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TARGETED NEXT-GENERATION RESEQUENCING PANEL IN INHERITED ATAXIAS

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INTRODUCTION

Hereditary ataxias

Hereditary cerebellar ataxias are a group of neurodegenerative diseases with clinical and genetic heterogeneity and variable age at onset. They usually present with incoordination of movement, unsteadiness, hypotonia, dysarthria, and/or oculomotor disorder due to cerebellar dysfunction. Additional neurological (peripheral neuropathy, pyramidal tract dysfunction, extrapyramidal signs, oculomotor abnormalities, intellectual disability, cognitive impairment, epilepsy, optic atrophy) or extraneurological signs (endocrine dysfunctions, cardiomyopathy, cataract) often complete the clinical picture.

For a long time the most important classification of ataxias consisted of Anita Harding classification who divided degenerative cerebellar ataxias in two groups: the early onset forms, mainly characterized by an autosomal recessive transmission, and the late onset ataxias with autosomal dominant inheritance.

From the detection in 1993 of the CAG triplet repeat expansion in *ATXN1* gene, associated with Spinocerebellar ataxia type 1 (SCA1) (Orr et al., 1993), and in 1996 of the GAA expansion of *FXN* gene in Friedreich ataxia (Campuzano et al., 1996), advances in molecular genetics allowed the discovering of several new genes causing cerebellar and spinal ataxias, showing the limits of the old classification.

A pathogenic approach to classify hereditary ataxias did not consider genetic or natural history-base criteria, and divided the disorders into five main categories:

1) mitochondrial; 2) metabolic; 3) defective DNA repair; 4) abnormal protein folding and degradation; 5) channelopathies (De Michele et al, 2004).

Gradually, the old approach of linkage analyses in informative families, positional cloning and candidate gene testing were replaced or complemented by Next Generation Sequencing (NGS), and since 2010 an increasing number of new genes were associated to ataxias, and clinical phenotypes of old genes has been expanded.

Genetically, all transmission modes have been described, and diagnostic work-up remains a challenge. In autosomal dominant ataxias, referred to as spinocerebellar ataxias (SCA) in genetic nomenclature, at least 36 genes have currently been described, but in over 40% of cases the genetic cause remains unknown (Durr et al., 2010; Bird, 1998). Even more intricate is the genetic picture of autosomal recessive ataxias, with 45 genes in which ataxia is the core presenting feature, 22 genes in complex or multisystem recessive disorders that have prominent ataxia, and at least 12 genes associated with recessive disorders that may occasionally present with ataxia as secondary feature (Beaudin et al., 2017). The most common form is Friedreich ataxia, caused by GAA expansion, but numerous genes are involved in complex syndromes (Anheim et al., 2012). X-linked inheritance suggests consideration of the fragile X–associated tremor–ataxia syndrome or adrenomyeloneuropathy.

This wide genetic heterogeneity makes the molecular diagnosis of hereditary ataxias extremely difficult and economically costly, so many ataxic patients remain "orphans" of molecular diagnosis.

Next-generation sequencing: single-gene, gene panel, or exome/genome sequencing

The use of Sanger sequencing strategies for clinical investigation has become obsolete due to its time- and money-expensive features. In the past few years, Next-Generation Sequencing (NGS) has allowed to sequencing a large number of genes with increasing accuracy, speed and throughput of sequencing reducing also costs. NGS technology has revolutionized the paradigm of clinical diagnostics, helping to end the long search for a genetic cause often referred as "diagnostic odyssey" (Johnson, 2015), bringing an extremely wide application of NGS in biomedical domain (Figure 1). At the same time costs for genome sequencing dropped precipitously (Figure 2) and it is possible that in few years low-pass genome investigation will be a feasible option in clinical settings (Hayden 2014). Anyway, even if exome sequencing techniques are probably going to become the "gold standard" in mendelian diseases, to date multi-gene panels still represent an optimal time- and cost-effective first tier screening approach in rare disorders.

Four different molecular tests are currently available: Traditional Sanger Single-Gene Test, gold standard for sequencing, which is commonly used for investigation of disease-causing specific gene; Targeted Resequencing Panels (TRP), which consists in the analysis of the coding exons of a restricted number of genes; Exome Sequencing (ES) which includes the sequencing of the coding 1% of the entire human DNA, where lies up on the 85% of all pathogenic mutations; Whole Genome Sequencing (WGS), the most expensive, all-inclusive technique, detecting mutations in coding and non-coding regions and also copy number variations (CNV) (Hui, 2014; Xue et al., 2015). Single-gene test is preferred when distinctive clinical findings clearly point to a specific gene,

characterized by minimal locus heterogeneity. Single-gene test imply no incidental findings and a minimal number of variants of unknown significance (VOUS). VOUS is considered if the variant has never been reported in any of the databases for the assessment of variants' pathogenicity, and is not the kind with clear pathogenicity (i.e., frameshift, stop codon, or splice-site mutations).

TRP are the most cost-effective application, offering the possibility to make a differential diagnosis in disorders with overlapping phenotype and genetic heterogeneity, but well-defined disease-associated genes. Technical procedure of TRP needs Sanger confirmation and complementary assays to detect deletions/duplications. The overall coverage is higher than ES, VOUS are less found, and is less likely to incur in incidental findings than ES.

ES and WGS show success most often in cases with extreme clinical heterogeneity and nonspecific neurological disorder or in detection of de novo mutations (Yang et a., 2013). These latest methods have allowed the recognition of many novel genes not currently associated with diseases, and the connection of novel phenotypes with previously described genes. This fascinating role of ES and WGS is however burdened by the difficulty of discriminating, amongst the many non-disease causing variants identified, the true mutations. An accurate phenotyping of the patients is a prerequisite for the interpretation of the variants.

Another challenge when performing vast scale sequencing such as ES or WGS is potential detection of incidental findings, defined as genetic variants with medical or social implications that are discovered during genetic testing for an unrelated indication (Wolf et al., 2012). The rate of reportable incidental findings can range from 1 to 8.8%, depending on sequencing quality, variant selection, subject

cohort, and whether the laboratory is using the gene list recommended by the American College of Medical Genetics (ACGM) and Genomics Working Group (Berg et al., 2013; Lawrence et al., 2014). The ACMG subsequently established the Secondary Findings Maintenance Working Group to develop a process for curating and updating the list of high risk genes, supporting the reporting of known or expected pathogenic variants, excluding VOUS (Kalia et al., 2017).

Next-generation sequencing in ataxias

Routine procedure for testing hereditary ataxias generally include analysis for CAG expansions in autosomal dominant forms (SCA1, SCA2, SCA3, SCA6, SCA7, SCA17, DRPLA) and for the GAA intronic expansion in *FXN* gene associated with Friedreich ataxia, reaching a molecular diagnosis in about 40% of recessive cases and about 50% of dominant ones (Anheim et al., 2010; Durr, 2010). The repeat expansions can be easily and cheaply tested using standard PCR methods. Few other genes are commonly screened by Sanger sequencing because of prohibitive costs or limited gene test availability. So, the advent of NGS promised to revolutionize genetic testing and potentially could be part of standard evaluation of patients.

From 2013 to date, at least twelve NGS studies have investigated genetic etiology of large cohorts of ataxic patients, five using TRP (Nemeth et al, 2013; Mallaret et al., 2016; Hadjivassiliou et al., 2017; Coutelier et al., 2017; Iqbal et al., 2017) and seven ES approaches (Ohba et al., 2013; Sawyer at al., 2014; Fogel et al., 2014; Pyle et al., 2015; Keogh et al., 2015; van de Warrenburg et al., 2016; Marelli et al, 2016).

Targeted Resequencing Panels in ataxias: diagnostic yield and detected genes

- Nemeth et al. studied by TRP 50 ataxic patients with a positive family history or an age at onset below 50 years (Nemeth et al., 2013). The panel included a total of 58 genes known to be associated with human ataxia and additional 59 candidate genes derived from functional data or animal models. Clearly pathogenic mutations were found in nine cases with a detection rate of 18%. The most likely predictors of detecting a mutation were an adolescent age of onset (present in 8/9 positive cases), a family history, a complex and progressive phenotype. Pathogenic mutations were detected in *SETX* gene, the gene causative of Ataxia Oculomotor Apraxia 2 (AOA2), in two patients. This diagnosis was not previously been made, despite the suggestive clinical features and the presence of elevated levels of the biomarkers alfafetoprotein, because of lack of availability of genetic testing in UK. The other genes detected, in single cases, were *SACS* gene (responsible of ARSACS, Autosomal Recessive Spastic Ataxia of Charlevoix-Saguenay), *MRE11A* gene associated with Ataxia-Teleangectasia-like Disorder, *SCA11*, *SCA13*, *SCA14*, *SPARCA1* and *DARS2*. The last gene is responsible of autosomal recessive Leukoencephalopathy with Brainstem and Spinal cord involvement and Lactate elevation (LBSL). In the index case only one mutation has been found, but clinical phenotype was very consistent, so diagnosis was considered evident.
- 145 unrelated patients with autosomal recessive or sporadic progressive cerebellar ataxia and age at onset before 60 years were analyzed by TRP included 57 ataxia genes (Mallaret et al., 2016). A molecular diagnosis was made in 27/145 patients

(19%). Genetic analysis identified two pathogenic mutations in *ANO10* (six patients), in *SETX* (four patients), in *SYNE1* and *ADCK3* (three each), in *SACS* and *APTX* (two each) and in *TTPA*, *CYP27A1*, *POLG* (one each).

- From June 2014 to September 2016 at the Sheffield Ataxia Centre of UK 146 ataxic patients have been tested with a TRP including 42 ataxia genes (Hadjivassiliou et al., 2017). Of these, 54 patients had autosomal dominant family history and 17 a family history consistent with autosomal recessive inheritance. Confirmed pathogenic mutations were found in 35 cases with a diagnostic yield of 24%. VOUS were detected in eleven cases. Positive results were obtained in 46% of patients with autosomal dominant inheritance, in 24% of patients with a family history consistent with recessive inheritance, and in 27% of patients with sporadic ataxia. More frequent genetic diagnosis included Episodic Ataxia type 2 (EA2, *CACNA1A* gene) recognized in eleven cases, SCA14 in five cases, SCA5 and Spastic Paraplegia 7 (SPG7) in four patients each, ARSACS and SCA35 in three patients each.
- After exclusion of CAG expansions in SCA genes, 412 index cases with dominantly inherited cerebellar ataxia, from SPATAX (The Network of hereditary forms of SPAstic paraplegias and cerebellar ATAXias) cohort, underwent a 65 genes TRP and TaqMan PCR assay for deletions in *ITPR1* gene, responsible for SCA15 (Coutelier et al., 2017). Relevant genetic variants were identified from panel sequencing in 12% of patients, including confirmed pathogenic variants and probably/possible variants. With the combined approach of panel sequencing and TaqMan PCR assay, the diagnostic yield reached 14%, as deletions in *ITPR1* gene were detected in eleven patients. 89 VOUS were identified. Apart from diagnosing SCA15, the most frequently mutated gene was *CACNA1A* in 16

unrelated patients. Next in frequency were *SPG7* biallelic variants (in nine patients), monoallelic *AFG3L2* mutations responsible of SCA28 (in seven patients), and monoallelic *KCND3* mutations in three cases associated with SCA19/22.

- Recently, Iqbal et al. described the results of a study of 105 index cases with hereditary cerebellar ataxia or spastic paraplegia, analyzed by a gene panel targeting 159 neurodegenerative disorders genes, including 91 genes implicated in classical hereditary ataxias and spastic paraplegias (Iqbal et al., 2017). About the results of the 58 ataxic patients, eight probands carried pathogenic or likely-pathogenic variants, with detection rate of 14%. VOUS were detected in eight cases. Definite molecular diagnosis have been detected in ARSACS, SCA5, SCA14, SCA15, SCA19/22, SCA28, SCA35, and EA2.

Exome Sequencing in ataxias: diagnostic yield and detected genes

- Ohba et al. performed a family-based ES in 23 index cases with cerebellar atrophy in childhood on brain MRI (Ohba et al., 2013). They found three cases with de novo mutations in *CACNA1A* (two each) and *ITPR1* genes, and six cases with compound heterozygous mutations in *FOLR1* (Neurodegeneration from cerebral folate transport deficiency), *C5orf42* (Joubert syndrome), *POLG*, *TPP1* (SCAR7) and *PEX16* (Zellweger syndrome), reaching a diagnostic yield of 39%.
- Pediatric-onset cerebellar ataxia was investigated within a Canadian project to identify novel genes for rare childhood disorders using ES. Analysis of the 28 families included in the study resulted in a molecular diagnosis for eleven families in a known disease gene (ARSACS, AOA2, Holmes syndrome related to *RNF216* gene, Perrault syndrome, Congenital Disorders of Glycosylation type Ia,

Neurodegeneration with Brain Iron Accumulation related to *PLA2G6* gene), and two new disease genes were identified. The combined molecular diagnosis rate was of 46%. In detail, all consanguineous families investigated reached a diagnosis; the diagnostic yield for patients born to nonconsanguineous parents with more than one affected child was of 43%, while for simplex cases of 27% (Sawyer at al., 2014).

- A clinical ES of 76 ataxic patients, mainly with adult- and sporadic-onset, made a definitive genetic diagnosis in 21% of cases and identified probable variants in an additional 40% of cases (Fogel et al., 2014). *SYNE1* and *SPG7* were the most frequent genes detected.
- Pyle et al. performed an ES in 22 probands with suspected inherited ataxia (Pyle et al., 2015). Twelve of 22 individuals (54%) had family history, suggestive of dominant inheritance in 5/12, and autosomal recessive in 7/12. Confirmed pathogenic variants were found in 9/22 probands, with diagnostic yield of 41%. Possible pathogenic variants were identified in 5/22 probands and VOUS in two cases. Three cases were positive for *SACS* mutations and two cases for *TUBB4A* gene mutations, responsible for Hypomyelinating Leukodystrophy 6. One case was positive for *SPG7*.
- Twelve sporadic adult-onset (> 30 years of age) ataxic patients were investigated with ES in a UK study (Keogh et al., 2015). In 33% of patients were identified a pathogenic mutations (two cases of *SPG7*, one each of *ANO10* and *SYNE1* genes) and VOUS were found in additional two cases (17%).
- van de Warrenburg et al. reported the results of a clinical ES performed in 76 patients with cerebellar ataxia and spastic paraplegia, filtered for a "movement

disorders" bioinformatic gene panel, including about 200 genes implicated in cerebellar ataxias, spastic paraplegia, parkinsonism, choreas and other hyperkinetic movement disorders (van de Warrenburg et al., 2016). Of the 28 ataxic patients nine had causative variants, with a diagnostic yield of 32%. The most frequent pathogenic variant were detected in *SPG7* gene (three cases). A possible causative variant were detected in one additional patient.

- A strategy of TRP, extended, for negative cases, to a mini-ES, designed for genomic analysis of the coding regions of 4813 genes, and coupled to a bio-informatic approach for Copy Number Variations detection, was applied to 33 patients with progressive cerebellar ataxia and onset before the age of 50 (Marelli et al., 2016). Confirmed pathogenic variants were found in 14 patients (42%), more frequently in cases with positive family history. VOUS were found in eight patients (24%). A positive family history, followed by younger age at onset, were the most important factors associated with positive results. Disease causing mutations were identified in five recessive ataxia genes (*SETX*, *NPC1*, *ATM*, *HSD17B4*, *ERCC4*) and in three dominant ataxia genes (*ATXN2*, *PRKCG*, *PDYN*), with mutations in more than one family for *SETX* (five probands), *NPC1* (two probands) and *PRKCG* genes (two probands).

Considering all TRP studies, the average diagnostic rate was 17%. Including VOUS as representing relevant genetic information, the diagnostic rate would rise ranging between 26% and 34%. The average diagnostic yield of ES studies was 36%. Including VOUS the value raised to 53% (Figure 3).

However, the number of patients recruited for TRP analysis is widely larger compared to those who underwent ES (an average of 172 and 32 in TRP and ES,

respectively), suggesting a possible selection bias in ES cohorts and an average diagnostic rate quite overestimated than a less selected cohort. Moreover, the cohorts of the different studies were, for the most part, not naïve and largely nonconsecutive. Most patients, in fact, have already been variably screened for several genes, although possible is the lacking of systematicity in which genes were excluded before NGS. Moreover, the diagnostic power of a TRP depends heavily on the number of the genes included in the test panel, and if these genes are the right genes. A multi-gene panel is usually preferred considering the relatively higher deep coverage. ES has the power to identify causal variants also in newly described disease genes, or in extremely rare disease genes not present in targeted capture assay, discovering new genes and broadening the phenotype. Even if ES can bypass the selection a set of candidate genes, the interpretation of the huge amount of data depends to a focused and efficient assessment of the patient. The collaboration between the laboratory researchers and the clinicians is therefore of primary importance.

In the twelve NGS studies analyzed, an overall of 53 different genes were detected, in both autosomal dominant (35%) and recessive (65%) genes. Interestingly, 66% of the variants detected lie in only eleven different genes: *CACNA1A* (Episodic Ataxia type 2 - EA2), *SPG7* (Spastic Paraplegia 7), *SETX* (Ataxia Oculomotor Apraxia type 2 - AOA2), *SACS* (Autosomal Recessive Spastic Ataxia of Charlevoix-Saguenay - ARSACS), *PRKCG* (Spinocerebellar Ataxia type 14 - SCA14), *AFG3L2* (Spinocerebellar Ataxia type 28 - SCA28), *ANO10* (Spinocerebellar ataxia Autosomal Recessive 10 - SCAR10), *KCND3* (Spinocerebellar Ataxia type 19 - SCA19), *SPTBN2* (heterozygous mutation responsible of Spinocerebellar Ataxia type 5 - SCA5; homozygous mutation

responsible of Spinocerebellar ataxia Autosomal Recessive 14 - SCAR14), *SYNE1* (Spinocerebellar ataxia Autosomal Recessive 8 - SCAR8), and *KCNC3* (Spinocerebellar Ataxia type 13 - SCA13). About 64% mutations have been found in genes traditionally known as cause of hereditary ataxias, whereas 30% fall in genes recently discovered because of NGS strategies, and only 6% occur in genes not typically considered associated with ataxia. This frequency estimates, however, could be not reliable because of some genes were not available for testing before NGS in some centers, such as *SETX* gene that is not routinely tested in UK and France, or few families have been screened for *SYNE1* gene as the gene is very large at 146 exons in length.

PROJECT AIMS

The aim of our research was to investigate the role of a Targeted Next-Generation Resequencing Panel for ataxias in clinical practice, studying a heterogeneous group of patients with inherited ataxia who were a diagnostic challenge and are representative of the range of cases referred for serial genetic testing.

SUBJECTS AND METHODS

Inclusion criteria

Thirty-nine index patients were recruited from the Ataxias Clinic of Neuroscience, Reproductive and Odontostomatological Science Department of the Federico II University. The cohort was largely nonconsecutive. No restriction on age at onset or inheritance pattern were made, but there had to be a clinical suspicion of a genetic etiology, based on the combination of cerebellar ataxia phenotype plus either an onset age below 45 years or a positive family history. Also congenital ataxias were included. Most patients had already undergone extensive genetic testing, with a mean of 3.6 genes tested. Moreover, mutations in the most likely genes had to be excluded. All patients provided written consent for the study.

Inherited Ataxias Progression Scale

Progression of the disease was evaluated using the Inherited Ataxias Progression Scale (IAPS) (Campanella et al., 1980).

This scale is composed of four phases:

- Phase I: asymptomatic patient, identified among sibs or sons of a proband;
- Phase II: ataxia but independent;
- Phase III: permanently dependent on walking aids;
- Phase IV: permanently dependent on wheelchair.

DNA extraction

DNA samples were obtained from peripheral blood and treated with anticoagulant ethylenediaminetetraacetic acid (EDTA). DNA extraction was performed using nucleic acid isolation automated system *MagNA pure compact* (Roche). Genomic DNA was then qualitatively tested in 1,5% agarose gel.

Preliminary investigations

Prior to undergo TRP, all patients were tested for pathological expansions in SCA1, 2, 3, 6, 7, 8, 12, 17 and for the intronic GAA expansion in *FXN*.

Pathological CAG expansions in SCA1, 2, 3, 6, 7, 8, 12, 17 have been investigated using validated fluorescently-based TP PCR methodologies (Warner et al., 1996) (Cagnoli et al., 2006). Capillary electrophoresis was performed with *3130xl Genetic Analyzer* (Applied Biosystems) and fragment analysis was performed using software *GeneMapper 4.0* (Applied Biosystems).

Pathological intronic GAA expansion in *FXN* was tested using a long-PCR method (Campuzano et al., 1996). PCR products were tested in 0,8% agarose gel and molecular weight analysis was made using *Image Lab* (Bio-Rad). Thermocycler *GeneAmp PCR System 9700* (Applied Biosystems) was used in both investigation, and also in Sanger sequencing for confirmatory studies and in *SureSelect Target Enrichment System* (Agilent Technologies).

Confirmatory studies

Pathological mutations identified with NGS were confirmed by Sanger sequencing (Sanger et al., 1977). Amplicons of interest were obtained by PCR and amplified products were purified with *Exo-SAP* (exonuclease I-shrimp alkaline phosphatase). Nucleotide sequences were determined performing capillary electrophoresis using automated sequencer *3500 Genetic Analyzer* (Applied Biosystems) in association with *BigDye Terminator v3.1 Cycle Sequencing Kits* (Life Technologies) and *G50 Dye terminator removal kit* (RBC Bioscience). Electropherograms analysis was performed with software *SeqScape v2.7* (Life Technologies).

Ataxia targeted resequencing panel

Gene panel creation

A gene panel containing 273 genes known or supposed to be related to hereditary ataxias was created using *SureDesign* (Agilent Technologies) software (Table 1).

Sample library preparation

DNA samples were processed adopting *SureSelect Target Enrichment System* (Agilent Technologies). First, genomic DNA (gDNA) was quantitatively tested with *Qubit dsDNA Broad Range Assay* (Invitrogen). 2 µl of 25 ng/µl genomic DNA was hence enzymatically fragmented and adaptors were added to ends of the fragments, and furthermore adaptor-tagged gDNA libraries were repaired and PCR-amplified (Figure 4, A). Once DNA library amplicons were prepared, hybridization with regions of interest was performed using biotinylated RNA

probes (Figure 4, B). Streptavidin coated magnetic beads were then used in order to capture biotinylated probes bounded to gDNA of interest and to wash out the rest (Figure 4, C). Captured target DNA libraries were then amplified using dual indexing primers, a truly important process because samples were then multiplexed for sequencing, so was necessary to maintain a correspondence between each amplicon and its corresponding patient. Since libraries were pooled, amplicons were ready to be sequenced. *2200 TapeStation* (Agilent Technologies) was used to assess DNA quality and quantity (*D1000 assay* was used for pre-hybridization libraries and *High Sensitivity D1000 assay* for post-hybridization libraries).

Sequencing

A *MiSeq* Illumina platform was used for high-throughput sequencing in this study. *MiSeq* Illumina sequencing adopts a "bridge amplification" approach. *MiSeq reagent kit v2 (2x150bp)* and *4.5 Gb flow cell* were used in every sequencing. First, capture libraries previously prepared from each patient were pooled for multiplex sequencing (Figure 5, 1). A variable multiplexed pool concentration ranging between 6 pM and 10 pM were used for every sequencing, and 1% PhiX (a phage short genome used as sequencing control) was added to the sample pool. Hence, DNA was chemically denaturated and sequencing got start. Single-stranded fragments randomly bound the surface of the *flow cell*, a solid support where lie immobilized complementary oligonucleotides (Figure 5, 2). Nucleotides and enzyme were then added to initiate solid-phase bridge amplification (Figure 5, 3) and so double-stranded bridges were obtained (Figure 5, 4). Following denaturation left single-stranded templates anchored to the substrate (Figure 5, 5) and in this way several millions dense clusters of double-stranded DNA were

generated in each channel of the flow cell. Since the added nucleotides were marked with four different fluorophores, every time a base complementary to a single strand was incorporated, a laser excited the molecule and the light signal was recorded in order to identify which base had just been added (Figure 6). This process occurred at the same time for every strand attached to the flow-cell, and leaded to the identification of the nucleotidic sequence of every amplicon.

Data analysis

Data analysis was performed using software *SureCall* (Agilent Technologies) which allows to align sequence raw data with reference human genome sequence (hg19). Differences between these two inputs represent the variants identified in DNA samples used for sequencing. After that, variants found were analyzed using *Ingenuity variant analysis* (Qiagen) and filtered using the following parameters:

- *Call quality*: A score which represents the probability that a base call is true. In this study, variants with QC<30 (corresponding to a base call accuracy>99.9%) were discarded.
- *Read depth:* Represents how many times a variant has been sequenced. In this study, variants with read depth<20 were discarded.
- *Frequency*: Only rare variants were considered, so those with population frequency higher than 1% were discarded.

  Main database we referred to was *Genome Aggregation Database (gnomAD)* (http://gnomad.broadinstitute.org), a online database which contains frequencies of genetic variants in over 120.000 exome sequences and 15.000 genome sequences from unrelated individuals.
- *False positives*: Some variants may be present in several samples, and is

unlikely if we are considering a group of rare disease. Indeed, these are often NGS sequencing bias. In order to solve this problem, variants shared by 3 samples or more were discarded.

Moreover, in order to evaluate the functional impact of protein mutations, algorithms *SIFT (Sorting Intolerant From Tolerant) (*http://sift.jcvi.org) and *PolyPhen2* (*Polymorphism Phenotyping) (*http://genetics.bwh.harvard.edu/pph2*)* were used, both predicting the impact of missense mutations using parameters like sequence homology and phylogenetic information.

Splicing variant predictions were also performed using algorithms *NetGene2* (http://genetics.bwh.harvard.edu/pph2) and *BDGP* (http://www.fruitfly.org) for intronic variants downstream and upstream 20 nucleotides from each exon considered. Mutations in acceptor and donor splicing site were considered pathogenetic.

RESULTS

Clinical characteristics of the patients

54% of probands were male. The mean age at disease onset was 28 years (standard deviation 15.3; range 1-55 years). Sixteen out of 39 patients (41%) presented an onset before the age of 25, four of which patients presented with a congenital ataxia. Mean disease duration was 20 years (standard deviation 11.3; range 2-46 years). Fifteen out of the 39 patients (38%) had a positive family history, with 20% of cases with known consanguineous parents. The majority of patients had additional neurological features that included spasticity, peripheral neuropathy, cognitive impairment/decline, tremor, hyperkinetic choreiform movements, epilepsy, myoclonus, and dystonia, or extraneurological features included hypo or hypergonadotropic hypogonadism, skeletal deformities, cataract, and hypoacusia. Most patients had already undergone extensive genetic testing, with a mean of 3.5 genes. Clinical features and the genetic results are shown in table 2, 3 and 4.

Genetic Results

High quality sequencing data was obtained with an average of 96% bases covered >100x and 82% bases covered >200x in the targeted regions (Figure 7). By applying our filtering criteria, we identified eighteen probands out of 39 (46%) carrying pathogenic variants (Table 2). VOUS were found in nine probands (23%) (Table 3). In the remaining twelve out of 39 cases (31%) no candidate variants or variants with unlikely pathogenicity were identified (Table 4).

The comparison between the patients with positive diagnostic results and patients with VOUS or negative results showed a more frequent positive family history (66% vs 38%) in patients with confirmed pathogenic variants.

Patients with confirmed pathogenic variants (table 2)

Disease causing mutations were identified in 18 cases (17 recessive ataxias and one autosomal dominant). We recognized ten different genes: *SPG7* (five probands), *PNPLA6* (three probands), *SYNE1* (two probands), *SETX* (two probands), *RNF216*, *ZFYVE26*, *ANO10*, *PMM2*, *ATP13A2* and *TGM6* (one proband each). The most frequent cause of ataxia in our cohort was SPG7 (spastic paraplegia 7). All of the five cases (PT 1-5) had a prominent ataxic phenotype with cerebellar atrophy at brain MRI (Figure 8), but in three of them pyramidal signs were also present (PT 2, 3, 4) and in one case associated with ophthalmoparesis (PT 2). However, two of the five patients had no pyramidal signs, but rather a pure cerebellar ataxia (PT 1, 5). Both these patients carried a common variant in *SPG7* (p.Ala510Val), one in compound heterozygosity, one in homozygosity, confirming that the pure ataxia phenotype is not dependent to a specific genetic variant, but it is part of the SPG7 disease spectrum (van de Warrenburg et al., 2016).

Second gene for frequency detected was *PNPLA6* (patatin-like phospholipase domain-containing protein 6), a gene associated with Gordon Holmes/Boucher-Neuhäuser syndrome/ spastic paraplegia 39 (SPG39). The clinical spectrum of *PNPLA6* mutations unfolds along four different neurological key features: ataxia, chorioretinal dystrophy, hypogonadotropic hypogonadism and motor neuron disease (upper motor neuron disease with or without additional lower motor

neuropathy). Gordon Holmes syndrome is classically described as an early-onset autosomal recessive cerebellar ataxia with hypogonadotropic hypogonadism associated with brisk reflexes (Holmes, 1907). Cognitive impairment completes the phenotype. In Boucher-Neuhäuser syndrome the ataxia with hypogonadotropic hypogonadism is additionally associated with chorioretinal dystrophy (Boucher and Gibberd, 1969; Neuhauser and Opitz, 1975). In SPG39 spastic paraplegia is associated with distal upper and lower extremity wasting.

In 2013-2014 three different NGS studies recognized three genes associated with ataxia and hypogonadotropic hypogonadism: *RNF216* either alone or in combination (digenic mutations) with mutations in *OTUD4* (Margolin et al., 2013), *STUB1* (Shi et al., 2014), and *PNPLA6* (Synofzik et al., 2014).

Two of the three probands (PT 6 and 7) here detected presented with a classical phenotype, and sexual hormones assessment revealed a hypogonadotropic hypogonadism (Table 5). Brain MRI showed cerebellar atrophy (Figure 9). In both patients visual assessment showed a chorioretinal dystrophy, with visual acuity of 7/10 in both eyes for PT 6 and of 1/120 and 1/50 in PT 7. Cognitive evaluation revealed in both patients a mild intellectual disability. The third *PNPLA6* case (PT 8) showed only pyramidal signs associated with cerebellar ataxia, with normal cognitive and visual functions. In all three cases, and also in the affected brother of PT 7, a downbeat nystagmus was evident.

The phenotype of *RNF216*-mediated neurodegeneration initially associated with Gordon Holmes syndrome, have recently been expanded to include Huntington-like disorder with prominent chorea, behavioral problems, severe dementia and low gonadotropin serum levels (Santens et al., 2015). Also, in our *RNF216* case (PT 13) pyramidal signs were associated with hyperkinetic choreiform

movements, and mild cognitive impairment. A re-evaluation of the patients revealed reduced values of FSH and LH, with normal values of estradiol, progesterone, and prolactin.

We recognized two *SYNE1* cases (PT 9, 10). In one patient clinical picture is that of an adult-onset cerebellar ataxia with slight cerebellar atrophy at MRI, associated with a sensory peripheral neuropathy (PT 9). In the second case (PT 10) a mild cerebellar ataxia with prominent dysarthria and slight pyramidal signs was associated with a hypergonadotropin hypogonadism. Brain MRI showed marked cerebellar atrophy (Figure 10). She had an affected brother with early-onset syndrome characterized by a moderate intellectual disability, mild cerebellar ataxia and normogonadotropin hypogonadism. These data confirm that recessive ataxia due to *SYNE1* mutations is characterized by a phenotype variability from a slowly progressive, relatively pure cerebellar ataxia with only mild and infrequent extra-cerebellar symptoms starting in adult age, to a severe multisystemic neurodegenerative syndrome, also including intellectual disability (Synofzik et al., 2015). This is the first report of hypogonadism associated with *SYNE1* mutations.

Two cases of Ataxia with Oculomotor Apraxia 2 (AOA2) due to mutations in *SETX* gene were detected in our cohort (PT 11 and 12). In both patients oculomotor apraxia was absent, but strabismus and peripheral neuropathy was evident in the first (PT 11). The second case (PT 12) was not suspected for AOA2 because of clinical picture was characterized by prominent tremor and hyperkinetic choreiform movements without peripheral neuropathy. Furthermore, a family history positive for tremor was evident in father and a sister.

A complicated neurodegenerative syndrome was present in PT 14, positive for compound heterozygous mutations in *ZFYVE26*, associated to spastic paraplegia

15 (SPG15). Clinical phenotype was characterized by spastic ataxia with age at onset at 30 years, and progressive development of extrapyramidal signs, executive and psychiatric signs with hallucinations and marked dysphagia requiring a percutaneous endoscopic gastrostomy. Typical thin corpus callosum (TCC) and periventricular white matter abnormalities were not present at MRI. However, the occurrence of TCC and white matter abnormalities is variable in SPG15 (Hanein et al., 2008; Boukhris et al., 2008).

The *ANO10* case (PT 15) was a Romanian patient with progressive spastic-ataxia syndrome with onset at 30 years of age. At 31 years she developed retrobulbar optic neuritis leading to the diagnosis of multiple sclerosis, confirmed by the presence of oligoclonal bands in cerebrospinal fluid and white matter alteration at MRI. We decide however to include the patient in our study because of a marked cerebellar atrophy at MRI (Figure 11).

Two brothers with mild intellectual disability, strabismus and congenital non-progressive ataxia with marked cerebellar hypoplasia (proband PT 16) showed compound heterozygous mutations in *PMM2*, the most frequent gene associated with Congenital Disorder of Glycosylation (CDG Ia). The phenotype of CDG Ia varies from very severe to mild. Neurological symptoms include cerebellar ataxia, intellectual disability, retinitis pigmentosa and peripheral neuropathy. However, mild neurological presentations have also been reported (Drouin-Garraud et al., 2001).

In a patient with cerebellar ataxia associated to myoclonus, mild cognitive impairment and pyramidal signs (PT 17) we detected compound heterozygous mutations in *ATP13A2* gene. Disease onset was in the fourth decade. No extrapyramidal signs or vertical gaze palsy were present. Molecular defects in this

gene have been causally associated with Kufor-Rakeb syndrome, an autosomal recessive form of juvenile-onset parkinsonism, and neuronal ceroid lipofuscinosis, a neurodegenerative disorder characterized by the intracellular accumulation of autofluorescent lipopigments. Recently mutations in this gene has been associated to a hereditary complicated spastic paraplegia (SPG78), presenting as adult-onset combined pyramidal-cerebellar syndrome complicated by cognitive impairment, axonal motor and sensory polyneuropathy, and a marked cerebellar atrophy at MRI (Estrada-Cuzcano et al., 2017).

Only one case with autosomal dominant mode of inheritance was successfully solved, recognized as SCA35 (c.1953_1955dupACA, p.Q652dup) (PT 18). Clinical phenotype is characterized by an adult-onset ataxia associated with pyramidal signs, hyperkinetic choreiform movements, mild cognitive impairment, and anorexia. SCA35 was first described in Chinese families as a slowly progressive ataxia associated to hyperreflexia, tremor, sometimes dystonia and cognitive impairment. Recently SCA35 has been described also in few European cases. In particular in a European patient with ataxia associated with myoclonus has been detected a *TGM6* mutation c.1951_1952insAAC that showed the same effect on the protein transglutaminase 6 (p.Q652dup) of our mutation (Tripathy et al., 2017).

Patients with variants of uncertain significance (table 3)

A homozygous mutation in *RNF170* gene was detected in a patient (PT 19). This gene has been associated with autosomal dominant sensory ataxia 1 (SNAX1) that results in a progressive ataxia caused by degeneration of the posterior columns of the spinal cord, without cerebellar involvement. Clinical features of our patient (a

congenital non-progressive mild cerebellar ataxia with evident cerebellar atrophy at MRI, mental retardation and strabismus) was discordant to the *RNF170* phenotype.

A young woman with congenital downbeat nystagmus, primary amenorrhea for ovarian failure, and tremor with onset at 18 years of age was evaluated for a very mild non-progressive ataxic disturbance (PT 20). She had a sister with the same clinical picture. Compound heterozygous mutations in *AARS2* (alanyltRNA synthetase 2) gene was detected. Mutations in this gene were associated with a LeuKoENcephalopathy Progressive with ovarian failure (LKENP) in females, a clinical presentation previously described as ovarioleukodystrophy (Schiffmann et al., 1997). Neurological phenotype was characterized by progressive ataxia and cognitive/behavioral impairment, although in few cases no motor dysfunctions or only postural tremor have been reported (Dallabona et al., 2014). Congenital nystagmus, tremor and ovarian failure fit well to the *AARS2* mutations, whereas the absence of leukoencephalopathy at MRI and normal cognitive function do not. In the same patient we also detected a single mutation in *SYNE1* and in *ZFYVE26* gene, genes associated to autosomal recessive disorders, and in *ABCB7* gene, associated to X-linked sideroblastic anemia and ataxia (Shimada et al., 1998).

Three mutations in *HARS* (histidyil-tRNA synthetase) gene were detected in a patient with very early onset of psychomotor delay and slow progression of ataxic syndrome (PT 21). Severe mental delay, strabismus, microcephaly, short stature, ligamentous laxity completed the clinical phenotype. Cerebral MRI was normal. She had a sister with same clinical features. Heterozygous mutations in *HARS* gene have been associated with autosomal dominant inherited peripheral neuropathy (Charcot-Marie-Tooth disease, axonal, type 2W) (Safka Brozkova et

al., 2015). The Usher syndrome Type IIIB, an autosomal recessive disorder characterized by deafness and blindness, is, instead, associated with compound heterozygous or homozygous mutation in *HARS* gene (Puffenberger et al., 2012). The discordance of the clinical picture led us to consider the pathogenicity of these mutations uncertain. Single mutation in *CLP1*, associated with the autosomal recessive Pontocerebellar hypoplasia, type 10 (Karaka et al., 2014) have been also detected in this proband.

Compound heterozygous mutations in *HSD17B4* gene was detected in a patient with ataxia associated with pyramidal signs and demyelinating sensorimotor neuropathy with onset at 18 years old (PT 22). The *HSD17B4* product, D-bifunctional protein (DBP), multifunctional enzyme of the peroxisomal ß-oxidation of fatty acids, consisting of three domain: dehydrogenase, hydratase and sterol carrier protein-2. Mutations were found in the dehydrogenase as well as in the hydratase domain; no mutations were observed yet in the sterol carrier protein-2 domain. Patients with DBP deficiency die mostly in their first year of life. Typical clinical observations are hypotonia, facial dysmorphism, psychomotor delay, neonatal seizures, neuronal migration defects or demyelination. Biochemical findings are highly elevated levels of very long chain fatty acids (VLCFA) in plasma (Möller et al., 2001). Few cases of slowly progressive DBP deficiency has been described, characterized by juvenile onset of cerebellar ataxia, peripheral neuropathy, pyramidal signs and deafness. Blood level of VLCFA, pristanic acid and phytanic acid were often normal (Matsukawa et al., 2017). Furthermore, *HSD17B4* gene has been associated to Perrault syndrome, a disorder characterized by sensorineural deafness and ovarian dysgenesis in females. Cerebellar ataxia, mental delay and peripheral neuropathy are often associated

(Pierce at al., 2010). Clinical picture of our case was not suggestive, first of all for the normal auditory function. Furthermore, one mutation of our patient (c.727G>T; p.V243L) affected the dehydrogenase domain; the second one (c.2191C>T p.Q731*) occurred within the sterol carrier protein-2 domain, strongly suggesting that is a polymorphism.

A mutation in *CAMTA1* gene, associated to Cerebellar Ataxia, Non Progressive, with Mental Retardation (CANPMR), an autosomal dominant neurodevelopmental disorder characterized by mild mental retardation and early onset ataxia (Thevenon et al., 2012), has been identified in a case of our cohort (PT 23). Clinical phenotype of our patient was not matching because of a mild slight progressive adult-onset ataxia without cognitive impairment, associated with chronic coughing and a probable recessive inheritance. A similar reasoning can be applied for the mutation detected in the same patient in *IFRD1* gene, associated to Spinocerebellar ataxia 18 (SCA18) (Brkanac et al., 2009). Apart from the dubious autosomal dominant pattern of inheritance, clinical picture of SCA18 is characterized by ataxia associated with a sensorimotor peripheral neuropathy. Nerve conduction studies of the proband and his sister were normal. Single mutations in autosomal recessive disease-associated genes (*WFS1* and *PNPLA6*) have been also detected in the same proband.

In a patient with very mild ataxia with prominent cerebellar dysarthria and onset in the fourth decade (PT 24) we detected heterozygous mutations in five genes: *AFG3L2*, *ITPR1, SPAST, ABHD12*, and *NOL3*. Heterozygous mutations in *AFG3L2* gene have been associated to Spinocerebellar ataxia type 28 (SCA28) (Cagnoli et al., 2010) whereas heterozygous mutations in *ITPR1* gene have been associated to Spinocerebellar ataxia type 29 (SCA29) (Synofzik et al., 2011), both

adult-onset autosomal dominant spinocerebellar ataxia slowly progressive. Nerve conduction studies were usually normal in both SCAs, as in our patient. Further analysis of segregation are needed to recognize the value of these mutations. *SPAST* gene is associated to the most frequent autosomal dominant form of spastic paraplegia (SPG4). The absence of pyramidal signs in the patient, strongly suggest that this variant is a polymorphism. The autosomal recessive disorder Polyneuropathy, Hearing loss, Ataxia, Retinitis pigmentosa, and Cataract (PHARC) caused by mutations in *ABHD12* gene (Fiskerstrand et al., 2010), is not suggestive for clinical phenotype and pattern of inheritance. Autosomal dominant Familial Cortical Myoclonus due to mutations in *NOL3* gene (Russell et al., 2012) is characterized by a clinical picture more complicated than that of our case.

In a complicated case of cerebellar ataxia, associated with myoclonus, an episode of generalized seizure, neurosensorial hypoacusia, cataract, neurogenic bladder, dolichocolon and rectal atony, we detected two missense mutations in *PLA2G6* gene, and heterozygous mutations in *SLC9A1* and *DNMT1* genes. *PLA2G6*-associated neurodegeneration (PLAN) (Kurian et al., 2008) comprises a continuum of three autosomal recessive phenotypes: Infantile Neuroaxonal Dystrophy, characterized by very early onset and progressive form of psychomotor regression or delay, and spastic tetraparesis; Atypical Neuroaxonal Dystrophy, associated to childhood till second decade onset of ataxia and neuropsychiatric disorders; and *PLA2G6*-related dystonia-parkinsonism with variable age of onset of dystonia, parkinsonism and neuropsychiatric changes and often cognitive decline (Gregory et al., 2008). Clinical phenotype of our patient was not congruous to *PLA2G6*-associated disorders, raising doubts about the value of the mutations found. In the same patient we also detected a heterozygous

mutation of *SLC9A1* gene, associated to the autosomal recessive Lichtenstein-Knorr syndrome. Clinical features of the syndrome comprise severe progressive sensorineural hearing loss and cerebellar ataxia with onset in childhood or young adulthood (Guissart et al., 2015). Auditory impairment is part of clinical features of the Autosomal Dominant Cerebellar Ataxia, Deafness, and Narcolepsy (ADCADN), due to mutation in *DNMT1,* characterized by adult onset progressive cerebellar ataxia, narcolepsy/cataplexy, sensorineural deafness and dementia. Despite the presence of ataxia and hypoacusia in our patient, the presence of seizure, myoclonus and intestinal dysfunction are not part of the clinical phenotype of both Lichtenstein-Knorr syndrome and ADCADN.

A patient with ataxia associated with oculomotor apraxia, diplopia and mild pyramidal signs (PT 26) presented compound heterozygous mutations in both *SACS* gene and in *SYNE1* gene, and a heterozygous mutation in *CACNA1G* gene. Autosomal Recessive Spastic Ataxia of Charlevoix-Saguenay (ARSACS), caused by compound heterozygous or homozygous mutations in *SACS* gene, is clinically characterized by an early onset spastic ataxia with a sensorimotor peripheral neuropathy (Bouchard et al., 1978). Retinal striation, mental delay, progressive myoclonic epilepsy and skeletal abnormalities have been also described as cases with absent pyramidal involvement or absent peripheral neuropathy (Ogawa et al., 2004; Muona et al., 2015; Armour et al., 2016). *SYNE1* gene was initially defined as a pure cerebellar ataxia, then recognized as associated to involvement of first and second motor neuron and cognitive delay/impairment. Clinical features of our patient could not be clearly associated with either *SACS* or *SYNE1* gene, in particular for the oculomotor apraxia that is a predominant feature. Autosomal dominant Spinocerebellar ataxia type 42 (SCA42) due to mutation in *CACNA1G*

gene, is a slowly progressive pure cerebellar ataxia, sometimes associated with pyramidal tract involvement. Few families have been described at the moment only with the same R2715H mutation (Coutelier et al., 2015; Morino et al., 2015). Clinical features of our patient and absent positive familial history make the pathogenicity of our mutation unlikely.

In a young woman (PT 27) with ataxia associated with mild pyramidal signs, and a congenital strabismus, we detected a heterozygous mutation in *CACNA1A* gene, found to be responsible for three disorders with autosomal dominant inheritance: Episodic Ataxia type 2 (EA2), Familial Hemiplegic Migraine type 1 (FHM1), and Spinocerebellar ataxia 6 (SCA6). EA2 is mainly characterized by episodes of ataxia, vertigo, and nausea lasting for minutes to hours, often associated with progressive ataxia, and clinical overlap with FHM1 and SCA6 have been described (Romaniello et al., 2010). The same patient presented a heterozygous mutation also in *SPTBN2* gene. Heterozygous missense or in-frame mutations in *SPTBN2* cause autosomal dominant Spinocerebellar ataxia 5 (SCA5), in contrast, stop codon homozygous or compound heterozygous mutations are responsible of the Spinocerebellar ataxia 14, autosomal recessive (SCAR14). De novo mutations in *SPTBN2* have also been detected in congenital form of ataxia (Parolin Schnekenberg et al., 2015). SCA5 is a relatively pure cerebellar ataxia with onset generally in the third or fourth decade of life. SCAR14 is characterized by a congenital ataxia and cognitive impairment with additional mild spasticity (Elsayed et al., 2014). Also a single mutation in *SYNE1* has been identified in the proband.

DISCUSSION

Single Gene Standard Sequencing in disorders with high genetic heterogeneity, such as hereditary ataxias, has a low diagnostic yield per gene. After exclusion of repeat expansion ataxias, as SCAs for autosomal dominant forms, and Friedreich ataxia for recessive ones, over 80 other genes associated with various forms of ataxia remain candidates. Furthermore, since 2010 the use of NGS techniques has increased the number of new genes associated to ataxias, and clinical phenotypes of old genes has been expanded. We used a Targeted Next-Generation Resequencing Panel containing 273 genes, known or supposed to be related to hereditary ataxias, as a tool to investigate 39 heterogeneous patients with ataxia without a molecular diagnosis. Our cohort was largely non-consecutive and most patients had already been investigated for different genes based on clinical features. The selected patients had a clinical suspicion of a genetic etiology, based on the combination of cerebellar ataxia phenotype plus either an age at onset below 45 years or a positive family history. Patients with congenital ataxias were also included. Most patients of our cohort had a complex phenotype, in which ataxia was associated with neurological and extraneurological features.

The diagnostic yield of our study (46%) is highest than those obtained in previous TRP ataxia studies, which had an average diagnostic rate of 17%, and also than of ES studies that reached a 36% of diagnostic yield. This positive result surely depends on the significantly higher number of genes selected in our panel (273 genes) respect to the mean of 88 genes (range 42-159) of the other previous ataxia panels. A key role has also been played by the efficient clinical assessment of the patients, necessary for the confirmation of the pathogenicity of the different

variants detected. The presence of a positive family history remained the most important isolated feature associated with the finding of a pathogenic mutation. In particular seven out of eight patients with known consanguineous parents reached a positive result.

The most frequent gene detected in our cohort was *SPG7* confirming the important causative role of this gene in undiagnosed ataxias (Pfeffer et al., 2015). However, noteworthy is the fact that in two of these patients (PT 3 and 4), during the study process we identified the same mutations in *SPG7* gene through Single Gene Testing, just for the growing evidence in literature of the role of this gene in ataxias associated also with mild pyramidal signs, such as in our patients.

Next in frequency was *PNPLA6* gene, not commonly detected in other ataxic cohorts of previous TRP or ES studies. This result depends on the fact that in our cohort six out of 39 cases (15%) presented a hypogonadism, and the "new genes" associated with hypogonadism (*RNF216*, *OTUD4, STUB1*, and *PNPLA6*) had not previously been investigated in our patients because of lack of availability of genetic testing. However, one of the *PNPLA6* patients (PT 8) had not hypogonadism, and also the patient detected with compound heterozygous mutations in *RNF216* gene (PT 13) was not identified as hypogonadic, and only a re-evaluation of the case showed low FSH and LH levels with normal estradiol, progesterone and prolactin levels. In one patient with hypogonadotropic hypogonadism (PT 2) the diagnosis was SPG7, and in one case with hypergonadotropic hypogonadism and a positive family history for hypogonadism in a brother (PT 10), the gene detected was surprisingly *SYNE1*. This endocrinological finding has never been associated to *SYNE1* gene, expanding the phenotype of the disease.

The clinical phenotype of our *RNF216* patient characterized by hyperkinetic choreiform movements confirms the precedent description of a Huntington-like disorder associated with this gene (Santens et al., 2015).

Lastly, in one woman with ovarian failure (PT 20) possible pathogenic variants were detected in *AARS2* gene, associated with Leukoencephalopathy progressive with ovarian failure, and in a young man with hypergonadotropic hypogonadism (PT 33), variants with unlikely pathogenicity were identified.

Mutations in *SYNE1* and in *SETX* genes has been identified in two probands each. These genes have frequently been detected in previous TRP and ES ataxia studies too (Nemeth et al., 2013; Mallaret et al., 2016; Sawyer et al., 2014; Fogel et al., 2014; Marelli et al., 2016), because few families have been screened for *SYNE1* gene as the gene is very large, and *SETX* gene is not routinely tested in UK and France whereas in our probands clinical features were atypical.

Mutations in *SACS* gene, responsible for ARSACS, have been frequently detected in other TRP and ES studies (Nemeth et al., 2013; Mallaret et al., 2016; Hadjivassiliou et al., 2016; Iqbal et al., 2017; Sawyer et al., 2014; Pyle et al., 2015). In our cohort none has been recognized as ARSACS, probably because in a previous study we tested for *SACS* gene a cohort of ARSACS-like patients from our Ataxias Clinic (Criscuolo et al., 2004).

In nine patients (23%) we found possible pathogenic variants of uncertain significance, pointing to the difficulty of the determination of the pathogenicity of a given variant, and to the necessity of further testing and family studies for validation or exclusion of genetic findings. However, the sharing of VOUS and clinical phenotypes will allow a possible future validation of the pathogenicity of variants.

Finally, in 31% of cases no candidate variants or variants with unlikely pathogenicity were identified. Possible reasons include the presence of cases in which causal variants are present in novel genes that are yet to be identified or were found during the study period. Such newly identified genes can be added into TRP on a regular basis. Furthermore, some disease-causing variants might be localized in the promotor, introns, or other non-coding DNA or could be indels or copy number variations that are not analyzed in our study.

The patients in which we could not detect confirmed pathogenic variants are candidates for exome sequencing studies.

CONCLUSION

The clinical use of NGS techniques is becoming more widespread; our findings suggest that after exclusion of repeat expansion ataxias, ataxia TRP, eventually followed by full exome analyses if first approach was negative, could be a good first tier diagnostic line, when a specific single gene is not immediately suspected to be causative. Our TRP provides high-quality sequencing data with excellent coverage of the selected genes and a very high diagnostic yield.

TABLES AND FIGURES

Table 1. List of the 273 genes included in the ataxia panel

GENES	MIM	GENES	MIM	GENES	MIM	GENES	MIM
AARS2	612035	*C12ORF65*	613541	*DKC1*	300126	*GRM1*	604473
ABCB7	300135	*C9ORF72*	614260	*DNAJC19*	608977	*HARS*	142810
ABCD1	300371	*CA8*	114815	*DNAJC3*	601184	*HARS2*	600783
ABHD12	613599	*CACNA1A*	601011	*DNMT1*	126375	*HEXA*	606869
ACO2	100850	*CACNA1G*	604065	*EEF2*	130610	*HEXB*	606873
ADCK3	606980	*CACNB4*	601949	*EIF2B1*	606686	*HIBCH*	610690
ADGRG1	604110	*CAMTA1*	611501	*EIF2B2*	606454	*HSD17B4*	601860
AFG3L2	604581	*CASK*	300172	*EIF2B3*	606273	*INPP5E*	613037
AHDC1	615790	*CC2D2A*	612013	*EIF2B4*	606687	*ITPR1*	147265
AHI1	608894	*CCDC88C*	611204	*EIF2B5*	603945	*KCNA1*	176260
ALDH5A1	610045	*CD40LG*	300386	*ELOVL4*	605512	*KCNA2*	176262
ALG3	608750	*CDK5*	123831	*ELOVL5*	611805	*KCNC1*	176258
ALG6	604566	*CEP104*	616690	*ERCC4*	133520	*KCNC3*	176264
AMACR	604489	*CEP290*	610142	*ERCC8*	609412	*KCND3*	605411
AMPD2	102771	*CEP41*	610523	*EXOSC3*	606489	*KCNJ10*	602208
ANO10	613726	*CHMP1A*	164010	*EXOSC8*	606019	*KCTD7*	611725
APOB	107730	*CLCN2*	600570	*FA2H*	611026	*KIF1A*	601255
APTX	606350	*CLN5*	608102	*FARS2*	611592	*KIF1C*	603060
ARL13B	608992	*CLN6*	606725	*FASTKD2*	612322	*KIF7*	611254
ARSA	607574	*CLN8*	607837	*FGF14*	601515	*LAMA1*	150320
ATCAY	608179	*CLP1*	608757	*FLVCR1*	609144	*LMNB2*	150341
ATG5	604261	*COA7*	615623	*FMR1*	309550	*LYST*	606897
ATL1	606439	*COQ2*	609825	*FOLR1*	136430	*MARS2*	609728
ATM	607585	*COQ4*	612898	*FXN*	606829	*MED17*	603810
ATP13A2	610513	*COQ9*	612837	*GALC*	606890	*MFN2*	608507
ATP1A3	182350	*SPP1*	166490	*GAN*	605379	*MFSD8*	611124
ATP2B3	300014	*CSTB*	601145	*GBA2*	609471	*MKS1*	609883
ATP7B	606882	*CTBP1*	602618	*GBE1*	607839	*MMACHC*	609831
ATP8A2	605870	*CTSD*	116840	*GDAP1*	606598	*MME*	120520
BEAN1	612051	*CWF19L1*	616120	*GFAP*	137780	*MRE11A*	600814
BRAT1	614506	*CYP27A1*	606530	*GJB1*	304040	*MTPAP*	613669
BRF1	604902	*CYP7B1*	603711	*GJC2*	608803	*MTTP*	590075
C10ORF2	606075	*DARS*	603084	*GLB1*	611458	*MVK*	251170
C12ORF65	613541	*DARS2*	610956	*GOSR2*	604027	*NAGLU*	609701
C5ORF42	614571	*DDHD2*	615003	*GRID2*	602368	*NDUFS1*	157655

GENES	MIM	GENES	MIM	GENES	MIM	GENES	MIM
NDUFS7	601825	*POLR3A*	614258	*SLC2A1*	138140	*TMEM237*	614423
NEU1	608272	*POLR3B*	614366	*SLC33A1*	603690	*TMEM240*	616101
NOL3	605235	*PPT1*	600722	*SLC35A2*	314375	*TMEM67*	609884
NOP56	614154	*PRICKLE1*	608500	*SLC52A2*	607882	*TOP1*	126420
NPC1	607623	*PRKCG*	176980	*SLC6A19*	608893	*TPP1*	607998
NPC2	601015	*PRNP*	176640	*SLC9A1*	107310	*TRNT1*	612907
NPHP1	607100	*PRPS1*	311850	*SLC9A6*	300231	*TRPC3*	602345
OFD1	300170	*PRRT2*	614386	*SMPD1*	607608	*TSEN2*	608753
OPA1	605290	*PSAP*	176801	*SNAP25*	600322	*TSEN34*	608754
OPA3	606580	*PSEN1*	104311	*SNX14*	616105	*TSEN54*	608755
OPHN1	300127	*PTF1A*	607194	*SPAST*	604277	*TTBK2*	611695
OTUD4	611744	*PTRH2*	608625	*SPG11*	610844	*TTC19*	613814
PAX6	607108	*QARS*	603727	*SPG7*	602783	*TTC21B*	612014
PDE6D	602676	*RAB3GAP1*	602536	*SPTBN2*	604985	*TTPA*	600415
PDHA1	300502	*RARS*	107820	*SRD5A3*	611715	*TUBB3*	602661
PDSS1	607429	*RARS2*	611524	*STS*	300747	*TUBB4A*	*602662*
PDSS2	610564	*RELN*	600514	*STUB1*	607207	*UBA5*	610552
PDYN	131340	*RNF170*	614649	*STXBP1*	602926	*UBR4*	609890
PEX10	602859	*RNF216*	609948	*SURF1*	185620	*UCHL1*	191342
PEX16	603360	*RPGRIP1L*	610937	*SYNE1*	608441	*VAMP1*	185880
PEX2	170993	*RUBCN*	613516	*SYNE2*	608442	*VARS2*	612802
PEX6	601498	*SACS*	604490	*SYT14*	610949	*VLDLR*	192977
PEX7	601757	*SAMD9L*	611170	*TBC1D23*	617687	*VRK1*	602168
PHYH	602026	*SCN1A*	182389	*TCTN1*	609863	*VWA3B*	614884
PIK3R5	611317	*SCN2A*	182390	*TCTN2*	613846	*WDR81*	614218
PLA2G6	603604	*SCN8A*	600702	*TCTN3*	613847	*WFS1*	606201
PLEKHG4	609526	*SCYL1*	607982	*TDP1*	607198	*WWOX*	605131
PLP1	300401	*SEPSECS*	613009	*TGM6*	613900	*XPA*	611153
PMM2	601785	*SETX*	608465	*THG1L*	Nd	*XRCC4*	194363
PMPCA	613036	*SIL1*	608005	*TINF2*	604319	*ZFYVE26*	612012
PNKP	605610	*SLC17A5*	604322	*TMEM138*	614459	*ZFYVE27*	*610243*
PNPLA6	603197	*SLC1A3*	600111	*TMEM216*	613277	*ZNF423*	604557
POLG	174763	*SLC25A46*	610826	*TMEM231*	614949	*ZNF592*	613624

Table 2. Clinical features and genetic results of patients with confirmed pathogenic variants

PT	Sex	AE y	Onset y	Inheritance	IAPS/age at reaching phase	Additional features	MRI	Gene	Variant DNA; protein	Zygosity	Disorder
1	F	55	30	SP	II/30	urinary incontinence	slight CA	*SPG7*	c.1529C>T; p.A510V c.1940C>A; p.A647E	ch	SPG7
2	M	68	36	AR	III/59	pyramidal signs, opthalmoparesis, urinary incontinence, hypogonadotric hypogondism	CA	*SPG7*	c.73_80delCCAGGCCC; p.P25fs*46SPG7 c.1940C>A; p.A647E	ch	SPG7
3	F	61	52	AR	II/52	pyramidal signs	CA, slight supratentorial atrophy	*SPG7*	c.1529C>T; p.A510V c.1972G>A; p.A658T	ch	SPG7
4	F	69	45	SP	III/65	pyramidal signs, urge incontinence	CA	*SPG7*	c.1231G>A; p.D411N c.679C>T; p.R227*	ch	SPG7
5	M	71	49	AR Cons	II/49		slight CA, slight supratentorial atrophy	*SPG7*	c.1529C>T; p.A510V	ho	SPG7
6	M	32	19	SP	II/19	mild intellectual disability, hypogonadotric hypogondism, gynecomastia, chorioretinal dystrophy	CA	*PNPLA6*	c.3365C>T; p.P1122L c.4081C>T; p.R1361*	ch	Gordon Holmes/ Boucher-Neuhäuser syndrome
7	M	41	26	AR Cons	II/26	mild intellectual disability, hypogonadotric hypogondism, cryptorchidism, pyramidal signs, chorioretinal dystrophy	CA	*PNPLA6*	c.1880C>T; p.A627V	ho	Gordon Holmes/ Boucher-Neuhäuser syndrome
8	F	63	44	AR Cons	III/56	pyramidal signs	CA	*PNPLA6*	c.3385G>A; p.G1129R	ho	Gordon Holmes/ Boucher-Neuhäuser syndrome
9	F	68	47	AR Cons	III/64	sensory neuropathy	slight CA	*SYNE1*	c.14676G>C; p.E4892D	ho	SCAR8
10	F	37	24	AR Cons	II/24	mild pyramidal signs, hypergodatropin hypogonadism; a brother with ataxia, hypogonadism and moderate intellectual disability	marked CA	*SYNE1*	c.4609C>T; p.R1537*	ho	SCAR8

(continued)

Table 2. Continued

PT	Sex	AE y	Onset y	Inheritance	IAPS/age at reaching phase	Additional features	MRI	Gene	Variant DNA; protein	Zygosity	Disorder
11	F	80	35	AR Cons	IV/62	neuropathy, pyramidal signs, strabismus	CA, slight supratentorial atrophy	*SETX*	c.3242T>C; p.F1081S	ho	AOA2
12	M	51	11	SP/ AD for tremor	IV/34	sensorymotor neuropathy, tremor	CA	*SETX*	c.7292dupA; p.N2431fs*19	ho	AOA2
13	F	43	37	SP	II/37	pyramidal signs, tremor, hyperkinetic choreiform movements, mild cognitive impairment	CA	*RNF216*	c.1890+3A>G c.1678A>G; p.M560V	ch	Gordon Holmes/ Boucher-Neuhäuser syndrome
14	M	53	30	SP	II/30	pyramidal signs, esecutive dysfunction, resting tremor, bradykinesia, retrocollis, vertical opthalmoparesis, marked dysphagia (PEG), hallucinations	pontoCA	*ZFYVE26*	c.3722G>A; p,R1241Q c.3970T>A; p.S1324T	ch	SPG15
15	F	41	30	SP	III/40	pyramidal signs, retrubalbar optic neuritis at 31 y, OCB in CSF	WMA, marked CA	*ANO10*	c.206T>A; p.L69*	ho	SCAR10
16	M	32	1	AR	II/1	pyramidal signs, mild mental retardation, strabismus	marked CA	*PMM2*	c.422G>A; p.R141H c.323C>T; p.A108V	ch	CDG IA
17	M	75	38	AR Cons	IV/60	myoclonus, pyramidal signs, slight cognitive impairment	CA, supratentorial atrophy	*ATP13A2*	c.1190C>T; p.T397M	ho	Kufor-Rakeb syndrome
18	F	67	55	AD	III/66	pyramidal signs,hyperkinetic choreiform movements, mild cognitive impairment, anorexia	CA, slight supratentorial atrophy	*TGM6*	c.1953_1955dupACA; p.Q652dup	he	SCA35

PT patient; AE age at examination; AO age at onset; y years; IAPS Inherited Ataxias Progression Scale; MRI magnetic resonance images; F female, M male;
SP sporadic; AR autosomal recessive; AD autosomal dominante; Cons consanguineity; CA cerebellar atrophy; he heterozygous; ho homozygous; ch compound heterozygous.
SPG7 Spastic Paraplegia type 7 (MIM 607259); Gordon Holmes syndrome (MIM 212840); Boucher-Neuhäuser syndrome (MIM 215470); SCAR8 Spinocerebellar ataxia, autosomal recessive 8 (MIM 610743);
AOA2 Ataxia-oculomotor-apraxia-2 (MIM 606002); SPG15 Spastic Paraplegia 15 (MIM 270700); SCAR10 Spinocerebellar ataxia, autosomal recessive 10 (MIM 613728);
CDG IA Congenital Dysorders of Glycosylation IA (MIM 212065); Kufor-Rakeb syndrome (MIM 606693); SCA35 Spinocerebellar ataxia 35 (MIM 613908).

Table 3. Clinical features and genetic results of patients with VOUS

PT	Sex	AE y	Onset y	Inheritance	IAPS/age at reaching phase	Additional features	MRI	Gene	Variant DNA; protein	Zygosity	Disorder
19	F	26	3	SP	II/3	mild intellectual disability, strabismus, mild pyramidal signs	CA	*RNF170*	c.566T>G p.F189C	he	SNAX1
20	F	35	18	AR	II/18	congenital downbeat ny, primary amenorrhea, tremor	normal	*AARS2* - *SYNE1* *ZFYE26* *ABCB7*	c.446G>A pC149Y; c.385A>C p.T129P; c.13849A>C p.N4617H; c.1844C>T p.S615F; c.246+1G>A	ch - he he he	LKENP - SCAR8 SPG15 ASAT
21	F	38	1	AR	II/1	intellectual disability, strabismus, microcephaly, short stature, ligamentous laxity	normal	*HARS* - *CLP1*	c.90+4A>C c.1393A>C p.I465L; c.910_912dupTTG p.L305dup; c.898C>T p.R236C	ch - - he	Usher s. type IIIB - - PCH10
22	M	30	18	SP	II/18	pyramidal signs, demyelinating sensorimotor neuropathy	CA	*HSD17B4*	c.2191C>T p.Q731*; c..727G>T p.V243L	ch	Perrault s.
23	M	59	50	AR	II/50	cough, RLS	mild CA	*CAMTA1* *IFRD1* *WFS1* *PNPLA6*	c.4886G>A p.S1629N; c.1256G>A p.R419H c.712+16G>A; c.82G>T p.E28*	he he he he	CANPMR SCA18 Wolfram s. Gordon Holmes/ Boucher-Neuhäuser s.
24	M	43	35	SP	II/35		normal	*AFG3L2* *ITPR1* *SPAST* *ABHD12* *NOL3*	c.1895G>A p.R632Q; c.5576T>G p.V1859G; c.138T>G p.H46Q; c.62C>T p.S21F; c.179C>A p.A60D	he he he he he	SCA28 SCA15 SPG4 PHARC FCM

(continued)

Table 3. Continued

PT	Sex	AE y	Onset y	Inheritance	IAPS/age at reaching phase	Additional features	MRI	Gene	Variant DNA; protein	Zygosity	Disorder
25	F	47	21	SP	III/47	myoclonus, seizure, tremor, sensorineural hypoacusia, cataract, dolicholoIon, rectal atony, neurogenic bladder	CA, suvratentorial atrophy, WMA	*PLA2G6* - *SLC9A1* *DNMT1*	c.2346C>G p.L782L; c.1952T>G p.L651R; c.2435C>T p.P812; c.1619A>G p.Y540C	ch - he he	PLAN - Lichtenstein-Knorr s. Cerebellar ataxia, deafness, narcolepsy, AD
26	M	63	29	SP	III/31	oculomotor apraxia, diplopia, mild pyramidal signs	normal	*SACS* - *SYNE1* - *CACNA1G*	c.4717A>T p.M1573L; c.1417A>G p.N473D; c.25342G>A p.D8448N; c.3536A>C p.E1179A; c.6298C>T p.P2100S	ch - ch - he	ARSACS - SCAR8 - SCA42
27	F	30	20	SP	II/20	strabismus, mild pyramidal signs	CA	*CACNA1A* *SPTBN2* *SYNE1*	c.5861C>T p.T1954M; c.2728G>T p.A910S; c.11271_11272delGG	he he he	EA2 SCA5/SCAR14 SCAR8

PT patient; AE age at examination; AO age at onset; y years; IAPS Inherited Ataxias Progression Scale; MRI magnetic resonance images; F female, M male;
SP sporadic; AR autosomal recessive; CA cerebellar atrophy; he heterozygous; ch compound heterozygous; RLS restless legs syndrome;
SNAX1 Ataxia, sensory, 1, autosomal dominant (MIM 608984); LKENP Leukoencephalopathy, progressive with ovarian failure (MIM 615889);
SCAR8 Spinocerebellar ataxia, autosomal recessive 8 (MIM 610743); SPG15 Spastic Paraplegia 15 (MIM 270700); ASAT Anemia, sideroblastic, with ataxia (MIM 301310);
Usher syndrome type IIIB (MIM 614504); PCH10 Pontocerebellar Hypoplasia type 10 (MIM 615803); Perrault syndrome (MIM 233400);
CANPMR Cerebellar Ataxia Non Progressive, with Mental retardation (MIM 614756); SCA18 Spinocerebellar ataxia type 18 (MIM 607458); Wolfram syndrome (MIM 222300);
Gordon Holmes syndrome (MIM 212840); Boucher-Neuhäuser syndrome (MIM 215470); SCA28 Spinocerebellar ataxia type 28 (MIM 610246); SCA15 Spinocerebellar ataxia type 15 (MIM 606658);
SPG4 Spastic paraplegia 4 (MIM 182601); PHARC Polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, and cataract (MIM 612674); FCM Myoclonus, familial cortical (MIM 614937);
PLAN PLA2G6-associated neurodegeneration (MIM 256600-610217-612953); Lichtenstein-Knorr syndrome (MIM 616291); Cerebellar ataxia, deafness, and narcolepsy, autosomal dominant (MIM 604121)
ARSACS Spastic ataxia, Charlevoix-Saguenay type (MIM 270550); SCA42 Spinocerebellar ataxia type 42 (MIM 616795); EA2 Episodic ataxia type 2 (MIM 108500);
Spinocerebellar ataxia type 5 (MIM 600224); SCAR14 Spinocerebellar ataxia 14, autosomal recessive (MIM 615386).

Table 4. Clinical features and genetic results of patients with variants with unlikely pathogenicity or no candidate variants

PT	Sex	AE y	Onset y	Inheritance	IAPS/age at reaching phase	Additional features	MRI	Gene	Variant DNA; protein	Zygosity	Disorder
28	M	20	1	SP	II/1	mild intellectual disability, hyperkinetic choreiform movements, pyramidal signs	normal	*SYNE1* *SACS* *PNPLA6*	c.14263C>T p.L4755F; c.6511G>A p.A2171T; c.656G>A p.G219D	he he he	SCAR8 ARSACS Gordon Holmes/ Boucher-Neuhäuser s.
29	M	42	39	AR, Cons	II/39	tremor	normal	*KIF1C*	c.2734C>T p.R912W	he	SPAX2
30	M	60	42	AR	II/42		CA	*ATM* *GRID2* *MTPAP*	c.5727G>A p.M1909I c.2587G>A p.V863I c.1060G>T p.V354L	he he he	Ataxia-telangiectasia SCAR18 SPAX4
31	F	48	36	SP	II/36	sensory neuropathy, hypoacusia, ofthalmoparesis, pyramidal signs, head dystonia	slight CA	*SETX*	c.5051C>G p.S1684C	he	AOA2
32	M	71	25	SP	III/68	sensorymotor axonal neuropathy, scoliosis	normal	*SYNE1*	c.17203-5C>T	he	SCAR8
33	M	24	18	SP	II/18	mental delay, hypergonadotropic hypogonadism, pyramidal signs, periferal neuropathy, urinary incontinence	vermian hypoplasia	PLEKHG4 SYNE1	c.2626G>C p.E876Q c.1141G>A p.D381N	he he	previous correlation to SCA31 SCAR8

(continued)

Table 4. Continued

PT	Sex	AE y	Onset y	Inheritance	IAPS/age at reaching phase	Additional features	MRI	Gene	Variant DNA; protein	Zygosity	Disorder
34	F	58	28	AR	II/28	head tremor	mild vermian and spinal cord atrophy	*SETX*	c.1468G>A p.V490I	he	AOA2
35	M	33	13	SP	II/13	few epileptic seizures, pyramidal signs, dorsal lipomas	slight WMA, mild spinal cord atrophy	*ATM*	c.6495dupT p.V2166fs*9	he	Ataxia-telangiectasia
36	M	55	30	AD for tremor	II/30	tremor, writer's cramp	slight CA	*SIL1* *WFS1* *SYNE1*	c.944G>A p.V332M c.577A>C p.K193Q c.21826G>A p.D7276N	he he he	Marinesco-Sjogren s. Wolfram syndrome; SCAR8
37	M	14	12	SP	II/12		CA	no variants			
38	F	24	1	SP	II/1	intellectual disability	cerebellar hypoplasia	no variants			
39	M	67	43	AR	III/50	cognitive impairment	CA	no variants			

PT patient; AE age at examination; AO age at onset; y years; IAPS Inherited Ataxias Progression Scale; MRI magnetic resonance images; F female, M male;
SP sporadic; AR autosomal recessive; AD autosomal dominante; Cons consanguineity; CA cerebellar atrophy; he heterozygous; ch compound heterozygous.
SCAR8 Spinocerebellar ataxia, autosomal recessive 8 (MIM 610743); ARSACS Spastic ataxia, Charlevoix-Saguenay type (MIM 270550); Gordon Holmes syndrome (MIM 212840);
Boucher-Neuhäuser syndrome (MIM 215470); SPAX2 Spastic ataxia 2, autosomal recessive (MIM 611302); Ataxia-telangiectasia (MIM 208900); SPAX4 Spastic ataxia, autosomal recessive (613672);
AOA2 Ataxia-oculomotor-apraxia-2 (MIM 606002); SCA31 Spinocerebellar ataxia type 32 (MIM 117210); Marinesco-Sjogren syndrome (MIM 248800); Wolfram syndrome (MIM 222300).

Table 5. Sexual assessment in patients with *PNPLA6* mutations

	PT 6	PT 7	PT 7 brother
LH mU/ml (nv 5-18)	0.1	10	3.2
FSH mU/ml (nv 5-18)	0.1	7.4	5.9
Testosteron ng/dl (nv 300-900)	35	182	256
Androstenedion ng/ml (nv 1.1-3.5)	0.88	1.85	1.81
DHEA-S µg/dl (nv 80-560)	148	173	308
17 β Estradiol pg/ml (nv 0-56)	59.9	35.4	not done
17 OH Progesteron ng/ml (nv 0.6-3.3)	0.71	0.08	0.76
Testicular echography	4 ml (each)	Left hypotrophic undescended testis, reduced volume of the right testis	Reduced volume of both testes

Figure 1. Cost per genome since 2001 (courtesy of www.genome.gov)

Figure 2. The large increase of published NGS reports in biomedical domain since 2008 (Chaitankar et al., 2016)

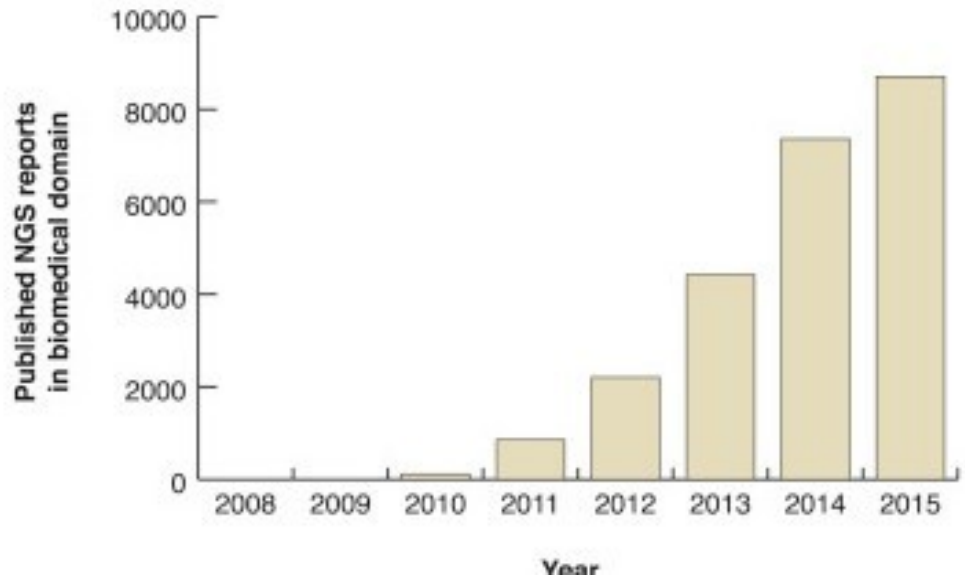


Figure 3. Diagnostic yield in literature hereditary ataxias patients cohorts (Galatolo et al., 2017)

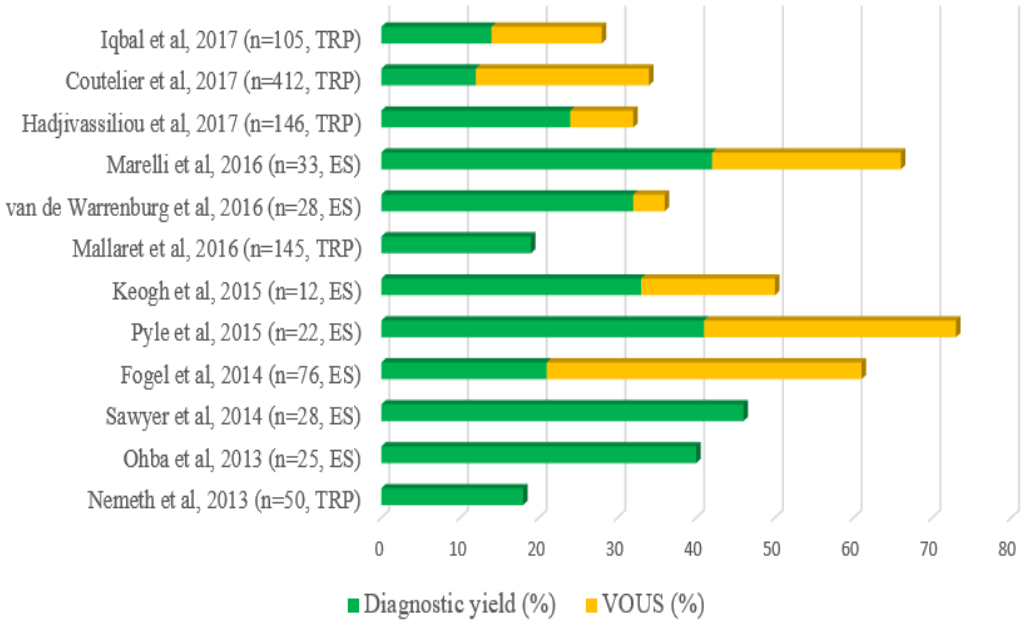


Figure 4. Workflow of *SureSelect Target Enrichment system* for library preparation (Image courtesy of www.agilent.com)

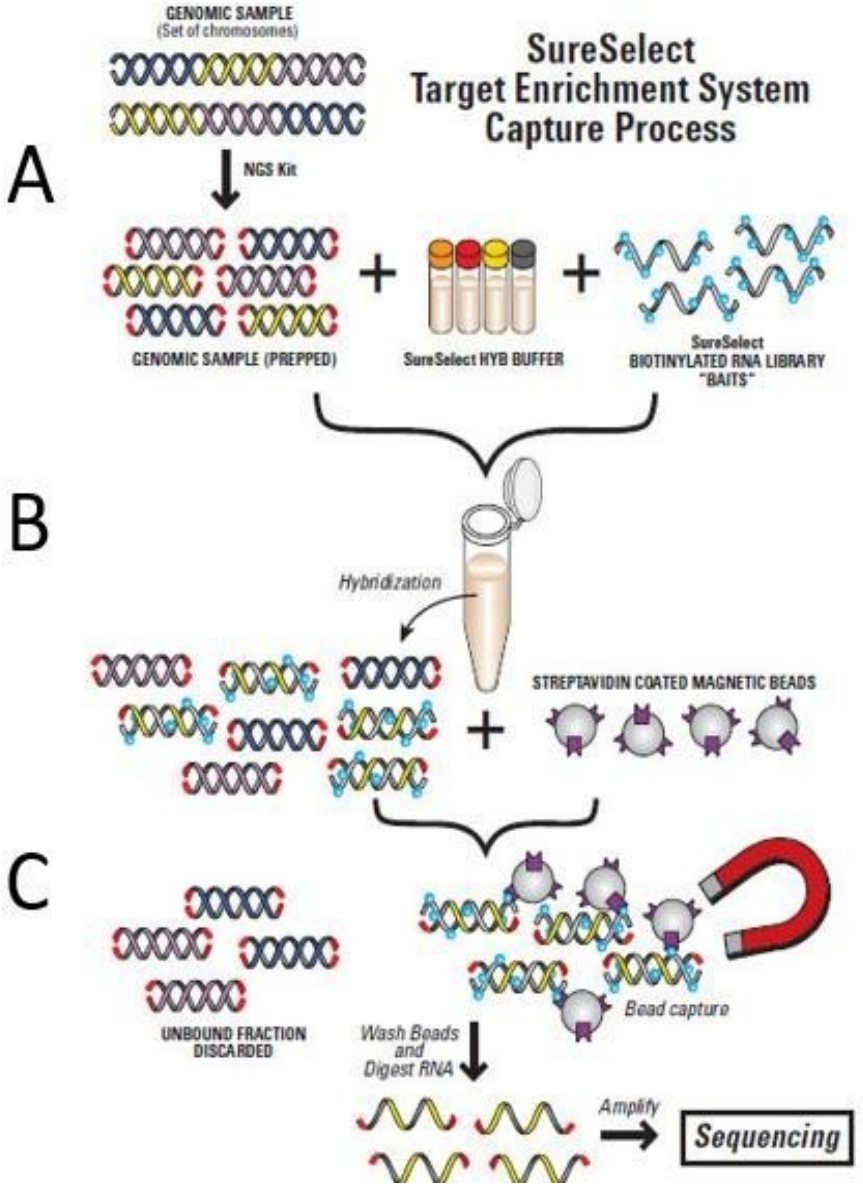
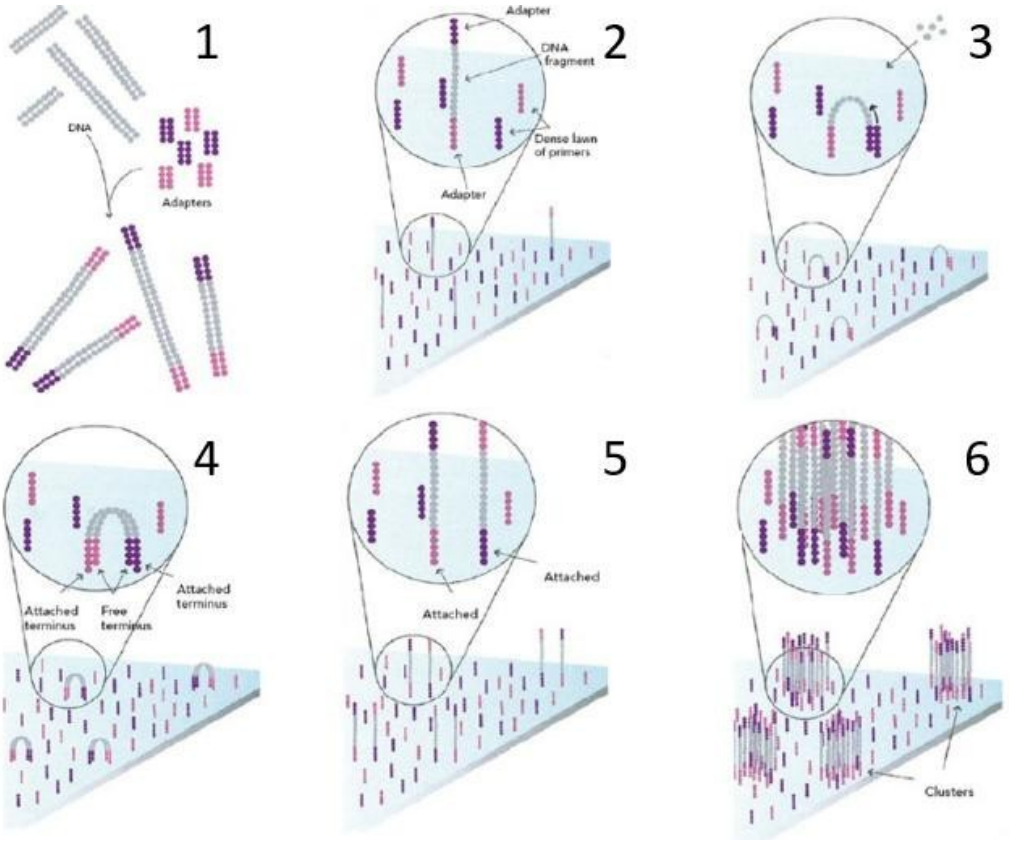
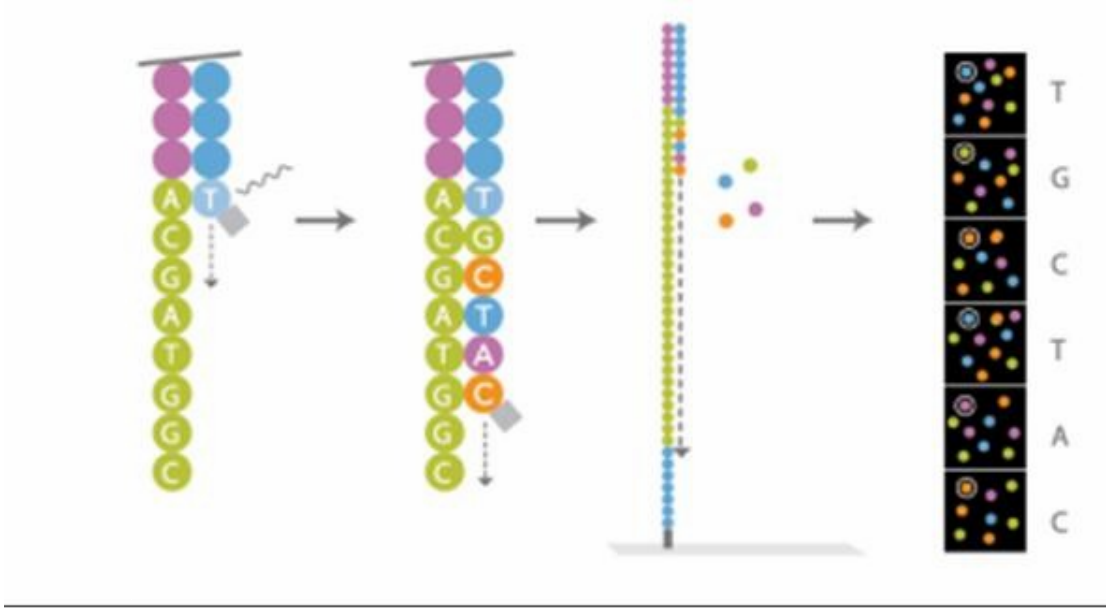


Figure 5. Bridge amplification and cluster generation for Illumina sequencing (Image courtesy of www.illumina.com)



1. Prepare Genomic DNA Sample: randomly fragment genomic DNA and ligate adapters to both ends of the fragments. 2. Attach DNA to Surface: bind single-stranded fragments randomly to the inside surface of the flow cell channels. 3. Bridge Amplification: add unlabeled nucleotides and enzyme to initiate solid-phase bridge amplification. 4. Fragments Become Double Stranded: the enzyme incorporates nucleotides to build double-stranded bridges on the solid-phase substrate. 5. Denature the Double-Standed Molecules: denaturation leaves single-stranded templates anchored to the substrate. 6. Complete Amplification: several million dense clusters of double-stranded DNA are generated in each channel of the flow cell.

Figure 6. Sequencing and base calling for Illumina sequencing (Ansorge, 2009)



Amplified DNA fragments are subjected to sequencing-by-synthesis using 3' blocked labelled nucleotides: extend first base, read, and deblock; repeat step above to extend strand; generate base calls.

Figure 7. Chart reporting sequencing quality. An average of 99% target regions were sequenced almost 50 times (50X), 96% 100X and 82% 200X

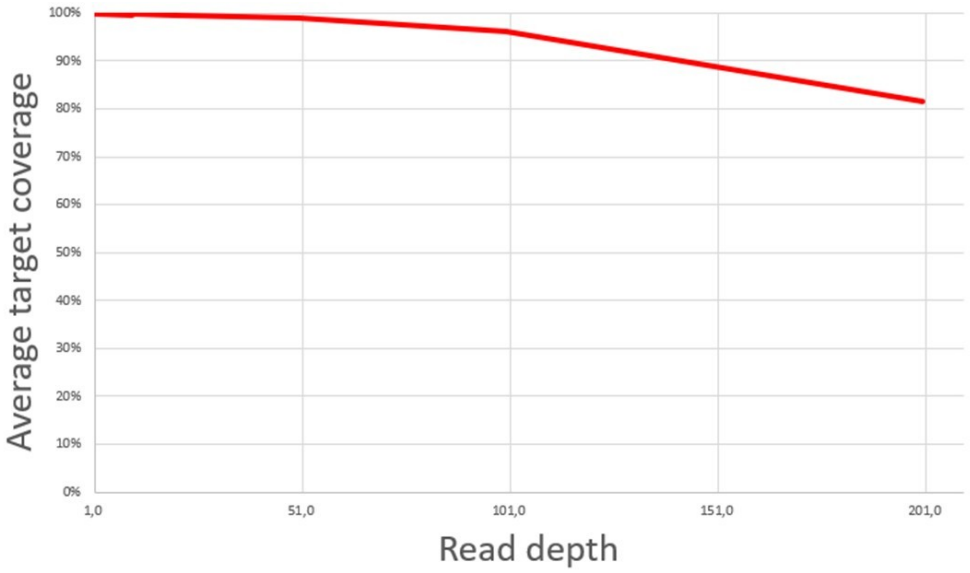


Figure 8. Brain MRI of patient 2 carrying mutations in *SPG7* gene.
T_2-weighted sagittal and axial brain MRI showing cerebellar atrophy

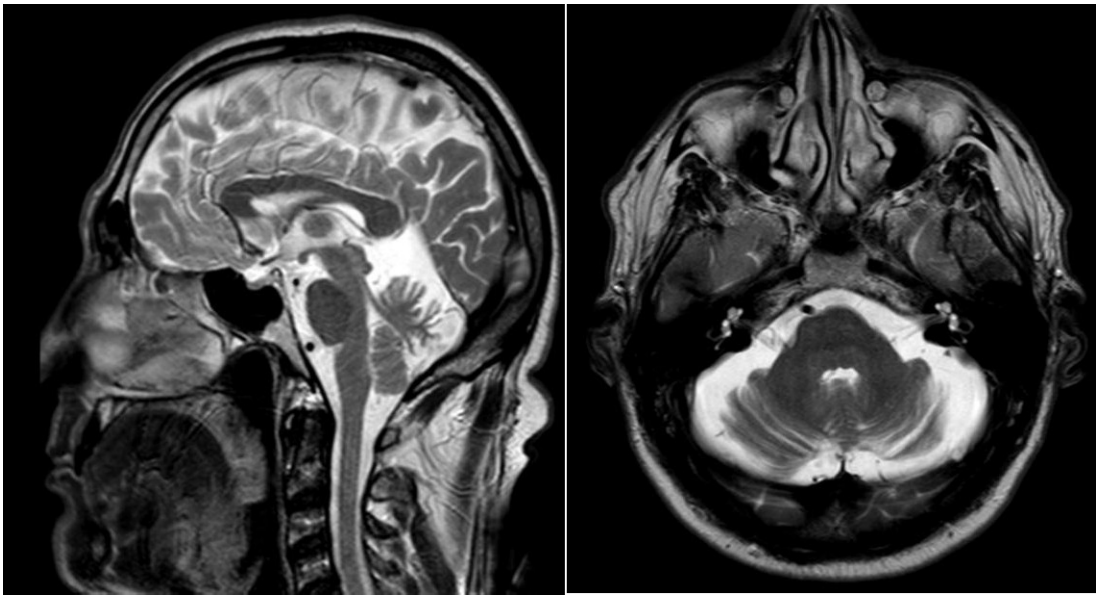


Figure 9. Brain MRI of patient 6 carrying mutations in *PNPLA6* gene.
T_2-weighted sagittal and axial brain MRI showing marked cerebellar atrophy

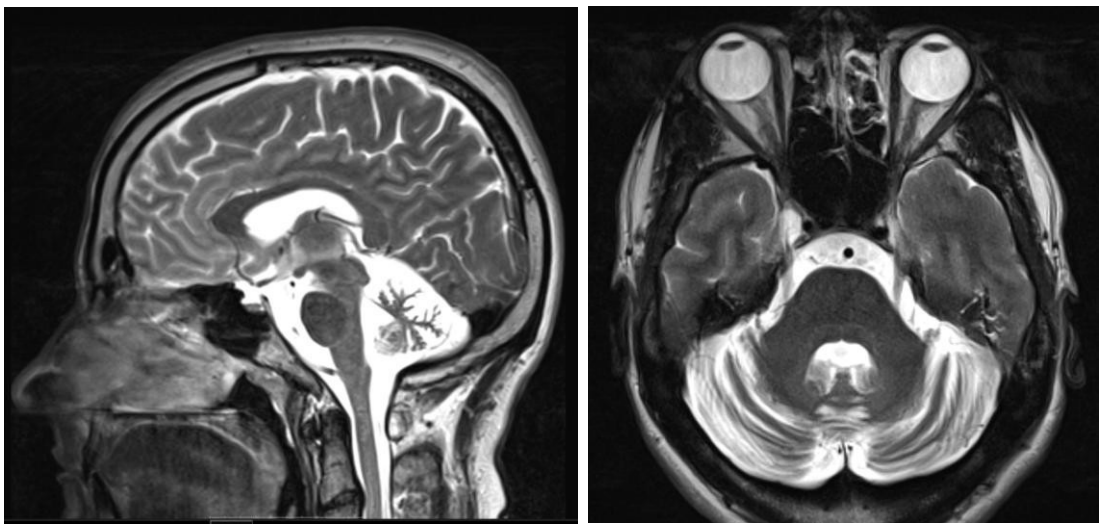


Figure 10. Brain MRI of patient 10 carrying mutations in *SYNE1* gene.
T_2-weighted sagittal and axial brain MRI showing marked cerebellar atrophy

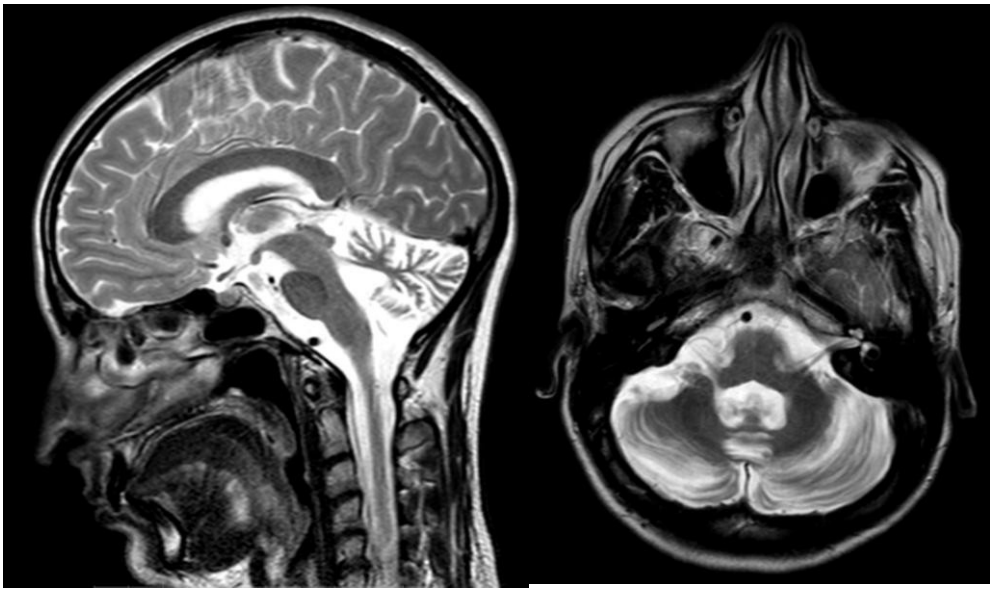
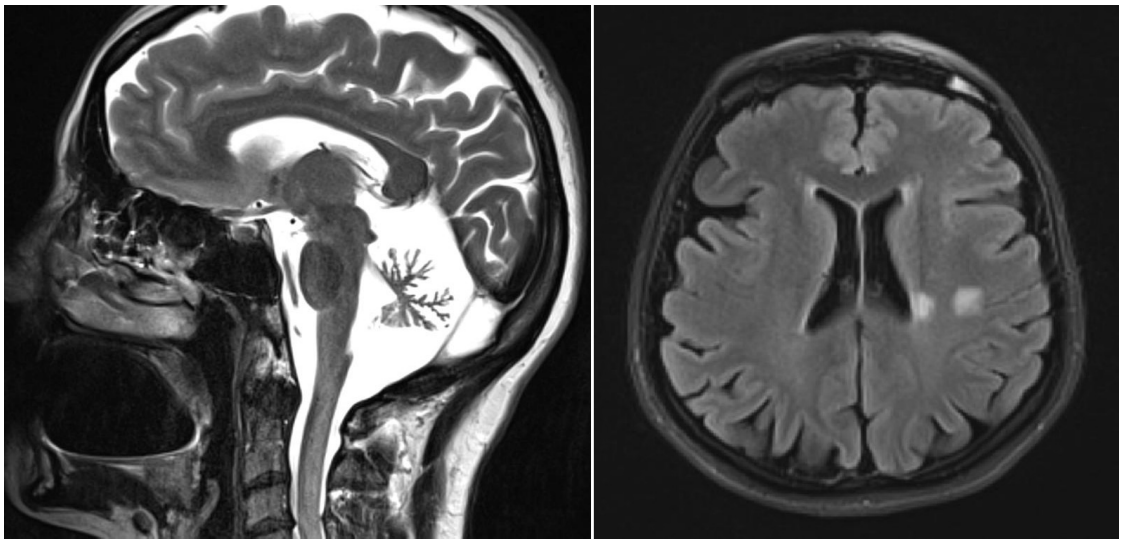


Figure 11. Brain MRI of patient 15 carrying mutations in *ANO10* gene.
T_2-weighted sagittal brain MRI showing marked cerebellar atrophy and FLAIR axial brain MRI showing white matter abnormalities.



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