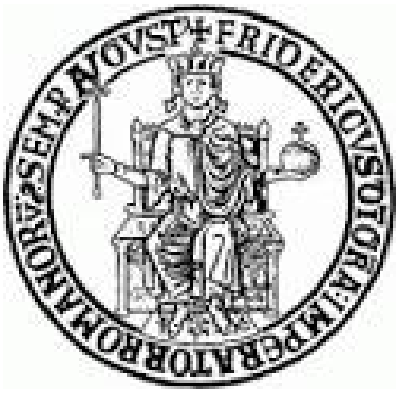
UNIVERSITY OF NAPLES FEDERICO II

DEPARTMENT OF NEUROSCIENCE, REPRODUCTIVE

AND ODONTOSTOMATOLOGICAL SCIENCE



PhD Program in Neuroscience - XXXII Cycle

Prof. Maurizio Taglialatela

NEXT GENERATION SEQUENCING IN UNDIAGNOSED SPORADIC AND INHERITED ATAXIAS

PhD Student

Dr Maria Lieto

Tutor

Prof. Alessandro Filla

INDEX

1. INTRODUCTION

1.1 Inherited And Sporadic Ataxias

The inherited cerebellar ataxias are clinically and genetically heterogeneous, presenting at any age, and usually without features pointing to a specific molecular diagnosis (Anheim et al., 2010). The clinical features are characterized by slowly progressive ataxia, poor coordination of hands, speech and eye movement. In addition, patients may develop ophtalmoplegia, spasticity, neuropathy, cognitive impairment, epilepsy or extraneurological signs. Hereditary ataxia may result from one or any combination of the dysfunction of the cerebellum and its associated systems, lesions in the spinal cord, peripheral sensory loss. The cerebellar syndrome may present in variable combination with other neurological signs such as pyramidal or extrapyramidal signs, ophthalmoplegia, cognitive impairment, intellectual disability, epilepsy, optic atrophy or extraneurological signs such as cataract, cardiomyopathy, endocrine dysfunctions. Atrophy of the cerebellum and brainstem are most often the prominent features, but other structures can be affected, leading to a substantial range of phenotypes.

Prevalence of the autosomal dominant cerebellar ataxias is estimated to be approximately 1-5:100,000 population (van de Warrenburg et al., 2002, Ruano et al., 2014). Of the autosomal dominant ataxias, SCA3 is the most common worldwide, followed by SCA1, 2, 6, and 7. Autosomal recessive types of hereditary ataxia account for approximately 3:100,000 (Ruano et al., 2014) with Friedreich ataxia, ataxia-telangiectasia, and ataxia oculomotor apraxia being most common (see AOA1, AOA2). The prevalance of genetic childhood ataxia varies from 0.1 to 10 cases per 100,000 population (Musselman et al., 2014).

For a long time the most important classification of ataxias consisted of Anita Harding classification. She divided the hereditary ataxias in four categories: congenital, metabolic, associated with defective DNA repair, and degenerative, the last group being the largest. The degenerative ataxias were further classified, according to the onset below or after 25 years, in early onset, with usually autosomal recessive inheritance, and late onset forms, with usually autosomal dominant inheritance. Harding's classification made a simple classification, mainly phenotype-based, that may be still useful in clinical setting (Harding, 1983).

In the last decade, the advances in molecular genetics have shown some limits of the previous classification: 1) the broadening of the phenotypes after gene cloning, as happened for Friedreich ataxia; 2) the unexpected high genetic heterogeneity in some forms, such as the dominant spinocerebellar ataxias.

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).

At the same time also the pathogenic classification has some limits because for some inherited ataxias multiple pathogenic mechanisms may be known and for others pathogenesis may be unknown. A strict genetic classification is cumbersome and of little clinical usefulness.

To simplify, we might refer to the following classification:

- Autosomal dominant spinocerebellar ataxia (SCA) is a historical term first used in the 1950s based on Friedreich ataxia as a model. SCA now refers to autosomal dominant hereditary ataxia, and the numbers are assigned in the order in which the disease was identified (initially by linkage analysis and more recently by gene discovery). In some SCAs ataxia is the only phenotypic finding (e.g., SCA6), whereas other SCAs may have a complicated phenotype (e.g., SCA3). Some SCAs have spinal cord involvement, but many do not. Some of the complicated forms have not been given an SCA number (e.g., DRPLA). Synonyms for ADCA used prior to the identification of the molecular genetic basis of these disorders were Marie's ataxia, inherited olivopontocerebellar atrophy, cerebello-olivary atrophy, or the more generic term, spinocerebellar degeneration.

  It si useful classify SCAs considering the type of mutation responsible for them (Durr, 2010):

  - Polyglutamin expansion SCAs: SCA 1, 2, 3, 6, 7, 17 and dentato-rubral-pallidum-luysian atrophy (DRPLA).
  - Non coding expansion SCAs: SCA 8, 10, 12, 31, 36.
  - Conventional mutation SCAs: 5, 11, 13, 14, 15/16, 20, 27, 28, 35.

- Autosomal recessive cerebelar ataxia (ARCA) refers a heterogeneous, complex, disabling inherited neurodegenerative diseases that are manifested mostly in children and young adults. Cerebellar ataxia may be associated with the involvement of both the central and peripheral nervous systems, as well as with many systemic signs (oculomotor impairment, movement disodrders, mental retardation/cognitive decline, epilepsy). Important neurologic signs other than cerebellar ataxia include peripheral neuropathy. Clinically is useful distinguish different groups (Anheim et al., 2012):

- ARCA with pure sensory peripheral neuropathy: Friedreich's ataxia (*FXN*); Sensory axonal neuropathy with dysarthria and ophthalmoplegia (POLG), Ataxia with Vitamin E deficiency (*TTPA*); Abetalipoproteinemia (*MTP*).
- ARCA with sensorimotor axonal neuropathy: Ataxia teleangiectasia (ATM); Ataxia with oculomotor apraxia type 1 (APTX), Ataxia with oculomotor apraxia type 1 (SETX), Lateonset GM gangliosidosis (HEX), Congenital disorder of glycosylation type IA (PMM2), ARASACS (SACS), Refsum's disease (PEX7), Cerebrotendinous xanthomatosis (CYP27)
- ARCA without peripheral neuropathy: ARCA1, caused by mutation in *SYNE1*; ARCA2, caused by mutation in *ADCK3*; and Niemann-Pick type C.

In addition, in the clinical practice it may be useful to consider:

- Ataxia and hypogonadism such as Boucher-Neuhäuser/Gordon Holmes syndrome, caused by mutation in PNPLA6 or RNF216 genes, Marinesco-Sjogren syndrome
- Ataxia with Ocular involvement Friedreich's ataxia, Wolfram syndrome, aceruloplasminemia and ceroidlipofuscinosis

- X-linked inheritance cerebellar ataxia: it is quite uncommon except for fragile X tremor ataxia syndrome (FXTAS) (Zanni & Bertini, 2011).
- Episodic Ataxias (EA) characterized by periods (minutes to hours) of unsteady gait often associated with nystagmus or dysarthria (Jen et al., 2007). Myokymia, vertigo, or hearing loss may occur in some of the subtypes. Permanent ataxia and even cerebellar atrophy may result late in the disease course.
- SPAX refers to ataxias that often have a prominent component of spasticity mainly caused by mutations in SPG7, AFG3L2, KIF1C
- Ataxia associated with mutation of mitochondrial DNA (mtDNA) including MERRF (myoclonic epilepsy with ragged red fibers), NARP (neuropathy, ataxia, and retinitis pigmentosa) (Finsterer 2009), and Kearns-Sayre syndrome.

However, in most patients with adult-onset progressive ataxia, the condition manifests without an obvious familial background. Sporadic adult onset ataxias (SAOA) include degenerative non-hereditary, hereditary, and acquired ataxias. Multiple system atrophy and idiopathic late cerebellar ataxia are degenerative non-hereditary ataxias. Late-onset Friedreich's ataxia, spinocerebellar ataxia type 6 and 2, and fragile X-associated tremor/ataxia syndrome account for most sporadic hereditary ataxias. Alcoholic cerebellar degeneration, paraneoplastic and other autoimmune cerebellar degeneration, vitamin

deficiencies, and toxic-induced and infectious cerebellar syndrome are the main causes of acquired cerebellar degeneration (Lieto et al., 2019).

Thus, nearly a thousand mutations mapping to over a hundred different genes have been identififed in hereditary ataxias. In particular, 47 forms of SCAs have been characterized, and the pathogenic genes of 35 SCAs identified, the most common being SCA1-2-3-6-7, all featuring pathological CAG triplet expansions. Nonetheless, 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. The recessive forms (ARCAs) have been thus far linked to more than 90 genes, the most common being FXN (the one causing Friedreich ataxia (Anheim et al., 2012).). (Anheim et al., 2012). ARCAs include clinical phenotype ranging from congenital to late-adulthood onset and pose more difficulties in medical diagnosis because of the often complex manifestations. X-linked inheritance suggests consideration of the fragile X–associated tremor–ataxia syndrome or adrenomyeloneuropathy. A further level of complexity comes from the observation that the limit between cerebellar ataxias and hereditary spastic paraparesis is ill-defined, and the spectrum of ataxia spasticity disorders is growing rapidly (Synofzik and Schule 2017).
The molecular diagnosis of inherited cerebellar ataxia is important but challenging, due to genetic heterogeneity, phenotypic variability, and high cost, so many ataxic patients remain "orphans" of molecular diagnosis (Durr 2010; Anheim et al., 2012).

Ataxia-spasticity spectrum

Hereditary spinocerebellar ataxias and hereditary spastic paraplegia (HSPs) each define a genetically heterogeneous group disoreders characterized respectively by the progressive degeneration of the cerebellar Purkinje cells and spinocerebellar tracts (ataxias) and corticospinal tracts (HSPs), respectively. They were traditionally designated in separate clinicogenetic disease classifications, according to the predominant disease phenotype on first gene locus description and to the mode of inheritance: SCAs refers to autosomal dominant spinocerebellar ataxia, SCARs/ARCAs refers to autosomal recessive spinocerebellar ataxia, SPGs refers to spastic paraplegia; SPAX refers to ataxias that often have a prominent component of spasticity.
This traditional classification bears in itself some problems considering that a large phenotipic, genetic and pathophysiological overlap exists between ataxias and spastic paraplegias.
Yet, the clinical use of next generation sequencing in the past decades has increasingly expanded the clinical spectrum of each of this two groups, revealing the limitations of this

classification system. Discovery of increasing number of new genes causing both cerebellar and pyramidal phenotype has raised awareness of a substantial overlap between these two diseases classifications. In addition, various genes (eg, SPG7, SYNE1, PNPLA6) traditionally rooted in either the ataxia or hereditary spastic paraplegia classification systems have now been shown to cause ataxia on the one end of the disease continuum and hereditary spastic paraplegia on the other.

Mutations in SPG7, identified as a cause of HSP in 1998 (Casari et al., 1998) and scholastically classified as a HSP gene, only 15 years later have been associated with ataxia (van Gassen et al., 2012). Yet, within the past years has been recognized as one of the most common cause of autosomal recessive e ataxia (Pfeffer et al., 2015; Choquet et al., 2016).

Recessive mutations in SYNE1 were identified as a cause of cerebellar ataxia in 2007 and consequently designated ARCA1 and SCAR8 (Gros-Louis et al., 2007). For almost a decade mutations in SYNE1 were thought to cause a slowly progressive, largely pure cerebellar ataxia, till 2016 when it was appreciated to be causative for a broad pleiotropic phenotypic spectrum, with corticospinal tract damage and even predominant complicated HSP presentations among the most frequent features (Synofzik et al., 2016).

Furthermore, mutations in SACS gene, first described in 1978 and causing autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS) (De Braekeleer et al., 1993), have been recently found in pure cerebellar ataxia (Synofzik et al., 2013).

A further example can be found for the gene PNPLA6, identified in 2008 responsible for SPG39, spastic paraplegia associated with distal upper and lower extremity wasting (Rainier et al., 2008). In 2014 mutations in this gene has been reported as a cause of autosomal recessive ataxia associated with hypogonadotropic hypogonadism (Boucher-Neuhauser/Gordon-Holmes syndrome), but more recently it has been shown that they can even be cause of pure cerebellar ataxia (Wiethoff et al., 2016).

HSP and ataxias, which share a substantial number of genes, might also be connected on a functional level via shared cellular pathways and pathomechanisms. This suggests a shared vulnerability of cerebellar and corticospinal neurons for common pathophysiological processes. It might be this mechanistic overlap that drives their clinical overlap.

Other genes such as GBA2 and KIF1C were almost simultaneously published as both a hereditary spastic paraplegia and and ataxia genes.

These examples are a part of a largely expanding list of genes causing ataxia and HSP on a phenotypic continuum. The implications for clinical and genetic diagnostic practice are apparent: the need to test for mutations in ataxia genes (“ataxia panels”) to also comprise

HSP genes; in addition, both ataxia and HSP gene panels should be expanded to cover not only the relevant genes "by classification," but need to go beyond classification systems to cover also genes not included in any of the classification systems. (Synofzik at al., 2017).

1.2 Next Generation Sequencing

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.

Gene identification in HA can be divided into three periods: the positional cloning and Sanger sequencing period, a phase of transition, and the latest NGS era (Figure 1) (Galatolo et al., 2018). In the first period, from the start of the 1990s, a large number of genes causing inherited ataxias were discovered thanks to positional cloning and direct capillary sequencing. Even though these methods are now almost obsolete, they represented a milestone in the genetics of ataxia. Indeed, the genes responsible for Spinocerebellar ataxia type 1 (SCA1), a frequent autosomal dominant HA (Orr et al., 1993), and Friedreich ataxia, the most common recessive form of inherited ataxia (Campuzano et al., 1996), were both identified in the early 1990s. Both diseases are caused by a pathologically expanded trinucleotide repeat that cannot be detected by NGS methodologies (Marelli et al., 2016).

The advent of NGS in the first decade of the 2000s has revolutioned the field of genomics, and indeed, in the last 5 years, has come to the forefront of testing cases with suspected genetic causes of spinocerebellar ataxia or spastic paraplegia once the more common repeat expansion mutations have been ruled out (Fogel et al., 2014). WES offers cost effective broad-coverage testing of almost all known coding variants due to single nucleotide changes, small insertions/deletions, or proximal splice site variants. It also offers opportunities for simultaneous analysis of comparator DNA (i.e., from parents or siblings) to facilitate identification of de novo or compound heterozygous mutations and rule out rare familial benign polymorphisms based on segregation with disease status. to the discovery of several new genes causing cerebellar or sensory ataxia. Since, at the time, the old approach of linkage analyses in informative families, positional cloning, and candidate gene testing was still working well, there was a transition period during which older techniques were gradually replaced or complemented by NGS at some point along the gene discovery pathway. Just a few years ago, in 2013, NGS entered its latest era, which has increased our knowledge of the genetics of ataxia, uncovering new genes in

larger groups of families, and a broader spectrum of associated clinical phenotypes (Ngo et al., 2019). Furthermore, in recent years, NGS technologies, allowing the screening of large cohorts of patients, have made it possible to detect new variants in known ataxia genes (Keogh et al., 2015) and to define the worldwide prevalence of gene mutations initially considered confined to specific populations (Ruano et al., 2014). "These technologies also offered the possibility to replace the strategy of candidate gene analysis by hypothesis-free approaches" (Helbig et al., 2016).

Indeed, in contrast to genomic testing, serial testing of single genes and multigene panel testing rely on the clinician developing a hypothesis about which specific gene or set of genes to test. Hypotheses may be based on one mode of inheritance, distinguishing clinical features, apparent anticipation for a nucleotide-repeat disorder; ethnicity (country/region of origin); distinctive age of onset; specific neurophysiological and neuroimaging findings.

Clinicians role is also crucial in the choice of the multigene panel to determine which one is most likely to identify the genetic cause of the condition at the most reasonable cost while limiting identification of variants of uncertain significance and pathogenic variants in genes that do not explain the underlying phenotype.

Thus, the use of Sanger sequencing strategies for clinical investigation has become obsolete due to its time- and money-expensive features. NGS provide high-throughput simultaneous testing of multiple genes and allow either the whole genome or parts of it (via exome sequencing or targeted panels) to be sequenced in hours, at great depth and increasing sensitivity. This technology has been rapidly adapted to clinical testing and is radically changing the paradigm of clinical diagnostics. In some cases, the technology helps end the lengthy search for a genetic cause, referred to as the "diagnostic odyssey." (Johnson 2015). Identifying molecular diagnoses in such genetically heterogeneous disorders is challenging. Usually multitier, expensive and time-consuming investigations are performed. Nevertheless, a large number of affected individuals remain without a molecular diagnosis.

Prior to 2008, the use of Sanger-based technologies meant that resequencing was substantially more expensive—for example, a human genome cost an estimated $20–25 million in 2006 (Figure 2). A recent study (Schwarze et al., 2018) suggests that the costs of genome sequencing and clinical analysis of a rare disease trio are £7050 (£2350 per genome). The costs of sequencing are yet to meet the desired $1000 per genome. However, this expectation likely only reflects the consumables component and does not consider the overall costs of the sequencing process, which include sample processing (including library preparation and sequencing), bioinformatic data processing and analysis, interpretation and

reporting of sequencing results, and data storage. Clinical interpretation in particular can be lengthy and costly. This dichotomy has led to descriptions of "the $1000 genome and the $100,000 analysis."

In conclusion, exome sequencing improves clinical care for several reasons. First, at a minimum, making a genetic diagnosis will bring to an end what has been for many patients and families an endless diagnostic odyssey. Indeed, a recent invastigation from Eurordis shows that 25% of patients had to wait between 5 and 30 years from early symptoms to confirmatory diagnosis of their diseases. Second, given the number of individual diagnostic tests and gene panels that are often performed before exome sequencing, the latter, if performed early on, may actually save cost to patients and the health care system. Third, and more importantly, especially in the case of families in which a first child has an obscure disease, a genetic diagnosis may enable more informed family planning in a timely manner. Fourth, there are rare, but clear-cut, examples in which genetic diagnosis through exome sequencing significantly affected treatment (Gomez and Das, 2014). From Eurordis we know that 40% of patients first received an erroneous diagnosis. This led to medical interventions (including surgery and psychiatric treatments) that were based on a wrong diagnosis.

Next Generation Sequencing: main clinical approaches

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 (Figure 3) (Yang et al., 2013; Rexach et al., 2019). 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). In conclusion, in spite of the obvious advantages of NGS technologies, we have to face with technical limitation related to procedures of PCR amplification, as well as the selective capturing of target regions, the gap in the coverage and, ultimately, the possibility of short read showinh unanticipated off-target effects (Meienberg et al., 2016). In addition, the Es and TRP methods are unable to read copy number variations (CNVs) and mutations in complex intronic regions, and this may be a further caveat for the interpretation of the results (Galatolo et al., 2018)

1.3 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). However, pathological repeat expansion

account for a limited amount (2-13 3%) of late onset sporadic ataxia (Abele et al., 2002). Considering that nucleotide repeat expansion are relatively common and cannot be detected using TRP and ES strategies, most patients with ataxia have this testing done even when the clinical phenotype makes the diagnosis unlikely, as in sporadic cases. 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 eighteen NGS studies have investigated genetic etiology of large cohorts of ataxic patients seven using TRP (Nemeth et al, 2013; Mallaret et al., 2016; Hadjivassiliou et al., 2017; Coutelier et al., 2017; Iqbal et al., 2017, Giordano et al., 2017, Arslan et al., 2019) and eight 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, Sun et al., 2019) and two a combination of both (Nibbeling et al., 2017, Shakya et al., 2019).

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.

Giordano et al in 2017 published a study about sporadic adult-onset degenerative ataxias. 194 patients who met specific inclusion criteria (progressive ataxia, ataxia onset after age 40, informative and negative family history and no estabilished acquired cause of ataxia) were tested. The genetic panel included 201 genes associated with ataxia. In 6 (3%), a definite genetic diagnosis was made. In 3 of them variants in recessivve genes were found (2xSPG7, 1xATM), in the remaining 3, in dominant genes (2xCACNA1A, TRPC3/SCA41). Likely pathogenic variants were found in 5 patients (ADCK3, POLG, SNX14, CACNA1A, OPA1). VUS were detected in 14 patients. The proportion of genetic diagnosis is higher compared previous studies (Fogel and Keogh).

84 pediatric patients aged 0-18 and predicted to carry the autosomal recessive gene were selected by Arslan et al in the study published 2019. A list of 111 target genes linked to ataxia was included in the panel. Molecular diagnosis was estabilished in 21/84 (25%) patients. The most common gene was PLA2G6 (7 cases), followed by APTX (5 cases), CLN5 (2 cases), SETX, EPM2A, TSEN54, SIL1, POL3A, SACS, VLDLR.

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 bioinformatic 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).

Sun et al. 2018 performed a targeted exome approach (exome sequencing followed by targeted analysis) to 170 patients ranged in age from 2 to 88 years with congenital to adult onset ataxia. Analysis was focused on 441 curated genes associated with ataxia and ataxia-like conditions. Pathogenic and suspected diagnostic variants were identified in 88 of the

170 patients, providing a positive molecular diagnostic rate of 52%. Forty-six different genes were implicated, with the six most commonly mutated genes being SPG7, SYNE1, ADCK3, CACNA1A, ATP1A3, and SPTBN2, which accounted for >40% of the positive cases. In many cases a diagnosis was provided for conditions that were not suspected and resulted in the broadening of the clinical spectrum of several conditions. This strategy of analyzing a large set of ataxia-related genes in all referred patients provided a high positive molecular diagnostic yield, and is particularly useful for making a molecular diagnosis in patients who have an atypical presentation for a particular disorder.

Nibbeling at al., in 2017 reported five novel spinocerebellar ataxia genes, FAT2, PLD3, KIF26B, EP300 and FAT1, identified through a combination of exome sequencing in genetically undiagnosed families and target resequincing of exome candidates in a cohort of singletons. Twenty families with AD cerebellar ataxia (40 individuals) who tested negative for SCA1,2, 3, 6, 7, 17, 19/22 and 23 underwent WES. Mutations in four known SCA genes (CACNA1A, PRKCG, TMEM240, AFG3L2), were identified in 4 out 20 families (20%). In addition, three novel candidate genes in which deleterious variants segregated were found (FAT2, PLD3, KIF26B). Interestingly, to validate these three gene sas novel SCA genes they proceeded screening an additional cohort of 96 unrelated ataxic patients with a dedicated gene panel. An additional variation in FAT2 was found. Furthermore, almost all genes were genetically validated, assessing damaging effects of the gene variants in cell models. No additional mutations were identified in PLD3 and KIF26B in this independent validation cohort. Two of the putative candidate genes FAT1 and EP300 were both mutated in multiple unrelated cases. In eight cases from this cohort we also identified mutations in known, but rarer, SCA genes including CACNA1A, PRKCG, KCND3, AFGL3L2, and TGM6, accounting for 8.3% (8/96) in this cohort.

A total of 141 early onset cerebellar ataxia patients from 98 families were included in the study conducted by Shakya et al in 2019. Criteria of inclusion were: age at onset before 25 years, not autosomal dominat inheritance or patients with clinical phenotype and diagnosis of any of the autosomal recessive cerebellar ataxia (ARCA) subtype. Whole exome sequencing was performed in 16 index cases with positive family history and recessive inheritance; Targeted gene Panel Sequencing (ARCA candidate genes, n = 41; targeted panel sequencing-recessive cerebellar ataxia (TPS-CAR)) in remaining 82 index cases with or without family history (without dominant inheritance). As result 9 index cases were identified from the T-WES approach (56%, n = 16) and 12 from the TPS-CAR approach (15%, n = 82). The most frequent found genes were SACS (7 cases), SETX (6 cases), ATM, TTPA, GRID2, ANO10, SYNE1, CYP27A1. To further solve the genetic

spectrum of remaining ARCA families, they adopted a family based design for WES; 10 additional related individuals from four families were selected for WES. From this fmailiy based WES two variants in FA2H and RARS2 were classified as likely pathogenic.

The diagnostic yield is also quite variable (from 18% to 46%) and globally higher for patients with early onset (Ohba et al., 2013; Sawyer et al., 2014; Fogel et al., 2014) or positive family history (Sawyer et al., 2014; Pyle et al., 2015)

The average diagnostic rate ranges from 3% to 25% in TRP, and from 20 % to 52 % in ES. However, the number of patients recruited for TRP analysis is widely larger compared to those who underwent ES (an average of 162 and 51 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.

Galatolo at al. in 2018 reported that in the twelve NGS studies analyzed (Figure 4), an overall of 53 different genes were detected. Interestingly, 66% of the variants detected resided 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). From this study it was observed that 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.

2. 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.

3. SUBJECTS

3.1 Inclusion criteria

One-hundred-twenty 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 was made. Also congenital ataxias were included. Most patients had already undergone extensive genetic testing. Moreover, mutations in the most likely genes had to be excluded. All patients provided written consent for the study.

3.2 Inherited Ataxias Progression Scale

The Inherited Ataxia Clinical Rating Scale (IACRS) score (Filla 1990) was used. IACRS has been validated in patients with hereditary ataxia and ranges from 0 (normal) to 38 (maximal impairment). 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.

4. METHODS

4.1 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.

4.2 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).

4.3 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).

4.4 Ataxia targeted resequencing panel

Gene panel creation

A gene panel containing 285 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 5, A). Once DNA library amplicons were prepared, hybridization with regions of interest was performed using biotinylated RNA probes (Figure 5, 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 5, 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 6, 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 6, 2). Nucleotides and enzyme were then added to initiate solid-phase bridge amplification (Figure 6, 3) and so double-stranded bridges were obtained (Figure 6, 4). Following denaturation left single-stranded templates anchored to the substrate (Figure 6, 5) and in this way several millions dense clusters of double-stranded DNA were generated in each channel of the flow cell (Figure 6, 6). 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 7). 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.

4.5 WES and segregation studies

WES was adopted in selected cases (Pt 2-8-57-79-116-120) using a SureSelect (Agilent, Santa Clara, CA) All Exon v6 exome capture kit and performed on the Illumina

sequencing platform, using a reported bioinformatics pipeline.

Segregation study helped us to reach a definite diagnosis in the following families: 1, 2, 3, 5, 6, 23, 25, 26, 33, 35, 37, 38.

In summary, NGS investigations were performed using TRP in all 120 patients and adopting WES only in 6 selected proband.

5. RESULTS

5.1 Clinical characteristics of the patients

A total of 120 patients belonging to 102 families were assessed. 55% of probands were male. The mean age at disease onset was 29 years (standard deviation 18.3; range 1-70 years). Fortysix out of 120 patients (38%) presented an onset before the age of 25, eighteen of which patients presented with a congenital ataxia. A total of 62/120 patients (52%) had a positive family history. Among these, 29/62 (47%) had autosomal dominant family history (belonging to 17 families), 32/62 (52%) had a family history consistent with autosomal recessive inheritance (belonging to 26 families). The remaining 57/120 (47%) had no family history (sporadic ataxia). In one patient family history was unknown. 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. Clinical features and the genetic results are shown in table 2, 3 and 4.

5.2 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 8). By applying our filtering criteria, we identified 55 probands out of 120 (46%), belonging to 38 out of 102 (37%) of the assessed families, carrying pathogenic variants (Table 2). Of the patients with confirmed pathogenic variants, 40/55 patients (73%) had a positive family history. Indeed, in 10/38 families there was an autosomal dominant transmission, in 13/38 families an autosomal recessive transmission and in 15/38 families family history was negative. If we selectively consider patients with a positive family history (40/62) the diagnostic rate dramatically increases (64 %). VOUS were found in seven probands (6%) (Table 3). In the remaining fiftyeight out of 120 cases (48%) 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 (72% vs 52%) in patients with confirmed pathogenic variants.

Patients with confirmed pathogenic variants (Table 2)

Disease causing mutations were identified in 55 cases (30 recessive ataxias and 25 autosomal dominant). We recognized ten different genes: STUB1 (sixteen probands in seven families), SPG7 (five unrelated probands), PNPLA6 (four probands in three families), SETX (two unrelated probands), SYNE1 (one family with two probands), PMM2 (one family with two probands), HARS (one family with two probands), EXOSC3 (one family with two probands), TGM6 (one family with two probands), CACNA1G (one family with two probands), PRKCG (one family with two probands), RNF216, AARS2, KIF1C, ZFYVE26, ATP13A2, KIF1A, POL3A, ANO10, RFC1, ADCK3, SLC2A1, SPG7/AFG3L2, HSD17B4, MMACHC.

The most frequent cause of ataxia in our cohort was STUB1. Mutations in *STUB1* were originally described in association with SCAR16 (Shi et al., 2013), a rare autosomal recessive form characterized by early-onset cerebellar ataxia, cognitive impairment, severe cerebellar atrophy, variably associated with hypogonadotropic hypogonadism, extrapyramidal or pyramidal tract signs. Subsequently, a new autosomal dominant ataxia, classified as SCA48, was shown segregating a heterozygous c.823_824delCT *STUB1* variant in a large Spanish kindred presenting symptoms of CCAS (Cerebellar Cognitive Affective Syndrome), sometimes preceding the motor cerebellar signs (Genis et al., 2018). We report seven families, widening its related phenotype (De Michele et al., 2019).

Initially, we identified the two families carried two different novel STUB1 (pt.1-6, 9, 10). One of the two mutations, p.(Gly33Ser) (pt 1-6), occurs in the TRP domain, whereas p.(Pro228Ser) (pt.9-10) is located in the ubiquitin ligase region (De Michele et al., 2019)

In the remaining five families we found two novel in/del mutations p.(Val264Glyfs*4) (pt.11-12); p.(Pro274Alafs*3) (pt.13-14), and one already reported mutation p.(Tyr230Cysfs*9) (pt.7-8) (Depondt et al., 2014), and two missense p.(Ala67Thr) (pt.15), p.(Pro57Leu) (pt. 16) (Lieto et al., 2019) The phenotype in these kindreds appeared more complex than observed in the previously described Spanish family. The clinical picture we have reported included a heterogeneous severity and age at onset of ataxia, associated with various combination of movement disorders such as chorea, parkinsonism, and dystonia, in addition to sphincter disturbances, and epilepsy. Moreover, psychiatric symptoms and cognitive were also resent, ranging from a predominant involvement of the executive

functions typical of the CCAS to a diffuse and more pronounced cognitive impairment.
Interestingly, brain MRI documented a significant cerebellar atrophy affecting all of them and some patients showed T2-weighted hyperintensity in both dentate nuclei (figure 9). This MRI finding could represent a useful imaging biomarker of the disease.
Moreover, lack of evidence of founder mutations in our cohort supports that SCA48, as other dominant SCAs, might be identified worldwide.

Next in frequency was SPG7 (spastic paraplegia 7). All of the five cases (pt. 17-21) had a prominent ataxic phenotype with cerebellar atrophy at brain MRI (Figure 10), but in three of them pyramidal signs were also present (pt.17-20-21) and in one case associated with ophthalmoparesis (pt. 20). However, two of the five patients had no pyramidal signs, but rather a pure cerebellar ataxia (pt.18-19). 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).

Third 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).

Three of the four probands (pt.22-23) here detected presented with a classical phenotype, and sexual hormones assessment revealed a hypogonadotropic hypogonadism. Brain MRI showed cerebellar atrophy (Figure 11). In both patients visual assessment showed a chorioretinal dystrophy, with visual acuity of 7/10 in both eyes for pt 22 and of 1/120 and 1/50 in pt 23. Cognitive evaluation revealed in both patients a mild intellectual disability.

The fourth PNPLA6 case (pt 24) showed only pyramidal signs associated with cerebellar ataxia, with normal cognitive and visual functions. In three out four cases a downbeat nystagmus was evident. Consanguineity was reported in two of the three families.

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). In our RNF216 case (pt.42) pyramidal signs were associated with hyperkinetic choreiform movements, and mild cognitive impairment. In contrast to the majority of the reported cases, our patient showed preserved fertility, normal peripheral nerve conduction, and atypical MRI findings. MRI showed severe cerebellar atrophy affecting both the vermis and the hemispheres, supratentorial atrophy predominantly affecting the parietal and occipital lobes; few and scattered supratentorial white matter hyperintense foci; mild hyperintensity of dentate nuclei, partly extending to the pons (Figure 12).

Two cases of Ataxia with Oculomotor Apraxia 2 (AOA2) due to mutations in SETX gene were detected in our cohort (pt.26-27). In both patients oculomotor apraxia was absent, but strabismus and peripheral neuropathy was evident in the first (pt.26). The second case (pt.27) was not suspected for AOA2 because prominent tremor and hyperkinetic choreiform movements without peripheral neuropathy characterized the clinical picture. Furthermore, a family history positive for tremor was evident in father and a sister.

We recognized two SYNE1 cases (pt.28-29) in one family. Consanguineity was reported. In the pt 28 a mild cerebellar ataxia with prominent dysarthria and slight pyramidal signs was associated with a hypergonadotropin hypogonadism. Brain MRI showed marked cerebellar atrophy (Figure 13). In the affected brother (pt.29) an early-onset syndrome characterized by a moderate intellectual disability, mild cerebellar ataxia and normogonadotropin hypogonadism were reported. 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 brothers with mild intellectual disability, strabismus and congenital non- progressive ataxia with marked cerebellar hypoplasia (pt.30-31) 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).

Two bi-allelic HARS (histidyil-tRNA synthetase) variants (c.1393A>C [p.Ile465Leu] and c.910_912dupTTG [p.Leu305dup]) were detected in two sisters (pt.32-33) with congenital ataxia, mild intellectual disability, and dystonic postures. Severe mental delay, strabismus, microcephaly, short stature, ligamentous laxity completed the clinical phenotype. Cerebral MRI was normal. All mutations are rare, segregate with disease, and are predicted to have a significant effect on protein function. Mutations in HARS were found to cause two different disorders through either autosomal dominant or autosomal recessive inheritance, a feature shared with only four other ARS enzymes (Meyer-Schuman and Antonellis, 2017). Puffenberger and collaborators (Puffenberger et al, 2012) initially identified a homozygous missense mutation in three Old Order Amish patients described with the Usher Syndrome type IIIB syndrome, an autosomal recessive disorder characterized by motor impairment, and progressive vision and hearing loss in childhood. Later, several studies (Vester et al, 2013) (Abbot et al, 2018) (Royer-Bertrand et al, 2019) revealed monoallelic mutations in HARS as the cause of dominantly inherited Charcot-Marie-Tooth disease type 2W (CMT2W), a motor and sensory peripheral neuropathy affecting both lower and upper limbs. Herein, again we expand the HARS-related phenotype.

In two siblings (pt.34-35) we report a homozygous mutation (p.D132A) in EXOSC3 gene that encodes a core component of the human RNA exosome complex. The mutation is responsible for a pontocerebellar hypoplasia type IB. Our cases presented as a rapidly progressive congenital ataxia with oculomotor apraxia, mental retardation and pyramidal signs. The phenotype caused by the same mutation consisted of neonatal onset of severe hypotonia, often with respiratory insufficiency, and global developmental delay, without achieving any motor milestones or speech, and progressive microcephaly. Other features included oculomotor apraxia, progressive muscle wasting, and distal contractures (Wan et al., 2012). Our patients represent a relatively mild form of the disorder, expanding the phenotipic spectrum associated with EXOSC3 mutations.

A family with autosomal dominant mode of inheritance was successfully solved, recognized as SCA35 (c.1953_1955dupACA, p.Q652dup) (pt.36-37). 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).

In one family a heterozygous mutation in *CACNA1G* gene was detected in two siblings (pt.38-39) with ataxia associated with dysarthria, tremor, areflexia, reduced vibration sense and dysphagia. CACNA1G gene is responsible for Autosomal dominant Spinocerebellar ataxia type 42 (SCA42), a slowly progressive pure cerebellar ataxia. The R2715H mutation we found has already been described in few families (Coutelier et al., 2015; Morino et al., 2015).

A heterozygous mutation in PRKCG gene, codifing for a protein kinase Cγ was detected in two siblings (pt.40-41) affected by a late onset slight ataxia. Mutation in this gene has been associated with SCA 14 characterized by slowly progressive cerebellar ataxia, dysarthria, and nystagmus. In one (pt.40) of the two patients we found hypogonadism and thrombocytopenia as additional features.

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.43). 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). In our case, a mild phenotype was detected showing congenital nystagmus, tremor and ovarian failure. Despite what reported we found the absence of leukoencephalopathy at MRI and normal cognitive function. This mutation was tested and confirmed in an affected sister, expanding the clinical spectrum AARS2-related.

We report a homozygous mutation in KIF1C (c.765delC p.D256fs*10) in a patient (pt..44) showing spastic ataxia and onset in second decade with tremor. MRI showed no atrophy of the cerebellum but white matter abnormalities. KIF1C gene has recently been identified as one of the genetic causes of HSP and associated with pure or complicated HSP (SPAX2/SPG58). Our patient is completely in line with the available data reported in literature (Yucel Yilmaz et al., 2018, 2018 Dor et al., 2014, Novarino et al., 2014)

A complicated neurodegenerative syndrome was present in pt 45, 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).

A novel deleterious homozygous mutation in *ATP13A2* (c.1205C>T p.Thr402Met) was found in a 65-year-old man (pt.46) from a consanguineous marriage who developed ataxia at 38 years and "jerky movements" at 55. Neurological examination showed action and massive reflex myoclonus, brisk knee jerks, lower limb increased tone, executive dysfunction. Brain MRI showed cerebellar and cortical atrophy and his EEG displayed slow activity without spikes. 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).

In one patient (pt.47) with congenital ataxia, optic atrophy, urinary incontinence, slight mental retardation and sensory neuropathy we found a heterozygous mutation in *KIF1A* gene. Recessive mutations have been described in 2 main syndromes: hereditary spastic paraplegia (HSP) and hereditary sensory and autonomic neuropathy (HSAN). Recently, Lee et al. and Esmaeeli Nieh et al. described heterozygous de novo mutations that are associated with a presentation at a younger age and more severe phenotypes Affected children invariably present developmental delay and intellectual deficiency with cognitive and language impairment. Microcephaly is inconstantly present. Axial hypotonia, peripheral spasticity with gradual regression of locomotor activity, and peripheral neuropathy are often present. Epilepsy can develop in early childhood. From an ophthalmic point of view, optic atrophy is almost always present. On MRI, our patient showed cerebellum and corpus callosum atrophy, consisten with this mutation.

In one patient (pt.48) showing a late onset spastic ataxia and nystagmus and onset in the fifth decade we detected a compound heterozygous mutation in POL3A gene. This gene codes for the largest subunit of RNA polymerase III (Pol III) and is associate with

hypomyelinating leukodystrophy characterized by varying combinations of four major clinical findings (neurological such as ataxia, spasticity, dystonia), abnormal dentition, endocrine and ocular abnormalities. Although onset is usually in childhood, late onset has also been reported (Bernard et al., 2011).

The ANO10 case (pt. 49) 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 14).

A patient (pt.50) with a cronic chough since the age of 30 associated with a slight slowly progressive ataxia (onset at the age of 50) presented two mutations in RFC1 gene, responsible for cerebellar ataxia, neuropathy, vestibular areflexia syndrome (CANVAS) Cortese 2019 At the brain MRI a mild cerebellar atrophy was evident. Infante (2018) has recently described five patients with CANVAS and with chronic cough and preserved limb muscle stretch reflexes (Wu et al., 2014). Szmulewicz and colleagues commented on that "we have found chronic cough and autonomic dysfunction to be variable features of CANVAS. Many patients report a relatively long-standing chronic, non-productive cough that may have preceded the onset of imbalance and somatosensory impairment" (Szmulewicz et al., 2014). Our patient perfeclty fits the phenotype RFC1-related.

Our study revealed a previously reported homozygous non-sense mutation in COQ8A [c.1042C > T (p.Arg 348*)]. Disease onset of our patient (pt.51) was at age 25 years with gait instability. First examination at 43 years of age revealed slight difficulty with tandem gait, slight dysmetria and dysarthria, brisk tendon reflexes with positive Trömner sign, flexor plantar response, and cervical dystonia with retrocollis. There was intermittent upper limb action and head tremor, and writing difficulties with abnormal dystonic pen grip. Parents were second-cousins. MRI showed mild vermian atrophy. Galosi Mutations in COQ8A represent the most common cause of primary CoQ10 deficiency and result in autosomal-recessive cerebellar ataxia type 2 (ARCA2; spinocerebellar autosomal recessive 9 [SCAR9]), a slowly progressive ataxic syndrome characterized by cerebellar atrophy and moderate CoQ10 deficiency in skeletal muscle. Variable associated clinical features include exercise intolerance, epilepsy, and intellectual disability. To date, 53 patients with 38 COQ8A mutations have been reported. Variable phenotypes, ranging from slowly progressive ataxia to infrequent severe forms with early onset ataxia, epilepsy and regressive course are described. Less obvious manifestations, including clumsiness, writing

difficulties and speech disorder, characterize a subset of patients with a milder phenotype and a more slowly progressive course. Interestingly, our case displayed an unusual phenotype with prominent writing deterioration and variable speech disorder with examination showing dystonic features with only mild ataxia (Galosi et al., 2019). The combination of writing difficulty, dystonia and ataxia may represent a distinctive constellation of COQ8A mutations. COQ10 supplemmentation may result in a clinical stabilization (Lagier-Tourenne et al., 2008, Mollet et al., 2008).

In a very young patient (pt.52) affected by a slight congenital ataxia associated with a mild mental retardation, hyperreflexia and childhood seizures, we detected a heterozygous mutation (p.E329K) in *SLC2A1* gene, codifing GLUT1 transporter. Glut-1 deficiency syndrome is a disorder of brain energy metabolism caused by impaired glucose transport into the brain mediated by the facilitative glucose transporter Glut-1. It is an autosomal dominant disorder characterized primarily by onset in childhood of paroxysmal exercise-induced dyskinesia. The dyskinesia involves transient abnormal involuntary movements, such as dystonia and choreoathetosis, induced by exercise or exertion, and affecting the exercised limbs. Some patients may also have epilepsy, most commonly childhood absence epilepsy, with an average onset of about 2 to 3 years. Mild mental retardation may also occur. Wang 2005 However, the presentation and course are variable. The hallmark of the disease is a low cerebrospinal fluid (CSF) glucose concentration in the presence of normoglycemia Treatment of the nutrient deficiency is based on providing ketone bodies as an alternative brain fuel (Wang et al., 2005).

We report a case (pt.53) with a slight ataxia and mild pyramidal signs where a heterozygous mutation in SPG7 co-occurred with a mutation in the SCA28/AFG3L2 gene. Mutations in AFG3L2 cause spinocerebellar ataxia type 28. SPG7 encodes paraplegin, a component of the mitochondrial AAA protease that closely interacts with its binding partner AFG3L2. Both paraplegin and AFG3L2 are highly expressed in Purkinje neurons. This might explain why the phenotypic spectrum of SPG7 includes a predominantly ataxic presentation and a hypothetical phenotypic effect in our patient with variants on both genes.

Compound heterozygous mutations in HSD17B4 gene was detected in a patient (pt.54) with ataxia associated with pyramidal signs and demyelinating sensorimotor neuropathy with onset at 18 years old. 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). The absence of the deafness in our patient indicates a milder phenotype. Clinical heterogeneity of Perrault syndrome is largely reported and with this case we further broad the phenotype. Segregation study was conducted and the mutations confirmed.

One patient (pt.55) presented since the second decade a spastic paraplegia reported a heterozygous mutation in *MMACHC* gene (c.271dupA) associated with methylmalonic aciduria and homocystinuria. The MMACHC gene provides instructions for making a protein that helps convert vitamin B12 into one of two molecules, adenosylcobalamin (AdoCbl) or methylcobalamin (MeCbl). The c.271dupA mutation accounted for at least 40% of all disease alleles (Lerner Ellis et al., 2006, Morel et al., 2006). This disorder is usually diagnosed in the early neonatal period. Patients with an adult onset and predominant neurological disorders have also been described.

Patients with variants of uncertain significance (Table 3)

A patient with ataxia associated with oculomotor apraxia, diplopia and mild pyramidal signs (pt 56) 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). SYNE1 gene was initially defined as a pure cerebellar ataxia, and 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 complicated case (pt 57) 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.

In a young woman (pt 58) 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.

A homozygous mutation in RNF170 gene was detected in a patient (pt 59). 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.

In a patient (pt 60) wih very mild ataxia with prominent cerebellar dysarthria and onset in the fourth decade 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.

We found a heterozygous mutation in ADCK3 gene, responsible for primary CoQ10 deficiency and result in autosomal-recessive cerebellar ataxia type 2 (ARCA 2). Our patient (pt 61) presented a congenital non progressive ataxia, slight developmental delay, dyslexia, telangiectasia, mild hyperkinetic movements and dystonia. On brain MRI vermis hypoplasia and megacisterna magna were present. The heterozygous mutation does not

fully explain our case.

In a patient (pt 62) with a predominant dystonia with torcicollis associated with spastic ataxia and tremor we found mutations in three different genes. A heterozygous mutation in SPG7 gene, responsible for one the most common cause of ataxia, was detected. The presence of a spastic ataxia phenotype makes this disease likely. A heterozygous mutation in MFN2 a gene related "Charcot-Marie-Tooth disease, axonal, type 2A2A", an autosomal dominant hereditary neuropathy, is unlikely in our patient because neuropathy is absent (Zuchner et al., 2004). The last was a missense mutation in ELOVL5 gene causing SCA38, a dominant spinocerebellar ataxia, that is improbable since consanguinity is reported (Di Gregorio et al., 2014).

6. 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. Currently, the use of Next Generation Sequencing techniques is significantly contributing to the gentic characterization of rare and genetically heterogeneous disorders, allowing identification of novel associated gene and widening the phenotypic spectrum and the pattern of inheritance associated with specific genes and bringing to an end what has been for many patients and families an endless diagnostic odyssey. In this study we used a Targeted Next-Generation Resequencing Panel containing 285 genes, known or supposed to be related to hereditary ataxias, as a tool to investigate 120 heterogeneous patients with ataxia without a molecular diagnosis. A total of 62/120 patients (52%) had a positive family history. Among these, 29/62 (47%) had autosomal dominant family history (belonging to 17 families), 32/62 (52%) had a family history consistent with autosomal recessive inheritance (belonging to 26 families). The remaining 57/120 (47%) had no family history (sporadic ataxia). 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. 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. WES in 6 probands and segregation studies were useful and necessary tools to reach the diagnosis in selected cases. 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. Confirmed pathogenic variants were identified in 38/102 (37 %) of the assessed families. Of these families, in 10/38 there was an autosomal dominant transmission, in 13/38 an autosomal recessive transmission and in 15/38 family history was negative.

This positive result surely depends on the significantly higher number of genes selected in our panel (285 genes) compared 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. Indeed, 40 of the 55 patients with confirmed pathogenic variants (73%) had a positive family history. If we selectively consider patients with a positive family history (40/62) the diagnostic rate dramatically increases (64 %).

The most frequent gene detected in our cohort was STUB1, found in 7 different families and totally in 16 patients. The frequency in our cohort is surprisingly high (16%) and suggests that this disorder would not be uncommon among SCAs, at least in Italy. The phenotype in these kindreds appeared more complex than observed in the previously described Spanish family where a cerebellar cognitive affective syndrome with onset in late adult life was associated with ataxia. At large, the clinical picture we observed also mimicked SCA17, a dominantly inherited ataxia with expansion of the CAG/CAA trinucleotide repeats in the TATA-binding protein (TBP). SCA17 is associated with cognitive and psychiatric abnormalities, extrapyramidal features, and seizures. SCA17 was indeed the initial clinical diagnosis in patients 2-9. Taking in consideration that, previously, biallelic mutations in STUB1 had been associated in about thirty families with SCAR16, a very severe autosomal recessive form of spinocerebellar ataxia, it remains an open question why STUB1-related ataxias can act through different pattern of transmission. This constitutes another exemple of coexistence of autosomal recessive and dominant inheritance like observed in other genes (i.e., KIF1A, SPG7) suggesting that zygosity is no longer a barrier to defining a molecular diagnosis in inherited ataxias.

Next in frequency is 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.

The third 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 seven out of 120 cases (6%) 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 25) had not hypogonadism, and also the patient detected with compound heterozygous mutations in RNF216 gene (pt 42) was not identified as hypogonadic, indeed, she had two successful pregnancies. 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) whereas the presence of fertility suggests that hypogonadism may not be a sentinel sign of the disease. In one patient with hypogonadotropic hypogonadism (pt 20) the diagnosis was SPG7, and in one case with hypergonadotropic hypogonadism and a positive family history for hypogonadism in a brother (pt 28), the gene detected was surprisingly SYNE1. This endocrinological finding has never been associated to SYNE1 gene, expanding the phenotype of the disease.

Mutations in SYNE1 and in SETX genes have 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 seven patients (6%) 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 48 % 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 who we could not detect confirmed pathogenic variants are candidates for exome sequencing studies.

7. CONCLUSION

Next-generation sequencing approaches allow for rapid and inexpensive large-scale genomic analysis, creating unprecedented opportunities to integrate genomic data into the clinical diagnosis and management of neurological disorders. 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. Our TRP provides high-quality sequencing data with excellent coverage of the selected genes and a very high diagnostic yield.
Finally, this study further highlights the significance of NGS applications for the molecular characterization of rare inherited neurological disorders, being a crucial step to understand their pathogenesis and, hopefully, offering the way to future therapeutic options.

8. TABLES AND FIGURES

Table 1. List of the 285 genes included in the ataxia panel

GENI	MIM	GENI	MIM	GENI	MIM	GENI	MIM
AARS	612035	*ATP1A3*	182350	*CHMP1A*	164010	*EEF2*	130610
ABCB7	300135	*ATP2B3*	300014	*CLCN2*	600570	*EIF2B1*	606686
ABCD1	300371	*ATP7B*	606882	*CLN5*	608102	*EIF2B2*	606454
ABHD12	613599	*ATP8A2*	605870	*CLN6*	606725	*EIF2B3*	606273
ACO2	100850	*BEAN1*	612051	*CLN8*	607837	*EIF2B4*	606687
ADCK3	606980	*BRAT1*	614506	*CLP1*	608757	*EIF2B5*	603945
ADGRG1	604110	*BRF1*	604902	*COA7*	615623	*ELOVL4*	605512
AFG3L2	604581	*C10ORF2*	606075	*COQ2*	609825	*ELOVLF*	611805
AHDC1	615790	*C12ORF65*	613541	*COQ4*	612898	*ERCC4*	133520
AHI1	608894	*C5ORF42*	614571	*COQ9*	612837	*ERCC8*	609412
ALDH5A1	610045	*C12ORF65*	613541	*CSPP1*	166490	*EXOSC3*	606489
ALG3	608750	*C9ORF72*	614260	*CSTB*	601145	*EXOSC8*	606019
ALG6	604566	*CA8*	114815	*CTBP1*	602618	*FA2H*	611026
AMACR	604489	*CACNA1A*	601011	*CTSD*	116840	*FARS2*	611592
AMPD2	102771	*CACNA1G*	604065	*CWF19L1*	616120	*FASTKD2*	612322
ANO10	613726	*CACNB4*	601949	*CYP27A1*	606530	*FGF14*	601515
APOB	107730	*CASK*	300172	*CYP7B1*	603711	*FLVCR1*	609144
APTX	606350	*CC2D2A*	612013	*DARS*	603084	*FMR1*	309550
ARL13B	608992	*CCDC88C*	611204	*DARS2*	610956	*FOLR1*	136430
ARSA	607574	*CD40LG*	300386	*DDHD2*	615003	*FXN*	606829
ATG5	604261	*CDK5*	123831	*DKC1*	300126	*GALC*	606890
ATL1	606439	*CEP104*	616690	*DNAJC19*	608977	*GAN*	605379
ATM	607585	*CEP290*	610142	*DNAJC3*	601184	*GBA2*	609471
ATP13A2	610513	*CEP41*	610523	*DNMT1*	126375	*GBE1*	607839
GDAP1	606598	*MKS1*	609883	*PIK3R5*	611317	*SCN2A*	182390
GFAP	137780	*MMACHC*	609831	*PLA2G6*	603604	*SCN8A*	600702
GJB1	304040	*MME*	120520	*PLEKHG4*	609526	*SCYL1*	607982
GJC2	608803	*MRE11A*	600814	*PLP1*	300401	*SEPSECS*	613009
GLB1	611458	*MTPAP*	613669	*PMM2*	601785	*SETX*	608465
GOSR2	604027	*MTTP*	590075	*PMPCA*	613036	*SIL1*	608005
GRID2	602368	*MVK*	251170	*PNKP*	605610	*SLC17A5*	604322
GRM1	604473	*NAGLU*	609701	*PNPLA6*	603197	*SLC1A3*	600111

HARS	142810	*NDUFS1*	157655	*POLG*	174763	*SLC25A4*	610826
HARS2	600783	*NDUFS7*	601825	*POLR3A*	614258	*SLC2A1*	138140
HEXA	606869	*NEU1*	608272	*POLR3B*	614366	*SLC33A1*	603690
HEXB	606873	*NOL3*	605235	*PPT1*	600722	*SLC35A2*	314375
HIBCH	610690	*NOP56*	614154	*PRINCKLE1*	608500	*SLC52A2*	607882
HSD17B4	601860	*NPC1*	607623	*PRKCG*	176980	*SLC6A19*	608893
INPP5E	613037	*NPC2*	601015	*PRNP*	176640	*SLC9A1*	107310
ITPR1	147265	*NPHP1*	607100	*PRPS1*	311850	*SLC9A6*	300231
KCNA1	176260	*OFD1*	300170	*PRRT2*	614386	*SMPD1*	607608
KCNA2	176262	*OPA1*	605290	*PSAP*	176801	*SNAP25*	600322
KCNC1	176258	*OPA3*	606580	*PSEN1*	104311	*SNX14*	616105
KCNC3	176264	*OPHN1*	300127	*PTF1A*	607194	*SPAST*	604277
KCND3	605411	*OTUD4*	611744	*PTRH2*	608625	*SPG11*	610844
KCNJ10	602208	*PAX6*	607108	*QARS*	603727	*SPG7*	602783
KCTD7	611725	*PDE6D*	602676	*RAB3GAP1*	602536	*SPTBN2*	604985
KIF1A	601255	*PDHA1*	300502	*RARS*	107820	*SRD5A3*	611715
KIF1C	603060	*PDSS1*	607429	*RARS2*	611524	*STS*	300747
KIF7	611254	*PDSS2*	610564	*RELN*	600514	*STUB1*	607207
LAMA1	150320	*PDYN*	131340	*RNF170*	614649	*STXBP1*	602926
LMNB2	150341	*PEX10*	602859	*RNF216*	609948	*SURF1*	185620
LYST	606897	*PEX16*	603360	*RPGRIP1L*	610937	*SYNE1*	608441
MARS2	609728	*PEX2*	170993	*RUBCN*	613516	*SYNE2*	608442
MED17	603810	*PEX6*	601498	*SACS*	604490	*SYT14*	610949
MFN2	608507	*PEX7*	601757	*SAMD9L*	611170	*TBC1D23*	617687
MFSD8	611124	*PHYH*	602026	*SCN1A*	182389	*TCTN1*	609863
TCTN2	613846	*TMEM67*	609884	*TTPA*	600415	*WDR81*	614218
TCTN3	613847	*TOP1*	126420	*TUBB3*	602661	*WFS1*	606201
TDP1	607198	*TPP1*	607998	*TUBB4A*	602662	*WWOX*	605131
TGM6	613900	*TRNT1*	612907	*UBA5*	610552	*XPA*	611153
THG1L	Nd	*TRPC3*	602345	*UBR4*	609890	*XRCC4*	194363
TINF2	604319	*TSEN2*	608753	*UCHL1*	191342	*ZFYVE26*	612012
TMEM138	614459	*TSEN34*	608754	*VAMP1*	185880	*ZFYVE27*	610243
TMEM216	613277	*TSEN54*	608755	*VARS2*	612802	*ZNF423*	604557
TMEM231	614949	*TTBK2*	611695	*VLDLR*	192977	*ZNF592*	613624
TMEM237	614423	*TTC19*	613814	*VRK1*	602168		
TMEM240	616101	*TTC21B*	612014	*VWA3B*	614884		

Table 2. Patients with confirmed pathogenic variants

Family	PT	Sex	AE y	AO y	Inheritance	IAPS/ age at reaching phase	Additional features	MRI	Gene	Variant DNA; protein	Zygosity	Disorder
1	1	F	41	28	AD	II/28	chorea, dystonia, increased tendon jerks, mild cognitive dysfunction, psychiatric disorders	moderate CA	*STUB1*	c.97G>A;p.G33S		SCA48
1	2	M	61	45	AD	II/49	Psychiatric disorders, Seizures, Parkinsonism, Cognitive dysfunction, dysphagia	marked CA	*STUB1*	c.97G>A;p.G33S	he	SCA48
1	3	M	44	5 (seizure) 22 (ataxia)	AD	II/22	Seizures, dystonia, mild psychiatric and cognitive disorders	marked CA	*STUB1*	c.97G>A;p.G33S	he	SCA48
1	4	F	59	12 (seizure) 40 (ataxia)	AD	II/40	Seizures, dyphagia, mild psychiatric and cognitive disorders	marked CA	*STUB1*	c.97G>A;p.G33S	he	SCA48
1	5	M	54	44	AD	II/44	dizziness, hearing loss,psychiatric disorders, mild cognitive dysfunction, dysphagia	CA	*STUB1*	c.97G>A;p.G33S	he	SCA48
1	6	F	60	50	AD	II/50	tremor, chorea, psychiatric disorders, mild cognitive dysfunction		*STUB1*	c.97G>A;p.G33S	he	SCA48
2	7	F	67	48	AD	III	dysphagia, dystonia, chorea, psychiatric and cognitive disorders, parkinsonism, urinary incontinence	moderate CA	*STUB1*	c.689_692_delACCT p.Y230C fs*9	he	SCA48

Table 2. (Continued)

2	8	M	50	31	AD	II/31	tremor, dystonia	CA, cortical atrophy	*STUB1*	c.689_692_delACCT p.Y230C fs*9	he	SCA48
3	9	M	60	55	AD	II/55	choreic movements, mild cognitive impairment, mild psychiatric disorder	CA	*STUB1*	c.682 C>T; p.P228S	he	SCA48
3	10	M	39	35	AD	II/35	tremor, cognitive and psychiatric disorders, dysphagia	CA	*STUB1*	c.682 C>T; p.P228S	he	SCA48
4	11	F	70	40	AD	II/40	slow saccades, chorea, cognitive decline		*STUB1*	c.791_792delTG p.Val264Glyfs*4	he	SCA48
4	12	M	66	56	AD	II/56	chorea, psychiatric and cognitive disorders	moderate CA	*STUB1*	c.791_792delTG p.Val264Glyfs*4	he	SCA48
5	13	F	37	34	AD	II/34	chorea, cognitive decline	CA	*STUB1*	c.818_819dupGC p.Pro274Alafs*3	he	SCA48
5	14	F	67	42	AD	III/	Chorea,dystonia, psychiatric and cognitive disorders, dysphagia, urinary incontinence	moderate CA	*STUB1*	c.818_819dupGC p.Pro274Alafs*3	he	SCA48
6	15	F	51	37	SP	III/48	hands hyperkinetic movements, RLS	CA	*STUB1*	c.199G>A p.A67T	he	SCA48
7	16	M	52	43	AD	II/43	dysphagia, tongue hypotrophy, slow saccades, Dysautonomia	CT CA	*STUB1*	c.170C>T p.Pro57Leu	he	SCA48
8	17	F	69	45	SP	III/65	pyramidal signs, urge incontinence	CA	*SPG7*	c.1231G>A; p.D411N c.679C>T; p.R227*	ch	SPG7
9	18	F	55	30	SP	II/30	urinary incontinence	slight CA	*SPG7*	c.1529C>T; p.A510V c.1940C>A; p.A647E	ch	SPG7

Table 2. (Continued)

10	19	M	71	49	AR Cons	II/49		slight CA, slight supratent atrophy	*SPG7*	c.1529C>T; p.A510V	ho	SPG7
11		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
12	21	F	61	52	AR	II/52	pyramidal signs	CA, slight supratent orial atrophy	*SPG7*	c.1529C>T; p.A510V c.1972G>A; p.A658T	ch	SPG7
13	22	M			AR Cons	II			*PNPLA6*	c.1880C>T; p.A627V	ho	Gordon Holmes/ Boucher-Neuhäuser syndrome
13	23	M	41	26	AR Cons	II/26	mild mental retardation, hypogonadotric hypogondism, cryptorchidism, pyramidal signs, retinitis pigmentosa	CA	*PNPLA6*	c.1880C>T; p.A627V	ho	Gordon Holmes/ Boucher-Neuhäuser syndrome
14	24	M	32	19	SP	II/19	mild mental retardation?, hypogonadotric hypogondism, gynecomastia, chorioretinal dystrophy	CA	*PNPLA6*	c.3365C>T; p.P1122L c.4081C>T; p.R1361*	ch	Gordon Holmes/ Boucher-Neuhäuser syndrome
15	25	F	63	44	AR Cons	III/56	pyramidal signs	CA	*PNPLA6*	c.3385G>A; p.G1129R	ho	Gordon Holmes/ Boucher-Neuhäuser syndrome
16	26	F	80	35	AR Cons	IV/62	neuropathy, pyramidal signs, strabismus	CA, slight supratent orial	*SETX*	c.3242T>C; p.F1081S	ho	AOA2

Table 2. (Continued)

								atrophy				
17	27	M	51	11	SP	IV/34	sensorymotor neuropathy, tremor	CA	*SETX*	c.7292dupA; p.N2431fs*19	ho	AOA2
18	28	F	37	24	AR Cons	II/24	mild pyramidal signs, hypergodatropin hypogonadism; a brother with ataxia, hypogonadism and mental retardation	marked CA	*SYNE1*	c.4609C>T; p.R1537*	ho	SYNE1/SCAR8
18	29	M	41	2	AR Cons	II/	mental retardation,dystonia, no neuropathy, brisk tendon jerks, cryptorchidism, valgus knee, epicanthus,	marked CA	*SYNE2*	c.4609C>T; p.R1537*	ho	SYNE1/SCAR8
19	30	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
19	31	M	31	1	AR	II/1	pyramidal signs, mild mental retardation, strabismus, dystonia	marked CA	*PMM2*	c.422G>A; p.R141H c.323C>T; p.A108V	ch	CDG IA
20	32	F	38	1	AR	II/1	mental delay, strabismus, microcephaly, short stature, ligamentous laxity	normal	*HARS*	c.90+4A>C c.1393A>C p.I465L; c.910_912dupTTG p.L305dup;	ch	Usher s. type IIIB
20	33	F	37	1	AR	II/1	mental delay, strabismus, microcephaly, short stature, hypoacousia	enlargement lower cerebella cystern	*HARS*	c.90+4A>C c.1393A>C p.I465L; c.910_912dupTTG p.L305dup;	ch	Usher s. type IIIB
21	34	M	16	1	AR	III-IV	aprassia oculare, mental delay, ROT vivaci, Babinski		*EXOSC3*	c.395A>C p.D132A	ho	Pontocerebellar Hypoplasia

Table 2. (Continued)

												IB
21	35	F	13	1	AR	IV/	mental delay, strabismus, ocular apraxia, brisk tendon jerks		*EXOSC3*	c.395A>C p.D132A	ho	Pontocerebellar Hypoplasia IB
22	36	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
22	37	F			AD				*TGM6*	c.1953_1955dupACA; p.Q652dup	he	SCA35
23	38	F	58	35	AD	II/35	mild ataxia, areflexia, tremor, dysarthria	CA	*CACNA1G*	CACNA1G c.5144G>A p.R1715H	he	SCA42
23	39	M	40	22	AD	II/22	mild ataxia, reduced vibration sense, dysarthria, dysphagia	marked CA	*CACNA1G*	CACNA1G c.5144G>A p.R1715H	he	SCA42
24	40	M	49	44	AD	II/44	mild ataxia, hypogonadism, thrombocytopenia	CA	*PRKCG*	PRKCG c.358C>T p.L120F	he	SCA14
24	41	M	54	50	AD	II/50	slight ataxia,	slight CA	*PRKCG*	PRKCG c.358C>T p.L120F	he	SCA14
25	42	F	43	37	SP	II/37	pyramidal signs, tremor, hyperkinetic choreiform movements	CA	*RNF216*	c.2061+3A>G; c1849A>G p.M617V	ch	Gordon Holmes/ Boucher-Neuhäuser syndrome

Table 2. (Continued)

26	43	F	35	18	AR	II/18	congenital downbeat ny, primary amenorrhea, tremor	normal	*AARS2*	c.446G>A p.C149Y; c.385A>C p.T129P	ch	Leukoencephalopathy, progressive, with ovarian failure
27	44	M	27	13 (tremor) 16 (ataxia)	SP	II/16	tremor, spastic ataxia	white matter abnormalities	*KIF1C*	*KIF1C c.765delC p.D256fs*10*	ho	SPAX2/SPG58
28	45	M	53	30	SP	II/30	pyramidal signs, esecutive dysfunction, resting tremor, bradykinesia, retrocollis, vertical opthalmoparesis, marked dysphagia (PEG), hallucinations	pons atrophy CA	*ZFYVE26*	c.3722G>A; p,R1241Q c.3970T>A; p.S1324T	ch	SPG15
29	46	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
30	47	M	42	congenital	SP	III/5; IV/19	optic atrophy, urinary incontinence, slight mental retardation, absent reflexes, sensory neuropathy	CA >vermis, Hypoplasia CC	*KIF1A*	*c.609_610delGAinsAAAAG*	he	Mental retardation, autosomal dominant 9
31	48	M	64	40	SP		spasticity, nystagmus, reduced strenght, one brother affected		*POL3A*	*c.3839dupT(p.M1280fs*20); c.1909+22G>A*	ch	Pol3-related Leukodystrophy
32	49	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	ANO10/SCAR 10
33	50	M	59	50	AR	II/50	cough, RLS	mild CA	*RFC1*			CANVAS

Table 2. (Continued)

34	51	M	53	25	SP	II/25	mild ataxia, retrocollis, brisk tendon jerks	slight vermian atrophy, gliosis	*ADCK3*	c.1042C>T p.R348*	ho	ARCA2
35	52	F	13	1	SP	II/1	slight mental delay, slight ataxia,brisk tendon jerks, epilepsy		SLC2A1	SLC2A1 c.985G>A p.E329K	he	GLUT1 deficiency syndrome
36	53	M	63	25	SP	II/25	bris tendon jerks	CA	*SPG7* *AFG3L2*	SPG7 c.1529C>T p.A510V AFG3L2 c.1712T>G p.V571G	he he	SPG7/SCA28
37	54	M	30	18	SP	II/18	pyramidal signs, demyel. sensorimotor neuropathy	CA	*HSD17B 4*	c.2191C>T p.Q731*; c..727G>T p.V243L	ch	Perrault syndrome
38	55	M	22	20	AD	II/20	Spastic paraparesis	normal	*MMACH C*		he	Methilmaloni c aciduria

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 dominant; Cons consanguinity; CA cerebellar atrophy; WMA white matter abnormalities; he heterozygous; ho homozygous; ch compound heterozygous.

Table 3. Patients with variants of uncertain significance

Family	PT	Sex	AE y	AO y	Inheritance	IAPS/age at reaching phase	Additional features	MRI	Gene	Variant DNA; protein	Zygosity	Disorder
39	56	M	63	29	SP	III/31	oculomotor apraxia, diplopia, mild pyramidal signs	normal	*SACS* - *SYNE1* - *CACNA 1G*	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
40	57	F	47	21	SP	III/47	myoclonus, seizures, tremor, sensorineural hypoacusia, cataract, dolichololon, rectal atony, neurogenic bladder	CA, supratent atrophy, WMA	*PLA2G6* - *SLC9A1* *DNMT1* *SYNJ1 (EX)* *DHDDS (EX)* *ADRA2 B (EX)* *IFIH1 (EX)*	c.2346C>G p.L782L; c.1952T>G p.L651R; c.2435C>T p.P812; c.1619A>G p.Y540C p.P1238S p.D95N p.E301EEEE p.E868D		PLAN - Lichtenstein-Knorr s. Cerebellar ataxia, deafness, narcolepsy, AD

Table 3. (Continued)

41	58	F	30	20	SP	II/20	strabismus, mild pyramidal signs	CA	*CACNA 1A SPTBN2 SYNE1*	c.5861C>T p.T1954M; c.2728G>T p.A910S; c.11271_11272delGG		EA2 SCA5/SCAR14 SCAR8
42	59	F	26	3	SP	II/3	mild mental delay, strabismus, mild pyramidal signs	CA	*RNF170*	c.566T>G p.F189C	he	SNAX1
43	60	M	43	35	SP	II/35	ataxia	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		SCA28 SCA15 SPG4 PHARC FCM
44	61	M	20	1	SP	II/1	slight mental delay, brisk tendon jerks, dyslexia, telangectasia, hyperkinesia, dystonia	megacisterna, vermis hypoplasia	*COQ8A*	COQ8A c.547C>T p.Q183*	he	
45	62	M	46	26	AR cons	II/26	spasticity, tremor, dystonia, Thrombocytopenia	slight CA and supratentorial atrophy	*MFN2 ELOVL5 SPG7*	MFN2 c.2119C>T p.R707W ELOVL5 c.952G>T p.V318L	he he	CMT2A SCA38 SPG7

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 dominant; Cons consanguinity; CA cerebellar atrophy; WMA white matter abnormalities; he heterozygous; ho homozygous; ch compound heterozygous.

Table 4. Patients with variants with unlikely pathogenicity or no candidate variants.

Family	PT	Sex	AE y	AO y	Inheritance	IAPS/age at reaching phase	Additional features	MRI	Gene	Variant DNA; protein	Zygosity
46	63	M	77	70	SP	IV/75	Absent reflexes, urinary disturbances	slight vermis atrophy	*GDAP2* *KIF1A* *SETX* *SPTBN2*	*GDAP2 c.1486delT p.Y496fs* *KIF1A c.2806G>A p.D936N* *SETX c.103G>A p.E35K* *SPTBN2 c.1350+17C>T*	*he he* *he* *he*
47	64	F	63	16	AD?	III/59	episodic ataxia, no neuropathy, headache	CA	*CACNA1A*	*p. R1019.R; p.E992V; p.F1287F*	
48	65	M	53	47	SP	II/47	slight ataxia, no neuropathy	normal	*HARS2, SYNE1, CACNA1G*	*p.Q418H; p.Q3239E0; p.V2143I*	*he he* *he*
49	66	F	58	28	AR	II/28	head tremor	mild vermian and spinal cord atrophy	*SETX*	c.1468G>A p.V490I	he
50	67	F	66	52	SP	IV/65	peripheral neuropathy	CA, WM abnormalities	no variants		
51	68	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

Table 4. (Continued)

52	69	M	67	53	SP	III/67	neuropathy, Dat-scan 30%, decreased leve LH e Test	marked CA	*COQ8A* *SETX* *KIF1A*	*ATP13A2 c.1353+20T>G* *ATP13A2 c.1039+6C>T COQ8A c.1106A>G p.N369S* *KIF1A c.4927G>A p.D1643N* *SETX c.482T>G p.V161G*	*he* *he* *he*	*he*
53	70	M	55	30	SP	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
54	71	F			AR		one brother with ataxia, one sister with Parkinson		*HARS*	*HARS c.202C>T p.R68W*	*he*	
55	72	F	68	47	AR Cons	III/64	sensory neuropathy	slight CA	*SYNE1*	c.14676G>C; p.E4892D	ho	
56	73	M	12	conge nital	SP	II/1	ptosis, intellectual disability, early puberty, cryptorchidism	possible vermian hypoplasia, megacisterna magna CA	*CACNA1G* *TGM6*	*CACNA1G c.1048-5C>G TGM6 c.2078A>G p.D693G*	*he*	*he*
57	74	M	42	4	SP	II/4			*PLA2G6* *OPHN1*	*PLA2G6 c.710G>A p.R237H* *OPHN1 c.784C>A p.L262I*	*he*	*ho*
58	75	F	68	1	SP	II/1	decelopmental delay, gait disturbances, hyperkinesia	cerebellar hypodensity	*SYNE1* *SACS*	*SYNE1 c.15337G>A p.V5113I* *SACS c.10880T>G p.I3627S*	*he*	*he*
59	76	M	57	47	a sister affected	II/47	RBD, urinary incontinence, impotence, hypogonadism, hypoacusia,	pontocerebell ar ataxia, HBS	*CWF19L1*	*CWF19L1 c.1300G>C p.D434H*	*he*	

Table 4. (Continued)

60	77	M	49	34	SP	II/34	spastic ataxia, pharingeal dystonia, polydipsia	slight vermis atrophy	*KCND3* *KIF1A*	*KCND3 c.1646G>A p.R549H* *KIF1A c.1897C>T p.R633C*	*he* *he*	*he* *he*
61	78	M	33?		a sister affected		mental delay, camptocormia, areflexia, rigidity, hypersensibility to ldopa, increased ck	cerebellar atrophy, hyperintensity caudatus	*SLC33A1* *SAMD9L*	*SLC33A1 c.1054A>G p.M352V* *SAMD9L*	*he*	*he*
61	79	F	39	9	one brother affected	II/14	mental delay, psychiatric disorders, tremor, myoclonus, parkinsonism,	CA, riduzione caudato, iperintensità putamen	*VWA3B (EX)* *WNK1 (EX)*	*p.E91K* *p.M720V*		
62	80	F	48	36	SP	II/36	sensory neuropathy, hypoacusia, ofthalmoparesis, pyramidal signs, head dystonia	slight CA	*SETX*	c.5051C>G p.S1684C	he	
63	81	F	20	48	SP, a cousin with FRDA	II/20	abent reflexes, slight ataxia, possible neuropathy	marked vermis atrophy, less hemispheric	*ATP13A2* *CACNA1G* *CACNA1A*	*ATP13A2 c.2939G>A p.R980H* *CACNA1G c.2740G>A p.V914I* *CACNA1A c.3052G>A p.E1018K*	*he* *he* *he*	*he*
64	82	F	55	47	SP	II/47	axonal sensory neuropathy, Msa-like, RBD, RLS, cough	CA	*CCDC88C* *KCNC3*	*CCDC88C c.5251G>A p.V1751I* *KCNC3 c.1051G>A p.V351M*	*he*	*he*

Table 4. (Continued)

65	83	M	56	29	two affected brothers (one decesead) and a cousin	III/51	corioretinosis (also in the brother), reduction visus	CA and suvratentorial atrophy	*ANO10* *TRCP3* *TUBB3*	*ANO10 c.1021G>C p.V341L* *ANO10 c.1009T>G p.F337V* *ANO10 c.289delA p.M97** *TRPC3 c.1529C>T p.T510I* *TUBB3 c.832A>G p.K278E*	*he* *he* *he* *he*	*he* *he*
66	84	F	49	44	SP, a cousin with CMT1A	II/44	spastic ataxia, cough, strabismus	slighyt CA	*ZFYVE26*	*ZFYVE26 c.7129-17_7129-16insGAA* *CACNA1G c.6400-11A>C*	*he* *he*	
67	85	M	47	40	SP	II/40	erectile dysfunction, urinary disturbancs, brisk tendon jerks	normal	*SACS*	*SACS c.1373C>T p.T458I*	*he*	
68	86	M	47	18	AD (a brother and a nephiew with spastic paraparesis)	III/45	hyperreflexia, ptosis, III cn palsy	CA	TRPC3 SAMD9L OPA3	TRPC3 c.2547+3A>G SAMD9L c.632T>C p.V211A OPA3 c.143-6C>A	*he* *he*	*he*
69	87	F	43	40	SP	II/40	slight ataxia, no neuropathy, anxiety, itch sine materia,	CA	*SLC1A3* *SYNE1* *CACNA1G*	*SLC1A3 c.188T>C p.I63T* *SYNE1 c.17203-5C>A* *CACNA1G c.2153C>T p.P718L*	*he* *he*	*he*
70	88	M	42	39	AR, Cons	II/39	tremor	normal	*KIF1C*	c.2734C>T p.R912W	he	
71	89	M	20	1	SP	II/1	mild mental delay, 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
72	90	M	67	43	AR	III/50	cognitive impairment	CA	no variants			

Table 4. (Continued)

73	91	F	65	47	AR cons	II/47	hyperkinesia, apraxia, pyramidal signs, MMSE 24/30, brachial plexus lesion	CA	*ANO10* *SETX* *ATP7B* *TTBK2*	*ANO10 c.132dupA p.D45fs*9* *SETX c.7972A>G p.R2658G* *ATP7B c.445G>A p.V149M* *TTBK2 c.1319T>G p.V440G* *TTBK2 c.1271T>C p.I424T*	*he* *he* *he* *he* *he*	*he*
74	92	F	62	58	adoptive	II/58	spastic ataxia, nystagmus	CA	*CLCN2* *SETX* *CACNA1A*	*CLCN2 c.1856-3C>T* *SETX c.4982C>G p.P1661R* *CACNA1A c.6778G>C p.E2260Q*	*he* *he*	*he*
75	93	F	52	32	SP	IV/36	oculomotor apraxia, skoliosis	normal	*SYNE1* *ABCB7*	*SYNE1 c.3670-14_3670-13delTC* *ABCB7 c.604G>A p.A202T*	*he*	*he*
76	94	F	56	50	SP	II/50	diabetes, hypoacusia, sensorymotor axonal and demyelinated neuropathy	marked cortical and bulbopontocerebellar atrophy	*MFN2* *SPG11* *GFAP* *POLG* *CACNA1G*	*MFN2 c.809T>C p.M270T* *MFN2 c.1403G>A p.R468H* *SPG11 c.4744-6T>C* *SPG11 c.2897G>A p.R966H o* *POLG c.3421G>A p.V1141M* *GFAP c.88C>T p.R30C t* *CACNA1G c.6446G>A p.R2149Q*	*he* *he* *he* *he*	*he* *he*
77	95	F	24	1	SP	II/1	mental delay	cerebellar hypoplasia	no variants			
78	96	F	17	congenital	SP	II/1	mild mental delay (QI 81), mild dystonia, reduced hot sensation	cortical cerebellar hyperintensities	*SLC2A1* *SPAST* *SCN1A* *SLC1A3*	*SLC2A1 c.1108G>A p.V370M* *SPAST c.326C>G p.P109R* *SCN1A c.1388C>G p.T463R* *SLC1A3 c.1045A>T p.I349F*	*he* *he* *he*	*he*
79	97	M	73	59	AD	II/59	absent reflexes	CA	*GALC*	*GALC c.673G>A p.A225T* *GALC c.9G>T p.G3G*	*ch*	

Table 4. (Continued)

80	98	F	22	1	SP	II/1	tremore, mental delay	vermian atrophy	*SCN2A*	*SCN2A c.5042A>G p.N1681S*	*he*
81	99	M	45	42	SP	II/42	eyelid retraction	mild CA	*TGM6* *SACS* *ARSA*	*SACS c.2927G>A p.R976H* *TGM6 c.1953_1955dupACA p.Q652dup* *ARSA c.868C>T p.R290C* *ARSA c.728G>C p.R243P*	*he* *he* *ch*
82	100	F	64	52	AD	IV/55	spastic ataxia, hypoacusia	hypointensity pallidum, bulbar olive hyperintensity, CA	GBE1 PRNP	GBE1 c.1492G>A p.E498K GBE1 c.986A>G p.Y329C PRNP c.305C>T p.P102L	ch he
83	101	F	16	congenital	SP	II/1	strabismus, epicantus, incontinence, mental delay, areflexia	normal	*CACNA1A* *ATP1A3* *CAMTA1*	*CAMTA1 c.4245G>C p.E1415D* *CACNA1A c.4939G>A p.D1647N* *ATP1A3 c.70C>A p.P24T*	*he* *he* *he*
84	102	F	67	50	SP	II/1	chorea, dystonia, tremor, areflexia	supratentorial atrophy	*PLA2G6*	*PLA2G6 c.209G>A p.R70Q*	*he*
85	103	M			SP				*CAMTA1* *GJC2* *OPA1* *SPTBN2*	*CAMTA1 c.1240C>T p.P414S* *GJC2 c.1234C>T p.H412Y* *OPA1 c.1286G>A p.G429D* *SPTBN2 c.2456G>A, c.2457C>A p.R819H-p.R819R (su alleli diversi), p.R819Q (su stesso allele)*	*he* *he* *he*

Table 4. (Continued)

86	104	F	64	62	AD	II/62	late onset ataxia	CA	*UBR4* *SLC1A3* *SYNE1* *CACNA1G*	*UBR4 c.14112-4G>C* *UBR4 c.4313-13G>A* *SLC1A3 c.28A>G p.K10E* *SYNE1 c.25381G>A p.E8461K* *SYNE1 c.3670-14_3670-13delTC* *CACNA1G c.3000A>C p.E1000D*	*he* *he* *he* *he*	*he* *he*
87	105	F	58	1	congenital/ SP		pure ataxia	CA	*SETX* *KCNC3*	*SETX c.4433C>A p.A1478E* *KCNC3 c.1268G>A p.R423H*	*he* *he*	
88	106	M	79	30	AD	II/30	pure ataxia	CA, WMA	*MFN2* *UBR4* *KCND3* *KIF1A* *SETX*	*MFN2 c.1403G>A p.R468H* *UBR4 c.8731+16G>A* *UBR4 c.508+4G>A*	*he* *he*	*he*
89	107	M	17	7	SP	II/7	ocular teleangectasiae, slight ocular apraxia, mild mental delay	marked CA	*ZFVYE26*	*ZFYVE26 c.4797+3A>G*	*he*	
90	108	F	26	1	father ataxia, neuropathy, leukoma; brother with corneal leukoma; a sister with oral and	II/1	corneal leukoma, mental delay, ptosisi, tremor, brisk tendon jerks	CA	*GRM1* *AFG3L2* *AFG3L2*	*c.1610G>A p.R537Q* *c.2105G>A p.R702Q c.293-14_293-13delTT*	*he* *he*	*he*

Table 4. (Continued)

					anal pailloma and myastenia						
91	109	M	68	40	AR	III/57	laryngeal dystonia, pyramidal signs	mild CA, posterior WMA	*KIF1C*	*KIF1C c.940+17C>A*	*he*
92	110	M	51	46	SP	II/1	brisk tendon jerks	pons and medium cerebellar peduncle atrophy	*GRM1* *SYNE1*	*GRM1 c.1982G>A p.R661H* *SYNE1 c.25091C>T p.P8364L* *SYNE1 c.14984G>A p.C4995Y*	*he* *he* *he*
93	111	M	71	25	SP	III/68	sensorymotor axonal neuropathy, scoliosis	normal	*SYNE1*	c.17203-5C>T	he
94	112	F	44	15	SP	II/15	hyperreflexia	CA	*GLB1* *CYP7B1* *TWNK*	*TWNK c.2045G>A p.R682H* *GLB1 c.1051C>T p.R351**	*he* *he*
95	113	M	52	23	SP	II/23	orizontal ophtalmoplegia	normal	no variants		
96	114	M	46	14	SP	II/14	pyramidal signs, possible rethinopathy	CA	*KCNC1* *SPG11* *SPG7*	*KCNC1 c.683G>T p.R228L* *SPG11 c.1675T>A p.S559T* *SPG7 c.773_774delTG p.V258fs*30*	*he* *he* *he*
97	115	M	47	41	SP	II/41	nystagmus, hyperreflexia	CA	*SYNE1*	*SYNE1 c.706C>T p.R236**	*he*
98	116	M	33	13	SP	II/13	few epileptic seizures, pyramidal signs, dorsal lipomas	slight WMA, mild spinal cord atrophy	*ATM* *LRSAM1 (EX)* *CACNA1A (EX)* *RTN2 (EX)* *AHDC1 (EX)*	c.6495dupT p.V2166fs*9 p.R594C pE2372V p.P313P? P.T943P p.V463L	he he he he he he

Table 4. (Continued)

									CEP290 (EX)		
99	117	M	14	12	SP	II/12	pure ataxia	CA	no variants		
100	118	F	67	50	SP	II/50	optic atrophy, hyperkinesia, congenital cataract, slight yramidal signs	slight CA	*SLC1A3 ELOVL4 TTBK2*	*SLC1A3 c.1589A>G p.N530S ELOVL4 c.370-18C>T TTBK2 c.2434G>A p.V812M*	*he he he*
101	119	M	41	11	AR Cons	III/19	pyramidal signs, spasticity, reduced vibration sense	normal		no variants	
102	120	M	60	42	AR	II/42	pyramidal signs, staring eyes	marked CA	*ATM GRID2 MTPAP FAT2(EX) YARS (EX) ATM (EX)*	c.5727G>A p.M1909I c.2587G>A p.V863I c.1060G>T p.V354L p.R2905Q p.S338G p.1054R	he he he he he

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 dominant; Cons consanguinity; CA cerebellar atrophy; WMA white matter abnormalities; he heterozygous; ho homozygous; ch compound heterozygous.

Figure.1 Timeline of the discovery of genes involved in hereditary spinocerebellar ataxia (Galatolo et al., 2018)

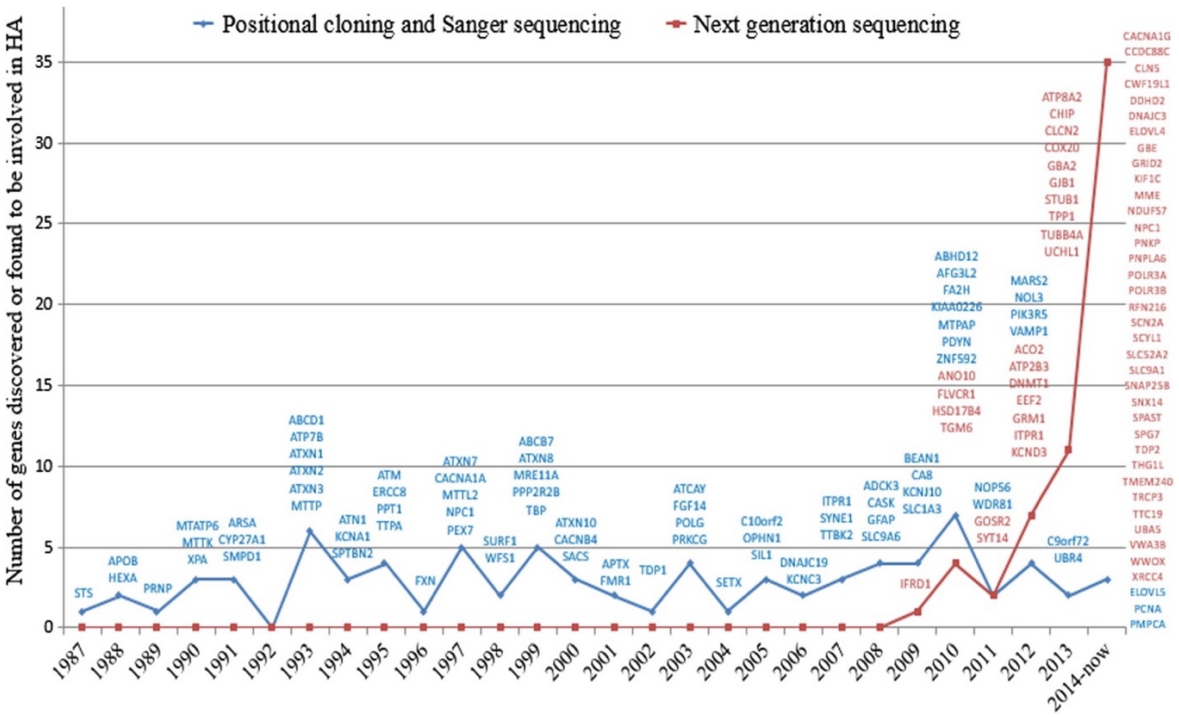


Figure 2. Cost per genome since 2001 (courtesy of www.genome.gov)

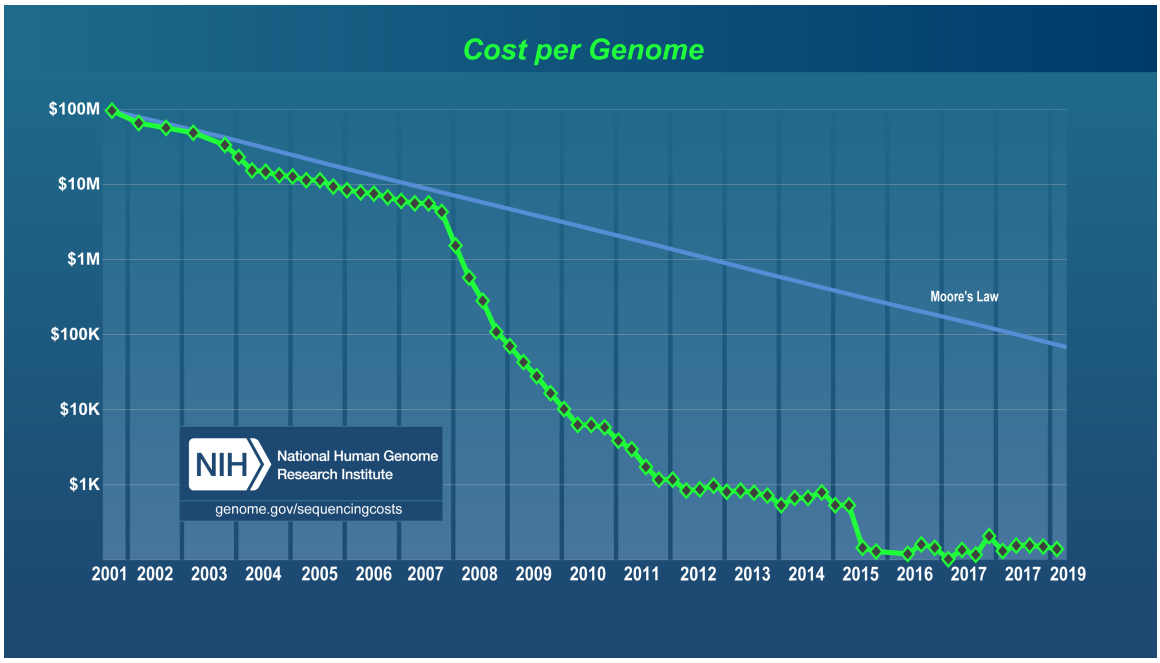


Figure 3: Proportion of patients molecularly diagnosed with various neurological diseases by whole exome sequencing (Rexach et al. 2019)

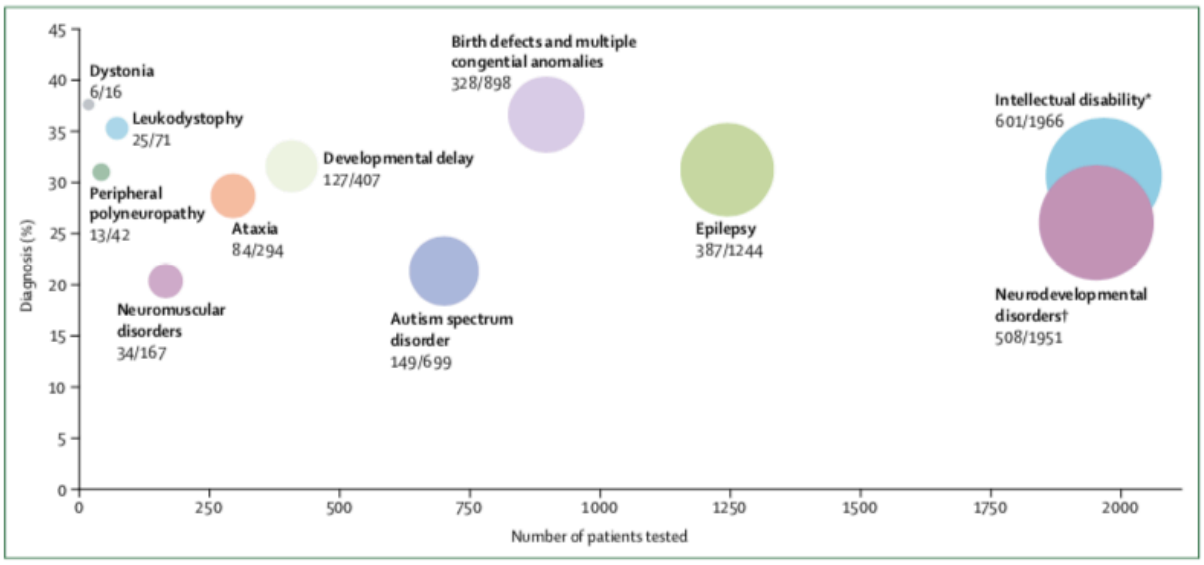


Figure 4. Diagnostic yield reported in published cohorts of patients with hereditary ataxia (Galatolo et al., 2018)

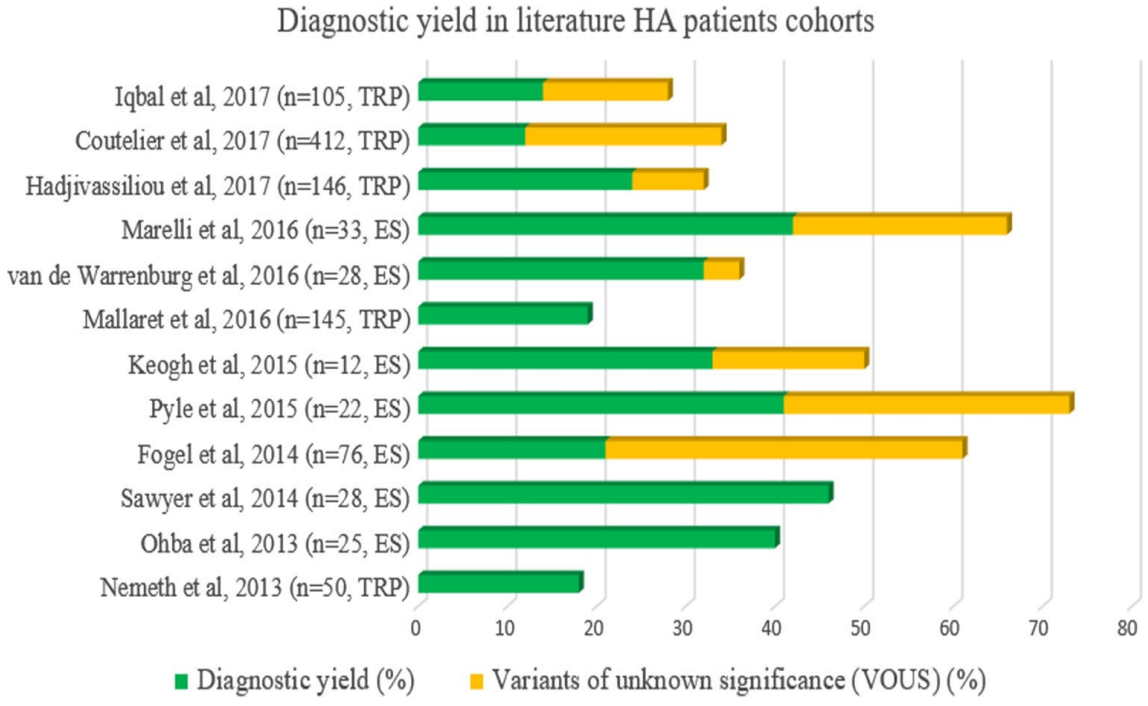


Figure 5. Workflow of SureSelect Target Enrichment system for library preparation (Image courtesy of www.agilent.com)

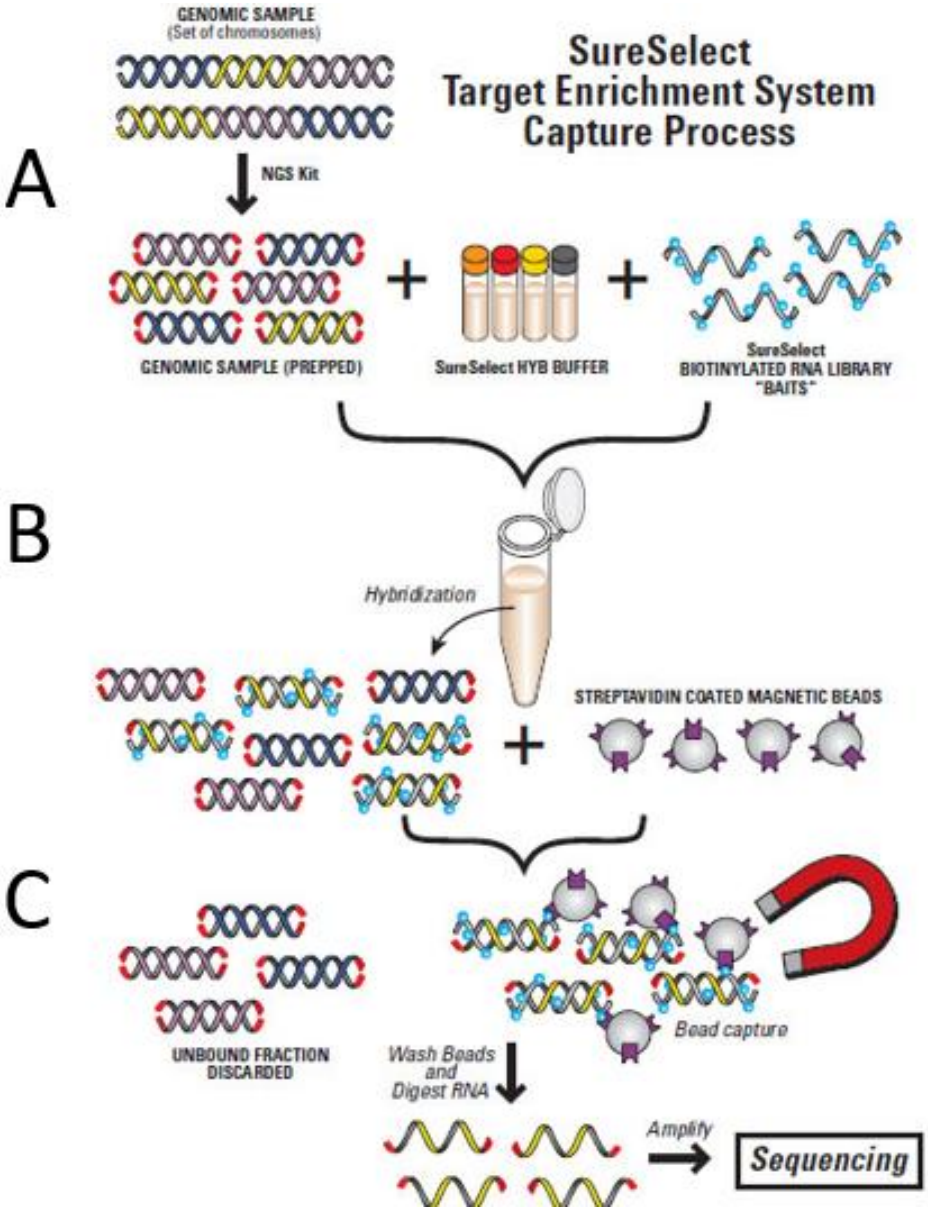


Figure 6. Bridge amplification and cluster generation for Illumina sequencing (Image courtesy of www.illumina.com)

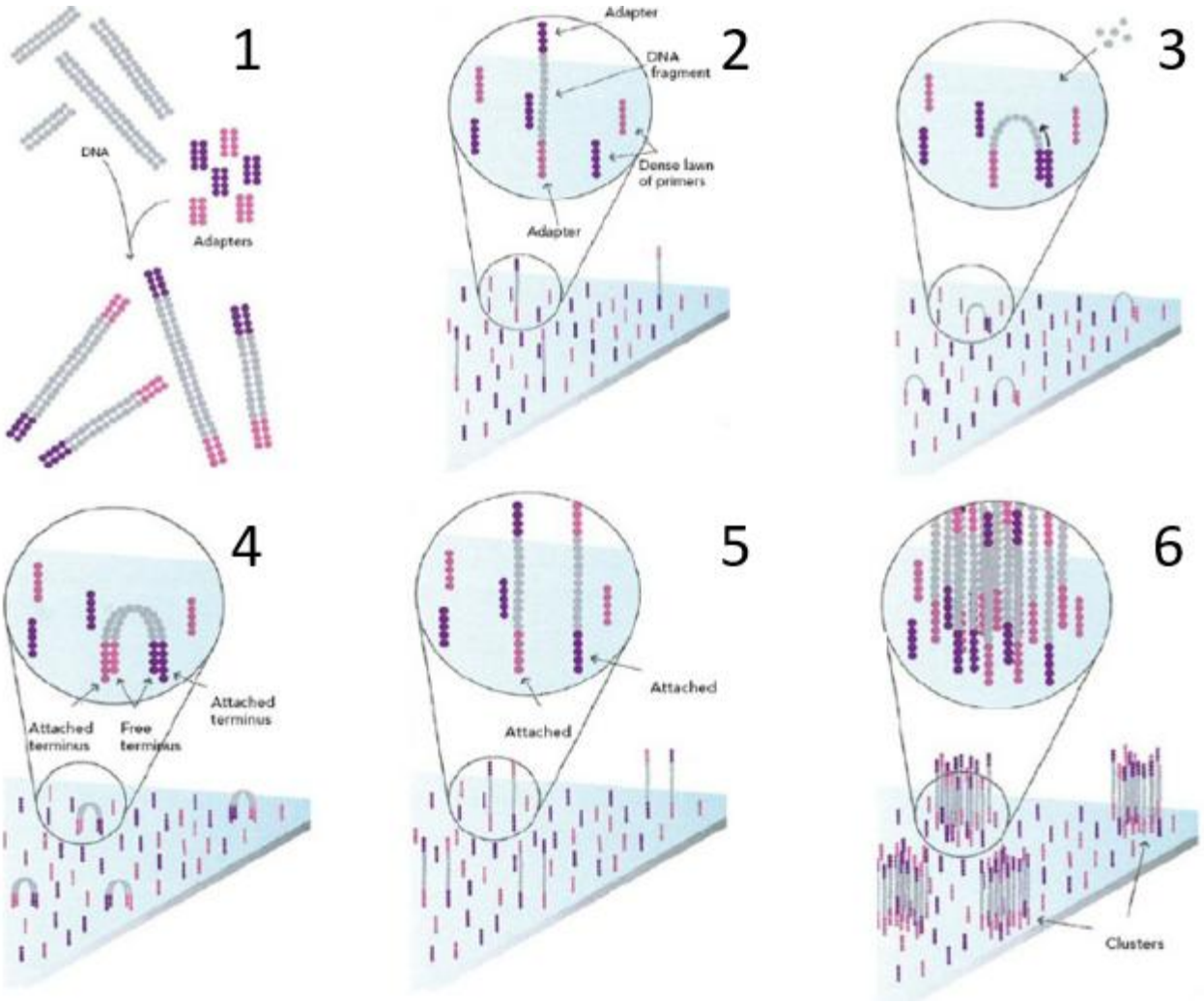
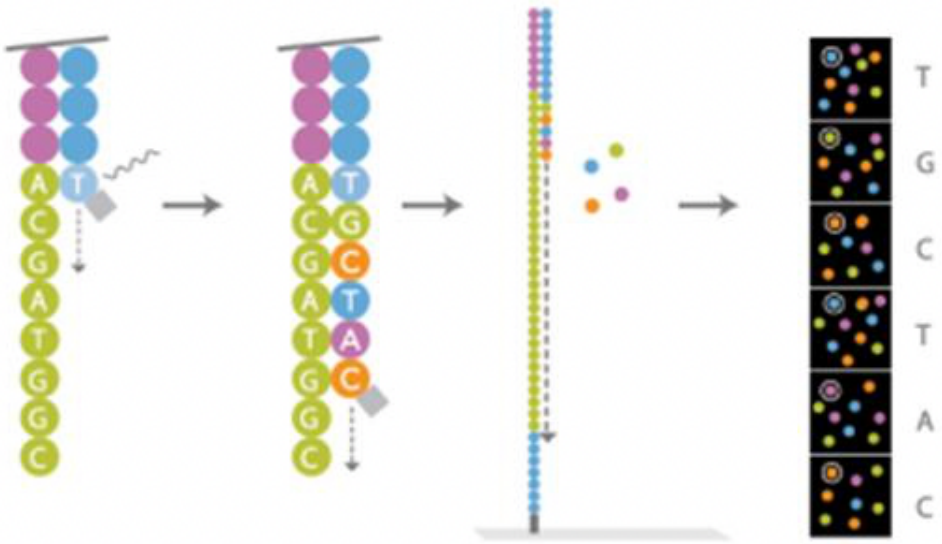


Figure 7. Sequencing and base calling for Illumina sequencing (Ansorge, 2009)



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

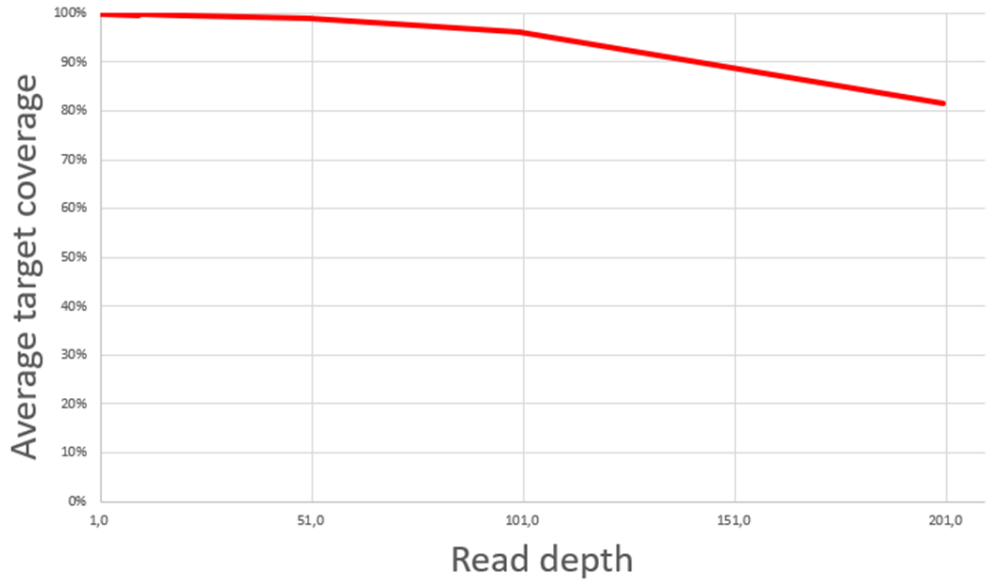


Figure 9. Brain MRI of three patients carrying mutations in STUB1 gene. Selected coronal and axial T2-weighted slices, along with axial T1-weighted images, showing typical MRI findings in three SCA48 patients (TOP-BOTTOM: 1.III-14, 1.IV-1, 2.III-2). In all cases, a significant atrophy of both vermis (blue arrows) and cerebellar hemispheres, more pronounced at the level of the posterior and lateral cerebellar areas (i.e. Lobule VI, Crus I, Crus II and Lobule VIII, red arrows), was present. Furthermore, hyperintensity of both dentate nuclei was found, extending to medial portion of the middle cerebellar peduncles (green arrows). (De Michele at al, 2019)

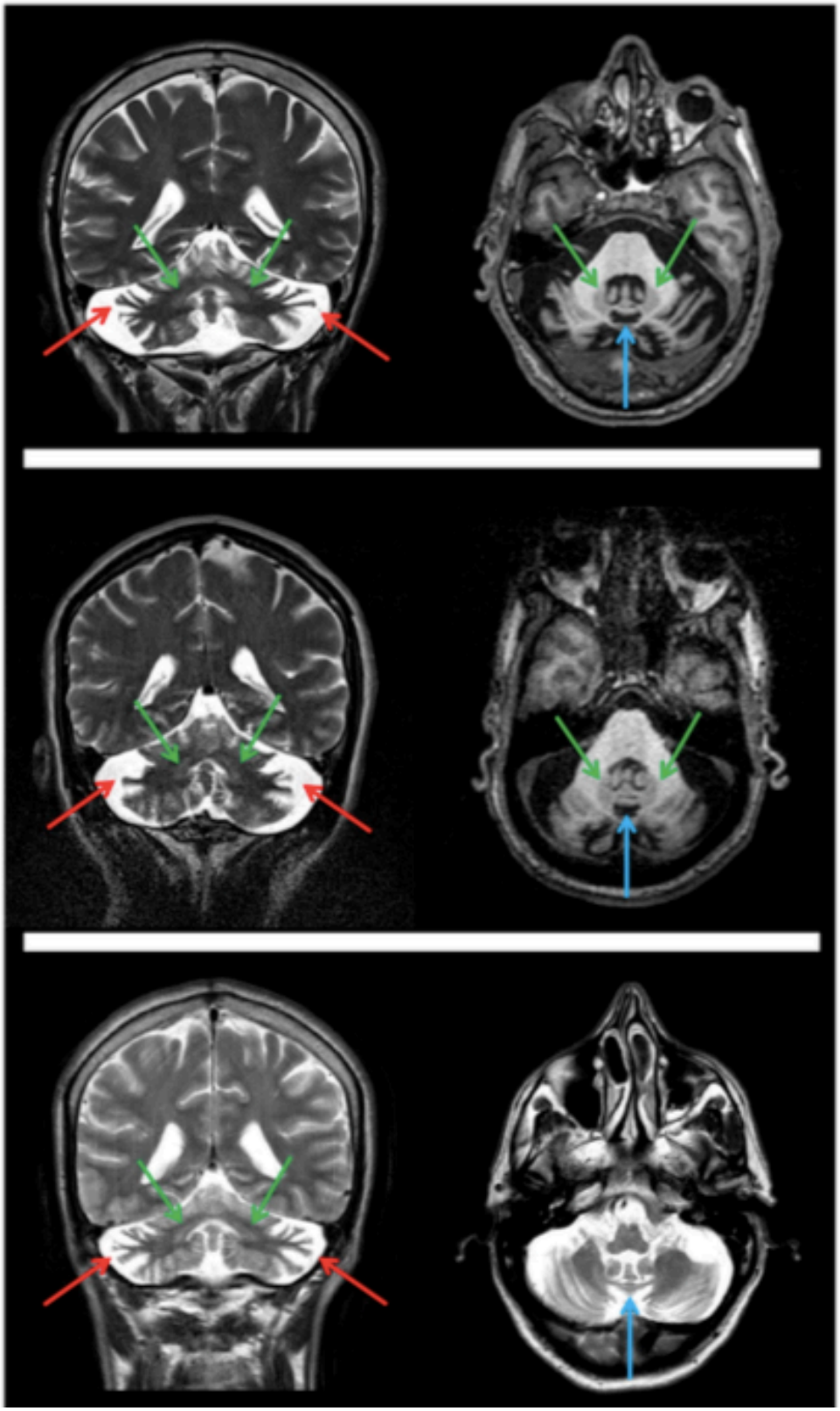


Figure 10. Brain MRI of patient 20 carrying mutations in SPG7 gene. T_2-weighted sagittal and axial brain MRI showing cerebellar atrophy

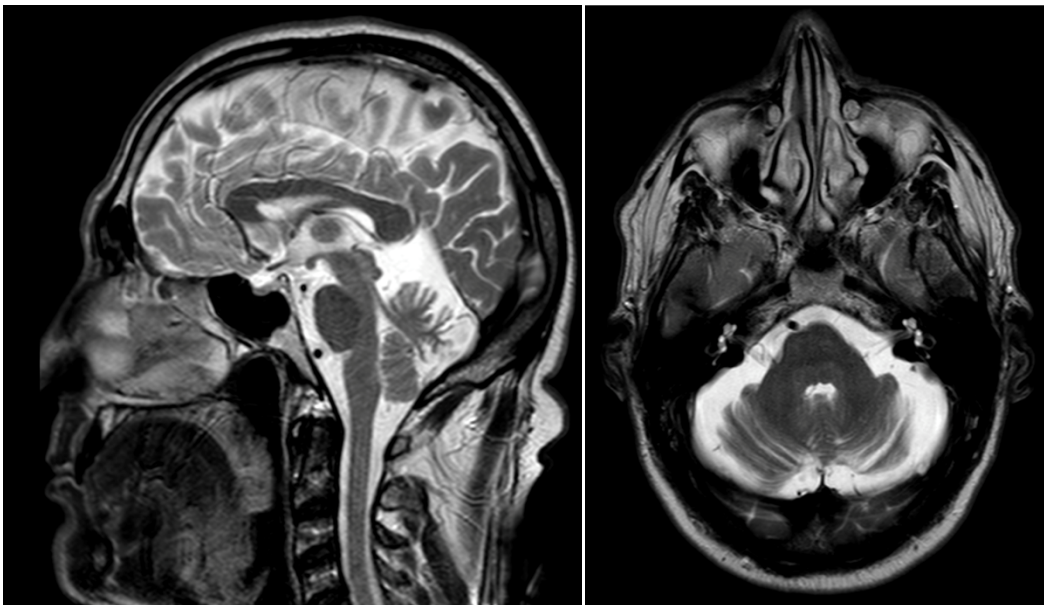


Figure 11. Brain MRI of patient 24 carrying mutations in PNPLA6 gene. T_2-weighted sagittal and axial brain MRI showing marked cerebellar atrophy

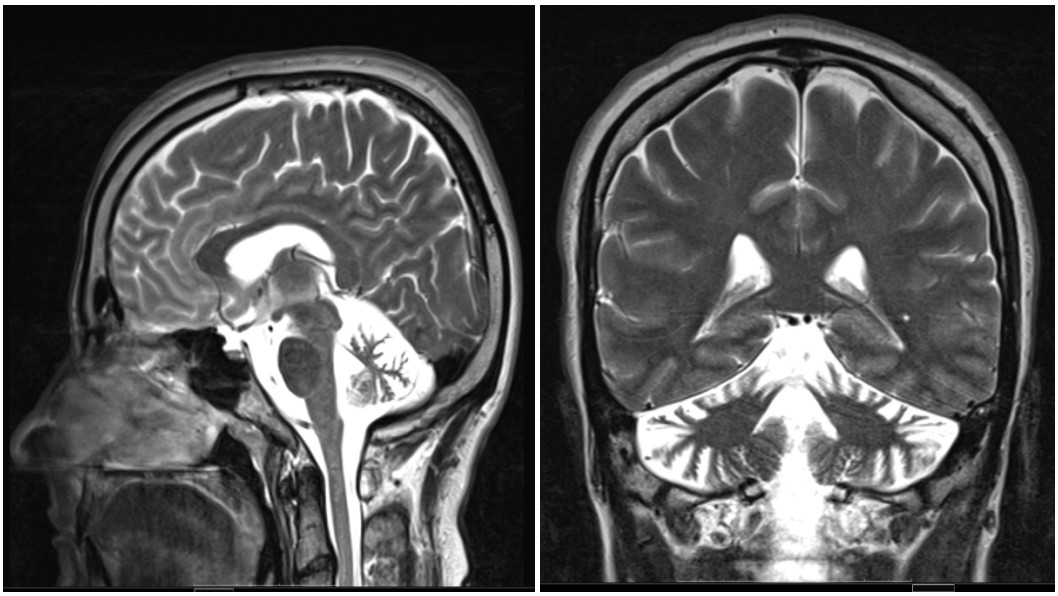


Figure 12. Brain MRI of patient 42 carrying mutations in RNF216 gene. 1. Coronal T2-weighted image showing diffuse cerebellar atrophy, increased signal of dentate nucleus (white arrow), and parietal atrophy. 2. Sagittal T1-weighted image showing atrophy of the cerebellum, with sparing of the pons and midbrain. 3-4. Axial fluid attenuated inversion recovery images showing the presence of scattered white matter lesions affecting both t he deep and periventricular white matter.

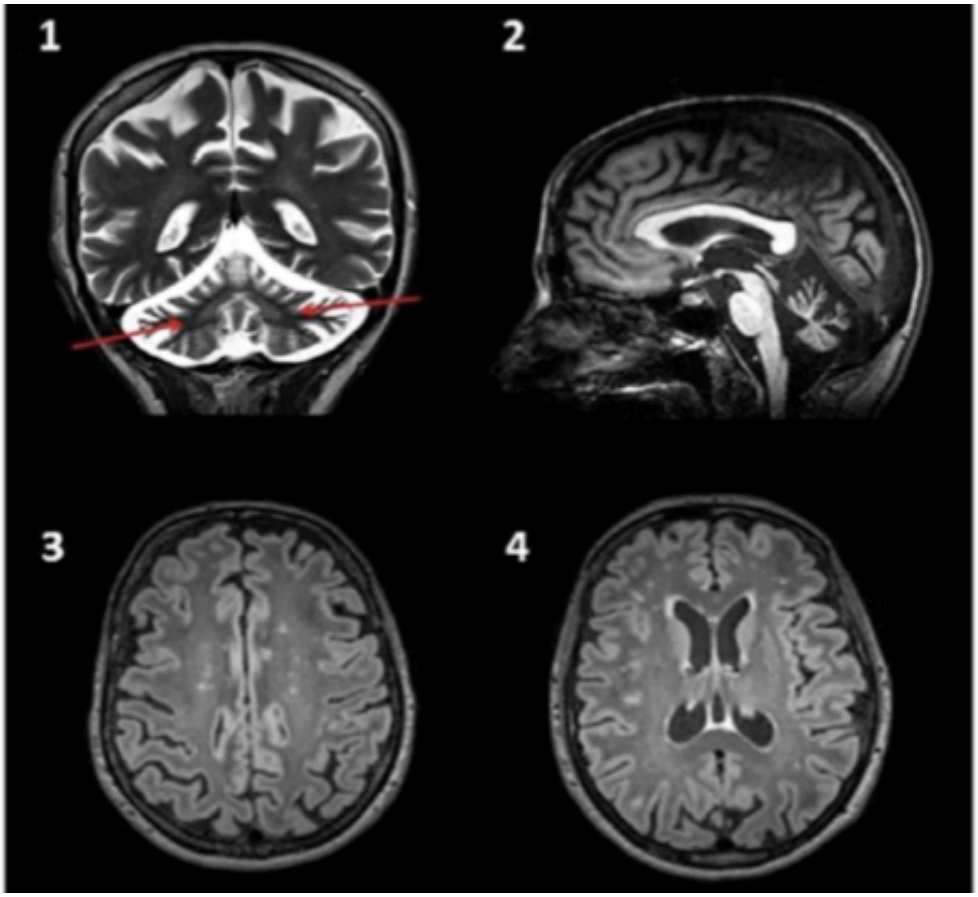


Figure 13. Brain MRI of patient 28 carrying mutations in SYNE1 gene. T_2-weighted sagittal and axial brain MRI showing marked cerebellar atrophy

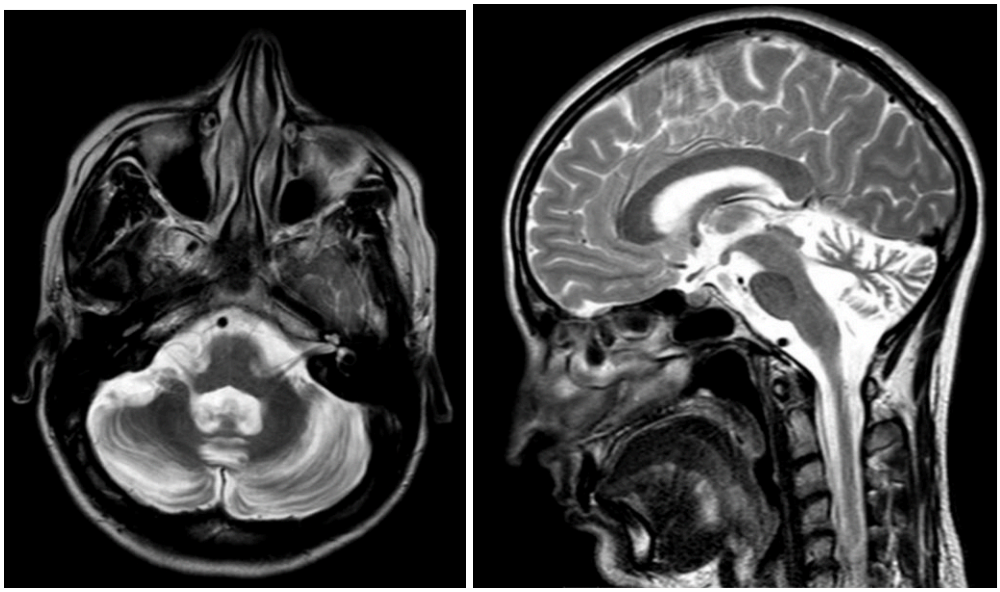
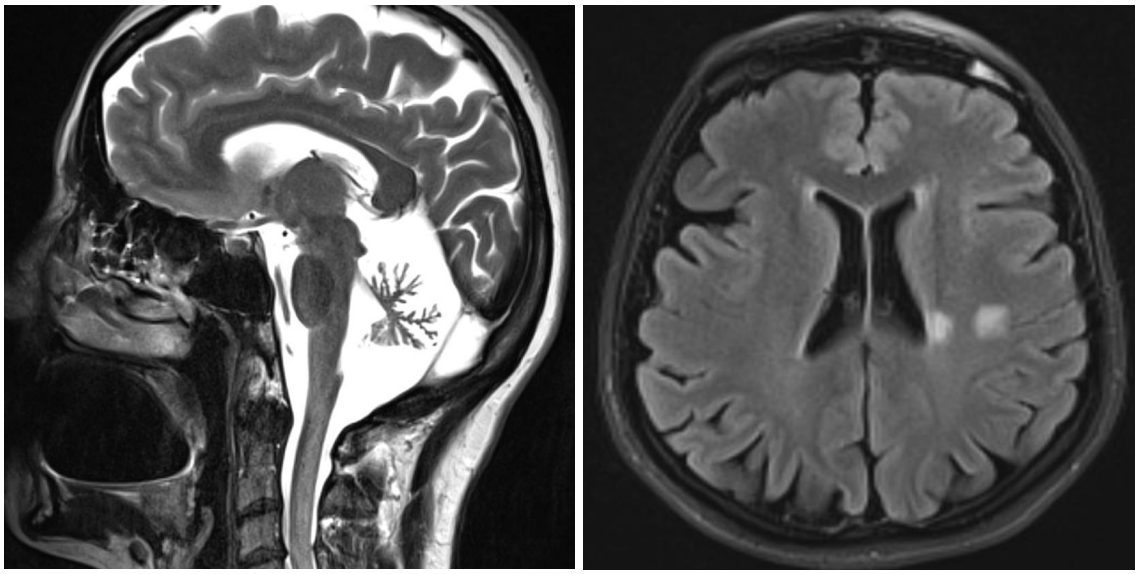


Figure 14. Brain MRI of patient 49 carrying mutations in ANO10 gene. T2-weighted sagittal brain MRI showing marked cerebellar atrophy and FLAIR axial brain MRI showing white matter abnormalities.



9. REFERENCES

- Abbott JA, Meyer-Schuman R, Lupo V, et al. Substrate interaction defects in histidyl-tRNA synthetase linked to dominant axonal peripheral neuropathy. Hum Mutat. 2018 Mar;39(3):415-432. doi:10.1002/humu.23380.
- Abele M, Burk K, Schols L, Schwartz S, Besenthal I, Dichgans J, Zuhlke C, Riess O, Klockgether T (2002) The aetiology of sporadic adult-onset ataxia. Brain 125(Pt5):961–968. https://doi.org/10. 1093/brain/awf107
- Anheim M, Fleury M, Monga B, et al. Epidemiological, clinical, paraclinical and molecular study of a cohort of 102 patients affected with autosomal recessive progressive cerebellar ataxia from Alsace, Eastern France: implications for clinical management. Neurogenetics. 2010; 11: 1-12.
- Anheim M, Tranchant C, Koenig M. The autosomal recessive cerebellar ataxias. N Engl J Med. 2012 Feb 16;366(7):636-46. doi: 10.1056/NEJMra1006610.
- Arslan EA, Öncel İ, Ceylan AC, et al. Genetic and phenotypic features of patients with childhood ataxias diagnosed by next-generation sequencing gene panel. Brain Dev. 2019. pii: S0387-7604(18)30608-9. doi:10.1016/j.braindev.2019.08.004.
- Berg JS, Adams M, Nassar N, at al. An informatics approach to analyzing the incidentalome. Genet Med. 2013; 15: 36-44.
- Bernard G, Chouery E, Putorti ML, et al. Mutations of POLR3A encoding a catalytic subunit of RNA polymerase Pol III cause a recessive hypomyelinating leukodystrophy. Am J Hum Genet. 2011;89:415–23.
- Bird TD. Hereditary Ataxia Overview. 1998 [updated 2019].
- Boucher BJ, Gibberd FB. Familial ataxia, hypogonadism and retinal degeneration. Acta Neurol Scand 1969; 45: 507–10.
- Boukhris A, Stevanin G, Feki I, et al. Hereditary spastic paraplegia with mental impairment and thin corpus callosum in Tunisia: SPG11, SPG15, and further genetic heterogeneity. Arch Neurol. 2008; 65: 393-402.
- Shi C H, Schisler J C, Rubel C E, et al. Ataxia and hypogonadism caused by the loss of ubiquitin ligase activity of the U box protein CHIP, Hum. Mol. Genet. 23 (2014) 1013–1024.
- Cagnoli C, Stevanin G, Brussino A, et al. Missense mutations in the AFG3L2 proteolytic domain account for ~1.5% of European autosomal dominant cerebellar ataxias. Hum Mutat. 2010; 31: 1117-24.

- Cagnoli C, Stevanin G, Michielotto C et al. Large pathogenic expansions in the SCA2 and SCA7 genes can be detected by fluorescent repeat-primed polymerase chain reaction assay. J Mol Diagn. 2006; 8(1):128-32.
- Campuzano V, Montermini L, Moltò MD, et al. (1996) Friedreich's ataxia: autosomal recessive disease caused by an intronic GAA triplet repeat expansion. Science 271(5254):1423– 1427. https://doi.org/10.1126/science.271.5254.1423
- Casari G, De Fusco M, Ciarmatori S, et al. Spastic paraplegia and OXPHOS impairment caused by mutations in paraplegin, a nuclear- encoded mitochondrial metalloprotease. Cell 1998;93:973-983.
- Choquet K, Tetreault M, Yang S, et al. SPG7 mutations explain a significant proportion of French Canadian spastic ataxia cases. Eur J Hum Genet 2016;24:1016-1021.
- Cortese A, Simone R, Sullivan R, et al. Biallelic expansion of an intronic repeat in RFC1 is a common cause of late-onset ataxia. Nat Genet. 2019 Apr;51(4):649-658. doi:10.1038/s41588-019-0372-4.
- Coutelier M, Blesneac I, Monteil A, et al. A Recurrent Mutation in *CACNA1G* Alters Cav3.1 T-Type Calcium-Channel Conduction and Causes Autosomal-Dominant Cerebellar Ataxia. The American Journal of Human Genetics 2015; 97: 726-737.
- Coutelier M, Coarelli G, Monin ML, et al. A panel study on patients with dominant cerebellar ataxia highlights the frequency of channelopathies. Brain. 2017; 140: 1579-1594.
- Criscuolo C, Banfi S, Orio M, et al. A novel mutation in SACS gene in a family from southern Italy. Neurology. 2004; 62: 100-2.
- Dallabona C, Diodato D, Kevelam SH, et al. Novel (ovario) leukodystrophy related to AARS2 mutations. Neurology. 2014; 82: 2063-71.
- De Braekeleer M, Giasson F, Mathieu J, et al. Genetic epidemiology of autosomal recessive spastic ataxia of Charlevoix-Saguenay in northeastern Quebec. Genet Epidemiol 1993;10(1):17–25.
- De Michele G, Coppola G, Cocozza S, Filla A. A pathogenetic classification of hereditary ataxias: is the time ripe?. J Neurol. 2004; 251(8):913-22.
- De Michele G, Lieto M, Galatolo D et al. Spinocerebellar ataxia 48 presenting with ataxia associated with cognitive, psychiatric, and extrapyramidal features: A report of two Italian families. Parkinsonism Relat Disord. 2019 May 14. pii: S1353-8020(19)30222-6. doi: 10.1016/j.parkreldis.2019.05.001
- Depondt C, Donatello S, Simonis N et al. Autosomal recessive cerebellar ataxia of adult onset due to STUB1 mutations. Neurology. 2014 May 13;82(19):174950

- Di Gregorio, E., Borroni, B., Giorgio, E., et al. ELOVL5 mutations cause spinocerebellar ataxia 38. Am. J. Hum. Genet. 95: 209-217, 2014.[PubMed:
- Dor, T., Cinnamon, Y., Raymond, et al. KIF1C mutations in two families with hereditary spastic paraparesis and cerebellar dysfunction. J. Med. Genet. 51: 137-142, 2014.
- Drouin-Garraud V, Belgrand M, Grünewald S, et al. Neurological presentation of a congenital disorder of glycosylation CDG-Ia: implications for diagnosis and genetic counseling. Am J Med Genet. 2001; 101: 46-9.
- Dürr A. Autosomal dominant cerebellar ataxias: polyglutamine expansions and beyond. Lancet Neurol. 2010;9:885–94.
- Elsayed SM, Heller R, Thoenes M, et al. Autosomal dominant SCA5 and autosomal recessive infantile SCA are allelic conditions resulting from SPTBN2 mutations. Eur J Hum Genet. 2014; 22: 286-8.
- Esmaeeli Nieh S, Madou MR, Sirajuddin M, et al. De novo mutations in KIF1A cause progressive encephalopathy and brain atrophy. Ann Clin Transl Neurol. 2015;2:623-35.
- Estrada-Cuzcano A, Martin S, Chamova T, et al. Loss-of-function mutations in the ATP13A2/PARK9 gene cause complicated hereditary spastic paraplegia (SPG78). Brain. 2017; 140: 287-305.
- Finsterer J. Mitochondrial ataxias. Can J Neurol Sci. 2009b;36:543–53.
- Fiskerstrand T, H'mida-Ben Brahim D, Johansson S, et al. Mutations in ABHD12 cause the neurodegenerative disease PHARC: An inborn error of endocannabinoid metabolism. Am J Hum Genet. 2010; 87: 410-7.
- Fogel BL, Lee H, Deignan JL, et al. Exome sequencing in the clinical diagnosis of sporadic or familial cerebellar ataxia. JAMA Neurol. 2014; 71: 1237-46.
- Fogel, B. L., Lee, H., Deignan, J. L., et al. (2014). Exome sequencing in the clinical diagnosis of sporadic or familial cerebellar ataxia. JAMA Neurol, 71(10), 1237-1246. doi:10.1001/jamaneurol.2014.1944
- Galatolo D, Tessa A, Filla A, Santorelli FM. Clinical application of next generation sequencing in hereditary spinocerebellar ataxia: increasing the diagnostic yield and broadening the ataxia-spasticity spectrum. A retrospective analysis. Neurogenetics. 2018;19(1):1-8. doi: 10.1007/s10048-017-0532-6.
- Galosi S, Barca E, Carrozzo R, et al. Dystonia-Ataxia with early handwriting deterioration in COQ8A mutation carriers: A case series and literature review. Parkinsonism Relat Disord. 2019 Sep 28;68:8-16. doi:10.1016/j.parkreldis.2019.09.015.

GeneReviews® Available from http://www.ncbi.nlm.nih.gov/books/NBK1138/

- Genis D, Ortega-Cubero S, San Nicolás H et al. Heterozygous STUB1 mutation causes familial ataxia with cognitive affective syndrome (SCA48). Neurology. 2018 Nov 20;91(21)
- Giordano I, Harmuth F, Jacobi H, et al. Clinical and genetic characteristics of sporadic adult-onset degenerative ataxia. Neurology. 2017 5;89(10):1043-1049. doi:10.1212/WNL.0000000000004311.
- Gomez CM, Das S. Clinical exome sequencing: the new standard in genetic diagnosis. JAMA Neurol. 2014;71:1215-6. doi:10.1001/jamaneurol.2014.2015.
- Gregory A, Kurian MA, Maher ER, et al. PLA2G6-Associated Neurodegeneration. 2008 [updated 2017]. In: Adam MP, Ardinger HH, Pagon RA, et al GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2017.
- Gros-Louis F, Dupre N, Dion P, et al. Mutations in SYNE1 lead to a newly discovered form of autosomal recessive cerebellar ataxia. Nat Genet 2007;39(1):80–85.
- Guissart C, Li X, Leheup B, et al. Mutation of SLC9A1, encoding the major Na+/H+ exchanger, causes ataxia-deafness Lichtenstein-Knorr syndrome. Hum Mol Genet. 2015; 24: 463-70.
- Hadjivassiliou M, Martindale J, Shanmugarajah P, et al. Causes of progressive cerebellar ataxia: prospective evaluation of 1500 patients. J Neurol Neurosurg Psychiatry. 2017; 88: 301-309.
- Hanein S, Martin E, Boukhris A, et al. Identification of the SPG15 gene, encoding spastizin, as a frequent cause of complicated autosomal-recessive spastic paraplegia, including Kjellin syndrome. Am J Hum Genet. 2008; 82: 992-1002.
- Harding AE. Classification of the hereditary ataxias and paraplegias. Lancet. 1983; 1:1151-5.
- Helbig I, Tayoun AAN (2016) Understanding genotypes and phe- notypes in epileptic encephalopathies. Mol Syndromol 7(4):172– 181. https://doi.org/10.1159/000448530
- Holmes G. A form of familial degeneration of the cerebellum. Brain 1907; 30: 466–89.
- https://doi.org/10.1007/s00415-015-7772-x
- Hui P (2014) Next generation sequencing: chemistry, technology and applications. Top Curr Chem 336:1–18. https://doi.org/10. 1007/128_2012_329.
- Iqbal Z, Rydning SL, Wedding IM, et al. Targeted high throughput sequencing in hereditary ataxia and spastic paraplegia. PLoS One. 2017; 12: e0174667.
- Johnson NE. Whole-exome sequencing in neurologic practice: Reducing the diagnostic odyssey. Neurol Genet. 2015 Dec 24;1(4):e37. doi: 10.1212/NXG.0000000000000037. eCollection 2015 Dec. PubMed PMID: 27066574; PubMed

- Kalia SS, Adelman K, Bale SJ, et al. Recommendations for reporting of secondary findings in clinical exome and genome sequencing, 2016 update (ACMG SF v2.0): a policy statement of the American College of Medical Genetics and Genomics. Genet Med. 2017; 19: 249-255.
- Keogh MJ, Steele H, Douroudis K, (2015) Frequency of rare recessive mutations in un-explained late onset cerebellar ataxia. J Neurol 262(8):1822–1827.
- Kurian MA, Morgan NV, MacPherson L, et al. Phenotypic spectrum of neurodegeneration associated with mutations in the PLA2G6 gene (PLAN). Neurology. 2008; 70: 1623-9.
- Lagier-Tourenne C, M. Tazir, L.C. López, et al., ADCK3, an ancestral kinase, is mutated in a form of recessive ataxia associated with coenzyme Q10 deficiency, Am. J. Hum. Genet. 82 (2008) 661–672.
- Lawrence L, Sincan M, Markello T, at al. The implications of familial incidental findings from exome sequencing: the NIH Undiagnosed Diseases Program experience. Genet Med. 2014; 16: 741-50.
- Lee JR, Srour M, Kim D, et al. De novo mutations in the motor domain of KIF1A cause cognitive impairment, spastic paraparesis, axonal neuropathy, and cerebellar atrophy. Hum Mutat. 2015;36:69-78. 2.
- Lerner-Ellis JP, Tirone JC, Pawelek PD, Doré C, Atkinson JL, Watkins D, et al. Identification of the gene responsible for methylmalonic aciduria and homocystinuria, cblC type. Nat Genet 2006;38(1):93–100.
- Lieto M, Riso V, Galatolo D, et al. The complex phenotype of spinocerebellar ataxia type 48 in eight unrelated Italian families. Eur J Neurol. 2019 Sep 30. doi: 10.1111/ene.14094. [Epub ahead of print] PubMed PMID: 31571321.
- Lieto M, Roca A, Santorelli FM, et al. Degenerative and acquired sporadic adult onset ataxia. Neurol Sci. 2019; 40:1335-1342. doi: 10.1007/s10072-019-03856-w.
- Mallaret M, Renaud M, Redin C, et al. Validation of a clinical practice-based algorithm for the diagnosis of autosomal recessive cerebellar ataxias based on NGS identified cases. J Neurol. 2016; 263: 1314-22.
- Marelli C, Guissart C, Hubsch C, et al., (2016) Mini-exome coupled to read-depth based copy number variation analysis in patients with inherited ataxias. Hum Mutat 37(12):1340–1353. https://doi.org/10.1002/humu.23063
- Margolin DH, Kousi M, Chan YM, et al.Ataxia, dementia, and hypogonadotropism caused by disordered ubiquitination. N Engl J Med. 2013; 368: 1992-2003.
- Matsukawa T, Koshi KM, Mitsui J, et al. Slowly progressive d-bifunctional protein deficiency with survival to adulthood diagnosed by whole-exome sequencing. J Neurol Sci. 2017; 372: 6-10.

- Meienberg J, Bruggmann R, Oexle K, Matyas G. Clinical sequencing: is WGS the better WES? Hum Genet. 2016 Mar;135(3):359-62. doi: 10.1007/s00439-015-1631-9.
- Meyer-Schuman R, Antonellis A. Emerging mechanisms of aminoacyl-tRNA synthetase mutations in recessive and dominant human disease. Hum Mol Genet. 2017;26(R2):R114-R127. doi: 10.1093/hmg/ddx231.
- Möller G, van Grunsven EG, Wanders RJ, Adamski J. Molecular basis of D- bifunctional protein deficiency. Mol Cell Endocrinol. 2001; 171: 61-70.
- Mollet J, A. Delahodde, V. Serre, et al. CABC1 gene mutations cause ubiquinone deficiency with cerebellar ataxia and seizures, Am. J. Hum. Genet. 82 (2008) 623–630.
- Morel CF, Lerner-Ellis JP, Rosenblatt DS. Combined methylmalonic aciduria and homocystinuria (cblC): phenotype genotype correlations and ethnic-specific ob- servations. Mol Genet Metab 2006;88:315–21.
- Morino H, Matsuda Y, Muguruma K, et al. A mutation in the low voltage- gated calcium channel CACNA1G alters the physiological properties of the channel, causing spinocerebellar ataxia. Mol Brain. 2015; 8: 89.
- Musselman KE, Stoyanov CT, Marasigan R, et al. Prevalence of ataxia in children: A systematic review. Neurology. 2014;82:80–9.
- Németh AH, Kwasniewska AC, Lise S, et al. Next generation sequencing for molecular diagnosis of neurological disorders using ataxias as a model. Brain. 2013; 136: 3106-18.
- Neuhauser G, Opitz JM. Autosomal recessive syndrome of cerebellar ataxia and hypogonadotropic hypogonadism. Clin Genet 1975; 7: 426–34.
- Ngo KJ, Rexach JE, Lee H, et al. A Diagnostic Ceiling for Exome
- Nibbeling EAR, Duarri A, Verschuuren-Bemelmans CC, et al. Exome sequencing and network analysis identifies shared mechanisms underlying spinocerebellar ataxia. Brain. 2017Brain. 2017 1;140(11):2860-2878. doi: 10.1093/brain/awx251.
- Novarino, G., Fenstermaker, A. G., Zaki, M. S., at al. Exome sequencing links corticospinal motor neuron disease to common neurodegenerative disorders**.** Science 343: 506-511, 2014.
- Ohba C, Osaka H, Iai M, et al. Diagnostic utility of whole exome sequencing in patients showing cerebellar and/or vermis atrophy in childhood. Neurogenetics. 2013; 14: 225-32.
- Orr HT, Chung MY, Banfi S, et al. (1993) Expansion of an unstable trinucleotide CAG repeat in spinocerebellar ataxia type 1. Nat Genet 4(3):221–226. https://doi. org/10.1038/ng0793-221

- Pfeffer G, Pyle A, Griffin H, et al. SPG7 mutations are a common cause of undiagnosed ataxia. Neurology 2015;84:1174-1176.
- Pfeffer G, Pyle A, Griffin H, et al. SPG7 mutations are a common cause of undiagnosed ataxia. Neurology. 2015; 84: 1174-6.
- Pierce SB, Walsh T, Chisholm KM, et al. Mutations in the DBP-deficiency protein HSD17B4 cause ovarian dysgenesis, hearing loss, and ataxia of Perrault Syndrome. Am J Hum Genet. 2010; 87: 282-8.

  PubMed PMID: 20301317.
- Puffenberger EG, Jinks RN, Sougnez C, et al. Genetic mapping and exome sequencing identify variants associated with five novel diseases. PLoS One. 2012; 7: e28936.
- Pyle A, Smertenko T, Bargiela D, et al. Exome sequencing in undiagnosed inherited and sporadic ataxias. Brain. 2015; 138: 276-83.
- Rainier S, Bui M, Mark E et al. Neuropathy target esterase gene mutations cause motor neuron disease. Am J Hum Genet. 2008; 82:780-785.
- Rexach, J., Lee, H., Martinez-Agosto, et al., (2019). Clinical application of next-generation sequencing to the practice of neurology. Lancet Neurol, 18(5), 492-503. doi:10.1016/S1474- 4422(19)30033-X
- Romaniello R, Zucca C, Tonelli A, et al. A wide spectrum of clinical, neurophysiological and neuroradiological abnormalities in a family with a novel CACNA1A mutation. J Neurol Neurosurg Psychiatry. 2010; 81 :840-3.
- Royer-Bertrand B, Tsouni P, Mullen P, et al. Peripheral neuropathy and cognitive impairment associated with a novel monoallelic HARS variant. Ann Clin Transl Neurol. 2019;6(6):1072-1080. doi: 10.1002/acn3.791
- Ruano L, Melo C, Silva M.C., Coutinho P., The global epidemiology of hereditary ataxia and spastic paraplegia: a systematic review of prevalence studies, Neuroepidemiology 42 (2014) 174–183.
- Ruano L, Melo C, Silva MC, Coutinho P (2014) The global epide- miology of hereditary ataxia and spastic paraplegia: a systematic review of prevalence studies. Neuroepidemiology 42(3):174–183. https://doi.org/10.1159/000358801
- Russell JF, Steckley JL, Coppola G, et al. Familial cortical myoclonus with a mutation in NOL3. Ann Neurol. 2012; 72: 175-83.
- Sanger F; Nicklen S; Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. 1977; 74 (12): 5463–7

- Santens P, Van Damme T, Steyaert W et al. RNF216 mutations as a novel cause of autosomal recessive Huntington-like disease. Neurology. 2015; 84:1760-1766.
- Sawyer SL, Schwartzentruber J, Beaulieu CL, et al. Exome sequencing as a diagnostic tool for pediatric-onset ataxia. Hum Mutat. 2014; 35: 45-9.
- Sawyer SL, Schwartzentruber J, Beaulieu CL, et al. Exome sequencing as a diagnostic tool for pediatric-onset ataxia. Hum Mutat. 2014; 35: 45-9.
- Schiffmann R, Tedeschi G, Kinkel RP, et al. Leukodystrophy in patients with ovarian dysgenesis. Ann Neurol. 1997; 41: 654-61.
- Schwarze K, Buchanan J, Taylor JC, Wordsworth S. Are whole-exome and whole-genome sequencing approaches cost-effective? A systematic review of the literature. Genet Med. 2018;20(10):1122-1130. doi: 10.1038/gim.2017.247.
- Sequencing in Cerebellar Ataxia and Related Neurological Disorders. Hum Mutat. (2019). doi: 10.1002/humu.23946.
- Shakya S, Kumari R, Suroliya V, et al. Whole exome and targeted gene sequencing to detect pathogenic recessive variants in early onset cerebellar ataxia. Clin Genet. 2019 Aug 20. doi: 10.1111/cge.13625.
- Sun M, Johnson AK, Nelakuditi V, et al.. Targeted exome analysis identifies the genetic basis of disease in over 50% of patients with a wide range of ataxia-related phenotypes. Genet Med. 2019;21(1):195-206. doi: 10.1038/s41436-018-0007-7.
- Synofzik M, Gonzalez MA, Lourenco CM, et al. PNPLA6 mutations cause Boucher-Neuhauser and Gordon Holmes syndromes as part of a broad neurodegenerative spectrum. Brain. 2013; 137(Pt 1):69–77.
- Synofzik M, Schüle R. Overcoming the divide between ataxias and spastic paraplegias: Shared phenotypes, genes, and pathways. *Mov Disord*. 2017; 32:332–345.
- Synofzik M, Smets K, Mallaret M, et al. SYNE1 ataxia is a common recessive ataxia with major non-cerebellar features: a large multi-centre study. Brain 2016;139(Pt5):1378–1393.
- Synofzik M, Smets K, Mallaret M, et al. SYNE1 ataxia is a common recessive ataxia with major non-cerebellar features: a large multi-centre study. Brain 2016;139(Pt5):1378–1393.
- Synofzik M, Soehn AS, Gburek-Augustat J, et al. Autosomal recessive spastic ataxia of Charlevoix Saguenay (ARSACS): expanding the genetic, clinical and imaging spectrum. Orphanet J Rare Dis. 2013; 8:41.
- Szmulewicz DJ, McLean CA, MacDougall HG, Roberts L, Storey E, Halmagyi GM (2014) CANVAS an update: clini- cal presentation, investigation and management. J Vestib Res 24:465–474

- Tripathy D, Vignoli B, Ramesh N et al. Mutations in TGM6 induce the unfolded protein response in SCA35. Hum Mol Genet. 2017; 26(19):3749-3762.
- van de Warrenburg BP, Schouten MI, de Bot ST, et al. Clinical exome sequencing for cerebellar ataxia and spastic paraplegia uncovers novel gene- disease associations and unanticipated rare disorders. Eur J Hum Genet. 2016; 24: 1460-6
- van de Warrenburg BP, Sinke RJ, Verschuuren-Bemelmans CC, et al. Spinocerebellar ataxias in the Netherlands: prevalence and age at onset variance analysis. Neurology. 2002; 58:702–8.
- van Gassen KL, van der Heijden CD, de Bot ST, et al. Genotype- phenotype correlations in spastic paraplegia type 7: a study in a large Dutch cohort. Brain 2012;135:2994-3004.
- Vester A, Velez-Ruiz G, McLaughlin HM, et al. A loss-of-function variant in the human histidyl-tRNA synthetase (HARS) gene is neurotoxic in vivo. Hum Mutat. 2013;34(1):191-9. doi: 10.1002/humu.22210.
- Wan J, Yourshaw M, Mamsa H, et al. Mutations in the RNA exosome component gene EXOSC3 cause pontocerebellar hypoplasia and spinal motor neuron degeneration. Nat Genet. 2012 Apr 29;44(6):704-8. doi: 10.1038/ng.2254.
- Wang, D., Pascual, J. M., Yang, H., et al., Glut-1 deficiency syndrome: clinical, genetic, and therapeutic aspects. Ann. Neurol. 57: 111-118, 2005.
- Warner JP, Barron LH, Goudie D et al. A general method for the detection of large CAG repeat expansions by fluorescent PCR. J Med Genet. 1996; 33(12):1022-6.
- Wiethoff S, Bettencourt C, Paudel R, et al. Pure Cerebellar Ataxia with Homozygous Mutations in the PNPLA6 Gene. *Cerebellum*. 2016; 16(1):262–267.
- Wolf SM, Crock BN, Van Ness B, et al. Managing incidental findings and research results in genomic research involving biobanks and archived data sets. Genetics in medicine : official journal of the American College of Medical Genetics. 2012; 14: 361–384.
- Wu TY, Taylor JM, Kilfoyle DH, et al. (2014) Autonomic dysfunction is a major feature of cerebellar ataxia, neuropathy, vestibular areflexia 'CANVAS' syndrome. Brain 137:2649–2656
- Xue Y, Ankala A, Wilcox WR, Hegde MR (2015) Solving the molecular diagnostic testing conundrum for Mendelian disorders in the era of next-generation sequencing: single-gene, gene panel, or exome/genome sequencing. Genet Med 17(6):444–451. https://doi.org/10.1038/gim.2014.122
- Shi Y, Wang J, Li J D, et al, Identification of CHIP as a novel causative gene for autosomal recessive cerebellar ataxia, PLoS One 8 (2013) e81884.
- Yang Y, Muzny DM, Reid JG, et al. Clinical whole-exome sequencing for the diagnosis of mendelian disorders. N Engl J Med. 2013; 369: 1502-11.

- Yücel-Yılmaz D, Yücesan E, Yalnızoğlu D, et al. Clinical phenotype of hereditary spastic paraplegia due to KIF1C gene mutations across life span. Brain Dev. 2018 Jun;40(6):458-464. doi:10.1016/j.braindev.2018.02.013.
- Zanni G, Bertini ES. X-linked disorders with cerebellar dysgenesis. Orphanet J Rare Dis. 2011;6:24.
- Zuchner, S., Mersiyanova, I. V., Muglia, M., et al. Mutations in the mitochondrial GTPase mitofusin 2 cause Charcot-Marie-Tooth neuropathy type 2A**.** Nature Genet. 36: 449-451, 2004.