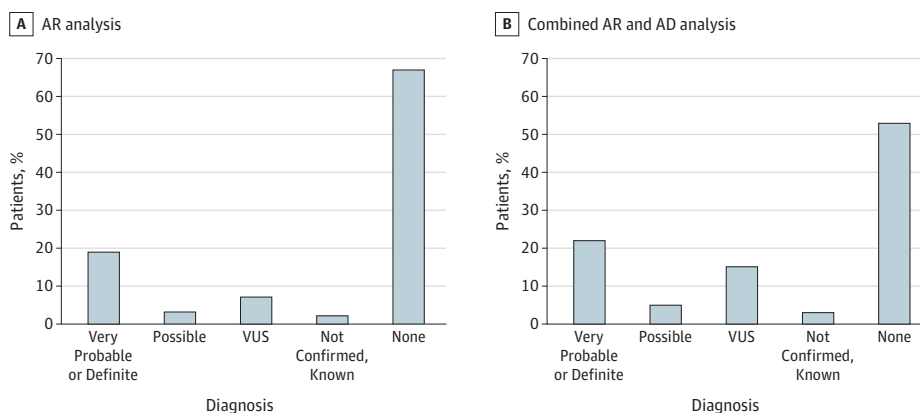
| Clinical Group | No. of Index Patients | No. (%) of Patients | | | Consanguinity | Very Probable or Definite Diagnosis | Very Probable or Definite Diagnosis When Age at Onset <25 y | Very Probable or Definite Diagnosis When Age at Onset >40 y | Possible Diagnosis |
|---|-----------------------|---------------------|--------------------|------------|------------------------|-------------------------------------|---|---|--------------------|
| | | Age at Onset <25 y | Age at Onset >40 y | | | | | | |
| AOA-like phenotype (increased α -fetoprotein level, low albumin level, ocular apraxia) | 17 | 15 (88.2) | 1 (5.9) | 7 (41.2) | 6 (35.3) | 6 (35.3) | 0 | 2 (11.8) | |
| Late complex (MSA-like, parkinsonism, not pyramidal) | 30 | 0 | 20 (66.7) | 7 (23.3) | 3 (10.0) | 0 | 0 | 1 (3.3) | |
| Metabolic (mitochondrial features, white matter changes) | 70 | 45 (64.3) | 6 (8.6) | 24 (34.3) | 16 (22.9) | 10 (14.3) | 0 | 2 (2.9) | |
| Pure (only cerebellar signs, slow progression) | 62 | 20 (32.3) | 13 (21.0) | 19 (30.6) | 9 (14.5) | 5 (8.1) | 0 | 6 (9.7) | |
| Sensory ataxia (FRDA/PolG-like) | 19 | 9 (47.4) | 8 (42.1) | 8 (42.1) | 2 (10.5) | 2 (10.5) | 0 | 2 (10.5) | |
| Spastic ataxia (cerebellar and pyramidal signs) | 100 | 42 (42.0) | 15 (15.0) | 36 (36.0) | 35 (35.0) | 18 (18.0) | 4 (4.0) | 6 (6.0) | |
| All ^a | 298 | 131 (44.0) | 63 (21.1) | 101 (33.9) | 72 (22.6) ^b | 41 (31.3) | 4 (6.4) | 19 (5.9) | |

Abbreviations: AOA, cerebellar ataxia with oculomotor apraxia; FRDA, Friedreich ataxia; MSA, multisystemic atrophy; PolG, polymerase gamma.

^b Includes 1 patient with known *SPG7* mutations lacking sufficient clinical information for classification.

^a Includes the 298 patients for whom complete clinical information was available.

Figure 1. General Study Results



Variants were sorted based on classic criteria (detailed in the Variant Analysis subsection of the Results section), then analyzed assuming an autosomal recessive (AR) transmission mode (including homozygous and heterozygous compound variants), which allowed identifying possibly to definitely causative variants in 74 patients (23.2%). Combining this analysis with one based on

presumed autosomal dominant (AD) transmission (including heterozygous variants and a frequency $\leq 0.1\%$) improved this number to 91 patients (28.5%) for AD and AR inheritance modes, with 10 additional patients carrying very probable to definite pathogenic mutations. VUS indicates variant of unknown significance.

Variants Processing

Biallelic (homozygous or compound heterozygous) variants were first sought because of the likelihood of AR inheritance. Very rare ($\leq 0.1\%$ frequency in the aforementioned databases) heterozygous variants were also considered in ADCA genes (eTable 1 in the Supplement and Figure 1). The plausibility of genotype-phenotype correlations was evaluated for all flagged variants.

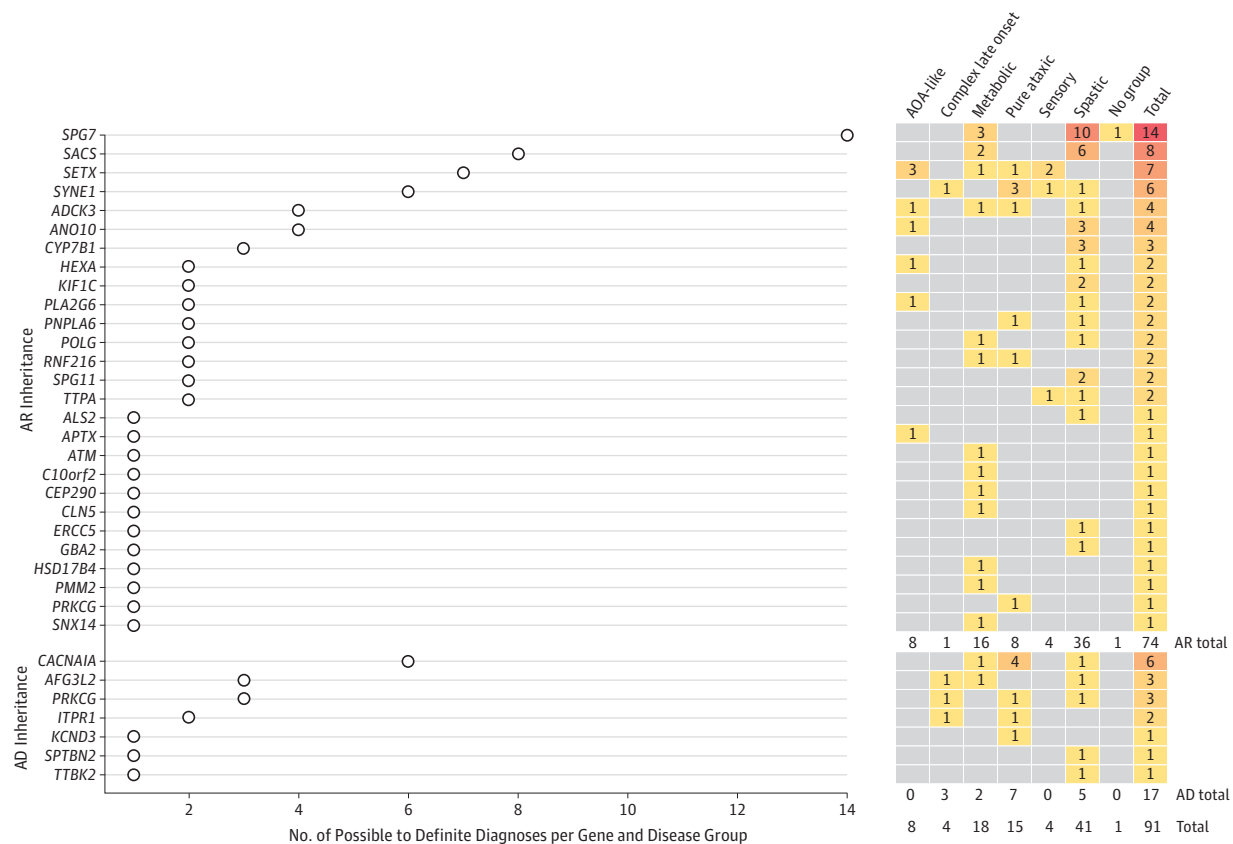
Sanger sequencing was performed if (1) the variant read depth was below $10 \times$, (2) informative family members were available for segregation (other affected members [$n = 13$] or

healthy relatives without biallelic mutations [$n = 6$]), or (3) it allowed determining the *cis*- or *trans*-localization of compound heterozygous variants ($n = 7$). All Sanger resequenced variants and segregation results are reported in eTable 2 in the Supplement.

Results

The 319 included patients had equal sex distribution (160 female [50.2%] and 159 male [49.8%] patients). Mean (SD) age

Figure 2. Number of Possible to Definite Diagnoses per Gene and Disease Group



The left part of the Figure plots the number of patients identified with possible to definite mutations for each gene listed. The right part shows the repartition of these patients among the 6 disease subgroups. Disease groups are described

in the Patient Recruitment and Clinical Evaluation subsection of the Methods section. AD indicates autosomal dominant; AR, autosomal recessive; AOA, cerebellar ataxia with oculomotor apraxia.

at onset was 27.9 (18.6) years; the age at onset was younger than 25 years for 131 of 298 patients (44.0%) with complete clinical data.

Sequencing

Approximately 8 gigabases of alignable sequence data were generated per sample. On average, 95% of exome capture baits had at least a 10 × depth and 70% at least a 30 × depth. A total of 488 393 single-nucleotide polymorphisms and 69 480 indels were included. We deliberately retained patients with low coverage to avoid distorting the percentage of positive findings.

Variant Analysis

The aforementioned analysis (Figure 1) provided 0 to 4 (AR) or 0 to 2 (AD) candidate genes per patient. We defined the following 4 pathogenicity groups: (1) probable or definitive causative variant(s), including a known or a recurrent variant, 3 or 4 concordant pathogenicity predictions by sorting intolerant from tolerant (SIFT), polymorphism phenotyping 2 (PolyPhen2) HumDiv, LRT, and Mutation Taster and Genomic Evolutionary Rate Profiling (GERP++) score of greater than 3 and plausible genotype-phenotype association, or compatible biomarker values (such as hexaminidase levels); (2) possibly caus-

ative variant(s), including most of the above but at least 1 criterion missing, or a slightly discordant phenotype for the gene spectrum; (3) variant(s) of clinically unknown significance (VUS); and (4) no diagnosis, including no variant in a known gene or a variant in known genes without convincing genetics arguments to match the clinical phenotype or the adequate inheritance mode.

We obtained very probable or definite diagnoses for 72 patients (22.6%; including 62 with AR and 10 with AD inheritance) (Figure 1). We identified possibly causative variants for an additional 19 patients (6.0%; 12 with AR and 7 with AD inheritance). The most frequently encountered recessive mutations were in *SPG7* (n = 14), followed by *SACS* (n = 8), *SETX* (n = 7), and *SYNE1* (OMIM 608441) (n = 6) (Figure 2). Dominant mutations were most numerous in *CACNA1A* (n = 6), followed by *PRKCG* (OMIM 176980) (n = 3). Possible to definite variants are listed in eTable 2 in the Supplement, with arguments for their classification; VUS are available in eTable 3 in the Supplement.

Diagnostic Yield per Clinical Group

The best diagnostic yields were found for AOA-like presentations with positive biological biomarkers (6 of 17 [35.3%]) and

spastic ataxias (35 of 100 [35.0%]) as very probable or definite and 2 of 17 (11.8%) and 6 of 100 (6%), respectively, as possible (Table). Patients with late complex CA then were the least frequently diagnosed, with 3 of 30 (10.0%) as very probable or definite and 1 of 30 (3.3%) as possible. Early disease onset and consanguinity were associated with better chances of diagnoses (41 of 131 [31.3%] with very probable or definite with onset before 25 years vs 4 of 63 [6.4%] after 40 years; 31 [30.7%] with consanguinity vs 41 [18.8%] without).

The predefined clinical group was generally concordant with the genetic diagnosis (Figure 2), with some exceptions. One patient with an AOA-like presentation (AAR-211-3) had elevated α -fetoprotein levels, no variant in the *APTX* gene, and pathogenic mutations in the *ANO10* gene (NM_018075.4:c.132dupA:p.D45fs;c.1537T>C:p.C513R). Patients with *ANO10* mutations usually develop cerebellar symptoms in adulthood (aged 17-43 years¹⁹), whereas patients with AOA show earlier onset. Onset at 24 years of age in this patient was coherent with *ANO10* mutations. We classified the diagnosis as possible because the clinical presentation was slightly unusual. Other patients with biallelic loss-of-function *ANO10* variants had classic spastic ataxia.

One patient (AFT-76-10) with epilepsy, thus classified in the metabolic group, carried a *CACNA1A* frameshift (NM_001127222.1:c.2493dupC:p.N832fs). Autosomal recessive inheritance was first assumed because of a consanguineous pedigree, but a dominant history later appeared, with ataxic signs in the daughter of a cousin, for whom no DNA was available for testing.

One patient with spastic ataxia (SAL-399-573) harbored 2 nonsense variants in *SYNE1* (NM_182961.3:c.14273T>G:p.L4758X;c.23346G>A:p.W7782X). Pyramidal signs were classically limited in the *SYNE1* spectrum, but recent results have established that spasticity is part of the phenotype.²⁰

Reports of Peculiar Phenotypes

We classified a previously reported *PDYN* variant (NM_001190898.2:c.616C>T:p.R206C²¹) as VUS. Its frequency in the Exome Aggregation Consortium (0.026%, or 16 heterozygous individuals), the lack of an effect on prodynorphin processing to dynorphin A and B,²¹ and the discordant phenotype of this French patient (AAR-287-4 with childhood-onset ataxia, intellectual disability, and behavioral abnormalities) relative to the previously reported late-onset spastic ataxia cast its pathogenicity in doubt.

We found a possibly causative *KCND3* variant (NM_004980.4:c.641A>G:p.K214R) in a patient with an unusual episodic gait disorder. The onset of relapsing limb paraesthesia at 30 years of age first evoked multiple sclerosis. Vertigo and episodic occurrences of gait disorder appeared thereafter without interictal ataxia. Increased reflexes, the Hoffmann sign, unilateral extensor plantar reflexes, and saccadic ocular pursuit were observed. Magnetic resonance imaging showed superior cerebellar vermis atrophy. The unaffected mother carried the variant, suggesting incomplete penetrance. This finding might be in accordance with its prevalence in public databases (28 of 119 072 alleles; ie, 28 heterozygous among 59 536 individuals [0.05%]).

One patient (AAR-180-11) harbored a previously reported homozygous frameshift variant of *ANO10* (NM_018075.4:c.132dupA:p.D45fs).¹⁹ This specific variant was always found in compound heterozygosity with others and assumed to be too severe when homozygous. The onset at 22 years in this homozygous patient and a mild spastic ataxic phenotype (stage 1 disability with no functional handicap) after 5 years of disease evolution is in accordance with previous reports for *ANO10* showing it to not be more severe.

Finally, 2 siblings (AAR-73-3 and AAR-73-4) with spastic ataxia from a consanguineous pedigree carried a causative homozygous nonsense *ERCC5* variant (NM_000123.3:c.3004C>T:p.Q1002X). The index patient (AAR-73-4) presented with spastic paraparesis, proximal and distal weakness, and severe sensory-motor demyelinating neuropathy at 24 years of age. She used a walking aid at 48 years of age, was wheelchair bound at 52 years of age, and had limited upward gaze and cognitive impairment at 54 years of age. The Scale for the Assessment and Rating of Ataxia score (18 of 40 at 46 years of age and 29 of 40 at 54 years of age) denoted clear progressivity. Magnetic resonance imaging of the brain showed slight cerebellar atrophy and white matter anomalies (Figure 3). Her brother (AAR-73-3) started to show signs of the disease at 15 years of age, used a walking aid at 39 years of age, and was bedridden and died at 51 years of age. The *ERCC5* gene belongs to a DNA repair pathway; biallelic mutations have been associated with xeroderma pigmentosum, Cockayne syndrome, and combined xeroderma pigmentosum-Cockayne syndrome phenotypes.^{22,23} Major characteristics from classic xeroderma pigmentosum-Cockayne syndrome presentations (increased photosensitivity, skin pigmentation abnormalities, enophthalmos, progressive microcephaly, deafness, or retinitis) were absent. Our finding is nevertheless consistent with the ataxic gait of some patients with *ERCC5* mutations.²⁴

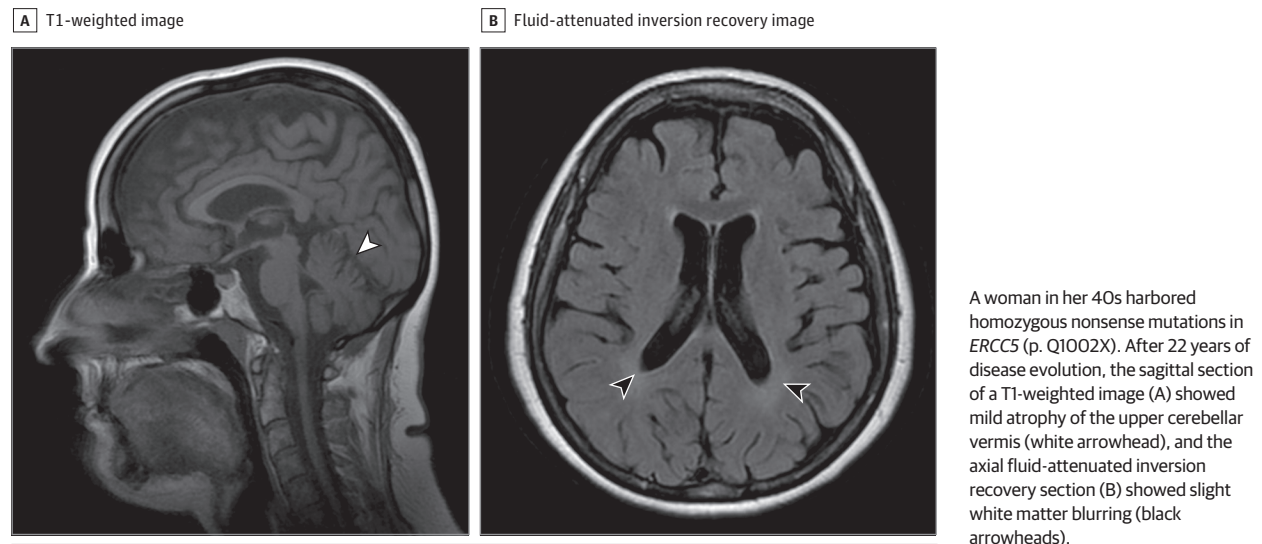
Discussion

We used exome-targeted capture sequencing followed by targeted analysis of genes broadly linked to CA to study 319 patients with undiagnosed CA, the largest such cohort to date, to assess the possibility of detecting mutations with this approach and their prevalence. Previously reported cohorts included no more than 76 patients.⁹

Our diagnostic yield was 22.6% (72 of 319 patients), with 5.9% (19 of 319) possible additional diagnoses (Figure 1). This rate might be lower because our cohort, recruited through a specialized university-based network of reference centers for rare diseases, has undergone extensive screening previously. However, these findings are similar to those of previous reports using targeted gene panels (18%⁸) or exome sequencing (21%-46%^{8-12,25}) and other mendelian diseases (34% diagnoses in heterogeneous patients²⁶; 33% in hereditary spastic paraplegia²⁷).

The diagnostic yield was highly variable among clinical subgroups, being the highest for AOA-like phenotypes and spastic ataxias and the lowest for late-onset complex cases. It was also lower in patients with onset after 40 years of age

Figure 3. Magnetic Resonance Image of the Brain in a Patient



(6.4%), in accordance with other observations.^{8,25} This finding may be explained by less extensive knowledge of genes implicated in late-onset ataxias or by the more frequent involvement of nongenetic causes.

In 9 cases, the diagnosis was established during the study but not confirmed. Five patients carried disease-causing repeat expansions, which cannot be detected by short-read techniques owing to the limited read length. These 5 patients included 1 with a heterozygous *ATXN7* polyglutamine expansion, 1 with a heterozygous and 1 with a homozygous *ATXN3* polyglutamine expansion, and 2 with pathogenic expansions in *FXN*. In this predominantly AR-assumed population, CAG repeat expansions in *ATXN1*, *ATXN2*, *ATXN3*, *CACNA1A*, *ATXN7*, *TBP*, and *ATN1* were sought based only on suggestive clinical elements on examination, as were GAA repeats in *FXN*. A sixth patient harbored a homozygous 31-kb deletion in *ANO10* encompassing exon 12 that was not detected by our approach. Mutations in *NDRG1* (OMIM 605262) were reported in another patient during the study process. This gene, mostly implicated in Charcot-Marie-Tooth disease type 4D, was not included in our list. Finally, 2 patients flagged by quality check metrics were diagnosed with *APT*-linked and *SPG7*-linked CA, but low global coverage prohibited from detecting the variants.

Exome-targeted capture sequencing is widely available and has several advantages compared with other sequencing approaches. If targeted gene panel sequencing is a cost-effective alternative to focus on many genes in 1 run, it requires an additional design step and redesign as new genes are linked to CA. Conversely, exome capture allows post hoc reanalysis of data as other groups identify mutations in new (CA) genes. For example, 2 patients not included in the claimed diagnosed group carried mutations in *CAPN1*²⁸ and 2 others carried mutations in *SLC25A46*.²⁹

This approach also allows a broader range of genes to be tested. When AR inheritance is the prime hypothesis, genes with AD-transmitted mutations might be overlooked. Even without a dominant transmission history, AD analysis yielded

a significant amount of additional diagnoses (Figure 1 and Figure 2), including 3 in consanguineous pedigrees. Two diagnoses were pathogenic, including a previously reported *PRKCG* mutation and a *CACNA1A* frameshift, which is classic for this gene.³⁰ Possible explanations include the de novo occurrence of the variant, incomplete penetrance, a censor effect owing to premature death, or paternity issues. Autosomal dominant analysis significantly raised the number of VUS because missense heterozygous variations are difficult to interpret. Our results confirm *CACNA1A* as a prominent gene in CA, which we also confirmed in an independent AD cohort.³¹ We often did not have access to DNA from relatives because patients were from an adult neurologic clinic and were enrolled during the course of several years. Access to DNA from relatives would have allowed the detection of de novo variants or confirmation of the localization of heterozygous compound mutations in *trans*. Whenever possible, sequencing a trio might prove to be more efficacious.

In general, next-generation approaches circumvent the issue of gene prioritization based on phenotypic presumptions, with the limitation of the aforementioned expansions. This nonsystematic, time-consuming, classic approach missed the diagnosis in the prescreened patients in the absence of pathognomonic signs. Most genes mutated in our cohort were implicated in only 1 or 2 patients (Figure 2). Furthermore, exome-targeted capture sequencing allows a broader range of genes to be tested, including genes associated with neurologic diseases but not typically with ataxia. We enlarged the clinical spectrum linked to genes for which the phenotype is not primarily CA, such as *ERCC5*, *PLA2G6*, or *SPG7*. Careful examination of clinical records revealed suggestive signs of these syndromes in most cases. However, they were not sufficiently suggestive to have been evoked at first glance. Moreover, mutations in a given gene can lead to divergent phenotypes spread over several clinical groups (≤ 4 for *SYNE1*, *SETX*, and *ADCK3*). Capturing the exome makes it possible to avoid prior assumptions about the causative gene.¹⁰

Spastic paraplegia genes are the most frequent example of phenotype broadening, with mutations in *SPG7* and *SACS* as well as *CYP7B1*, *SPG11*, and *PLA2G6* (Figure 2 and eTable 2 in the Supplement). *SPG7* mutations were recently recognized to be a major cause of CA³²; most patients, however, develop pyramidal signs along with disease progression. In our cohort, 9 of 14 patients with *SPG7* mutations had a spastic phenotype (64.3%) and 2 of 14 had pyramidal signs, although they were classified as metabolic. Six of 8 patients with *SACS* mutations (75.0%) presented with spastic ataxia, and 1 presented with pyramidal signs and demyelinating neuropathy. One patient harbouring possibly causative variants had a less classic phenotype of peripheral neuropathy, epilepsy, and myoclonus. Testing for a trait biomarker, such as an alteration of mitochondrial network morphology in fibroblasts,³³ might help to reach a definite conclusion.

Convincing genetic data, however, usually correlated with a characteristic phenotype. In some cases, the clinical features reinforced the genetic diagnostic presumption. We did not use the clinicogenetic correlation as an exclusion criterion when the variant was genetically convincing, as suggested by others,^{9,34} although we classified patients with discordant clinical signs as possibly diagnosed, except for 1 *CACNA1A* frameshift. Instead, we used clinicogenetic correlation to strengthen evidence of pathogenicity. Genotype-phenotype correlations are highly challenged by next-generation sequencing,⁷ but clinical presentation, including neuroimaging findings, such as the degree of cerebellar atrophy or abnormal basal ganglia signal, should remain a prominent criterion in deciphering the pathogenicity of a given variant for a given patient.³⁵

When available, trait biomarkers also aid the establishment of the pathogenicity of variants, as shown by the well-known example of elevated α -fetoprotein levels in ataxia-telangiectasia linked to *ATM* mutations. Oxysterol levels in patients harbouring *CYP7B1* mutations are usually elevated, as in 1 patient carrying a homozygous nonsense variant (NM_004820.4:c.524G>A:p.W175X). Individuals with mutations in *HEXA* have low levels of hexaminidase A, as in 1 patient with an AOA-like presentation and a homozygous missense mutation (NM_000520.5:c.1511G>A:p.R504H) who was considered to be diagnosed despite discordant in silico pathogenicity estimates. Another patient with a spastic phenotype carried 2 missense variants (NM_000520.5:c.739C>T:p.R247W; c.1033G>A:p.G345S) for which pathogenicity could not be confirmed by hexaminidase dosage and that we thus labeled pos-

sibly pathogenic. *TTPA* mutations are usually accompanied by vitamin E deficiency. This was confirmed in one patient with a homozygous frameshift (NM_000370.3:c.744delA:p.E248fs) but not in another with compound heterozygous missense variants and a slightly divergent clinical profile (NM_000370.3:c.265C>T:p.P89S;c.515C>G:p.T172S). The characteristic magnetic resonance imaging sign of a pallidal T2-weighted hypointensity was observed on reexamination for 1 patient with *PLA2G6* mutations (NM_003560.3:c.1903C>T:p.R635X;c.T2411C:p.L804P), allowing us to classify the variants as probably causative. Trait biomarkers, although not obligatory, are invaluable diagnostic tools when present. In the absence of such biomarkers, it is often difficult to reach a definite conclusion, as for the 49 VUS that we identified (eTable 3 in the Supplement).

Limitations

The major limitations of our approach rely in the fact that we missed some variants (ie, trinucleotide repeats expansions that cannot be detected by short-read sequencing and variants in genes not included in our design). The first fallback is inherent to the sequencing technique and can only be avoided by choosing another sequencing approach. The second limitation is a bioinformatics issue that can be solved by updating the gene list. Compared with the panel techniques, the coverage of all human genes might also not be optimized and the sequencing might be more expensive.

Conclusions

We aimed at assessing the efficacy of exome-targeted capture sequencing followed by analysis of genes known to be broadly linked to ataxia to identify mutations in undiagnosed patients. This technique is not optimized for sequencing a specific subset of genes, contrary to focused resequencing arrays, but is commonly available and allows the post hoc reanalysis of data as new causative genes are identified and the inclusion of a broader range of genes in the study design. We focused on a set of 209 genes, which allowed 30% of additional diagnoses in our otherwise extensively prescreened cohort. Our results confirm the genetic heterogeneity of CA, with many genes still to discover in ongoing work with this large cohort. Our strategy may be efficacious for obtaining results for known genes and for future development.

ARTICLE INFORMATION

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Author Affiliations: Institut National de la Santé et de la Recherche Médicale (INSERM) U1127, Paris, France (Coutelier, Stevanin, Davoine, Mochel, Forlani, Vidailhet, Tallaksen, Le Ber, Brice, Durr); Centre National de la Recherche Scientifique, Unité Mixte de Recherche (UMR) 7225, Paris, France (Coutelier, Stevanin, Davoine, Mochel, Forlani, Vidailhet, Tallaksen, Le Ber, Brice, Durr); Unité Mixte de Recherche en Santé 1127, Université Pierre

et Marie Curie (Paris 06), Sorbonne Universités, Paris, France (Coutelier, Stevanin, Davoine, Mochel, Forlani, Vidailhet, Tallaksen, Le Ber, Brice, Durr); Institut du Cerveau et de la Moelle Epinière, Paris, France (Coutelier, Stevanin, Davoine, Mochel, Forlani, Vidailhet, Tallaksen, Le Ber, Brice, Durr); Laboratory of Human Molecular Genetics, de Duve Institute, Université Catholique de Louvain, Brussels, Belgium (Coutelier); Ecole Pratique des Hautes Etudes, Paris Sciences et Lettres Research University, Paris, France (Coutelier, Stevanin, Davoine); Laboratory of Neurogenetics, National Institute on Aging, National Institutes of Health, Bethesda, Maryland (Hammer, Ding, Gibbs,

Singleton); Centre de Référence de Neurogénétique, Hôpital de la Pitié-Salpêtrière, Assistance Publique-Hôpitaux de Paris (AP-HP), Paris, France (Stevanin, Morin, Mochel, Ewencyk, Charles, Brice, Durr); Service de Neurologie, Hôpital Gui de Chauliac, Centre Hospitalier Universitaire (CHU) de Montpellier, Montpellier, France (Labauge); Service de Génétique, Service de Neurologie, INSERM U1079, Rouen University Hospital, Rouen, France (Hannequin); UMR 1169, INSERM and University Paris Saclay, Le Kremlin-Bicêtre, France (Melki); Medical Genetics Unit, Centre Hospitalier Sud-Francilien, Corbeil Essonnes, France (Melki); Service de Génétique, Centre

Hospitalier Universitaire de Tours, INSERM U930, Faculté de Médecine, Université François Rabelais, Tours, France (Toutain); Service de Pédiatrie 1, Hôpitaux Universitaires de Strasbourg, Strasbourg, France (Laugel); Fédération de Médecine Translationnelle de Strasbourg, Université de Strasbourg, Strasbourg, France (Laugel, Anheim); Service de Neurologie C, Hôpital Neurologique Pierre-Wertheimer, Hospices Civils de Lyon, Bron, France (Broussolle, Thobois); Centre de Neurosciences Cognitives, Centre National de la Recherche Scientifique (CNRS)-UMR 5229, Bron, France (Broussolle, Thobois); Université de Lyon, Université Claude-Bernard-Lyon 1, Villeurbanne, France (Broussolle, Thobois); Service de Génétique et Centre de Référence Pour les Malformations et les Maladies Congénitales du Cervelet, AP-HP, Paris, France (Afenjar); Département de Neurologie, Hôpital de Haute-pierre, CHU de Strasbourg, Strasbourg, France (Anheim); Institut de Génétique et de Biologie Moléculaire et Cellulaire, INSERM U964, CNRS-UMR 7104, Université de Strasbourg, Illkirch, France (Anheim); Service de Génétique Médicale, CHU de Toulouse, Hôpital Purpan, Toulouse, France (Calvas); Service de Neurologie, CHU Caremeau, Nîmes, France (Castelnovo); Service de Neurologie, Centre Hospitalier de Saint-Denis, Saint-Denis, France (de Broucker); Département des Maladies du Système Nerveux, Hôpital de la Pitié-Salpêtrière, AP-HP, Paris, France (Vidaïlhet); Service de Neurologie, Fondation Ophtalmologique A. de Rothschild, Paris, France (Moullignier); private practice, Chelles, France (Ghnassia); currently affiliated with Department of Neurology, Oslo University Hospital; and Faculty of Medicine, Oslo University, Oslo, Norway (Tallaksen); Département de Génétique and Centre de Référence Déficiences Intellectuelles de Causes Rares, Groupe Hospitalier Pitié-Salpêtrière, AP-HP, Paris, France (Mignot); Laboratoire Maladies Rares, Génétique et Métabolisme, Université de Bordeaux, Bordeaux, France (Goizet); Service de Génétique Médicale, CHU Pellegrin, Bordeaux, France (Goizet); Service de Neurogénétique, Hôpital de la Croix-Rousse, Hospices Civils de Lyon, Lyon, France (Ollagnon-Roman); Centre de Référence des Maladies Neuromusculaires et de la Sclérose Latérale Amyotrophique, Assistance Publique-Hôpitaux de Marseille, Aix Marseille Université, Hôpital de La Timone, Marseille, France (Pouget).

Author Contributions: Drs Brice and Singleton contributed equally to this work. Dr Durr had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Coutelier, Hammer, Stevanin, Brice, Singleton, Durr.

Acquisition, analysis, or interpretation of data: Coutelier, Hammer, Stevanin, Monin, Davoine, Mochel, Labauge, Ewencyk, Ding, Gibbs, Hannequin, Melki, Toutain, Laugel, Forlani, Charles, Broussolle, Thobois, Afenjar, Anheim, Calvas, Castelnovo, de Broucker, Vidaïlhet, Moullignier, Ghnassia, Tallaksen, Mignot, Goizet, Le Ber, Ollagnon-Roman, Pouget, Singleton, Durr.

Drafting of the manuscript: Coutelier, Hammer, Stevanin, Brice, Singleton, Durr.

Critical revision of the manuscript for important intellectual content: Coutelier, Stevanin, Monin, Davoine, Mochel, Labauge, Ewencyk, Ding, Gibbs, Hannequin, Melki, Toutain, Laugel, Forlani, Charles,

Broussolle, Thobois, Afenjar, Anheim, Calvas, Castelnovo, de Broucker, Vidaïlhet, Moullignier, Ghnassia, Tallaksen, Mignot, Goizet, Le Ber, Ollagnon-Roman, Pouget, Brice, Singleton, Durr. **Obtained funding:** Stevanin, Brice, Singleton, Durr. **Administrative, technical, or material support:** Davoine, Mochel, Gibbs, Melki, Forlani, Broussolle, Tallaksen, Goizet, Pouget, Durr. **Study supervision:** Stevanin, Moullignier, Brice, Singleton, Durr.

Group Information: The following Spastic Paraplegia and Ataxia (SPATAX) network members contributed to the recruitment and phenotyping of all patients in the SPATAX cohort: Alexandra Durr (coordinator), Centre de Référence de Neurogénétique, Hôpital de la Pitié-Salpêtrière, Assistance Publique-Hôpitaux de Paris (AP-HP); Giovanni Stevanin, PhD, Centre de Référence de Neurogénétique, Hôpital de la Pitié-Salpêtrière, AP-HP; Soraya Belarabi, Service de Neurologie Centre Hospitalier Universitaire (CHU) Mustapha; Abdelmadjid Hamri, Service de Neurologie Hôpital Benbadis; Meriem Tazir, Service de Neurologie CHU Mustapha; Sylvia Boesch, Medical University, Innsbruck; Massimo Pandolfo, Service de Neurologie Université Libre de Bruxelles, Hôpital Erasme; Uriele Ullmann, Institute of Pathology and Genetics; Laura Jardim, Universidade Federal do Rio Grande do Sul; Velina Guergueltcheva, Clinic of Neurology, University Hospital Alexandrovska; Ivalo Tournev, Clinic of Neurology, University Hospital Alexandrovska; Bing-wen Soong, National University Yang-Ming; Olga Lucia Pedraza Linares, Departamento Neurociencias Facultad de Medicina, Bogota; Jørgen E. Nielsen, Department of Medical Genetics Section of Neurogenetics, The Panum Institute, University of Copenhagen; Kirsten Svenstrup, Section of Neurogenetics, The Panum Institute, University of Copenhagen; Maha Zaki, Clinical Genetics Department, Human Genetics and Genome Research Division, National Research Centre; Jean-Philippe Azulay, Service de Neurologie et Pathologies du Mouvement Hôpital d'Adultes de la Timone; Guillaume Banneau, Unité Fonctionnelle de Neurogénétique Moléculaire et Cellulaire, Hôpital Pitié-Salpêtrière; Odile Boesplug-Tanguy, Service de Neurologie Pédiatrique et des Maladies Métaboliques-Hôpital Robert Debré; Alexis Brice, Institut du Cerveau et de la Moelle Epinière; Andrea Burgo, Laboratoire Structure-Activités des Biomolécules, Institut National de la Santé et de la Recherche Médicale (INSERM) 829, Université d'Evry-Val d'Essonne; Cécile Cazeneuve, UF de Neurogénétique Moléculaire et Cellulaire, Hôpital Pitié-Salpêtrière; Perrine Charles, Consultation de Génétique Clinique, Hôpital Pitié-Salpêtrière; Frédéric Darios, Institut du Cerveau et de la Moelle Epinière; Christel Depienne, Institut du Cerveau et de la Moelle Epinière; Charles Duyckaerts, Hôpital Pitié-Salpêtrière; Bertrand Fontaine, Hôpital Pitié-Salpêtrière; Sylvie Forlani, DNA and Cell Bank of the Brain and Spine Institute, Hôpital Pitié-Salpêtrière; Cyril Goizet, Consultation de Neurogénétique CHU de Bordeaux, Hôpital Pellegrin; Didier Hannequin, Unité de Génétique Clinique Hôpital Charles Nicolle; Jamilé Hazan, INSERM U952, Université Pierre et Marie Curie; Michel Koenig, Laboratoire de Génétique Moléculaire, Institut Universitaire de Recherche Clinique; Pierre Labauge, Service de Neurologie Hôpital Caremeau; Cecilia Marelli, Service de Neurologie Comportementale et Centre

Mémoire de Ressources et de Recherche, Centre Hospitalier Régional Universitaire Gui de Chauliac; Karine N'guyen, Département de Génétique Médicale Unité de Génétique Clinique, Hôpital d'Enfants de la Timone; Diana Rodriguez, Service de Neuropédiatrie et Pathologie du Développement Hôpital Armand Trousseau-La Roche-Guyon; Annie Sittler, Institut du Cerveau et de la Moelle Epinière; Christophe VERNY, Service de Génétique Département de Neurologie, Unité Charcot, CHU d'Angers; Peter Bauer, Institute of Medical Genetics and Applied Genomics, University of Tübingen; Lüdger Schöls, Department of Neurology and Hertie-Institute for Clinical Brain Research University of Tübingen; Rebecca Schüle, Department of Neurology and Hertie-Institute for Clinical Brain Research University of Tübingen; Georgios Koutsis, Neurogenetics Unit First Department of Neurology, National and Kapodistrian, University of Athens, Eginition Hospital; Alexander Lossos, Department of Neurology Hadassah-Hebrew University Hospital; Antonella Antenora, Department of Neurosciences, Federico II University; Maria Teresa Bassi, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Eugenio Medea Associazione La Nostra Famiglia; Manuela Basso, Centre for Integrative Biology, University of Trento; Enrico Bertini, Molecular Medicine and Department of Neurosciences Osp. Bambino Gesù; Alfredo Brusco, Department of Genetics Biology and Biochemistry University of Turin; Carlo Casali, Medico-Surgical Sciences and Biotechnologies, Sapienza University of Rome; Giorgio Casari, Vita-Salute San Raffaele University and Human Molecular Genetics Unit San Raffaele Scientific Institute, Il Dipartimento di Biotechnologie; Chiara Criscuolo, Clinica Neurologica, Università Degli Studi Di Napoli Federico II; Alessandro Filla, Dipartimento Di Scienze Neurologiche Clinica Neurologica, Università Degli Studi Di Napoli Federico II; Maria Lieto, Department of Neurosciences, Federico II University; Laura Orsi, Department of Neuroscience and Mental Health, Neurological Clinic 1, University Hospital Torino; Filippo M. Santorelli, Molecolare e Malattie Neuromuscolari, Neurogenetics Laboratory Unità Organizzativa Complessa Neurodegenerativa e Medicina, Istituto di Neuropsichiatria Infantile, IRCCS Fondazione Stella Maris; Enza Maria Valente, Istituto di Genetica Medica; Marinela Vavla, IRCCS Eugenio Medea Associazione La Nostra Famiglia; Giovanni Vazza, Università degli Studi di Padova; André Megarbane, Unité de Génétique Médicale Université Saint-Joseph Faculté de Médecine, Campus des Sciences Médicales; Ali Benomar, Service de Neurologie B Laboratoire de Neurogénétique, Hôpital des Spécialités, Rabat Instituts; Richard Roxburgh, Neurology Department, Auckland City Hospital; Anne Kjersti Erichsen, Department of Neurology, Ullevål University Hospital; Chantal Tallaksen, Department of Neurology, Ullevål University Hospital; Isabel Alonso, UniGENE, Institute for Molecular and Cellular Biology (IBMC), University of Porto; Paula Coutinho, Serviço de Neurologia, Hospital S. Sebastião; José Léal Loureiro, Serviço de Neurologia, Hospital S. Sebastião; Jorge Sequeiros, UniGENE, IBMC University of Porto; Mustapha Salih, Department of Pediatrics, College of Medicine and King Khalid University Hospital; Vladimir S. Kostic, Institute of Neurology Clinical Centre of Serbia; Idoia Rouco Axpe, Italfarmaco, SA;

Samir Roumani, Service of Neurology, Damascus; Berry Kremer, H.P.H. Department of Human Genetics, Radboud University, Nijmegen Medical Centre; Willeke Van Roon-Mom, Leiden University Medical Center; Amir Boukhris, Unité de Neurogénétique Service de Neurologie, Hôpital Habib Bourguiba; Chokri Mhiri, Unité de Neurogénétique Service de Neurologie, Hôpital Habib Bourguiba; Arzu Karabay, Istanbul Technical University, Molecular Biology-Biotechnology and Genetics Research Center, Ayazaga Campus Maslak; Suran Nethisinghe, Institute of Neurology, London's Global University; Cahir Okane, Department of Genetics, University of Cambridge; Megan Oliva, Department of Genetics, University of Cambridge; Evan Reid, Department of Medical Genetics, University of Cambridge; Thomas Warner, University Department of Clinical Neurosciences, Royal Free and University College Medical School, University College London; and Nicholas Wood, Neurogenetics, Institute of Neurology University Department of Clinical Neurology, The National Hospital.

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