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Cerebellar and non-cerebellar symptoms in hereditary ataxias

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Cerebellar and non-cerebellar symptoms in hereditary ataxias

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To my parents and brother

To Helena, Clara and Joel

Esta tese foi preparada de acordo com a "legislação e regulamentos aplicáveis ao Doutoramento em Ciências Médicas: Regulamento Geral dos Terceiros Ciclos de Estudos da Universidade do Porto, aprovado por Despacho Reitoral de 10 de abril de 2015 e publicado em Diário da República, 2ª série, nº 90 de 11 de maio de 2015, por Despacho nº 4889/2015. Decreto-lei nº 63/2016 – Diário da República nº 176/2016, Série I de 13 de setembro de 2016 do Ministério da Ciência, Tecnologia e Ensino Superior. Plano de estudos do doutoramento em Ciências Médicas, do Instituto de Ciências Biomédicas Abel Salazar – Universidade do Porto."

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LIST OF ABBREVIATIONS

AD – Autosomal dominant

ADCA - Autosomal dominant cerebellar ataxias

AO – Age at onset

AOA - Ataxia with oculomotor apraxia

ACMG - American College of Medical Genetics and Genomics

AR – Autosomal recessive

ARSACS - Autosomal recessive spastic ataxia of Charlevoix Saguenay

AT - Ataxia-telangiectasia

ATX - Ataxia

AVED - Ataxia due to isolated deficiency of vitamin E

CAG - Cytosine, Adenine, Guanine

CAMRQ4 - Cerebellar ataxia and mental retardation with or without quadrupedal locomotion 4

CANVAS - Cerebellar ataxia, neuropathy and vestibular areflexia syndrome

CCA - Congenital cerebellar ataxia

CDG - Congenital disorders of glycosilation

CGG - Cytosine, Guanine, Cytosine

CGPP - Centro de Genética Preditiva e Preventiva

CNV – Copy-number variant

CNS – Central nervous system

CoQ10 - Coenzyme Q10

COVID19 - Coronavirus disease 2019

CT - Computed tomography

DNA - Deoxyribonucleic acid

DRPLA - Dentatorubral-pallidoluysian atrophy

EEG – Electroencephalography

EFACTS - European *Friedreich's* Ataxia Consortium for Translational Studies

EOCA - Early onset cerebellar ataxia

EMG – Electromyography

ESMI - European Spinocerebellar Ataxia Type 3/Machado-Joseph Disease Initiative

EUROSCA - European Integrated Project on Spinocerebellar Ataxias

MRI – Magnetic resonance imagining

FA - Friedreich ataxia

FACOMS - Friedreich Ataxia Clinical Outcome Measures Study

FAVED – Friedreich ataxia with vitamin E deficiency

FHM - Familial hemiplegic migraine

FXTAS - Fragile X tremor-ataxia syndrome

HCA - Hereditary cerebellar ataxia

HSA – Hospital de Santo António

HSP – Hereditary spastic paraplegia

IBM – International Business Machines

IBMC – Institute for Molecular and Cell Biology

ICBAS – School of Medicine and Biological Sience

INAS - Inventory of Non-Ataxia Signs

i3S – Instituto de Investigação e Inovação em Saúde

LOFA – Late onset Friedreich ataxia

m-AAA proteases - Matrix mitochondrial AAA proteases

MDS - Movement Disorders Society

MERRF - Myoclonic epilepsy with ragged red fibres

MJD - Machado-Joseph disease

mtDNA – Mithocondrial DNA

MxMD – Mixed movement disorder

NGS - Next generation sequencing

NUTS - Nomenclature des unités territoriales statistiques

OPCA - Olivopontocerebellar atrophies

PCR – Polymerase chain reaction

PEO - Progressive extrinsic ophthalmoplegia

POLG - DNA polymerase gamma

PolyQ – Polyglutamine

RNA - Ribonucleic acid

SANDO - Sensory ataxic neuropathy, dysphagia and ophthalmoplegia

SARA – Scale for Assessment and Rating of Ataxia

SNS – Serviço Nacional de Saúde

SPG - Spastic paraplegia

SPSS - Statistical Package for the Social Sciences

SCA – Spinocerebellar ataxia

SCANOP - Spinocerebellar ataxia non-polyQ

ULSSA - Unidade Local de Saúde de Santo António

UP – Universidade do Porto

UPDRS - Unified Parkinson's Disease Rating Scale

USA - United States of America

VLOFA – Very late onset Friedreich ataxia

VUS – Variants of unkown significance

WES – Whole exome sequencing

WGS – Whole-genome sequencing

XL - X-linked

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LIST OF GENES AND ENCODED PROTEINS

ANO10 - Anoctamin 10

APTX - Aprataxin

ATM - Serine/Threonine Kinase

ATN1 – Atrophin1

ATP13A2 - ATPase Cation Transporting 13A2

ATP1A3 - Alpha-3 catalytic subunit of the Na+/K(+)-ATPase

ATP8A2 - ATPase Phospholipid Transporting 8A2

ATXN1 – Ataxin 1

ATXN2 – Ataxin 2

ATXN3 – Ataxin 3

ATXN7 – Ataxin *7*

ATXN8 – Ataxin 8

CACNA1A – *Calciu*m voltage-gated channel subunit alpha1 A

CAPN1 - Calpain 1

COQ8A - Coenzyme Q8A

CPLANE1 - Ciliogenesis and Planar Polarity Effector 1

CTNNB1 - Catenin Beta 1

ELOVL4 - Elongation of very long chain fatty acids protein 4

FA2H - Fatty Acid 2-Hydroxylase

FXN – Frataxin

GBA2 - Glucosylceramidase Beta 2

GRM1 - Glutamate Metabotropic Receptor 1

HEXB - Hexosaminidase Subunit Beta

ITPR1 - Inositol 1,4,5-Trisphosphate Receptor Type 1

KCNA2 - Potassium Voltage-Gated Channel Subfamily A Member 2

KCNC3 - Potassium Voltage-Gated Channel Subfamily C Member 3

KIAA1840 - Spatacsin

KIF1A - Kinesin Family Member 1A

KIF1C - Kinesin Family Member 1C

KMT2B - Lysine Methyltransferase 2B

L2HGDH - L-2-Hydroxyglutarate Dehydrogenase

MAG - Myelin Associated Glycoprotein

MAN1B1 - Mannosidase Alpha Class 1B Member 1

MT-ATP6 - Mitochondrially Encoded ATP Synthase Membrane Subunit 6

MT-CO1 - Mitochondrially Encoded Cytochrome C Oxidase I

MT-CO2 - Mitochondrially Encoded Cytochrome C Oxidase II

MT-TN - Mitochondrially Encoded TRNA-Asn (AAU/C)

NDUFV2 - NADH:Ubiquinone Oxidoreductase Core Subunit V2

NPHP4 - Nephrocystin 4

PGN – Paraplegin

PMM2 - Phosphomannomutase 2

PNKP - Polynucleotide Kinase 3'-Phosphatase,

PNPT1 - Polyribonucleotide Nucleotidyltransferase 1

POLG - DNA Polymerase Gamma, Catalytic Subunit

PRKCG - Protein Kinase C Gamma

PRX - Periaxin

TMEM244 - Transmembrane Protein 244

RFC1 - Replication Factor C Subunit 1

RPGRIP1L - Retinitis Pigmentosa GTPase Regulator Interacting Protein 1 Like

SACS - Sacsin

SETX - Senataxin

SPAST – Spastin

SYNE1 - Spectrin Repeat Containing Nuclear Envelope Protein 1

SYNGAP1 - Synaptic Ras GTPase Activating Protein 1

ZFYVE26 - Zinc Finger FYVE-Type Containing 26

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ABSTRACT

Background Ataxia refers to a syndrome characterized by imbalance and hindered coordination of voluntary movements. It may stem from pathology of the cerebellum or its afferent/ efferent pathways (*cerebellar ataxias*), or of the proprioceptive input at the posterior columns, sensory ganglion or nerve (*sensory ataxias*). The term is used to describe a symptom/ sign, but also a group of diseases in which ataxia stands out as the most relevant feature. Ataxias may be idiopathic or have acquired (e.g., vascular, inflammatory, demyelinating, toxic) and genetic aetiologies.

Hereditary cerebellar ataxias (HCA) span from pure cerebellar to more complex presentations with involvement of pyramidal tracts, basal ganglia, spinal cord or peripheral nerve. These may be affected to a variable extent and at different stage of disease progression, shaping their overall expression. HCA may be classified as autosomal dominant (AD), autosomal recessive (AR), mitochondrial, or X-linked. The most common AD-HCA are caused by a $(CAG)_n$ expansion translated into an elongated polyglutamine (polyQ) in the respective protein, and are often referred as polyQ ataxias. Causal non-coding repeat expansions also occur in HCA. Conventional variants, as single nucleotide substitutions or small insertions/ deletions, traditionally seen as rare causes of ataxia, have been increasingly recognized since widespread use of next generation sequencing (NGS) techniques.

HCA are rare diseases, with certain world geographic clusters. Prevalence of each subtype is challenging to determine, also due to clinical and genetic heterogeneity. In Portugal, a population-based study found an overall prevalence of 8.9:100,000: 5.6 for AD-HCA, and 3.3:100,000 for AR-HCA. The most frequent AD forms were Machado-Joseph disease (MJD), followed by dentatorubral-pallidoluysian atrophy and ATX-*CACNA1A.* Among AR-HCA, Friedreich ataxia (FA) was the most common; ataxia with oculomotor apraxia (AOA) and ataxia associated with L2-hydroxyglutaric aciduria ranked second and third. Patients lacking a definite genetic diagnosis range from 30-70% across different studies, but declined with adoption of NGS diagnostic techniques in clinical practice.

Objectives The primary aims were to provide a detailed description of symptoms/ signs in HCA, with special focus on non-cerebellar symptoms, at onset and throughout disease progression; to establish correlations between genotype and phenotype; to assess clinical severity, and to delineate the occurrence of disability milestones.

Subjects and Methods A longitudinal, prospective, observational study was started in 2017. The established cohort included a) patients from the population-based study who had not received a conclusive genetic diagnosis by 2017 and had genomic DNA available; and b) patients observed at the Neurology department of Hospital de Santo António.

All patients underwent clinical assessment by the same neurologist, according to a structured protocol including baseline identification of age-of-onset (AO) of neurological symptoms/signs and motor disability milestones. Every six months, a questionnaire on new neurological symptoms and disability was administered. Baseline and follow-up examinations included evaluation of the Scale for the Assessment and Rating of Ataxia (SARA) and Inventory of Non-Ataxia Signs (INAS). Results of imaging, electroencephalogram, electromyogram, evoked potentials, autonomic studies, muscle biopsy and blood tests, based on clinical judgment, were recorded. Genetic testing followed a predefined flowchart, updated in 2019 after description of a new intronic expansion in AR-HCA. Only variants classified as *pathogenic* or *likely pathogenic* (according to the American College of Medical Genetics and Genomics) were considered for diagnostic purposes.

Results The cohort consisted of 252 patients (164 families), with 249 symptomatic and 3 presymptomatic persons; 51 families showed AD, 91 AR and 5 mitochondrial inheritance; in 17 families, inheritance mode could not be precisely determined. A final genetic diagnosis was obtained in 116 (70.7%) families, with 53 genes implicated. The most common AD-HCA were MJD (35.0%), ATX/PxMD-*CACNA1A* (10.0%) and SCA2 (7.5%). The most frequent AR-HCA were Friedreich ataxia (15.5%), ATX-*RFC1* (14.1%), autosomal recessive spastic ataxia of Charlevoix Saguenay (ARSACS) (11.3%) and ATX-*SYNE1* (8.5%).

Most patients (54.8%) exhibited cerebellar onset, notably gait unsteadiness. This was particularly so in AD-HCA (70.5%); non-cerebellar onset was more common in AR-HCA (55.8%). Motor delay and cough emerged as the prevailing non-cerebellar symptoms at onset, the latter associating to a specific genetic diagnosis (ATX-*RFC1*). Uncommon AO included early onset in AD-HCA (39.6%) and late onset in AR-HCA (39.1%) of patients.

Pure cerebellar ataxias were rare (3.6%), and associated with shorter disease duration. Pyramidal signs, movement disorders and neuropathy were the most frequent non-cerebellar symptoms over disease course. Spastic-ataxias, less common than ataxia with pyramidal signs, typically showed AR inheritance, earlier onset and longer time up to definitive genetic diagnosis. Non-ataxia movement disorders were more commonly linked

to dominant inheritance and MJD. Pyramidal signs, motor delay and intellectual disability associated with ARSACS, and neuropathy with Friedreich ataxia.

Ataxia with oculomotor apraxia could result from pathogenic variants in *MAG*. *KMT2B*, traditionally associated with generalized dystonia, might also play a role in adult-onset HCA. D*e novo* variants in *SPAST* may be linked to complex phenotypes, and *ATP8A2*-related ataxia may manifest as milder forms of congenital ataxia.

PolyQ AD-HCA typically had late-onset, cerebellar presentation, ocular and peripheral signs, while non-repeat expansion AD-HCA had early-onset, congenital forms and paroxystic symptoms. Also, polyQ AD-HCA had a faster disease progression than non-repeat expansion AD-HCA, and showed higher frequency of falls and wheelchair confinement.

Overall, the presence of (non-ataxia) movement disorders correlate with faster progression, higher frequency of falls and shorter disease duration until use of cane.

Conclusions This work provides a comprehensive characterization of over 250 patients with HCA, and established a prospective cohort with ongoing annual evaluations for continuous monitoring. We identified over 50 genetic causes in those patients, some for the first time, reinforcing the high genetic diversity of HCA. The most common forms were due to repeat expansions, still not easily detected by NGS, what emphasizes the need for diagnostic protocols that prioritize the study of expansions as a first step.

While cerebellar presentation was predominant, we identified some instances of non-cerebellar onset, particularly in AR-HCA. Our research contributes to expanding the classical perspective of AO in HCA; despite AR-HCA typically manifesting at an early age, over a third had late onset; in AD-HCA, traditionally linked to late onset, approximately 40% presented with early onset. A pure cerebellar phenotype was rare in this cohort, with additional neurological symptoms emerging as disease progressed. This reinforces the involvement of extra-cerebellar structures in HCA. The presence of additional symptoms associated with increased severity and disability.

This thesis emerges as the result of a systematic and detailed clinical observation of a large number of patients with HCA. We expect it will help constituting homogenous groups for future clinical trials, and raise awareness on the diverse manifestations of all its forms, providing valuable insight for the clinical practice of neurologists, paediatric neurologists, and (laboratory and clinical) geneticists.

RESUMO

Introdução: Ataxia corresponde a uma síndrome que se caracteriza por alterações na coordenação dos movimentos voluntários e desequilíbrio. Pode surgir na sequência de lesões cerebelosas ou das vias aferentes/ eferentes do cerebelo (*ataxias cerebelosas*) ou por atingimento da sensibilidade propriocetiva a nível dos cordões posteriores, gânglio sensitivo ou nervo (*ataxias sensitivas*). O termo ataxia é usado para descrever um sintoma/ sinal, mas também um grupo de doenças neurológicas em que a ataxia é a expressão clínica mais relevante. As ataxias podem ser idiopáticas, adquiridas (e.g. vasculares, inflamatórias, desmielinizantes, tóxicas) ou genéticas.

As ataxias cerebelosas hereditárias (ACH) variam desde formas puramente cerebelosas, até formas complexas com envolvimento piramidal, gânglios base, medula e/ ou nervos periféricos. Estas estruturas podem ser afetadas em diferentes graus e em diferentes estadios da progressão da doença, influenciando desta forma a expressão fenotípica. As ACH podem ser classificadas em autossómicas dominantes (AD), autossómicas recessivas (AR), mitocondriais e ligadas ao cromossoma X. As ACH mais comuns, de hereditariedade AD, são causadas por uma expansão $(CAG)_n$, que codifica uma glutamina incorporada na respetiva proteína; sendo também conhecidas como ataxias por poliglutaminas (polyQ). A ocorrência de expansões em regiões não-codificantes pode ser observada em formas AD e AR. Mutações convencionais, como substituições de nucleotídeos ou pequenas inserções/deleções, classicamente consideradas como causas raras de ACH, têm sido cada vez mais descritas desde a introdução das técnicas de sequenciação de nova geração (NGS) na prática clínica.

As ACH são doenças raras, embora com “nichos” em algumas regiões do mundo. Estabelecer a prevalência de cada forma é desafiante, devido à sua heterogeneidade clínica e genética. Em Portugal, dados de um estudo de base populacional revelaram uma prevalência geral de 8,9:100.000 habitantes: 5,6 para AD e 3,3:100.000 para AR. As formas AD mais frequentes foram a doença de Machado Joseph (DMJ), seguida da atrofia dentatorubro-palidoluysiana e ATX-*CACNA1A*. Nas ACH-AR, a ataxia de Friedreich (FA) foi a mais comum, seguida da ataxia com apraxia oculomotora e a ataxia associada à acidúria L2-hidroxiglutárica. A frequência de doentes sem diagnóstico genético definitivo varia entre 30 e 70%, de acordo com as séries, um valor que tem diminuído com o uso da NGS.

Objetivos: Os principais objetivos foram uma descrição detalhada dos sintomas/ sinais das ACH, com particular ênfase nos sintomas não cerebelosos presentes *ab initium* e

durante a progressão clínica; estabelecimento de correlações entre genótipo e fenótipo; avaliação da gravidade da síndrome cerebelosa e caracterização dos marcos de incapacidade.

Doentes e métodos: Em 2017 iniciou-se um estudo longitudinal, prospetivo e observacional. A coorte incluiu a) doentes do rastreio de ataxias e paraparésias, que não tinham diagnóstico genético definitivo até 2017 e que tinham DNA disponível, e b) doentes observados na consulta externa de Neurologia do Hospital de Santo António.

Todos os doentes foram avaliados pelo mesmo neurologista, através de um protocolo estruturado, incluindo a identificação detalhada da idade de início de diferentes sintomas/sinais neurológicos e marcos de incapacidade motora. Semestralmente foi administrado um questionário sobre novos sintomas neurológicos e marcos de incapacidade. A Escala de Avaliação e Classificação de Ataxia (SARA) e o Inventário de Sinais Não-Atáxicos (INAS) foram avaliados à inclusão e semestralmente. Estudos de imagem, eletroencefalograma, eletromiografia, potenciais evocados, estudos autonómicos, biópsia de músculo e estudos sanguíneos foram efetuados de acordo com a melhor pratica clínica, e os resultados registados. A investigação genética foi realizada de acordo com um fluxograma pré-estabelecido, atualizado em 2019 após a descrição de uma nova expansão intrónica nas ACH-AR. Apenas variantes patogénicas ou provavelmente patogénicas (de acordo com o Colégio Americano de Genética Médica e Genómica) foram consideradas para diagnóstico.

Resultados: Incluíram-se 252 doentes (164 famílias), 249 sintomáticos e 3 pré-sintomáticos. Das 164 famílias, 51 tinham hereditariedade AD, 91 AR, 5 mitocondriais e em 17, não se conseguiu determinar o tipo de hereditariedade. Em 116 (70,7%) famílias alcançou-se um diagnóstico genético, com 53 genes diferentes implicados nesta coorte. As ACH-AD mais frequentes foram a DMJ (35,0%), ATX/PxMD-*CACNA1A* (10,0%), e SCA2 (7,5%). Nas ACH-AR foram a FA (15,5%), ATX-*RFC1* (14,1%), ataxia espinocerebelosa autossómica recessiva de Charlevoix Saguenay (ARSACS) (11,3%) e ATX-*SYNE1* (8,5%).

Um início cerebeloso, particularmente instabilidade na marcha, foi observado na maioria (54,8%) dos doentes. Na análise por tipo de hereditariedade, observou-se a mesma tendência nas ACH-AD (70,5% com início cerebeloso), mas não nas ACH-AR, onde o início não cerebeloso foi mais frequente (55,8%). As apresentações não cerebelosas mais comuns foram atraso motor e tosse, mas apenas esta última se associou a um diagnóstico genético específico (ATX-*RFC1*). Idades de início menos frequentes, com

início precoce nas ACH-AD e início tardio nas ACH-AR, foram observadas em 39.6% e 39.1% dos casos, respetivamente.

As ataxias cerebelosas puras foram raras (3,6%) e associadas a menor duração da doença. Sinais piramidais, doenças do movimento e neuropatia foram os sintomas não cerebelosos mais frequentemente identificados ao longo da progressão da doença. As ataxias espásticas, menos comuns do que as ataxias com sinais piramidais, apresentaram tipicamente hereditariedade AR, início precoce e intervalo de tempo mais longo até o diagnóstico genético definitivo. As doenças do movimento associaram-se a hereditariedade dominante e a DMJ. Sinais piramidais, atraso motor e incapacidade intelectual associaram-se à ARSACS, enquanto a neuropatia se associou à FA.

As AOA podem ser causadas por variantes patogénicas no *MAG*. *KMT2B*, um gene classicamente associado a distonia generalizada, pode estar implicado em ataxia de início no adulto. Variantes de novo no *SPAST* podem estar associadas a fenótipos complexos e a ataxia relacionada com o *ATP8A2* pode corresponder a formas menos severas de ataxia congénita.

As ACH-AD polyQ apresentaram tipicamente início na idade adulta, sintomas cerebelosos *ab initium*, sinais oculares e periféricos, enquanto nas ACH-AD sem expansão de tripletos observou-se início precoce, incluindo com formas congénitas, e sintomas paroxísticos. Adicionalmente nas ACH-AD polyQ ocorreu uma progressão mais rápida dos sintomas cerebelosos, com maior frequência de quedas e confinamento a cadeira de rodas.

Em geral a presença de doenças do movimento associou-se a mais rápida progressão dos sintomas cerebelosos, maior frequência de quedas e uso mais precoce de bengala.

Conclusões: Esta tese caracteriza de forma sistemática mais de 250 doentes com ACH, estabelecendo uma coorte prospetiva com avaliações anuais, que continuará a ser seguida. Mais de 50 causas genéticas foram identificadas nas ACH, algumas pela primeira vez associadas a este fenótipo, reforçando a variabilidade genética destas doenças. As mais frequentes foram as expansões de tripletos, ainda não detetadas pelas técnicas de NGS, reforçando a necessidade de protocolos de diagnóstico em que o estudo de expansões surja como primeira linha.

Apesar das apresentações cerebelosas terem sido as mais frequentes, estabelecemos que um início não cerebeloso ocorre num número significativo de casos, particularmente nas ACH-AR. Contribuímos para ampliar a perspetiva clássica das idades de início nas ACH; embora as ACH-AR sejam associadas a início precoce, aproximadamente 40%

tiveram início na idade adulta; nas ACH-AD, classicamente com início tardio, o início precoce ocorreu em mais de um terço dos casos. Fenótipos cerebelosos puros foram raros nesta coorte, com sintomas neurológicos adicionais a surgirem com a progressão da doença. Esta observação reforça o envolvimento de estruturas extra-cerebelosas neste grupo heterogéneo de doenças neurológicas. A presença de sintomas não cerebelosos associou-se a maior gravidade e incapacidade.

Esta tese surgiu como resultado da observação clínica sistemática e detalhada de doentes com ACH. Esperamos que possa contribuir para aumentar o conhecimento sobre as diferentes manifestações deste grupo de doenças, e que seja útil na atividade clínica de neurologistas, neuropediatras e geneticistas (clínicos e laboratoriais).

I. GENERAL INTRODUCTION

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A. CONCEPT

Ataxia, derived from the Greek ατάξις, literally means “without order”, referring to movements that retain the normal motor planning, but are performed in a clumsy way.[1] In Neurology, it denotes a syndrome characterized by imbalance and impaired coordination of voluntary movements.[2] It may be related to pathology of the cerebellum or its afferent/ efferent pathways (cerebellar ataxias), or of the proprioceptive input at the posterior columns, sensory ganglion or nerve (sensory ataxias).[2,3]

The term ataxia is simultaneously used to designate a symptom/ sign (e.g., limb ataxia, gait ataxia) or a group of neurological diseases in which ataxia is the most relevant clinical expression.[4,5] Ataxic movements involve the correct body part, gross motor plan and sequence, but suffer a disruption of execution related to the speed of the maneuver.[1] When involving single joint, it may manifest as dysmetria (particularly hypermetria), while in multi-joint movements loss of synchrony occurs, with unintended direction changes and trajectory curves (dyssynergy).[1] Ataxia as a group of neurological diseases comprises very heterogeneous entities, classically classified into three major groups: 1) hereditary; 2) nonhereditary degenerative; and 3) acquired, due to exogenous or endogenous aetiologies.[6]

Autosomal dominant (AD), autosomal recessive (AR), mitochondrial and X-linked (XL) inheritance may be observed in hereditary cerebellar ataxias (HCA). Pseudodominance is also described, potentially misleading a genetic investigation, mimicking AD transmission. This typically occurs in couples where one partner is a homozygote or compound heterozygote, and the other carries at least one pathogenic variant in the same gene (with at least one partner being affected) for an autosomal recessive (AR) disease.[7] This phenomenon may arise under conditions of a) declared or "hidden" consanguinity, especially in endogamous populations where successive consanguineous mattings result in offspring being homozygous; or b) be due to the high frequency of carriers in the population, leading to offspring being either homozygotes or compound heterozygotes. Pseudodominance has been observed in a few neurological disorders such as Friedreich's ataxia (FA),[8] Wilson's disease,[9] and ATX-*RFC1* (unpublished data from our group). Isolated (or non-familial) cases can pose an additional challenge as, after excluding acquired (or sporadic, non-genetic) aetiologies, they may correspond to AR

(single patient in one family) or AD disorders (non-biological parents, incomplete penetrance, age-dependent penetrance with early parental death, or *de novo* pathogenic variants).

Hereditary cerebellar ataxias (HCA) include a wide spectrum of disorders, from pure cerebellar to complex forms with additional pyramidal, basal ganglia, spinal cord or peripheral nerve involvement. These structures may be affected to a variable extent and at different stages of disease progression.

B. HISTORICAL BACKGROUND

The first description of a hereditary ataxia was made by Nikolaus Friedreich (Figure 1). Across five articles, from 1863 to 1877, Friedreich characterized the clinical and pathological details of nine siblings from three families, affected by a degenerative atrophy of the posterior columns of the spinal cord.[10-14] This manifested as a progressive ataxia, with sensory loss and weakness, frequently associated with scoliosis, foot deformity and heart disease.[10,11] In 1891, Menzel described the first family with a dominant ataxia, highlighting the pathological differences (atrophy of the cerebellum, pons and olivary complex) with FA, but not mentioning their different mode of inheritance.[15] It was Pierre Marie, in *Sur l´hérédo-ataxie cérébelleuse,* who systematized the clinical and pathological characteristics of families with AD inheritance, adult onset and hyperreflexia, distinguishing them clearly from FA.[16] For a large part of the XXth century, dominant ataxias were designated as **Marie's cerebellar ataxia**. In 1983, Anita Harding developed a clinical classification of hereditary ataxias, based on age at onset (AO), mode of transmission, clinical presentation, and aetiology.[17] This classification was used in clinical practice for several years.

The term **spinocerebellar ataxia** (SCA), initially used by neuropathologists to define cerebellar ataxia with involvement of the spinal cord, was accepted in the late XXth century to designate dominant forms of cerebellar ataxia.[5,18,19] The **molecular investigation** of HCA was initiated in 1977, with mapping of one form of AD-HCA (now recognized as SCA1) to chromosome 6, through linkage to HLA.[20] In the 90s, the first genetic causes were found for SCA1 and for dentatorubral-pallidoluysian atrophy (DRPLA), both proven to be due to a $(CAG)_n$ expansion translating into a polyglutamine (polyQ) tract.[21,22]

Further advances in genetic methodologies facilitated the identification of a substantial genetic diversity within the HCA.

Machado-Joseph disease

In 1972, Nakano, Dawson and Spence described the Machado family.[23] Guilherme (William) Machado, born in Bretanha, São Miguel, emigrated to Massachusetts, United States of America (USA), in the late XIXth century. Most of his descendants were still living in that area:[23,24] 51 affected members were identified, 15 of whom observed and described with a "late-onset cerebellar ataxia with peripheral neuropathy";[23] an exception was a 30-year-old female with pyramidal signs.[23,25] The authors suggested this was a new form, not corresponding to previously reported ataxias, and named it *Machado disease*. Later in the same year, Woods and Schaumburg reported another Azorean family in the USA, with a "nigro-spinodentatal degeneration with nuclear ophtalmoplegia".[26] These patients were descendants of José Tomás (Thomas), born in Flores, and had an ataxia with pyramidal signs and external ophthalmoplegia, starting between the age of 17 and 46 years.[24,26] As in the Machado family, there was also an atypical patient in the Thomas family, with peroneal atrophy, who the authors believed could have a different genetic disorder.[25,26] In 1976, Rosenberg, Nyan and Bay described a third American-Azorean family, descending from Antone Joseph (born as António Jacinto Bastiana), from Flores. Patients had an early onset (around 25 years) dystonic-rigid syndrome, associated with pyramidal signs.[27] Even though all patients presented cerebellar ataxia, the authors gave little emphasis to it, recognizing ataxia as a major sign only several years later.[25,28] Clinical similarities were noted with nigro-spinodentatal degeneration and, in a patient aged 70 (with 28 years of progression), also with Machado disease; however, the authors considered it a distinct entity and later named it *Joseph disease*.[24,27,29]

In January 1976, Coutinho and Andrade travelled to the Azores to study the intriguing neurological disorders that had been described in Azoreans in the USA, after rumours that several families could also be affected in the islands.[24,25] After extensive field work, they reported 15 Azorean kindreds (40 patients) with an "AD system degeneration", involving cerebellar, pyramidal, extrapyramidal and spinal-cord motor functions.[30] Initially reported in *O Médico*[31] and, later, in *Neurology*,[30] the authors suggested that the previously described Azorean families from the USA were affected by the same genetic disease, characterized by a variable phenotypic expression, partially dependent on AO. Interestingly, Romanul *et al.* (1977) made the same suggestion, after observing a large Azorean kindred, also from Massachusetts.[32] Several non-Azorean families with a similar

disorder were later reported, in mainland Portugal (1979), African-Americans in the USA (1980), Japanese (1986), Australian aborigines (1993), Chinese (1994), and Yemenite Jews from Israel (1994).[33-39] In the "International Symposium on Autosomal Dominant Motor System Disorders in Persons of Portuguese Ancestry", held in Lisbon in 1980, a single entity and the designation of Machado-Joseph disease (MJD) were proposed by Coutinho and Sequeiros.[24] Nevertheless, controversy persisted for several years on the existence of one or more distinct entities.

In 1993, Takiyama *et al.* assigned the MJD gene (initially named *MJD1,* now *ATXN3*) to the long arm of chromosome 14[40], which was then confirmed in the Portuguese families;[41] one year later, another Japanese team reported a CAG repeat expansion as its underlying genetic defect.[42] In 1994, a French team, described a group of families with a "new" form of spinocerebellar ataxia, and named it SCA3;[43] this was later recognized to be MJD.[44,45] Still, the designation "SCA3" has gained supporters of the numerical classification of SCA, and continues to be used by some.

Earlier descriptions of MJD include the Drew family of Walworth (1929),[46] a Japanese family[47] and a black family from North Carolina,[48] both in 1971. Considered for years as having distinct disorders (e.g. Marie's ataxia, dominant spinopontine atrophy), the possibility of having the same disorder was later raised or confirmed.[49-52]

Other SCA

The history of **SCA1** started in 1950, with the report of six generations of a North-American family with a dominantly inherited disorder of the cerebellum and brainstem.[53] Schut recognized the phenotypic variability within this family, where cerebellar ataxia was the main trait, but additional signs justified its subclassification into type 1 - hereditary spinal ataxia (with absent deep tendon reflexes and skeletal abnormalities); type 2 - hereditary cerebellar ataxia (subdivided in type 2a with hypoactive reflexes and severe proprioception impairment, type 2b with pyramidal signs); and type 3 - hereditary spastic paraplegia (with prominent pyramidal signs, mainly restricted to the lower limbs).[53] A decrease in AO with each succeeding generation was also observed by Schut, but it was not clearly associated with the different subtypes.[53] After decades of investigation, Orr *et al.* (1993) identified the disease causing mutation: as anticipation of AO was prominent in SCA1, as was in myotonic dystrophy and Huntington's disease, they devised a method to screen for candidate trinucleotide repeats (previously identified as the genetic mechanism in these diseases).[21]

The first description of **DRPLA** dates from 1946, by Titica and van Boegaert, in a Belgian family with two patients affected by progressive choreoathetosis, ataxia and dementia.[54] The anatomopathological study of one patient revealed atrophy of both the pallidoluysian and dentatorubral systems.[54] While the initial reports were in Caucasian and African-Americans, the largest number of families were identified in Japan.[55] Naito and Oyanagi described the striking clinical heterogeneity, with juvenile-onset being characterized by ataxia, myoclonic epilepsy and intellectual deterioration; and adult-onset by ataxia, choreoathetosis and dementia.[56] The DRPLA gene (*ATN1*) was identified in 1994, and again a $(CAG)_n$ expansion in the coding region of the gene was the causative mutation.[22,57] Somehow it escaped the SCA number classification.

In 1969, Boller and Segarra reported the first family with (what would later be called) **SCA2**, the "W" family, Anglo-Saxons living in Rhode Island, extending through 5 generations, indicative of AD inheritance.[58] In a clinical and postmortem study, the authors highlighted the spinopontine degeneration in this family.[58] The typical phenotype with early and marked slowing of saccadic eye movements, was recognized by Wadia and Swami, in 1971, in nine families from India.[59] Research in SCA2 was later facilitated by the study of 100 patients descending from the same ancestor, living in Holguín, Cuba.[60] Gispert *et al.* (1993) assigned the locus to the long arm of chromosome 12, in this large kindred.[61] Later, Pulst *et al.* (1996) identified the *ATXN2* gene and isolated a genomic clone containing an expanded $(CAG)_n$.[62] Thus, this became the third to be recognized as a polyQ SCA.

Friedreich ataxia

Friedreich's reports were received with criticism, with several neurologists suggesting these patients had *tabes dorsalis*.[63] It was only in 1876 that the term "hereditary" was included in the articles' title.[13,14] This phenotype was later accepted as a new entity.[63] Friedreich received little recognition during his life time for his insightful descriptions. In the year of his death, Brousse suggested it should be named **Friedreich ataxia** (FA or FRDA),[64] with the French school of neurologists being responsible for the recognition of his work. Several patients with a similar condition were subsequently reported,[65-67] sparking debates on the inclusion of atypical forms with later onset or with retained reflexes under the label of FA.[67] Interestingly, early-onset ataxia with retained reflexes was initially considered a distinct disorder,[68] being only established as FA after mapping of the *locus*.[69]

In the 1970s, one of the mandatory diagnostic criteria of FA was onset before age 20 years.[66] Several diagnostic criteria for FA were created across the years, with Harding's being the last *prior* to genetics era: typical FA was considered when occurring before the age of 25, and atypical FA when presenting thereafter.[67,70,71] Overall, later-onset forms tended to present milder symptoms, slower progression and paucity of extra-neurological involvement.[71]

The locus was mapped to chromosome 9, in 1988;[72] eight years later, the gene (*FXN*) was identified, and the genetic mechanism established as a biallelic $(GAA)_n$ expansion in the first intron.[73] Later, Delatycki *et al.* (1999) stated that only 98% of FA cases were due to the expansion of the $(GAA)_n$ in intron 1 of *FXN*; 2% may be due to an expansion and a point mutation (they indicated 17 different variants described by then).[74]

Though FA is the most frequent AR-HCA in most populations, many other forms were later identified.

Other recessive ataxias

The first description of **ataxia-telangiectasia** (AT) was in 1926, by Syllaba and Henner, in three adolescent Czech siblings with progressive choreoathetosis and ocular telangiectasia.[75] Additional families were reported in subsequent decades, with susceptibility to sinopulmonary infections being described in 1958,[76] and predisposition to cancer in 1963. It was Boder and Sederick who introduced the term ataxia-telangiectasia, to differentiate it from other early onset ataxias.[76] The AT locus was mapped to chromosome 11q22-23, in 1988,[77] and the gene identified seven years later.[78]

Ataxia with oculomotor apraxia (AOA) is the second most frequent recessive ataxia in Portugal.[79] The term oculomotor apraxia was introduced by Cogan, referring to the incapacity to generate voluntary horizontal saccades, giving rise to a typical head-thrusting and synkinetic blinking, in children with congenital oculomotor apraxia.[80] This sign was described in other diseases, as Joubert syndrome, and a group of ataxias that resembled AT ("AT-like") but lacked ocular telangiectasias, recurrent infections or malignancies.[81] This group of AOA was distinguished and characterized in detail by *Barbot et al.* (2001, 2008), in 11 Portuguese families exhibiting this phenotype.[82,83] Three of the four genes now known to be associated with AOA were first described in these and other Portuguese patients identified during the national survey on ataxia and spastic paraplegia:[79] *APTX* (in AOA1), *SETX* (in AOA2) and *PNKP* (in AOA4).[84-86]

Autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS), one of the most frequent forms of spastic ataxia, was reported in 1978, by *Bouchard et al.*, as a distinctive early-onset form, affecting 42 patients from 24 sibships.[87] It was initially described in genetic isolates in Charlevoix and Saguenay, Québec, where more than 300 patients have been observed.[88] Later, additional kindreds were identified in other parts of the globe.[89] The responsible gene (*SACS*) was mapped to chromosome 13q11-12, and cloned two years later.[88,90]

Figure1: **Historical timeline of cerebellar ataxias**



C. EPIDEMIOLOGY

POPULATION GENETICS

The relative frequency of the various HCA shows marked geographical and ethnic variability, namely due to a number of population genetics' principles.[89] One is the *founder effect* that may occur when a small subpopulation breaks off from a larger population.[91] When one (or more) of a small number of founders carries a pathogenic variant, this may achieve a much higher frequency than in the population of origin.[91] Another factor may be the fortuitous increase (*genetic drift*) of a rare gene in small populations or genetic isolates. In ataxias, several examples are well known, as MJD in the Azores, mainland Portugal, Brazil, France and Japan; SCA2 in the province of Holguín, Cuba; SCA6 in northern Netherlands; DRPLA in Japan and in centre and south Portugal; or ARSACS in French-Canadians, among others. [89]

HAPLOTYPE ANALYSIS AND ANCESTRAL ORIGINS

Haplotype analysis in MJD suggested the presence of two ancestral mutational events, with an heterogenous distribution of the respective lineages.[89,92,93] The Joseph haplotype, which may have had origin in Asia (more than 6,000 to 16,000 years ago), was present in 72% of all MJD families studied, living or originating in the island of Flores, north-eastern mainland Portugal, Japan, west coast of the USA, France, Germany, and many other countries.[94,95] The Machado haplotype is almost exclusive of families originating from São Miguel and central Portugal (mainly the Tagus valley), identified elsewhere in a very small number of families (Spain, Brazil, British West Indies and Sudan).[89] The Machado haplotype is approximately 3,200 years old, and probably has a Portuguese origin (though the low number of families known in other countries makes this difficult to be asserted).[96]

A single ancestry in Europe has been suggested for SCA2.[97] Also, Portuguese and Japanese families with DRPLA were shown to share the same haplotype.[89,98] For FA, the first mutational event has presumably occurred in Africa, around 25,000 years ago, after a $(GAA)_9$ duplication that originated alleles with 16-18 GAA units.[89,99] Then, single-step mutations seem to have occurred, creating alleles with 12-25 GAAs, which spread to Europe or Middle East; later, a second duplication gave origin to alleles over 30 GAA repeats.[89,99]

OVERALL FREQUENCY OF HEREDITARY CEREBELLAR ATAXIAS

On a global scale, HCA are rare diseases with clusters of some forms in certain regions of the world.[89] Establishing prevalence of each form is challenging, chiefly because of its clinical and genetic heterogeneity. Also, the scarcity of epidemiological studies, as population-based surveys, and the variability of genes tested at different centres curtail knowledge on true prevalence of these disorders.[89] Most often, we can only refer to estimated population frequency or to relative frequency among forms.

Most studies report on frequency of HCA subtypes in tertiary ataxia centres, where only polyQ ataxias were being tested for many years.[5] PolyQ ataxias were the first to be genetically determined,[21,22,42,62] as they share the expansion of a $(CAG)_n$ in the coding region of the respective genes. They are diagnosed by tests more widely available, as PCR, fragment analysis and/ or multiplex ligation-dependent probe amplification (MLPA). Detection of HCA caused by intronic expansions or conventional mutations is more recent, as they require techniques that were not so readily available.[5] This may have led to underestimating prevalence of those forms, particularly at some centres.

A meta-analysis on the global epidemiology of HCA revealed a prevalence varying from 0.0 to 5.5:100,000 for AD-HCA; 0.0 to 7.2:100,000 for AR-HCA; and 1.1 to 8.4:100,000 isolated (non-familial) cases of suspected HCA.[100]

GEOGRAPHIC DISTRIBUTION OF HCA

AD-HCA

MJD is the most frequent AD-HCA worldwide. Its relative frequency is highest in Portugal, Brazil and China, where MJD corresponds to about half of all AD-HCA patients.[89] In Japan, France, Taiwan, USA and Netherlands, its relative frequency is lower (21-43%), though still the most common AD-HCA in those countries.[89] **SCA2** is, overall, the second most frequent around the world, being the most common AD-HCA in Cuba (87%), India (57%), United Kingdom (47%), southern Italy (47%), Mexico (43%), and Cantabria, Spain (30%).[89] **SCA6** has a particularly high frequency in Japan (10-33% of all SCA families), but also in Germany (22%), and Australia (17%).[89,100] **SCA1** is identified in many populations worldwide, particularly in Poland (relative frequency of 68%), South Africa (41%), Serbia (34%), Russia (33%) south India (33%) and northern Italy (25%).[5,89] **SCA7, SCA17** and **DRPLA** are the least frequent polyQ ataxias worldwide (0-3%).[5] Nevertheless, DRPLA is particularly prevalent in Japan (Pacific coast), Singapore, Korea

and central Portugal.[89] AD-HCA caused by untranslated repeat expansions, seem to account for a small percentage of families: **SCA12** is frequent in India (7-16%), **SCA36** in Japan (9%) and Spain (6%), and **SCA37** in Alentejo (14%) and southern Spain (Huelva and other provinces in Andalusia).[5,101,102] The recently described SCA27B (also ATX-*FGF14*) has a relative frequency varying from 10-61% in late-onset ataxia cohorts, according to ethnic background, with a high frequency in French-Canadians.[103] Relative frequency of SCA14 in Europe, USA, Japan and Australia has been estimated at 1-4% of AD-HCA, while SCA28 is present in 1-3% in European SCA cohorts.[5] In a recent multicentric study, ataxia due to conventional mutations in *CACNA1A* (ATX-*CACNA1A*),[104] was the most frequent among non-polyQ ataxias.[105] Other forms of SCA appear to be even rarer. Nonetheless, epidemiological data on newer forms are still scarce.

AR- HCA

Friedreich ataxia is the most frequent AR-HCA worldwide,[100] predominating amongst Europeans, but also the Middle East, southern Asia and northern Africa, with an estimated prevalence of 2-4:100,000.[89] FA has not been described in Amerindians or Orientals, and seems to be rare in sub-Saharan Africans.[89] Interestingly, it appears to exist a prevalence gradient of FA in Europe, with highest levels being observed in northern Spain, south of France and Ireland, and lowest in Scandinavia and Russia.[106] **Ataxia telangiectasia**, with an estimated prevalence of 1-2.5/100,000[107] has long been suggested to be the second most frequent AR-HCA; nevertheless, studies testing simultaneously AT and AOA, showed that **AOA** was more common in two (Portugal and Alsace), and AT only in one (Norway).[100] AOA is the most common AR-HCA in Japan, where FA appears to be absent.[108] **Ataxia due to isolated deficiency of vitamin E** (AVED) is more frequent in the Mediterranean region, particularly due to patients coming from North Africa.[89] **ARSACS** had an incidence of 1:1,932 live births through 1941-1985 in the Charlevoix County and Saguenay-Lac St. Jean, where it was first described;[89] other families have been reported outside Québec, particularly in Mediterranean countries, and its distribution around the globe is much wider than initially thought.[89] The relative frequency of cerebellar ataxia, neuropathy and vestibular areflexia syndrome (**CANVAS** or ATX-*RFC1*) in European hospital-based cohorts has been reported to be up to 92% in patients with the complete CANVAS phenotype,[109] 12-63% with ataxia and neuropathy,[109,110] and 0-6% with pure cerebellar ataxia.[111,112] Still, the frequency of the *RFC1* expansion is estimated to range 1-5% in the healthy population, meaning a frequency of biallelic expansions from

1:10,000 to 1:400, which is a much higher estimate than the number of patients reported thus far.[109]

Considering hospital-based studies on the relative frequency of AR-HCA, a multicentric cohort of 623 patients established mainly in central Europe, but also including Canada, Asia and Africa, identified ARSACS as the most frequent (24.2%), followed by FA (16.2%), HSP/ATX-*PGN* (6.6%), AOA2 (4.0%), ATX-*SYNE1* (3.8%), AVED (3.4%), ATX-*COQ8A* (3.0%), AT (2.9%) and MxMD-*POLG* (2.4%).[113] The higher frequency of ARSACS than FA was unexpected, but could possibly be due to the high inclusion of Canadian patients, together with the underrepresentation or exclusion of European countries with a higher frequency of FA (as Spain, France or the UK). Interestingly, a similar study (580 patients) conducted in South America identified FA as the most frequent AR-HCA (57%), followed by ATX-*RFC1* (8%), AT (7%), ARSACS (4%), Niemann Pick type C (4%), AOA2 (3%), AOA2 (2%), ATX-*SYNE1* (2%).[114]

X-LINKED HCA

X-linked ataxias (XL-HCA) are relatively rare. **Fragile X tremor-ataxia syndrome** (FXTAS), with an estimated frequency in men over 50 years of age of 1:30,000, is one of the most common HCA in the aging population.[89] Other XL-HCA are extremely rare.

MITOCHONDRIAL HCA

Epidemiological data of mitochondrial disorders, particularly ataxia, has been difficult to establish as these are highly heterogeneous, both from a clinical and genetic point of view. A study in an adult population with suspected mitochondrial disease, from the north-East of England, identified a minimum prevalence of mtDNA mutations of 6.57:100,000.[115] A Finish population-based study identified a prevalence of 16.3:100,000, specifically for the A3243G variant.[116] In an Italian cohort of childhood-onset mitochondrial diseases, ataxia was the most frequent presentation (49%).[117] Still, missing data on the distribution of mitochondrial ataxias preclude further conclusions on their frequency.

THE PORTUGUESE POPULATION-BASED SURVEY

From January 1994 to April 2004, a systematic population-based study was conducted with the aim of determining the prevalence and geographic distribution of hereditary

ataxias and spastic paraparesis in Portugal, and continued thereafter for a few more years.[79] The study was led by Professor Paula Coutinho and involved a comprehensive team of neurologists, geneticists, epidemiologists and public health specialists. Identification of patients was carried out through primary care centres in mainland Portugal and Madeira, in a close collaboration with family doctors and other neurologists.[79]

Overall prevalence of HCA was estimated at 8.9:100,000 inhabitants: 5.6 for dominant, and 3.3:100,000 for recessive ataxias.[79] A definite genetic diagnosis was obtained by then in 63.4% AD-HCA families and 62.0% AR-HCA families.[79] As expected, MJD was the most prevalent HCA, with an estimated value of 3.12:100,000.[79] Somewhat surprisingly, DRPLA was the second most common AD-HCA, with a prevalence of 0.33:100,000 inhabitants.[79] ATX-*CACNA1A* was then the third, followed by SCA2, SCA7, and SCA8; only one patient was identified with SCA6, SCA12, or SCA17.[79]

FA was the most common AR-HCA, with a prevalence of 1:100,000.[79] The second was AOA, with an overall prevalence of 0.40:100,000, slightly higher for AOA1 than for AOA2 or 4 (0.13 *vs.* 0.11:100,000).[79,84] Ataxia associated with L2-hydroxyglutaric aciduria showed a prevalence of 0.19:100,000, higher than usually described in HCA series.[79] AT had a prevalence of 0.15:100,000, similar to Joubert syndrome.[79]

D. PATHOGENESIS

Heterogeneity in HCA occurs at the clinical, genetic and pathological level. The precise molecular mechanisms are not yet completely understood, but the growing number of genes involved is unravelling common pathways for various HCA.

ABNORMAL PROTEIN CONFORMATION

Globally, the most common AD-HCA (SCA1, DRPLA, SCA2, MJD, SCA6), share an unstable $(CAG)_n$ expansion within the coding region of the respective genes as their mutational mechanism.[118] The CAG repeat is translated into a long polyQ tract incorporated in the respective proteins.[5] For that reason, these are also known as polyQ ataxias. They are an example of neurodegenerative diseases caused by altered protein configuration. Common to all polyQ disorders is the accumulation and aggregation of the

long proteins, forming intraneuronal deposits, particularly in the nucleus.[5] The most accepted theory relies on these aggregates being directly toxic, leading to premature cell death.[5,119] The correlation between pathogenic repeat length and aggregates' formation is highly suggestive that the abnormal protein structure and aggregation are their underlying pathogenic mechanism.[5] Still, some lines of research suggest that aggregates may be just a protective strategy of the cell to discard the abnormal proteins,[5,119] the pathogenic mechanism residing elsewhere.

Independently of the role aggregates might have, it is accepted that the continued production of a polyglutamine protein, unable to assume its functional structure, imposes a metabolic stress on neurons.[5,119] The events triggered by the aggregates will probably vary for each disease, depending on brain expression, function and subcellular location of the respective proteins.[5,119]

PROTEOTOXICITY

Toxicity induced by abnormal proteins occurs also in non-polyQ ataxias. In SCA35, the mutant protein induces deleterious translational frameshifts.[5] In SCA14, the pathogenic genetic variant promotes protein misfolding and aggregation;[120] while in SCA5, the abnormal protein interferes with the cytoskeletal dynamics, decreasing the stability of glutamate receptors in Purkinje neurons.[121] In ARSACS, loss of the normal chaperone activity of sacsin appears to induce protein misfolding.[122, 118]

RNA TOXICITY

HCA caused by moderate-to-large penta or hexanucleotide-repeat expansions in non-coding regions (e.g., SCA31, SCA36, SCA37, FXTAS) often exhibit a dominant phenotype, typical of a toxic RNA gain of function.[5,123] The expanded mRNA adopts an atypical secondary configuration, capturing key RNA-binding proteins to form insoluble nuclear foci.[123] As a result, splicing or other RNA-dependent processes are impaired, causing various cell defects.[123] As RNA toxicity interferes with protein homeostasis, distinction of protein or RNA toxicity-based HCA may be artificial, not capturing the full complexity of these processes.

TRANSCRIPTIONAL DEFICIENCY

In FA and SCA27B, both caused by an intronic $(GAA)_n$ expansion, a transcriptional deficiency of the encoded protein is observed.[73,103] The $(GAA)_{exp}$ results in a partial silencing of the gene, and consequently low levels of the protein.[63] In FA, healthy carriers have a 50% reduction in frataxin levels, while patients have up to 95% reduction,[63,124] whereas in SCA27B the decrease is of 22%.[103]

IMPAIRED DNA REPAIR

DNA-repair pathways are classically compromised in AR-HCA, such as AT and AOA. A defect in enzymatic mechanisms promoting genome protection and repair leads to chromosomal instability, neurodegeneration, premature aging, and increased risk for neoplasms.[125] Double-stranded DNA breaks are induced by ionizing radiation, chemical agents, or occur during replication of single-stranded DNA.[126] The CNS, particularly the cerebellum, is especially vulnerable to DNA damage, although the underlying reasons remain unknown.[125] Thus, those patients are hypersensitive to ionizing radiation, and show a higher risk for cancer. Single-stranded DNA breaks, the most frequent, may arise spontaneously during normal metabolism, from the action of reactive oxygen species.[126] Interestingly, several genes involved in DNA-repair and damage response appear to modulate AO in some polyQ HCA[127], again suggesting a high complexity and the interaction of pathogenic mechanisms among the HCA.

IMPAIRED BIOENERGETICS

Impaired mitochondrial homeostasis, caused either by disease-associated variants in mitochondrial DNA (mtDNA) or deficient mtDNA replication and repair, are also important pathogenic mechanisms in HCA.[128] Neurons and, particularly, Purkinje cells, are amongst the higher energy-demanding cells in the human body, thus being highly susceptible to variation in the production of mitochondria's energy.[129]

Disruption of metabolic pathways, other than mitochondrial ones**,** is a pathogenic mechanism more frequent in AR than in AD-HCA. Impairment in any metabolic pathway may lead to ataxia, usually in combination with other neurological or non-neurological signs.[130] Defective phospholipid (e.g., ATX/HSP-*PNPLA6*), sphingolipid (e.g., HSP/ATX-*FA2H,* HSP/ATX-*GBA2*), or lysosomal pathways (e.g., Nieman-Pick type C, MxMD-

ATP13A2), usually evolve with progressive cerebellar ataxia. Glycosylation impairment (as in congenital disorders of glycosylation) affects intra-uterine development of the cerebellum, leading to congenital ataxia.[130] In amino-acid disorders (such as propionic acidemia and methylmalonic aciduria), it is frequent for patients to have acute presentations of ataxia in the context of systemic disorders, fever or increased protein intake.[130]

CHANNEL DYSFUNCTION

Conventional ion-channel dysfunction is a classical cause of both episodic ataxia and HCA, as is the case with ATX-*CACNA1A*, ATX-*KCNC3*, ATX-*ITPR1*, ATX-*CACNA1G*, or ATX-*GRM1*.[131] Evidence suggests the respective ataxia-related genes are grouped around pathways concerned with calcium homeostasis, synaptic integration and membrane excitability.[131] The intrinsic fire rate of Purkinje cells, as well as the neuronal inputs they receive, are controlled by voltage-dependent and ligand-gated ion channels. Defective channel function would induce neuronal dysfunction and, possibly, degeneration. Ataxias due to pathogenic variants in ion channels or related pathways generally have very slow progression, and are either “pure” or with transient (paroxystic) neurological symptoms, cognitive impairment, or myoclonus.[5]

CILIAL DYSFUNCTION

Cilial dysfunction is typical of Joubert syndrome and related diseases. The primary cilia act as detectors of extracellular signals, transmitting them intracellularly to control tissue maintenance, polarity, and proliferation.[132] Most neurons have a primary cilium, with a major role in brain development and function.[132] Also, the important function of cilia in the kidneys, bile duct or retinal photoreceptors, explain the multiorgan involvement observed in these disorders, including male infertility.[132]

UNDETERMINED

In several ataxias, putative pathogenic mechanisms remain unknown, as in biallelic *RFC1* expansions. Altered RNA-splicing has been suggested, as a slight increase in the retention of intron 2 in pre-mRNA was reported in studies with very small samples,[109] though it lacked confirmation. Interestingly, in a recent report, compound heterozygotes

carrying an expansion and a truncating variant showed diminished levels of RFC1, supporting a loss-of-function mechanism in those cases.[133]

E. CLINICAL AND GENETIC CLASSIFICATIONS OF HEREDITARY ATAXIAS

Classification of HCA has been under debate since the first forms were recognized. Various clinical classifications have been proposed over time, though none of them is perfect. One of the difficulties is related to the high number of neurological and metabolic disorders presenting with ataxia, as well as the clinical and anatomopathological heterogeneity of forms now genetically defined. Actually, ataxia is the main clinical feature of several disorders that primarily affect the cerebellum, but is also present in hereditary spastic paraplegias, inborn errors of metabolism and encephalopathies.[134] In a field where the relative frequency of repeat expansion disorders still limits the use of NGS as the first diagnostic tool, a clear and concise classification is fundamental.

In 1954, Greenfield proposed the first classification for HCA, based on **anatomopathological findings**.[3] *Type 1*, "predominantly spinal", encompassed conditions like Friedreich's ataxia, abetalipoproteinemia, and hereditary spastic paraplegia. *Type 2*, "predominantly cerebellar", included ataxia telangiectasia, late-onset cerebellar atrophy (Holmes type), and Marinesco-Sjögren-Garland disease. *Type 3*, "combined spinocerebellar and other CNS parts" (e.g., brainstem), comprised olivopontocerebellar atrophies (OPCAs), hereditary spastic ataxia, Ramsay-Hunt dentatorubral degeneration, and hereditary periodic ataxia (Menzel type).[3] Later, Harding regrouped HCA according to AO, as a proxy for mode of inheritance, and **clinical findings**.[17] With several genetic and pathogenic discoveries occurring by the end of the XXth century, De Michele *et al.* proposed, in 2004, a modified HCA classification, according to the underlying **pathogenic mechanism:**[135] it comprised six main categories (mitochondrial, metabolic, defective DNA repair, abnormal protein folding and degradation, channelopathies, and others). All these classification systems included disorders with different forms of inheritance. It is now widely accepted, however, that the first classification branch of any classification of HCA should stand on the **mode of inheritance**, thus dividing it into autosomal dominant, autosomal recessive, XL and mitochondrial. Further subclassifications have been proposed since, with the AD-HCA generating more consensus than AR-HCA, largely due to the complexity and heterogeneity of this last group.

Since the first **autosomal dominant** families described by Pierre Marie, until the genetics era, several clinical classifications have been adopted in medical literature. Greenfield divided AD-HCA into two primary categories: type A (Menzel's type) for spinocerebellar forms, which would overlap with the category of OPCA, and type B (Holmes type) for predominantly cerebellar forms.[3] Anita Harding's classification on autosomal dominant cerebellar ataxia (ADCA) gathered a high level of consensus for several years.[136] It distinguished four main types of ADCA: *type I*, characterized by the presence of extra-cerebellar symptoms/signs (such as optic atrophy, ophthalmoplegia, dementia, extrapyramidal features or amyotrophy, amongst others); *type II*, with pigmentary retinal degeneration (with or without ophthalmoplegia) and/ or extrapyramidal features; *type III* or "pure" cerebellar ataxia with later onset;[17,136,137] and a *type IV*, in publications from 1982 and 1983, with mental retardation, myoclonus and deafness, but later diagnosed as a mitochondrial disorder and excluded from the classification.[137] The presence or absence of pyramidal signs was not considered in the original classification system, even though more recent versions of Harding's classification list pyramidal signs in ADCA type I.[18,138,139] Harding considered these could occur in the same disorder, depending on disease duration, thus adding little to the system of classification.[136] With the first discoveries of the underlying genetic defects, most dominant ataxias came to be classified as SCA followed by a number. These numbers were serially assigned, according to the chronological order of the identification of the disease locus or gene,[5] though with some inconsistency (DRPLA was the second SCA to be mapped,[22,57] after SCA1;[21] followed by SCA2;[62] and then MJD/ SCA3,[42] which was thus the fourth). DRPLA was the exception to this rule, as was MJD (in part), as both kept their original denominations; the later conclusion that "SCA3"[43-45] was the same as MJD introduced some conflict and confusion. ADCA type I would correspond to SCA1, SCA2, MJD, SCA4, 7-8, 10,12-15, 17-22, 25, 27-28, 31-32, 34-36, 38, 42-44, 46-47, *DNMT1*; while ADCA II corresponds only to SCA7; and ADCA III would now include SCA5-6, 11, 23, 26, 30, 37, 41 and 45.[18,139] This limited the clinical usefulness of Harding's ADCA classification. The numerical classification is still widely used, but it also has the disadvantage to include some "missing genes" (i.e., mapped loci were never confirmed, such as SCA18 and 20; or numbers "reserved" and never attributed, as SCA9); as well as allelic diseases, as SCA15/SCA16, SCA19/SCA22. Table 1 summarizes the most relevant classifications and includes a proposal for their correspondence and integration.

Table 1: Correspondence of the different classifications for AD-HCA

Pierre Marie (1893)[16]	Greenfield (1954)*[3]	Harding, ADCA (1983)[17]	SCA (1993)[β 21]	MDS, ATX+ (2016)[104,140,141]
Marie's hereditary cerebellar ataxia	Type A, **Menzel's type** – Spinocerebellar ataxia with OPCA degeneration	**Type I** – ADCA with ophthalmoplegia/optic atrophy/dementia/extrapyramidal features		Complex ataxia
			SCA1	ATX-*ATXN1*
			DRPLA	ATX-*ATN1*
			SCA2	ATX-*ATXN2*
			MJD	ATX- *ATXN3*
			SCA4	ATX-*ZFH3*
			SCA9	--
			SCA10	ATX-*ATXN10*
			SCA13	ATX-*KCNC3*
			SCA17	ATX-*TBP*
			SCA18	--
			SCA20	--
			SCA21	ATX-*TMEM240*
			SCA25	ATX-*PNPT1*
			SCA28	ATX-*AFG3L2*
			SCA29	ATX-*ITPR1*
			SCA31	ATX-*BEAN1*
			SCA32	--
			SCA34	ATX-*ELOVL4*
			SCA36	ATX-*NOP56*
			SCA40	ATX-*CCDC88C*
			SCA43	ATX-*MME*
			SCA46	ATX-*PLD3*
			SCA48	ATX-*STUB1*
			SCA49	ATX-*SAMD9L*
			SCA50	ATX-*NPTX1*
				ATX-*DNMT1*
				Relatively pure ataxia
			SCA8	ATX-*ATXN8OS*
			SCA12	ATX-*PPP2R2B*
			SCA14	ATX-*PRKCG*
			SCA15	ATX-*ITPR1*
			SCA16	ATX-*ITPR1*
			SCA19	ATX-*KCND3*
			SCA22	ATX-*KCND3*
			SCA27A	ATX-*FGF14*
			SCA35	ATX-*TGM6*
			SCA38	ATX-*ELOVL4*
			SCA42	ATX-*CACNA1G*
			SCA44	ATX-*GRM1*
			SCA47	ATX-*PUM1*
		Type II – ADCA with pigmentary retinopathy ± ophthalmoplegia/ extrapyramidal features)		Complex ataxia
			SCA7	ATX-*ATXN7*
	Type B, **Holme's type** – Predominantly cerebellar atrophy	**Type III** – Pure ADCA of later onset (over 50 years)		Pure ataxia
			SCA5	ATX-*SPTBN2*
			SCA6	ATX-*CACNA1A*
			SCA11	ATX-*TTBK2*
			SCA23	ATX-PDYN
			SCA26	ATX-*EEF2*
			SCA27B	ATX-*FGF14*
			SCA30	--
			SCA37	ATX-*DAB1*
			SCA41	ATX-*TRPC3*
			SCA45	ATX-*FAT2*

Legend: *only AD forms, [β] Even though the term spinocerebellar ataxia was introduced by Holmes in 1907, it has only been adopted as a classification system after the description of SCA1 in 1993.

Notes: SCA24, a complex form HCA, was later identified as being a recessive condition, due to pathogenic variants in *VPS13D*, and reclassified as SCAR4. SCA33 was never assigned.

There have also been numerous proposals for classification of the **autosomal recessive** ataxias, none gathering a general consensus. Anita Harding classified HCA in known and unknown causes: the first included disorders of metabolism and DNA repair (mainly AR); and the latter was subdivided according to AO.[17] Early-onset ataxias of unknown aetiology corresponded almost exclusively to AR forms; they were further subdivided according to additional clinical features.[17] After the first gene discoveries in the nineties, new nomenclatures were developed. In 2003, Michel Koenig proposed a classification according to topographic and pathophysiological criteria, but it failed to gain supporters.[142] In 2006, Francesc Palau and Carmen Espinós considered five types of AR ataxias, based on both clinical and genetic criteria: congenital, metabolic, DNA-repair defects, degenerative, and with other features (including disorders with known and unknown genetic defect).[107] More recently, in 2019, the Society for Research on the Cerebellum and Ataxias Task Force published a classification after clinical and pathophysiological criteria:[134] AR-HCA were divided in primary and other metabolic/ complex disorders with ataxia as an associated feature. The first were then subdivided into “pure”; with intellectual disability, developmental delay or dementia; metabolic or mitochondrial; with extrapyramidal involvement and oculomotor apraxia; with polyneuropathy; and with motor neuron involvement.[134]

X-linked ataxias are yet another genetic and clinical heterogenous group, for which no consistent classification has been developed. It includes diseases caused by expansions and conventional mutations, but also genomic rearrangements on the X chromosome.[143] The “SCA plus number” nomenclature, has also been adopted for XL ataxias, in the form of SCA-X. Still, this is incomplete, leaving out several forms, namely FXTAS, which is by far the most prevalent.

Mitochondrial ataxias include a large and heterogeneous group of often severe diseases, associated with failure of oxidative phosphorylation (OXPHOS).[129] Classifications evolved from a clinical to a morphological, biochemical and, for last, genetical basis. Mitochondrial ataxias are currently divided into disorders caused by primary mtDNA mutations (with mitochondrial inheritance), or due to abnormalities of the OXPHOS-related nuclear genes, including genes involved in mtDNA maintenance that may cause secondary mtDNA impairment (cell nucleus-driven mtDNA deletions, thus with Mendelian inheritance).[129] This group of ataxias may, thus, present with any mode of inheritance.

In 2016, the International Parkinson and Movement Disorders Society Task Force for the Nomenclature of Genetic Movement Disorders proposed a new classification based on

the **gene name associated to a prefix according to the predominant phenotype**.[104] For the HCA, the prefix "SCA" was initially suggested;[104] but this was later changed to "ATX",[141] allowing for a double or triple prefix, whenever more than one phenotype was prominent (e.g., HSP/ATX).[141] This is a very extensive and somewhat cumbersome classification, though it is particularly useful for the interpretation of genetic sequencing data; it is not primarily based on inheritance mode, described as a secondary feature. This might be an advantage, as an increasing number of both AR and AD phenotypes are known to be associated with the same gene.

Classifications need to be considered as a *work in progress*, as they are, anyhow, an artificial way to systematise both clinical and genetic information. With the continuous expansion of medical and scientific knowledge, there will be an unremitting need for their reclassification.

F. CLINICAL FEATURES

The main clinical features of some of the most frequent HCA, organized according to the type of inheritance, are described above.

AUTOSOMAL DOMINANT ATAXIAS

The most frequent AD-HCA (SCA1, DRPLA, SCA2, MJD and SCA6), are all caused by an unstable $(CAG)_n$ expansion.[118] Less frequently, AD-HCA (e.g., SCA31, SCA36 or SCA37) have intronic repeat expansions as genetic mechanism.[5] Conventional mutations (missense, nonsense mutations, insertions or deletions) have been considered rare causes of HCA, but are increasingly being recognized with the use of NGS.[105] We describe below some of the better known AD forms, by chronological order of their description.

SCA1 (ATX-*ATXN1*)

This disease typically begins around the age of 30 years (range: 4-74 years), with gait imbalance and upper motor-neuron signs.[144] Over disease course, there is progression of the cerebellar and pyramidal syndromes, with emergence of brainstem signs, as

progressive gaze palsy, dysphagia, and cough.[144] In some patients, sensory neuropathy, movement disorders (dystonia), and subcortical cognitive deterioration (executive function impairment) may also be observed.[144] The juvenile phenotype is characterized by a rapid progression and more severe phenotype, with ophthalmoplegia, basal ganglia and bulbar deficits, and diffuse amyotrophy.[144] Patients with onset after age 60 years tend to have a slowly progressive cerebellar syndrome, with minimal or absent extracerebellar involvement.[144]

Normal alleles typically possess 6-44 CAG repeats, with those greater than 21 repeats being characterized by 1-3 interruptions of CAT units, encoding histone.[21,145] Disease alleles consist of pure uninterrupted CAG repeats with 39-91 triplet units.[21,145] The absence of CAT interruptions in large wild-type allele destabilizes the repeat sequence, rendering it susceptible to meiotic instability towards expansion. So, individuals with 39-44 uninterrupted CAG repeats will develop SCA1, but those with CAT-interrupted $(CAG)_{39-44}$ will not exhibit signs of the disease.[145] The function of ataxin-1 (ATXN1), the encoded protein, is still unclear. Yet, there is some evidence that it is involved in transcriptional regulation, through binding to DNA and RNA in the nucleus.[146]

SCA2 (ATX-*ATXN2*)

SCA2 usually begins in the 4th decade of life (varying from 2 to over 70 years), with a static and kinetic cerebellar syndrome.[147-149] There is an early involvement of the pons (slowed saccadic eye movements), midbrain (Parkinsonism), spinal cord (hyperreflexia, ankle clonus and decreased vibration sense), and muscles (cramps).[147] Early and pronounced slowing of saccadic velocity is a key clinical feature of this form of ataxia;[150] saccade velocity appears to be influenced by polyglutamine size, but not by disease duration.[150] Hyperreflexia, frequent and prominent in the early phase, usually disappears over the course of the disease, as neuropathy emerges.[147] Movement disorders have been frequently described in SCA2, with an asymmetric dopa-responsive Parkinsonism being more common among Japanese and Chinese families. In European SCA2 patients, postural tremor is the most distinctive movement disorder.[147,151] Additionally, myoclonus may be identified in early stages, while chorea and dystonia seem to be rare and cluster in some families.[147,151] Mild cognitive deterioration may emerge with disease progression, but overt dementia is not a feature of SCA2.[147] As in other polyQ SCA, the phenotype is more severe in early onset, with an early presence of fasciculations, lower limb amyotrophy, areflexia, movement disorders (dystonia, chorea and myoclonus) and retinitis pigmentosa.[152,147]

The normal $(CAG)_n$ alleles ranged in size from 17 to 31 repeats in the Portuguese population, and from 14 to 31 worldwide.[145,153] Intermediate range repeats (i.e., 32-34 CAG units) show incomplete penetrance. Isolated cases are frequently explained by expansion from and intermediate-size allele.[147] Size of pathogenic alleles ranges from 35-500 CAGs.[145,147] The physiological role of ATXN2 is also not yet completely understood, some studies suggesting it may be related to RNA metabolism and cellular signalling pathways.[147]

Machado-Joseph Disease (ATX-*ATXN3*)

First symptoms emerge with a mode at 40 years old, with rare cases starting in childhood, or after the 8th decade of life.[24,25,154] Clinical expression is influenced by AO, but comprises, in most patients, gait ataxia, progressive extrinsic ophthalmoplegia (PEO), or nystagmus, at onset.[24,25] PEO is almost invariably accompanied by diplopia and is characterized by an early limitation of the upward vertical gaze, followed by limitation of convergence and lateral gaze, with downward vertical gaze being usually preserved until a later stage.[25] Bulging eyes, recognized by Coutinho, are the result of eyelid retraction, and often allow identification of affected members in ancient photos and reconstitution of affected kindreds.[25] Also typical, in some patients, are the tongue, face and eyelid fasciculations, particularly on muscle contraction.[24,25] At early stages, pyramidal signs are frequent, but may tend to disappear with disease progression, or be masked by peripheral signs.[24,25] Movement disorders may also be part of the phenotype, particularly in early-onset patients, with dystonia being the most frequently observed.[25] Involvement of cranial or peripheral nerves may occur, with variable frequency and severity.[24,25] Sleep disorders, particularly REM-sleep behaviour disorder, and restless-legs syndrome are frequent in MJD.[25,154]

Coutinho and Andrade's phenotypic classification grounded on influence of AO on clinical variability. [30] In all three types, cerebellar ataxia, PEO and pyramidal signs are present; in type 1 (predominant in the Joseph family), there is early onset and rapid progression, with very prominent pyramidal signs and dystonic postures;[30] in type 2 (typical of the Thomas family), onset, severity and progression are with intermediate;[30] in type 3 (predominating in the Machado family), onset occurs later progression is slower, and peripheral signs are more frequent.[30] A type 4, with later onset, Parkinsonian features, mild cerebellar signs and a distal motor sensory neuropathy or amyotrophy, was subsequently described.[155,156]

Most persons have up to 44 repeats in both alleles, whereas patients bear one allele usually with 61-87 CAGs.[145,157-159] Alleles in the intermediate range of 45-59 units are rare

and may show incomplete penetrance, but this still remains to be clearly established.[160-162] Ataxin-3 (ATXN3), the encoded protein, is conserved across different eukaryotic organisms; in humans, it is highly expressed in the CNS.[154] Its function is not yet fully understood, but various studies indicate its activity as a deubiquitinating enzyme, binding and cleaving long ubiquitin chains.[154]

SCA6 (ATX-*CACNA1A*)

SCA6 is considered a typical late-onset "pure" ataxia, usually starting by the 6th decade of life (range 19-71 years).[148,149,163] The most frequent presentation is with gait instability, rarely preceded by diplopia or episodic vertigo.[163] With disease progression, there is a difficulty in fixating moving objects, as well as horizontal and vertical nystagmus.[163] Nevertheless, non-cerebellar signs may also occur, with 40-50% patients displaying pyramidal signs and 25% movement disorders (dystonia).[163] Less frequently, periodic alternating nystagmus and rebound nystagmus, dysphagia, urinary dysfunction, and muscle atrophy may appear.[163] Cognition is generally preserved, but some impairment in executive functions has been described.[163]

SCA6 is distinguished by having smaller $(CAG)_n$ expansions (20-33, which is within the normal range for the remaining SCA).[145,164] Also, the expression of the encoding protein seems to be restricted to a few neuronal cells.[163] *CACNA1A*, the gene responsible for SCA6, encodes a subunit of the P/Q-type voltage-dependent calcium channels, which regulates calcium influx and, thus, release of neurotransmitter-containing vesicles into the synaptic cleft.[165] Its pathogenicity is also not fully understood, some authors suggesting it relates to impaired channel function, while others refer the formation of intraneuronal inclusions, as in other polyQ diseases.[163]

Ataxia due to conventional mutations in *CACNA1A* (ATX-*CACNA1A*)

Conventional mutations (non-repeat expansions) in *CACNA1A* were first identified in patients with episodic ataxia type 2 or familial hemiplegic migraine (FHM), with or without associated cerebellar ataxia.[166,167] Initially seen as a rare cause of ataxia, its identification has increased in the past years.

Phenotypic diversity, including in AO, is high. Patients with infantile onset usually present with epilepsy and intellectual disability, while in the juvenile form there is a higher frequency of psychiatric symptoms.[105] When onset is in adulthood, a pure cerebellar

ataxia is observed, although pyramidal signs may emerge after a longer disease duration.[105] Both early and late-onset forms show slow progression, with independent gait maintained over several years.[167]

Large deletions and altered splicing appear to be associated with onset before midlife. Most infantile forms associate with variants in segment S5 of repeat IV, affecting non-polar aminoacids without causing a change in charge.[105]

SCA7 (ATX-*ATXN7*)

The phenotypic expression of SCA7 is highly variable and dependent on the number of CAG repeats, and anticipation is very prominent.[168] It may be so dramatic that a child may be diagnosed with an (apparently) unrelated neurological disease years before a parent, or even a grandparent.[169,170] This infantile phenotype (>100-150 CAGs), starts between age 3 months and 3 years, and is very severe, with neurological and systemic disease.[171] The classical phenotype (50-55 repeat units) begins in adolescence, with loss of colour discrimination and progressive visual acuity worsening.[171] Cerebellar ataxia usually emerges in the 2nd-3rd decades of life,[148,149] associated with pyramidal signs and, later, with dysarthria and dysphagia.[171] In some patients, external ophthalmoplegia, eyelid ptosis, tongue atrophy, and motor/ sensory neuronopathy occur; cognitive deterioration is rare.[171]

Normal alleles range 4-19 repeats, uncertain from 28-33, reduced penetrance 34-35, while pathogenic range 36-460 CAGs. [145]ATXN7 is expressed in the brain, retina, and peripheral tissues (striated muscle, testes, and thyroid), some studies suggesting a role as a negative transcription regulator.[172]

SCA8 (ATX-*ATXN8OS*)

In SCA8, onset occurs usually in the 3rd-5th decades of life, with a reported range from 1 to 73 years.[173] Dysarthria and gait unsteadiness are the most common symptoms at onset, and with disease progression impaired eye movements may emerge (nystagmus, abnormal pursuit and saccades).[173] Osteotendinous hyperreflexia is a common finding, while Babinski sign is rarely registered. Brainstem signs as dysphagia and poor cough reflex are observed in later stages, and sensory neuropathy has been reported in 30-50% of patients.[174] Cognitive impairment may be present in up to half of patients, a cerebellar

cognitive-affective disorder characterized by executive dysfunction and psychomotor slowing.[174]

SCA8 is caused by the bidirectional transcription of an expanded CTG repeat in the 3' untranslated region of *ATXN8OS* and the complementary CAG repeat in the polyglutamine open reading frame of the *ATXN8* gene.[175,176] These result in expression of a CUG expansion mRNA transcript and a polyQ protein, respectively, indicating a toxic gain of function both at RNA and protein level.[176] ATXN8 function is still unknown. Unlike most others, SCA8 has the particularity of giving rise to repeat contraction upon paternal transmission and expansion upon maternal transmission.[177] Reduced penetrance is much more common in SCA8 than in other SCA and pathogenic ranges are very uncertain, with a large overlap of repeat sizes in patients and in asymptomatic persons (with no symptoms and no family history of ataxia).[145,173] Normal alleles span 14-42 repeat units, with pathogenic alleles ranging 74-1000 repeats (with reduced penetrance), still there is large debate on whether it should be tested on a routine basis.[145]

SCA14 (ATX-*PRKCG*)

SCA14 tends to present from early adulthood (mean 33.8 years, range 5-71), with gait unsteadiness.[178] In addition to a complete cerebellar syndrome, horizontal nystagmus or saccadic intrusions are commonly observed.[178] Decreased vibration sense may be observed in up to one-third of families, while deep tendon reflexes may be increased or decreased, and Babinski sign is rarely present.[178] Myoclonus has been pointed as a distinctive feature, even though not present in all patients. As for other movement disorders, rigidity, tremor, and dystonia were described in a few patienss.[178] Intellectual impairment, attention deficit, and impaired executive functioning may emerge with disease progression.[179]

SCA14 is caused by conventional mutations in *PRKCG*, a gene encoding for protein kinase C, a multifunctional family of kinases involved in various cell processes, from membrane-receptor signal transduction to control of gene expression.[180]

SCA17 (ATX-*TBP*)

Onset in SCA17 is typically in the 4th decade of life, varying from 3 to 55 years.[181] The wide phenotypic spectrum of SCA17, also known as "Huntington's disease-like type 4", increases the diagnostic challenge in this disorder. In addition to a cerebellar syndrome, patients may exhibit movement disorders, as Parkinsonism, chorea and dystonia.[182]

Dementia and psychiatric disorders are frequent at presentation, and pyramidal signs may present as disease progresses.[182] Epilepsy is seen in some patients, and oculomotor abnormalities include impaired smooth pursuit, abnormal saccadic accuracy, and gaze-evoked nystagmus.[182]

SCA17 is caused by CAG/CAA repeat expansion in *TBP* gene, which encodes the TATA box-binding protein, a transcription initiation factor.[183] Range of normal alleles is 25-42, 43-48 in intermediate, and 49-66 in pathogenic alleles.[145] A repeat size of 43-50 is linked to a Huntington's disease-like phenotype, while range 50-60 is associated with diminished intellectual function, dystonia, and pyramidal signs.[184,185] $(CAG/CAA)_{>60}$ are rare, and associate with childhood onset.[186] Unlike other SCAs, expansion size tends to remain stable upon transmission, possibly due to the of CAA interruptions present within the $(CAG)_n$ tract.

SCA27B (ATX-*FGF14*)

In early 2023, a $(GAA)_n$ expansion in the first intron of *FGF14* (the gene for fibroblast growth factor 14), in heterozygosity, was described as a frequent cause of late-onset AD cerebellar ataxia, SCA27B or ATX-*FGF14*.[103,187] It is characterized by gait ataxia, with downbeat nystagmus, neuropathy, and bilateral vestibulopathy.[103,187,188] In up to half of the patients, ataxia is preceded by transient episodes (minutes to days) of variable combinations of diplopia, vertigo, dysarthria, and ataxia. Episodes may be triggered by alcohol intake or exercise, and occur with a daily to monthly frequency.[103] Reduced pallaesthesia and abnormal Romberg test are present since its early stages; reduced ankle reflexes and dysautonomia increase with disease course.[189]

Function of FGF14 is not well understood, but studies on animal models suggest it may have a role in neuronal signalling, axonal trafficking, and synaptosomal function.[190]

SCA37 (ATX-*DAB1*)

SCA37 is characterized by an adult onset (varying from 18 to 59 years)[101,102], pure cerebellar ataxia, with dysarthria at onset. This manifesting symptom was recognized as a feature unique to these families by Professor Coutinho[79]. It was her detailed clinical characterization that allowed for the identification of the genetic defect, in Portuguese families.[79,101] Abnormal eye movement have been described in Spanish patients, including irregular and slow vertical smooth pursuit, dysmetric vertical saccades, slow vertical

optokinetic nystagmus, and oscillopsia (a visual disturbance where objects appear to oscillate).[102]

It is caused by the insertion of an unstable $(ATTTC)_n$ amidst an $(ATTTT)_n$ tract in the noncoding region of *DAB1*.[101] Normal alleles bear a ATTTT stretch of 7-400 repeats, uninterrupted by ATTTC units, while age-dependent penetrance alleles show an $(ATTTC)_{31-75}$ insertion.[101] Intergenerational instability of the $(ATTTC)_n$ insertion was observed, particularly when the father is the transmitting parent.[101] An inverse correlation between size of $(ATTTC)_n$ and AO is also seen.[101] DAB1 is an adaptor molecule mediating signalling from reelin receptors, which controls neuronal migration and synapses' maturation in the developing brain.[101]

AUTOSOMAL RECESSIVE ATAXIAS

Recessive ataxias are described here following the classification of Palau and Espinós,[79,82,107], and our own criteria (Table 2), as this allows a better organization of such a complex group of disorders, while keeping the classification used during the population-based survey.

Table 2: Classification of AR-HCA, adapted from Palau and Espinos[107]

AUTOSOMAL RECESSIVE ATAXIAS
Congenital ataxias
Joubert syndrome
Cerebellar ataxia, impaired intellectual development and disequilibrium
Metabolic ataxias
Friedreich ataxia/ ATX-*FXN*
Abetalipoproteinemia/ ATX-*MTTP*
Ataxia with vitamin E deficiency / ATX-*TTPA*
Cerebrotendinous xanthomatosis/ ATX-*CYP27A1*
Coenzyme Q10 deficiency
Congenital disorders of glycosylation
L2-hidroxiglutaric aciduria/ ATX-*L2HGDH*
HSP/ATX-*PGN*
MxMD-*POLG*
Niemann-Pick type C/ ATX-*NPC1*
Sandhoff disease/ ATX/HSP-HEXB
DNA repair defects
Ataxia telangiectasia/ ATX-*ATM*
Ataxia with oculomotor apraxia (type 1, type 2, type 3, type 4)
Xeroderma pigmentosum/ ATX-*ERCC4*
Other degenerative ataxias
ARSACS/ ATX-*SACS*
ATX-RFC1
ATX-*SYNE1*
ATX-*ANO10*
HSP/ATX-*KIF1C*
HSP/ATX-*CAPN1*
HSP/ATX-*GBA2*
HSP-*ZFYVE26*
HSP-*SPG11*
MxMD-*ATP13A2*

CONGENITAL ATAXIAS

Congenital ataxias are characterized by *in-utero* development anomalies of the cerebellum and/ or brainstem.[137] In the newborn period or infancy, cerebellar dysfunction leads to a condition of global hypotonia, and delayed motor milestones (frequently also language).[137,191] Congenital ataxias include a mixed group of diseases, both phenotypically and genotypically. They are frequently non-progressive, with many patients showing improvement on follow-up.[191] Thus, some authors use the term *nonprogressive congenital ataxias*.

Joubert syndrome is one of the most frequent congenital ataxias. It is characterized by a typical mid-hindbrain malformation, in the form of a *molar tooth* on MRI.[132] In addition to hypotonia, patients frequently show abnormal eye movements (nystagmus, strabismus, oculomotor apraxia), and an abnormal respiratory pattern, characterized by short alternate episodes of tachypnoea and apnoea, or tachypnoea alone.[132] There is a delay in motor and cognitive milestones, with some degree of intellectual disability (ID) in all patients.[132] Approximately 50% acquire independent, ataxic, gait.[132] Associated organ anomalies may be present, manifesting at different ages, the most frequent affecting the retina, kidney, and liver.[132] More than 20 genes have been associated with Joubert syndrome, all encoding proteins related with the primary cilium structure or functioning.[132]

Disease-causing variants in *ATP8A2* were initially associated with a severe phenotype of congenital ataxia, described as the “cerebellar ataxia, mental retardation and disequilibrium syndrome 4” (or “cerebellar ataxia and mental retardation with or without quadrupedal locomotion 4”, CAMRQ4, OMIM #615268).[192] Most patients have intellectual disability, quadrupedal locomotion or no ambulation skills, and no language acquisition.[192,193] ATP8A2 is and ATPase amino-phospholipid transporter which is determinant in the phospholipid bilayer curvature, vesicle trafficking and regulation of neurite outgrowth.[192]

METABOLIC ATAXIAS

Metabolic ataxias include diseases with progressive ataxia as the predominant characteristic, intermittent ataxia, or ataxia as a minor feature.[107] The first group comprises FA, AVED, abetalipoproteinemia, cerebrotendinous xanthomatosis, Refsum disease, Niemann-Pick type C disease, glycosylation disorders, and some mitochondrial diseases.[107] The second group, include aminoacidurias, lactate and pyruvate metabolism disorders, argininosuccinase deficiency, and arginase deficiency.[107] Finally, ataxia as

minor feature is seen in metachromatic leukodystrophy, adrenoleukodystrophy, and sphingomyelin storage disorders.[107]

Friedreich ataxia (FA) (ATX-*FXN*)

FA typically starts around puberty, but its AO may vary widely, even within the same sibship. The earliest onset is registered by 2 years of age, while the latest is in the 7th decade.[63,194] Some clinical features may also vary, often in agreement with AO.

It usually starts with gait unsteadiness, but occasionally scoliosis or hypertrophic cardiomyopathy may be diagnosed first. Ataxia is of a mixed type, both cerebellar and sensitive, affecting the trunk, with imbalance and falls, but also the limbs, with dysmetria and deterioration of fine motor skills.[63] Dysarthria occurs early in disease course, while dysphagia (particularly for liquids) with advancing disease.[70] Nystagmus is rare, while square-wave jerks are frequently observed.[70] Pyramidal signs are a common finding, but may be partially masked by the severe neuropathy (leading to the typical finding of areflexia with Babinski sign).[63,70] Muscle weakness and amyotrophy (particularly in the hands) are common.[63,70] In addition to scoliosis and cardiac disease, other non-neurological features include *pes cavus*, neurosensorial deafness, optic atrophy, and diabetes.[70]

Delayed-onset forms have been further subdivided in *LOFA*, when occurring from 25 until 40 years old; and *VLOFA*, when onset was after age 40.[71] In some recent studies, age range was subdivided as *early onset* (<14 years), *intermediate onset* (15-24), and *late onset* (>24).[194,195] Re-emergence of this debate on the best definition of age ranges was closely related with patients selection for clinical trials. Overall, later-onset forms tend to present milder symptoms, slower progression and paucity of extra-neurological involvement.[71] Interestingly, early onset cerebellar ataxia with retained reflexes was considered a distinct disorder,[68] and established as FA only after mapping of the *locus*.[196]

Frataxin, the protein encoded by *FXN*, is involved in the normal regulation of mitochondrial iron transport and synthesis of iron-sulphur (Fe-S) cluster containing enzymes.[197] Decreased levels of frataxin induce a deficiency of Fe-S cluster containing subunits of the mitochondrial complexes I, II and III, resulting in mitochondrial dysfunction.[197] This would ultimately lead to increased oxidative stress and cell damage.[198] Early, severe, involvement of the proprioceptive system in FA, particularly atrophy of sensory neurons at the dorsal root ganglia, leads to loss of sensory input to the cerebellum.[196] Purkinje neurons loss and cortical cerebellar atrophy are usually mild to moderate, and occur at a

late stage.[196] As such, no clear cerebellar atrophy is evident in brain MRI, whereas atrophy of the cervical medulla is often an early finding.[63] Progressive degeneration of the corticospinal tract occurs in a dying-back process.[196]

AVED (ATX-*TTPA*) and abetalipoproteinemia (ATX-*MTTP*)

In these two diseases, serum levels of vitamin E are very low.[126] Symptoms typically start before age 20, but AO may vary from early childhood to the 7th decade.[199] Gait instability is the most common presentation, followed by limb dysmetria and dysarthria.[199] Proprioceptive and vibratory sensation loss, pyramidal signs, and lower limb areflexia are universally present.[199] Gaze paralysis, saccadic eye movements, distal limb amyotrophy, tongue fasciculations, and retinitis pigmentosa may also be present.[200] Some patients exhibit dystonia, with rare reports of myoclonus.[199] Clinical similarities with FA, led initially some authors to term it “FA with vitamin E deficiency (FAVED)”.[201] Distinction and a correct diagnosis are, nevertheless, highly important, as supplementation of vitamin E improves symptoms and prevents progression of the disease.[199]

Alpha-tocopherol transport protein, encoded by *TTPA*, is involved in the incorporation of alpha-tocopherol into very low-density lipoproteins.[201] In abetalipoproteinemia, *MTTP* encodes a subunit of the triglyceride transport protein, leading intestinal lipid malabsorption due to the lack of chylomicron formation and subsequent absence of fat-soluble vitamins.[202] Vitamin E deficiency causes degeneration of the posterior columns, spinocerebellar tracts, and loss of large myelinated fibres in peripheral nerves.[199]

Congenital disorders of glycosilation (CDG)

The neurological presentation of CDG includes a non-progressive congenital ataxia of varying severity, with hypotonia and osteotendinous hyporeflexia.[203] Retinitis pigmentosa, seizures, and transient neurological deficits may be present throughout disease course.[203] Additionally, there is facial dysmorphism, typically strabismus, subcutaneous lipodystrophy, and hepatic dysfunction.[203]

CDG constitute an heterogenous group of disorders, at a biochemical and genetic level.[203] Several subtypes have been identified, with type Ia, associated with pathogenic variants in *PMM2,* being the most common.[204]

Ataxia associated with mitochondrial DNA-dependent DNA polymerase gamma (MxMD-*POLG*)

Different phenotypes have been associated with pathogenic variants in the DNA polymerase gamma (*POLG*) gene, including sensory ataxic neuropathy, dysphagia and ophthalmoplegia (SANDO); and mitochondrial spinocerebellar ataxia and epilepsy.[129] The latter has a high frequency in northern Europe, usually starts in adolescence with migraine or epilepsy, and later develops progressive ataxia.[205]

POLG is involved in mtDNA replication and repair.[206] Disease-causing variants in *POLG* are associated with reduced activity of the protein, and mtDNA depletion in neurons (including Purkinje cells and substantia nigra), leading to a cascade of events ending with respiratory chain dysfunction.[128]

Coenzyme Q10 deficiency with cerebellar ataxia

Coenzyme Q10 (CoQ10, ubiquinone) deficiency syndrome is characterized by an early-onset cerebellar ataxia, associated with low levels of CoQ10 in skeletal muscle.[107] The clinical picture may be complex, with developmental delay, epilepsy, pyramidal signs, and myoclonus.[207] Multisystemic involvement has also been reported, with cardiomyopathy, hearing loss, and retinopathy.[207]

Ten genes involved in the biosynthesis of human CoQ10 (e.g. *COQ4, COQ5, COQ6 and COQ8A*) have been associated to this syndrome.[128] CoQ10 is involved in the transfer of electrons from complex I to II, and from complex II to III.[128]

SPG7 (HSP/ATX-*PGN*)

SPG7 was initially described and considered, for many years, only a form of spastic paraplegia (SPG). The use of NGS increased the diagnoses of SPG7. This led to the recognition that a vast majority of patients display signs of ataxia; and, also, that ataxia may even be its unique or most prominent feature.[208,209] Symptoms emerge around the 4th decade of life (ranging 10-69 years), with a spastic ataxia phenotype. Other neurological signs, as ophthalmoparesis, optic neuropathy, or Parkinsonism are frequently present.[208,209]

Paraplegin, the mutated protein, assembles into a hexamer with AFG3L2 (involved in SCA28), functioning as an m-AAA metalloprotease complex, at the inner mitochondrial membrane, involved in maintenance of the mitochondrial proteome.[209]

ATAXIAS ASSOCIATED WITH DNA-REPAIR DEFECTS

The CNS, particularly the cerebellum, is particularly vulnerable to DNA damage, although the underlying mechanisms remain unknown.[125]

Ataxia-telangiectasia (AT)

AT is a multisystem disease, characterized by progressive cerebellar ataxia beginning in early childhood, associated with oculomotor apraxia, and, in some patients, choreoathetosis, generalized dystonia, and stimuli-sensitive myoclonus.[209] These movement disorders may precede ataxia by several years.[209] Ocular conjunctival and cutaneous telangiectasias, immunodeficiency, recurrent sinopulmonary infections, high risk of neoplasms, and increased sensitivity to ionizing radiation are frequent in these patients.[107,209] Elevated levels of α-fetoprotein are a helpful biological marker for AT (although occurring in AOA2).[209]

The AT-mutated kinase, encoded by *ATM*, acts in the recognition and response to double-stranded nuclear DNA breaks, and phosphorylation of protein substrates involved in cell-cycle checkpoints, nuclear localization, gene transcription and expression, oxidative stress response, and apoptosis.[210]

Ataxia with oculomotor apraxia (AOA)

AOA concerns a set of patients with cerebellar ataxia, in whom oculomotor apraxia is a major clinical sign.[82] There is some debate on the definition of this symptom and sign. The description by Aicardi is still accepted by many authors, and has been adopted here:[81] oculomotor apraxia is characterized by the inability to perform voluntary ocular movements, with preservation (at least in the initial stages) of spontaneous movements and opto-kinetic and vestibulo-ocular reflexes: on attempted refixation, the head turns first with consequent eye contraversion and accompanying head thrusts.[81] In Portugal, the most frequent AOA are AOA1, followed by AOA4 and AOA2.[79,84]

AOA1 (ATX-*APTX*) is characterized by early-onset (usually between age 2 and 6 years).[86,211] Some patients exhibit chorea and dystonia. A severe axonal sensorimotor neuropathy is present in all. In advanced stages, hypoalbuminemia and hypercholesterolemia may arise.[86,211] Aprataxin, the normal protein concerned in AOA1, encoded by *APTX*, is involved in single and double-stranded DNA repair.[85]

In AOA2 (ATX-*SETX*), onset occurs around age 15 (range 3 to 30 years), with cerebellar ataxia.[86,212] Axonal sensorimotor neuropathy, less severe than in AOA1, is present in over 90%, and oculomotor apraxia in approximately 50% of cases.[86,212] Movement disorders, as dystonia and chorea, are less frequent than in AOA1 and pyramidal signs may emerge with disease progression.[86,212] α-fetoprotein serum levels are increased in most patients.[86,212] Senataxin, the protein disturbed in AOA2, encoded by *SETX*, acts as a DNA/RNA helicase in single-stranded DNA repair.[86]

AOA3 was described in one Saudi family, with onset in the 2nd decade.[213] Its gene (*PIK3R5*) encodes a phosphoinositide-3-kinase key in some transduction pathways.[213]

AOA4 (ATX-*PNKP*) has an AO similar to AOA1 (average 4.3 years), with oculomotor apraxia being present in the majority of patients.[84] Dystonia, frequently identified, may precede cerebellar ataxia in some cases, while a severe axonal sensorimotor neuropathy is almost universal, as is in AOA1.[84] *PNKP*, the gene responsible for AOA4, encoding for polynucleotide kinase 3-prime phosphatase, an enzyme that plays a crucial role in multiple pathways involved with DNA repair.[84]

OTHER DEGENERATIVE ATAXIAS

Cerebellar ataxia with neuropathy and vestibular areflexia syndrome (CANVAS, ATX-*RFC1*)

Cerebellar ataxia, neuropathy, and vestibular areflexia syndrome typically presents in middle life, with progressive gait ataxia, oscillopsia, and sensory disturbance.[109] A dry cough, the second most frequent presentation after ataxia, may precede motor symptoms in up to 30 years.[214] It is very characteristic, what led Coutinho to recognize it a separate group.[79,215] It may even lead to the recognition by history (or by family members) of presymptomatic carriers. The phenotypic spectrum of ATX-*RFC1* ranges from typical CANVAS, to isolated cerebellar ataxia or sensory neuropathy.[214] Dysautonomia may be present in half of the cases, with movement disorders being rarely identified.[214]

Human replication factor C, the protein encoded by *RFC1* (replication factor C, subunit 1), plays a major role in the recognition of DNA damage and recruitment of DNA-repair enzymes.[109] Still, no evidence of increased susceptibility to DNA damage has been identified on ATX-*RFC1* patients' fibroblasts.[109]

Autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS/ ATX-*SACS*)

ARSACS is the classical example of a spastic ataxia. Most patients have onset before age 2 (though ranging 1 to 30 years),[107] with a delay in independent gait, resulting from unsteadiness in very young toddlers, as the most frequent presentation.[216] The classic phenotype is of a slowly progressive ataxia, followed by severe spasticity and, later, a sensorimotor neuropathy.[216] Approximately 80% will develop this classical triad throughout disease course.[209,217] With longstanding disease progression, pyramidal signs may be obscured by neuropathy. Epilepsy is present in up to 20%, and retinal myelinated fibres have been described in patients from Québec.[107,209] Even though intellectual disability is not common in ARSACS, patients often learn at a slower pace, further exacerbated by difficulties in handwriting.

Sacsin, the protein encoded by *SACS*, is expressed in brain motor and other tissues. It is believed to integrate the ubiquitin-proteasome system, being involved in chaperone-mediated protein folding, including of ATXN1.[107,209]

Spectrin repeat-containing nuclear envelope protein 1 ataxia (ATX-*SYNE1*)

In French-Canadians, ATX-*SYNE1* is characterized by onset in the 4th decade of life (median 31 years); and by adolescence or early adulthood onset in other populations (median 22 years).[209] A pure cerebellar syndrome is present in up to 20%, usually in later-onset patients, while the remaining exhibit additional features, as motor neuron signs or neuropathy.[209] It may rarely present by a severe early-onset form, with multisystem neurodegeneration, respiratory dysfunction, intellectual disability, and reduced lifespan.[218]

Synaptic nuclear envelope protein 1, the protein encoded by *SYNE1*, belongs to the spectrin family of structural proteins, linking the plasma membrane to the actin cytoskeleton.[209]

ATX-*ANO10*

ATX-*ANO10* is characterized by onset in adulthood (30-50 years, in the majority of patients) of a slowly progressive cerebellar ataxia, frequently associated with pyramidal signs. Abnormal eye movements include gaze-evoked downbeat nystagmus and hypermetric saccades, in a few families.[219] Epilepsy and a dysexecutive cognitive syndrome may also be present.[220] Deficit of CoQ10 was described in a few patients, as

well as elevated α-fetoprotein levels, but these have not been replicated in subsequent studies.[220]

ANO10 encodes anoctamin 10, a transmembrane protein, member of a family of calcium-activated chloride channels.[220]

MITOCHONDRIAL INHERITED ATAXIAS

Mitochondrial DNA differs from nuclear DNA in several aspects: exclusive maternal inheritance and its existence in numerous copies, within every cell of the body.[129] In typical circumstances, all copies of mtDNA within an individual are identical, referred to as *homoplasmy*. The occasional coexistence of different populations of mtDNA, such as wild-type and mutant variants, is termed *heteroplasmy*.[129] This brings important clinical differences.

Additionally, several mitochondrial proteins, including the ones involved in mtDNA maintenance, are encoded by nuclear genes (and thus show Mendelian inheritance) and imported to the mitochondria.[129]

Ataxias caused by conventional variants in mtDNA include a classical complex mitochondrial disease phenotype, of which ataxia is part, but also sometimes a "SCA-like" phenotype. The first includes neuropathy, ataxia, and retinitis pigmentosa (NARP) caused by pathogenic variants in *MT-ATP6,* but also *MT-ND6* or *MT-TV*; myoclonic epilepsy with ragged red fibres (MERRF), in which ataxia is also a prominent feature, caused by pathogenic variants in *MT-TK,* but also *MT-TF, MT-TL1, MT-TI ANS MT-TP;* and Leigh disease, a subacute necrotizing encephalomyelopathy in which ataxia is usually present.[128] In classical disorders of large mtDNA deletions, as chronic progressive external ophthalmoplegia, mild cerebellar ataxia might be observed.[129]Typically with childhood onset, these are usually complex, multisystemic disorders, with exacerbations induced by fever or metabolic stress. The second ("SCA-like" phenotype) may present with or without pyramidal signs or neuropathy, frequently in the absence of extra-neurological involvement.[221-224] Usually have onset in early adulthood, and paroxysmal neurological symptoms, as epilepsy or migraine, may also be present. Variants in *MT-ATP6, MT-TK* or *MT-TL1* have been associated with this phenotype.[221-224]

Nucleus-driven (Mendelian) mitochondrial ataxias, as described in the previous section (e.g., MxMD-*POLG, HSP/ATX-PGN*), usually present with a SCA-like phenotype.

X-LINKED (XL) ATAXIAS

XL ataxias are a small, tough growing group of clinically and genetically heterogeneous diseases, characterized by cerebellar dysgenesis (hypoplasia, atrophy, or dysplasia), due to genetic variants or genomic imbalances on the X chromosome.[143] Over 20 genes, mainly encoding proteins involved in brain development and synaptic function, have been connected to XL ataxias.[143] They usually present with hypotonia, delayed developmental milestones, intellectual disability, and cerebellar ataxia.[143] XL inheritance may be sorted into recessive and dominant.[225] XL-recessive diseases manifests almost exclusively in males, females being healthy carriers or having a very mild phenotype.[225] In the latter both males and females are affected, but females are more often affected, less severely so,[225] and with a much larger variability than males. Manifesting female carriers for XL-recessive disorders, as well as the large variability seen in women (both for recessive and dominant XL diseases) are explained, in part, by the phenomenon of Lyonization, the random inactivation of one of the woman two X chromosomes.

Fragile X-associated tremor/ataxia syndrome (FXTAS) is a X-linked dominant phenotype, more frequently identified in males, and characterized by adult-onset (typically in the early 7th decade) of kinetic tremor and gait ataxia, usually associated with cognitive decline with frontal executive dysfunction, peripheral neuropathy, and dysautonomia.[226] Other frequent findings include mild Parkinsonism and psychiatric symptoms (as depression, anxiety, agitation).[227] Female carriers may also be affected, with lesser severity and wider variability, less cognitive decline, and some different symptoms, as muscle pain.[227]

The cause is usually a “premutation” (ranging 55-200 units) in the $(CGG)_n$ within the fragile-X mental retardation 1 gene (*FMR1*).[227] As with other repeat diseases, clinical severity may vary with repeat expansion size.

G. NATURAL HISTORY

Apart from more benign cases in some forms, most HCA typically have a relentless course, leading to disability and premature death. Clinical progression may occur at a different pace, determining factors not being fully understood. It is well known, however, that different ataxias have a different rate of progression. Nevertheless, this may be also true for the same form of ataxia, even within the same family. A deeper knowledge on ataxias' clinical course and identification of the factors influencing disease progression are essential to provide appropriate advice to patients and at-risk relatives, as well as for the design of clinical trials.

For the past years, large multicentric collaborative efforts have been initiated with the aim of establishing larger HCA cohorts. Some were created for specific ataxia forms (as FACOMS, Friedreich Ataxia Clinical Outcome Measures Study; EFACTS, European Friedreich's Ataxia Consortium for Translational Studies; or ESMI, European Spinocerebellar Ataxia Type 3/Machado-Joseph Disease Initiative); while others combine various HCA forms (EUROSCA, European Integrated Project on Spinocerebellar Ataxias; or SCANOP, spinocerebellar ataxia non-polyQ). These collaborative initiatives are allowing for a deeper understanding on some of the factors influencing natural history of HCA.

MODIFIERS OF AGE OF ONSET (AO)

Mean AO in **dominant ataxias** tends to be in the 4th decade of life in SCA1, SCA2, and MJD; later (around the 6th decade) in SCA6; or earlier (3rd decade) in SCA7.[148,149] In ataxias caused by a $(CAG)_n$ expansion, as in other polyQ diseases, size of the expansion inversely correlates with AO. Expanded alleles are unstable, tending to increase upon transmission.[5,119] This may lead to earlier onset and increased severity in consecutive generations, a phenomenon known as (genetic) *anticipation*. A parental bias for the degree of anticipation of AO may exist; it is well known for some polyQ SCA, as MJD, SCA7, and DRPLA, in which anticipation is greater upon paternal transmission, while in SCA8 that occurs for maternal transmission.[5,158 177] Still, the number of CAG repeats is responsible for only 50-60% of the variability seen in AO,[149] as other genetic and environmental factors must also be implicated.[228]

One of those factors may be influence of the normal allele of the causal gene. In SCA1 and SCA6, a larger repeat size in the normal allele associates with earlier AO; while in SCA7 the same occurs for shorter or medium-size normal alleles.[149] Additionally, the negative correlation between AO and repeat size may be exacerbated by large normal repeats in other $(CAG)_n$-containing genes: this is the case with *ATXN7* in SCA2;[149] *ATXN2, ATN1* and *HTT* in MJD;[149,229,230] *ATXN1* and *ATXN3* in SCA6[149]; and *ATXN3* and *TBP* in SCA7.[149] HTT, TBP, and ATXN7 play a direct role in transcription and transcriptional regulation,[231-233] while ATXN1 and ATXN2 are implicated in RNA metabolism.[234,235] In MJD, population-specific factors are also involved in AO, and may account for up to 8.3% of variance: for the same size of the $(CAG)_n$ expansion, non-Portuguese Europeans have a significantly earlier onset than Portuguese or Brazilians.[228] This geographical influence may be attributed, to some extent, to specific *ATXN3* haplotypes and mutational origins, as different MJD populations exhibit varying haplotypic frequencies.[92,93,228] Still, further investigation is required to ascertain the relationship between $(CAG)_{exp}$ haplotypes and AO.[228]

In **recessive ataxias,** onset tends to occur in the 1st decade of life in ARSACS, AT, AOA, and ATX-*COQ8A*;[84-86,236] the 2nd decade in FA, AVED, and abetalipoproteinemia; [63,70,199]; the 3rd-4th decades in ATX-*SYNE1*;[209] the 4th decade for HSP/ATX-*PGN,* MxMD-*POLG* and ATX-*ANO10*;[236] and the 6th decade for *ATX-RFC1*.[109,236]

In FA, AO inversely correlates with the number of repeats in *FXN* alleles: onset occurs 2.3-2.7 years earlier for every 100 GAAs on the shorter allele.[194,195] No significant predictive value has been identified for the size of the larger allele.[194,195] In homozygotes with two alleles with the same expansion size, onset occurs 2.7 years earlier for every 100 GAA units increase.[194] Size of the smaller allele contributes only to 36-56% of variation in AO, while other potential factors include somatic mosaicism, other modifying genes, and environmental factors.[70,194,237] It has recently been identified that a 3' small interruption in the *FXN* GAA repeat, delayed onset by approximately nine years, when compared to patients without 5' or 3' interruptions.[238]

In ATX-*RFC1*, repeat size of both the smaller and larger alleles is a predictor of AO of gait unsteadiness.[239] Size of the shorter allele correlates also with onset of cough, while the larger allele had a marginal correlation with onset of sensory symptoms.[239]

Studies on factors modulating AO in other AR, mitochondrial and XL HCA are still scarce.

DISEASE PROGRESSION AND DISABILITY

In patients from the EUROSCA cohort, disease severity (evaluated with the scale for assessment and rating of ataxia (SARA))[240] at the first observation was influenced by size of the expanded allele, AO, and disease duration in **SCA1, SCA2, and MJD;**[148] and in **SCA6** only by AO and disease duration[148]

In the prospective phase of EUROSCA, progression was shown to be faster in SCA1, intermediate in SCA2 and MJD, and slower in SCA6 (annual increase of 2.11 points in SARA scores in SCA1, 1.49 in SCA2, 1.56 in MJD, and 0.80 in SCA6).[241] In SCA1, shorter follow-up, older age at inclusion, and larger expansions associated with faster progression.[241] Clinical progression of SCA2 was associated with shorter follow-up, earlier onset, and lower severity at baseline; in SCA6 it associated only with lower severity at baseline.[241] Intriguingly, no factors reflecting progression in the SARA scale were identified in MJD;[241] as the number of patients included was similar to other SCA, this could possibly be due to the high phenotypic variability of MJD. Another study assessed disease progression in SCA27B, as approximately 0.29 SARA points/year, slower than in the most frequent polyQ SCA.[189]

AO is one of the best predictors of progression of **FA**, with early-onset patients showing the most severe impairment on all clinical and functional measures.[194] Length of the smaller allele is more predictive of AO than of clinical progression.[195] Analysis of clinical features modulating progression identified axial functions (sitting posture, upright stability, and gait) as the major determinant of motor decline in the ambulatory phase.[195] When evaluating progression according to SARA, the overall increase was 1.49 points per year.[113] Still, a variation according to the total SARA score at baseline was observed, with patients who had a lower score at baseline having the highest annual increase: 1.60 points/year with a starting score of 10, and up to 0.14 points/year in patients with a starting score of 35.[242] This reflects a variation of the scale's sensitivity across different disease stages.[113,242] When comparing typical FA with early onset cerebellar ataxia (EOCA), disease progression was faster in the first case: wheelchair confinement occurred after a median progression of 11 years, while in EOCA this was 22 years after onset; approximately 75% of typical FA patients survived 34 years after onset, while disease duration was 41 years in EOCA.[243]

In other AR ataxias, the fastest disease progression was described for AT (1.4 SARA points/year), being slower for *ATX-RFC1* (0.9 SARA points/year), and ARSACS and HSP/ATX-*PGN* (0.6 SARA points/year, each).[236] After 10 years of disease progression, most patients with HSP/ATX-*PGN,* ATX-*ANO10,* ATX-*COQ8A* were still ambulatory, while

>40% of those with ARSACS, ATX-*SYNE1*, AOA2, and MxMD-*POLG* had lost independent gait.[236]

NON-CEREBELLAR MANIFESTATIONS

Non-cerebellar manifestations imply a further significant burden for patients with HCA, but may be under-recognized in clinical practice.[3,244,245]

In the EUROSCA cohort, pyramidal signs were more frequent in SCA1, and neuropathy in SCA2; in SCA6, non-cerebellar symptoms/signs were rare.[148] Movement disorders (myoclonus, rigidity, chorea/dyskinesias, and resting tremor) were rare in all SCA, except dystonia, present in 23.9% of MJD patients.[148] In SCA1, advanced age associated with more urinary dysfunction and cognitive deficit, than in other SCA; in SCA2, larger repeat expansions and early onset increased the likelihood of muscle atrophy and oculomotor disturbances; expansion size associated also with chorea and dystonia.[148] In MJD, larger expansions and earlier onset associated with spasticity and hyperreflexia; while later onset, older age and longer disease duration were related with peripheral neuropathy.[148]

Non-cerebellar signs progressed at a similar rate in SCA1, SCA2, and MJD, but faster than in SCA6.[241] In SCA1, paresis, muscle atrophy, urinary dysfunction, sensory symptoms, and oculomotor signs increased along follow-up. In SCA2, areflexia, spasticity, resting tremor, sensory symptoms, urinary dysfunction, and oculomotor signs increased over time. In MJD this occurred only for paresis and oculomotor signs. In SCA6, no increase of extracerebellar symptoms was observed with follow-up.[241]

Patients with typical FA had more non-cerebellar signs than later-onset patients. In the EFACTS cohort, the most frequent were sensory neuropathy (91.4%), abnormal eye movements (90.5%), scoliosis (73.7%), feet deformities (58.8%), urinary dysfunction (42.8%), and cardiomyopathy/ cardiac hypertrophy (40.3%).[246] Visual loss (36.8%), hearing loss (10.9%), and metabolic disorders (11.1%) were less frequent.[246]

In a large AR-HCA European cohort, including FA, only 4% of patients had a pure cerebellar ataxia.[236] Non-ataxia movement disorders occurred in 73%, pyramidal signs in 63%, neuropathy in 45%, and cognitive impairment in 35%.[113] For similar genotype, AO and disease duration, SARA score was higher in presence of motor deficit, cognitive impairment, altered vibration, urinary dysfunction, oculomotor signs, muscle atrophy, or spasticity.[113]

Motor symptoms in HCA have been the main focus of research for many decades. A greater attention has only recently been given to non-motor symptoms. Their spectrum is wide, with cognitive impairment (e.g., attention span, executive functions, episodic and semantic memory) described in SCA1, SCA2, SCA48, and MJD, among others;[247-249] sleep disorders were particularly reported in SCA2 and MJD;[244,245,250] and autonomic dysfunction has been observed in various forms of HCA (urinary problems in SCA2, SCA7, and MJD; gastrointestinal symptoms in SCA7; and thermoregulation impairment in SCA7 and MJD).[244,247]

H. Unsolved cases from the Portuguese population-based survey

In 2013, at the time of the original publication of the Portuguese population-based survey, 73 (36.6%) families (comprising 174 patients) with AD-HCA remained without specific diagnosis.[79] Most of those (43 families, 58.9%) had a pure cerebellar ataxia: in 28 the onset was with ataxia, in six with dysarthria, in another six with spasmodic cough, in one migraine, and in one with nystagmus.[79] Of the 30 (41.1%) with complex forms, two had a congenital ataxia; in the remaining, ataxia was associated with progressive external ophthalmoplegia in nine, dementia in seven, pyramidal signs in five, Parkinsonism in two, and other symptoms/signs in five patients.

In AR-HCA, 95 (38.0%) families (125 patients), had no definite genetic diagnosis.[79] Only four (4.2%) of those had a pure cerebellar ataxia, while 91 (95.8%) had a complex ataxia (22 had congenital ataxia, 25 with pyramidal signs, 14 with neuropathy, 12 with oculomotor apraxia, and 18 had other symptoms).[79]

Phenotypic organization into uniform groups, allowed the continued work of neurologists and researchers, leading to identification of the underlying genetic defect in several families. In AD families with pure cerebellar ataxia typically presenting with dysarthria, a complex intronic expansion in *DAB1* was described (SCA37).[251] *PNKP* biallelic mutations were for the first time associated with an AOA phenotype (AOA4), in patients ascertained in the survey[84] (adding to the previous discovery of AOA1 and 2,[85,86] during the years the survey was being conducted). CANVAS (ATX-*RFC1*), identified by *Cortese et al.* and *Rafehi et al.* (2019),[109,187] came to explain yet another clinically homogenous group of families, as previously established by Paula Coutinho (in fact, it was pseudodominance in those pedigrees, from an isolated region with a high frequency of consanguineous

mattings, that misled the researchers to believe those families had yet another “new” SCA, the gene for which could not be discovered at that time).

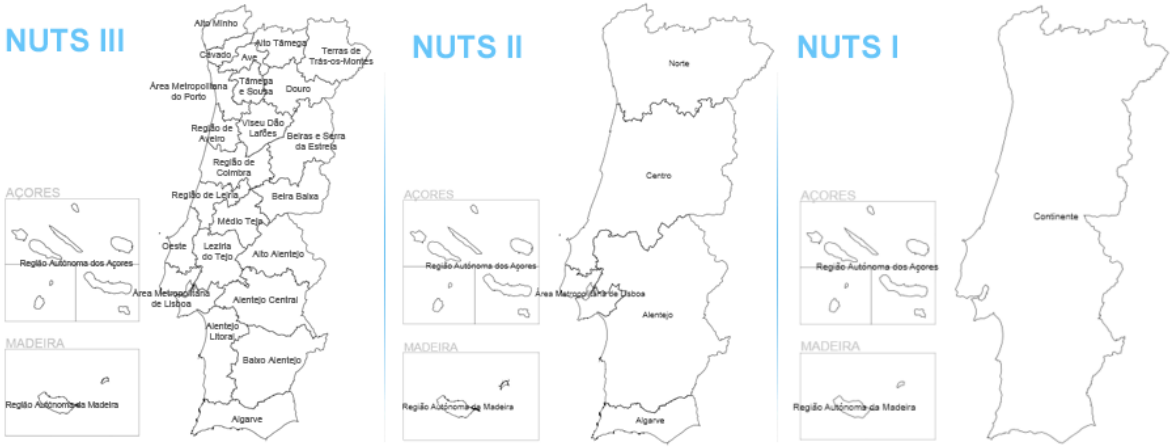
Still, several families remained without genetic diagnosis after the survey. With time, some DNA samples became degraded or insufficient for further studies. In all other families (with a sufficient amount and quality of DNA), clinical and genetic studies were carried out during this thesis work.

ADMINISTRATIVE DIVISION OF THE PORTUGUESE TERRITORY - NUTS

When the national survey was undertaken (1994-2004), the division of the Portuguese territory was made according to districts (18 in mainland, and 2 autonomous regions – Figure 2). In late 2003, following the European Union regulation number 1059/2003, the territories of all Member States were classified, for statistical purposes, in regions designated as NUTS (*nomenclature des unités territoriales statistiques*).[252] We adopted this for the present work.

The NUTS classification includes three levels (NUTS I, II and III) according to population, administrative and geographic criteria. NUTS I regions in Portugal include the Mainland, Autonomous Region of the Azores and Autonomous Region of Madeira. The Mainland is divided in 5 NUTS II regions: North (including Alto Minho, Cávado, Ave, Porto Metropolitan Area, Alto Tâmega, Tâmega e Sousa, Douro e Terras de Trás-os-Montes); Centre (including Aveiro, Coimbra, Leiria, Viseu Dão-Lafões, Beira Baixa, Médio Tejo, Beiras e Serra da Estrela); Lisbon Metropolitan Area (Lisbon Metropolitan Area); Alentejo (including Alentejo Litoral, Baixo Alentejo, Leziria do Tejo, Alto Alentejo, Alentejo Central); and Algarve. NUTS III regions of the Porto Metropolitan Area are Arouca, Espinho, Gondomar, Maia, Matosinhos, Oliveira de Azeméis, Paredes, Póvoa de Varzim, Porto, São João da Madeira, Santa Maria da Feira, Santo Tirso, Trofa, Vale de Cambra, Valongo, Vila do Conde, Vila Nova de Gaia.

Figure 2: NUTS Classification of the Portuguese territory



Source: Pordata

I. UNANSWERED QUESTIONS AND KNOWLEDGE GAPS

By the beginning of this work, many questions still remained to be answered in the field of HCA: from the physiological role of some wild-type proteins; pathogenic mechanisms underlying every HCA; the epidemiology of recently described HCA; difficulties in reclassifying VUS as benign *vs.* pathogenic; or understanding genetic modifiers and other factors that modulate disease progression; among many others.

We have identified some major questions in field of HCA, seen as critical by us, and tried to address them in this study:

- What is the frequency of recently described forms of HCA? Epidemiological data on classical HCA are well known in Portugal, but how has the introduction of NGS in clinical practice shaped further the genetic landscape of HCA in our country?
- Are the classical genotype-phenotype relationships still valid in this new era of massive parallel sequencing? Have the genotype-phenotype frontiers been fading? How have phenotype spectra evolved and expanded with new genetic knowledge?
- How frequent are, actually, non-cerebellar symptoms and signs in HCA? How do they reflect on disease natural history? How frequent are other (non-ataxia) movement disorders in HCA?
- Are HCA and hereditary spastic paraplegia distinct phenotypes, or just two ends of

the same spectrum? What is the phenotypical overlap between them and how frequent are mixed forms? Which genes are associated with spastic ataxia?

II. GENERAL OBJECTIVES

II. GENERAL OBJECTIVES

The objectives were set as the result of two complementary studies: (1) establishment of a new cohort of hereditary cerebellar ataxia based on the outpatient clinic; (2) follow-up and molecular diagnosis of patients who participated in the population-based survey, who remained without genetic diagnosis.

This thesis was the result of our clinical practice, guided by a detailed and structured observation. The ultimate goal was to contribute to the improvement of our patients' quality of life, by understanding the clinical features and progression of hereditary ataxias.

Thus, the main objectives were defined as follows:

1. Clinical characterization of symptoms and signs
   Detailed description of symptoms/ signs identified in cerebellar ataxias and analysis according to its type and genetic diagnosis. Analysis of symptoms/ signs at onset and during disease progression.

2. Frequency of non-cerebellar symptoms
   Evaluation of the frequency of non-cerebellar symptoms, present at onset and during disease progression.

3. Genotype-phenotype correlation
   Detailed characterization of the phenotypes identified in each genotype.

4. Disease progression
   4.1. Evaluation of cerebellar syndrome severity according to the SARA scale
   4.2. Disability milestones: characterization of the frequency and age at which the above-mentioned disability milestones were reached:
      4.2.1. Dysphagia
      4.2.2. Falls
      4.2.3. Loss of independent gait
      4.2.4. Loss of gait

III. SUBJECTS AND GENERAL METHODS

III. SUBJECTS AND GENERAL METHODS

Here, we describe the general methodology, underlying the overall project. The specific methods and patient material used for each study are detailed in the respective articles.

Methods of ascertainment The population was identified through two sources:

A. Patients from the national population-based survey (leaded by Prof Paula Coutinho, 1994-2004),[79] who had no genetic diagnosis until 2017 and who had genomic DNA available.

   Electronic databases, pedigree and patient files deposited at Centro de Genética Preditiva e Preventiva (CGPP), Instituto de Biologia Molecular e Celular (IBMC), Instituto de Investigação e Inovação em Saúde (i3S) - Universidade do Porto (UP) were accessed for this purpose. Contact with participants was made through their telephone number, referring family doctor or neurologist. Whenever the available contacts were outdated, a search on the electronic database of HSA-ULSSA and Registo Nacional de Utentes (RNU) was performed.

B. Patients observed at the Neurology Department of Hospital de Santo António – Unidade Local de Saúde de Santo António (HSA-ULSSA).

   A large number of patients was already being followed at the outpatient clinic for several years, some had taken part on the population-based survey. New referrals were performed by family doctors and neurologists from ULSSA, other hospitals from Serviço Nacional de Saúde (SNS) or private practice. Additional patients were identified after screening the families of probands for other affected relatives. Inclusion period lasted from September 2017 until December 2022.

Case definition All patients with hereditary ataxia were included, except those who were not able to provide informed consent and their legal tutors did not or were not able to consent on their behalf.

Study design All subjects were observed by the principal investigator at the Neurology outpatient clinic of ULSSA. A structured protocol was created for baseline evaluation (appendix 1), and follow-up every six months (appendix 2).

The baseline protocol comprised personal identification and medical history; for family history: family origin (defined as the origin of the oldest known family member affected), pedigree, identification of affected relatives, AO of the first symptoms and age at death of affected relatives. For patients it included: AO, age at first examination, age at genetic diagnosis, cognitive and motor development, symptoms/ signs at onset and through disease course (according to a list of cerebellar and non-cerebellar symptoms/ signs, and age when first identified), disability milestones (falls, use of cane, confinement to wheelchair) and strategies of treatment. Subsequent visits consisted of identification of new symptoms/ signs, disability milestones and (previous and ongoing) treatments. Magnetic resonance imaging (MRI), computed tomography (CT), electroencephalogram (EEG), electromyogram (EMG), evoked potentials, autonomic studies, muscle biopsy and blood tests were performed as per clinical judgement, and the results were registered in the protocol. At all visits, cerebellar symptoms severity was registered with SARA.[240] Also at all visits, non-cerebellar symptoms were registered according to the inventory of non-ataxia signs (INAS).[253] For movement disorders, a more detailed characterization was performed, particularly in the presence of Parkinsonism, where an observation was conducted according to the Unified Parkinson's Disease Rating Scale (UPDRS). Optional video recording was obtained, according to formal consent and a previously established script (appendix 3).

In patients from the national survey exclusively reached by telephone, only information that could be reliably obtained was included. In all cases (including those who could not be contacted), information obtained by neurologists and family doctors during the survey was transferred into the study protocol.

Phenotypic characterization *Age at onset* of the disorder was considered as the age of the first neurological symptom, regardless of its cerebellar or non-cerebellar nature, as observed or indicated by the patient or relative during the interview. Whenever the patient was being prospectively followed up since an asymptomatic stage, it was considered as the age at which the first symptoms were registered in clinical files. In patients whose symptoms had started before the first clinical observation ever, the information collected at the time of inclusion was checked with two first-degree relatives (whenever possible)

and clinical records. In case of discordance, possibly due to memory biases, clinical records information was preferred. AO of additional symptoms/ signs was considered according to the same strategy. As patients were observed every six months, all symptoms emerging after 2017 were prospectively registered.

Early onset was considered as onset before the age of 25 years, and *late onset* when starting at or after 25 years old (in line with the definition proposed by Palau and Espinós (2022)).[254]

Cerebellar signs were assessed through the SARA scale; and additional neurological signs registered according to INAS. *Pyramidal signs* were defined as hyperreflexia in four limbs or spasticity, or a Hoffman or Babinski sign. Spastic ataxia included only patients with overt spasticity (≥2 in modified Asworth scale).[255] *Epilepsy* was considered whenever the participant had two or more unprovoked seizures. *Movement disorders* (apart from ataxia) were classified according to the standards, and divided into hypokinetic (Parkinsonism) and hyperkinetic (tremor, dystonia, chorea, myoclonus, tics). The kinetic tremor was considered part of the cerebellar syndrome, while rest and postural tremor were registered under movement disorders. *Paroxysmal movement disorders or ataxia*, were considered whenever there were transient episodes of a single type of movement disorder or gait ataxia. *Motor delay* was defined when motor milestones occurred outside the 95% confidence interval according to World Health Organization, and *intellectual disability* as intellectual difficulties as well as difficulties in conceptual, social, and practical areas of living.[256] *Cognitive deterioration* included the loss of previously acquired cognitive skills. *Psychiatric symptoms* (anxiety, depression, psychosis) were considered as per clinical judgement.

Congenital cerebellar ataxia (CCA) was defined as a combination of developmental delay (motor, cognitive or both), and two or more additional neurological signs: oculomotor abnormalities, hypotonia or cerebellar signs (head or body titubation, dysarthria, nystagmus, tremor or ataxic gait).[191] Suspected CCA was defined as the combination of developmental delay and at least one of the above neurological signs.[191,257] For congenital ataxias, age at onset was considered as occurring before the age of 2 years (proposed by Palau and Espinós (2022)).[254]

Hemiplegic migraine was considered when in the presence of migraine with aura including fully reversible motor weakness and fully reversible visual, sensory and/ or speech/ language symptoms.

Severe dysarthria included scores of 5 or 6 on item “speech disturbance”, from SARA. *Dysphagia* was registered when occurring repeatedly and leading to burst of cough; solids and liquids dysphagia being registered independently. *Agraphia* was considered when the patients could no longer sign his/her name. *Falls* were when occurring repeatedly. *Loss of independent gait* corresponded to the use of cane (or stroller), while *loss of gait* to the use of wheelchair. *Age at occurrence* of the different milestones was recorded. *Age, cause and place of death* were documented, according to the medical records, if available, or as stated by first degree relatives, in the remaining cases.

Inheritance mode Dominant ataxias were considered as ataxia in patients with at least one affected relative in two or more generations (particularly if with male-to-male transmission); or as ataxia in patients with pathogenic variants in genes associated with dominant inheritance, independently of having or not other affected relatives (including isolated patients with presumably *de novo* pathogenic variants in genes associated with dominant inheritance). Variants were considered *de novo* if both parents had been studied and were not carriers. Whenever parents could not be tested and were described as healthy, *de novo* variants were considered if previously described as a recurrent cause of dominant ataxia. The transmitting parent was considered as the one having the same genetic diagnosis (either affected or presymptomatic). If a genetic diagnosis was not known within the family, the transmitting parent was defined as the one with a similar phenotype, either examined or described by at least two first degree relatives. In cases of early death (before disease onset), the transmitting parent was determined by the presence of the disease on his/ her ancestors. Whenever this approach was not sufficient, it was registered as unknown.

Recessive inheritance was determined when ataxic patients were present in the same generation, particularly when with parental consanguinity; or in cases with homozygous or compound heterozygous pathogenic variants in genes associated with recessive disorders.

Mitochondrial ataxias were defined as ataxia in patients with transmitting female relatives; or having pathogenic variants in genes from the mitochondrial DNA, independently of having or not other affected relatives.

An XL ataxia was considered when affected male patients were related through a carrier woman; or when carrying pathogenic variants in genes known to be associated with an XL ataxia.

Undetermined inheritance included cases with suspected affected relatives, either in the same or other generations, that could neither be observed nor the information be confirmed by at least two first degree relatives.

Geographic distribution of affected families Geographic origin was considered as the one of the most ancient family members affected by the disease, according to NUTS II or III classification.

Genetic tests A blood sample for DNA extraction was collected in patients who had not underwent genetic testing before. DNA (including from cases observed during the national) survey was stored at CGPP-IBMC, i3S biobank.

Single-gene test was performed in a few specific forms of HCA (e.g. for AD-HCA MJD due to the high prevalence in Portugal, SCA7 in the presence of retinopathy, ATX-*ITPR1* when congenital; for AR-HCA, FA or ATX-*RFC1* according to age of onset, phenotype and family history; for XL-HCA PCR and fragment analysis of *FMR1*). In the remaining probands, diagnostic strategy was set as the following:

1st line investigation

- PCR and fragment analysis of *ATXN1* (SCA1), *ATN1* (DRPLA), *ATXN2* (SCA2), *ATXN3* (MJD), *CACNA1A* (SCA6)*, ATXN7* (SCA7) and *TBP* (SCA17) genes, in patients with suspected dominant ataxia
- Next-generation sequencing (NGS) multigene panel for AOA (5 genes – *APTX, PIK3R5, PNKP, SETX, XRCC1*) whenever oculomotor apraxia was present
- NGS of whole-mitochondrial DNA in patients with suspected mitochondrial ataxias

2nd line investigation

- NGS panels, based on whole-exome sequencing (WES), either for dominant or for recessive ataxias, according to phenotype and pedigree. A (global) hereditary ataxia panel was performed whenever there were difficulties in defining the inheritance mode

3rd line investigation

- WES-based NGS panel for 1,411 genes associated with neurological disorders (neuroexome)

4th line investigation

- WES

All families from the national survey who remained without diagnosis, and whose DNA was still available, were studied directly through WES. Affected relatives of probands were studied through single gene testing.

Whenever the patients had no dominant neurological syndrome (i.e. ataxia was present, together with dystonia or pyramidal signs), neuroexome was performed as 1st line investigation.

Only pathogenic or likely pathogenic variants (according to the American College of Medical Genetics and Genomics, ACMG) were considered for diagnostic purposes. Variants of unknown significance (VUS) in which the bioinformatic analysis was suggestive of a deleterious effect were not considered for diagnostic purposes. The remaining variants (non-pathogenic or likely non-pathogenic) were registered but are not described.

Patients in whom a genetic diagnosis was not obtained through this strategy, were grouped according to phenotype, for future studies (including whole-genome sequencing, WGS).

Classifications The first branch used for ataxia classification was mode of inheritance. We then have subclassified according to the Movement Disorders Society (MDS) classification on genetic movement disorders,[104,140,141] but we have retained the adaptation from Palau and Espinos for recessive ataxias.[79,82,107] Also, for diseases such as Friedreich ataxia, MJD, SCA1 or SCA2, ARSACS, which are promptly recognized by its classical nomenclature, we used their original names instead. For genes not yet associated with an ataxic phenotype by the International Parkinson and Movement Disorder Society Task Force on Classification and Nomenclature of Genetic Movement Disorders, we used the suffix “-related ataxia” after the gene designation.

Complementary investigation MRI, CT scan, EEG, EMG, evoked potentials, autonomic studies, muscle biopsy, respiratory chain studies and additional blood tests were performed as per clinical judgement. Information was obtained for all patients, according to the routine reports.

Statistical analysis was conducted in collaboration with Departamento de Estudos de Populações of ICBAS, using IBM-SPSS version 29.[258]

Demographic data and analysis of phenotypic features were presented as frequency with percentage for qualitative variables. Mean (represented with standard deviation, SD) or median (represented with quartiles [Q1-Q3]) were used for quantitative variables, depending on data having or not a Normal distribution. Group comparisons were performed using the chi-squared test for qualitative variables; in small-size samples, the Monte Carlo method was adopted. For quantitative variables, when comparing two groups, the T student or Mann Whitney tests were used for Normal or non-Normal distribution. For comparisons of three or more groups, Analysis of Variance (ANOVA) or the Kruskal-Wallis test were used when variables had Normal or Non-normal distribution, respectively. Statistical tests were performed with a conventional two-tailed type I error of 0.05.

For each sub-study, specific methodologies and data analyses were adopted and are described in the respective articles.

Ethics The study was approved by *Comissão Nacional de Proteção de Dados* and the Ethics Committee from ULSSA (Appendix 4). Informed consent was obtained from all participants for clinical investigation, video-recording and genetic studies.

Two databases were created: one for demographic data and anonymization, only accessed by the principal investigator; another for clinical and genetic data, with cases identification through pseudonyms. Databases were stored at a computer located in Neurology Department of ULSSA, only accessed through identification and password, in a medical cabinet with restricted access (with the backup saved in a different computer with the same type of protection). DNA was stored at the biobank of CGPP-IBMC, i3S.

IV. RESULTS

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A. GENERAL CHARACTERIZATION OF THE COHORT

[Objective 1. Clinical characterization of symptoms and signs]
[Objective 3. Genotype-phenotype correlation]

The overall cohort was composed by 252 affected individuals (164 families): 62 patients (32 families) from the national survey[79] and 190 new patients (132 families). New patients' inclusion over the years occurred as follows: 106 patients from September 2017 until the end of 2018, 22 in 2019, 19 in 2020, 21 in 2021and 22 in the 2022. As for the 62 patients from the survey, 10 were re-evaluated (five through telephone interview and another five in person), 30 could not be reached (telephone numbers outdated and the referring physicians unreachable), and 22 had deceased (in 17 new clinical information could be retrieved from medical files).

Of the 164 families, 51 (31.1%) had forms with autosomal dominant, 91 (55.5%) with autosomal recessive and 5 (3.0%) with mitochondrial inheritance. In 17 (10.4%) families, without genetic diagnosis, we could not establish the inheritance mode more accurately.

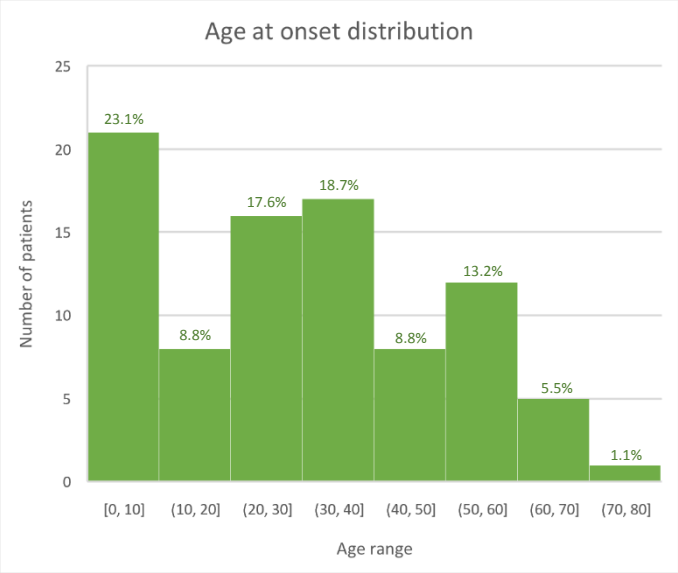
Age at inclusion was 48.0 [33.0-64.0] years; 110 males:142 females. Median follow-up of the overall cohort was, of 3.0 [1.0-5.0] years: 4.0 [2.0-5.0] for new patients and 0.0 [0.0-6.0] for those who had been identified during the survey (taking into consideration the large majority of these were observed only once); 37 (14.7%) patients died over the study course. Median disease duration was 27.0 [14.0-41.7] years, corresponding to the duration until the last visit or duration until death. Median age at death was 71.0 [IQR: 54.0-76.5] years, with overall survival after the first symptom being 35.6 (±15.1) years. The cause of death was known in 20 patients: infection in 19 and neoplasia in one.

FAMILIES WITH AUTOSOMAL DOMINANT INHERITANCE

Overall characterization of patients and families

There were 91 patients (53 females:38 males), belonging to 51 families with AD inheritance. With the exception of three, followed after pre-symptomatic genetic testing (two with MJD and one with SCA2), all other were already affected. In 13 (14.3%) there was no family history of neurological disorders. Age at first symptoms was 30 [11.5-43.75] years; when considering intervals for AO (Figure 3), even though there was a clear preponderance of adult onset, the interval with highest number of subjects was [0-10] years.

Figure 3: Age at onset distribution in AD-HCA



The majority (62, 70.5%) had a cerebellar onset, unsteadiness of gait being the most frequent presentation (45, 49.5%). When non-cerebellar onset, motor delay (8 (8.8%) patients with congenital ataxia) and hemiplegic migraine (6 (6.6%) patients from a family with *CACNA1A*-related ataxia) were the most common. Onset by cerebellar or non-cerebellar symptoms is shown on Figures 4 and 5.

Figure 4: Cerebellar symptoms at onset in AD-HCA

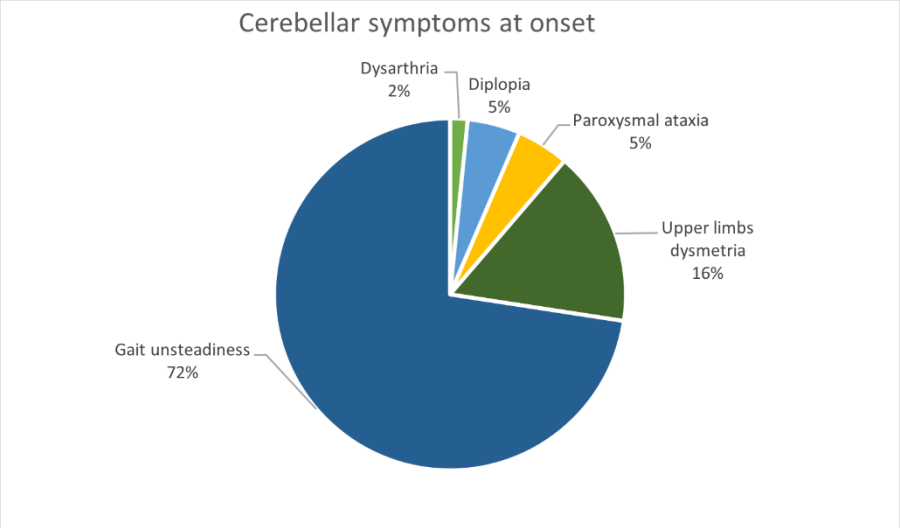
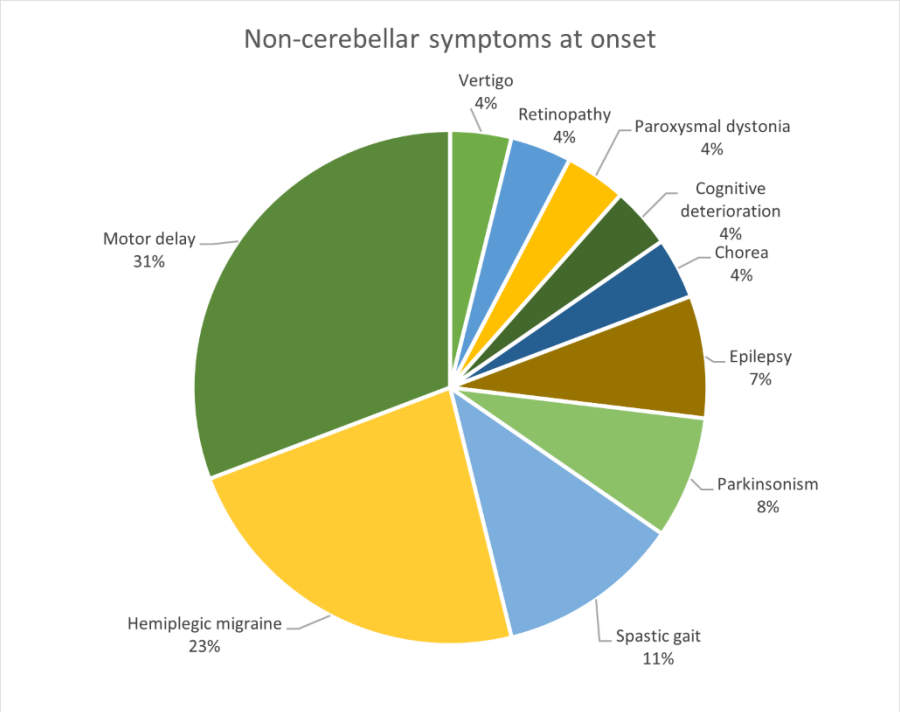


Figure 5: Non-cerebellar symptoms at onset in AD-HCA



Genetic diagnosis

A final diagnosis was achieved in 40 (78.4%) probands (77 total patients). It was obtained with single-gene testing in 20 (50.0%), PCR and fragment analysis (polyglutamine ataxias) in two (5.0%), multigene WES based panels in 18 (45.0%) (ataxia gene panel and neuroexome in nine patients each). Of the 11 (21.6%) probands without diagnosis, one was lost to follow up; all had been studied with PCR and fragment analysis of AD-HCA and 10 with multigene WES based panels. In three patients, variants of unknown significance (VUS) for which bioinformatics suggested a deleterious effect, were identified; these were not considered for diagnostic purposes.

Average time from onset up to genetic diagnosis was of 9.5 [4.0-25.2] years. MJD was diagnosed in 14 (35.0%) probands and ATX-*CACNA1A* ataxia in four (10.0%), one having an expanded CAG repeat (SCA6) and the remaining a point mutation. SCA2 was identified in three (7.5%) probands. With two (5.0%) probands each, *ATP1A3*-related ataxia [one with CAPOS (cerebellar ataxia, peripheral neuropathy and sensorineural hearing loss) syndrome, the other with paroxysmal dystonia and progressive ataxia], ATX-*ITPR1* (one congenital form, another adult-onset form), *ATX/HSP-KCNA2* and ATX-*PRKCG.* The remaining, ultra-rare, ataxias were each identified in one (2.5%) proband only (Table 3). Thirteen probands corresponded to isolated cases, in eight it was established the presence of *de novo* pathogenic variants.

Of the 11 probands who remained without genetic diagnosis, four had a pure cerebellar ataxia and the other a complex form (with pyramidal signs, myoclonus or neuropathy – Table 4).

When considering the total AD cohort, 12 had presymptomatic testing done [two (one family) with SCA2, 10 (five families) with MJD]: four had it performed at least five years before symptoms, eight after they experienced subtle symptoms.

Table 3: Genetic diagnosis of AD-HCA

	Number	
	Probands	**Total number of patients**
SCA type*		
MJD/ SCA3	14	29
ATX/PxMD-CACNA1A	4	11
SCA2	3	10
ATP1A3-related ataxia	2	4
ATX-*ITPR1*	2	3
ATX/HSP-*KCNA2*	2	3
ATX-*PRKCG*	2	2
ATX-*KCNC3*	1	4
ATX-TMEM240	1	2
SCA7	1	1
ATX-*ELOVL4*	1	1
ATX-*GRM1*	1	1
ATX-*STUB1*	1	1
HSP-*SPAST*	1	1
CTNNB1-related ataxia	1	1
HSP-*KIF1A*	1	1
KMT2B-related ataxia	1	1
SYNGAP1-related ataxia	1	1
Total	40	77

Legend: * adapted from the MDS classification.

Table 4: Phenotypic classification of AD-HCA patients without genetic diagnosis

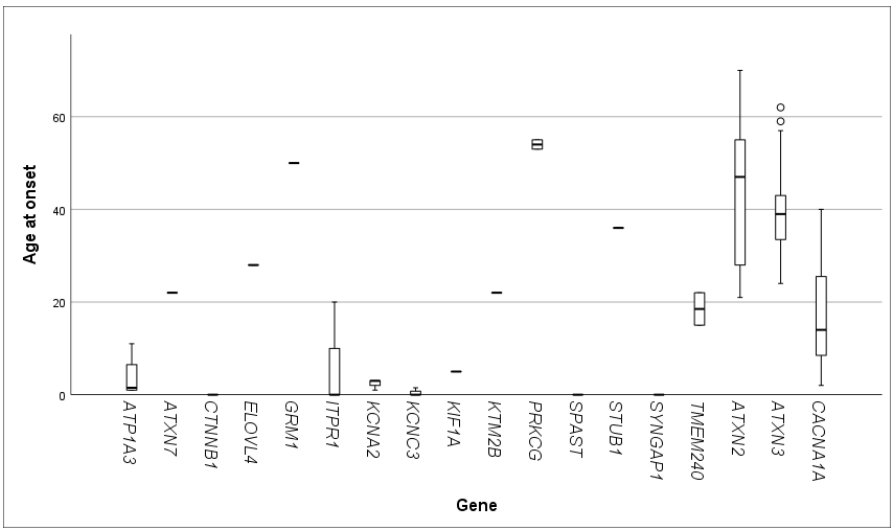
	Number	
	Probands	**Total number of patients**
Pure cerebellar ataxia	4	5
Complex cerebellar ataxia		
With pyramidal signs	3	5
With movement disorders	3	3
With neuropathy	1	1
Total	11	14

Onset according to genetic diagnosis

Age at onset

Onset in MJD occurred by 39.0 [33.0 - 43.0] and in SCA2 by 47.0 [25.5-57.0] years (Figure 6). In ATX/PxMD-*CACNA1A*, mean AO was by 14.0 [7.0-32.0] years, ranging from early to late-onset forms. Both *ATP1A3*-related ataxia and ATX-*KCNC3* had exclusively paediatric onset, the first at a mean of 1.5 years [1.0-8.75], the latter 0.0 [0.0-1.13] years. For ATX-*ITPR1*, two patients had congenital onset, while one had early-adulthood onset. In *ATX-TMEM240* it occurred in late adolescence or early adulthood. The remaining, less frequent ataxias, are depicted in Figure 6.

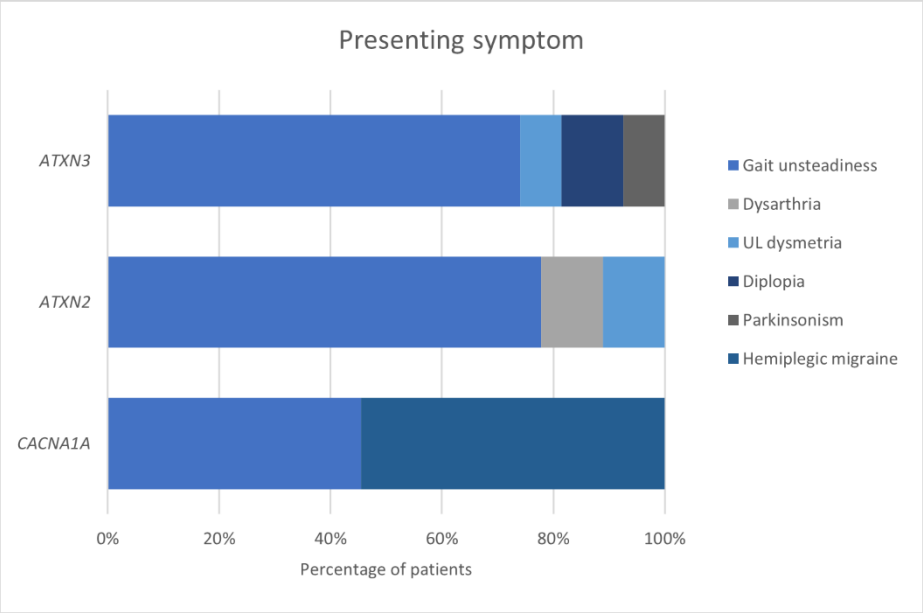
Figure 6: Age at onset by gene in AD-HCA



Presenting symptom

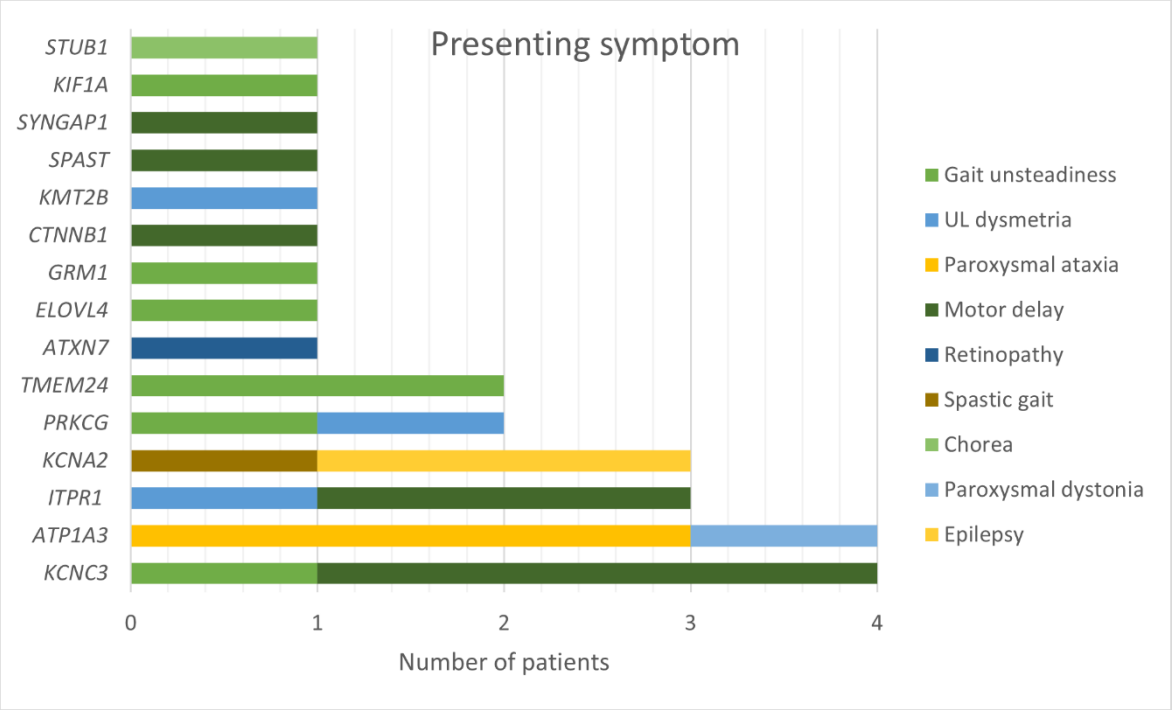
When analysing the first symptom, for which we had at least five patients per gene (Figure 7), gait unsteadiness was the most frequent in MJD (20, 74%) and SCA2 (7, 77.8%). Diplopia was the onset symptom in three (11.1 %) MJD patients, and Parkinsonism and upper limb dysmetria in two (7.4%) each. In SCA2, dysarthria and UL dysmetria were each the first symptom in one (11.1%) patient. In ATX-*CACNA1A*, hemiplegic migraine was the most common presentation (6, 54.5%), followed by gait unsteadiness (5, 45.4%).

Figure 7: Presenting symptom in AD-HCA with 5 or more patients per gene



Ataxias with less than five patients per gene had a more variable presentation. In ATX-*KCNC3*, three (75%) patients presented with motor delay, while one (25%) did with gait unsteadiness. Patients with *ATP1A3*-related ataxia had paroxysmal onset: three (75%) with ataxia and one (25%) with dystonia. In ATX-*ITPR1*, the two (66.6%) with congenital onset had motor delay, while the one (33.3%) with adult-onset showed UL dysmetria. In patients with ATX/HSP-*KCNA2*, presentation was by epilepsy in two (66.6%) and spastic gait in one (33.3%). Type of onset was homogenous within families. Presenting symptoms in less frequent ataxias are depicted in Figure 8.

Figure 8: Presenting symptom in AD-HCA with < 5 patients per gene



Phenotypic variation

Early-onset dominant ataxias

Thirty-six patients (39.6%), had onset before age 25 years; 13 (36.1%) had onset in the first two years of life, eight (22.2%) onset between 2-10 years, and 15 (41.7%) between 11-24 years (Table 5).

In 33 (91.7%) a genetic diagnosis was obtained: in 4 (12.1%) an oligonucleotide expansion, in 29 (87.9%) a conventional mutation. Genes involved in early-onset AD-HCA included *CACNA1A* (eight patients, 24.2%), *KCNC3* and *ATP1A3* (four patients each, 12.1%), with the less frequent genes being depicted in Table 5. *ATP1A3, CTNNB1, ITPR1, KCNA2, KCNC3, KIF1A, SPAST* and *SYNGAP1* were identified only in patients with early-onset. Time to diagnosis was of 21.5 [15.0-34.7] years. Twelve (33.3%) patients were isolated cases.

The majority of patients with onset in the first two years of life had motor delay as presenting symptom, except one with epilepsy and three with paroxysmal ataxia. For the remaining onset ages, paroxysmal symptoms (either hemiplegic migraine, epilepsy or paroxysmal dystonia/ ataxia) were more frequent in the youngest patients, while the frequency of cerebellar symptoms at onset increased with age (Table 5).

When comparing early-onset AD ataxias with late-onset AD ataxias (Table 6), the first were significantly associated with non-repeat expansion genetic defects, while the latter with oligonucleotide expansions ($p<0.001$). A longer time to diagnosis was observed in this group. In spite of early-onset having a significantly longer disease duration at baseline visit, there was no difference in total SARA score between groups. The use of cane occurred at a shorter disease duration in the late-onset group.

Table 5. Early-onset AD-HCA

Disorder[1]	Families (N)	Subjects (N)	*De novo* variants	AO (Y)	AD (Y)	First symptom/sign (N)	Ataxia (N)	AAO (Y)	Developmental milestones (M)
Onset < 2 year									
CTNNB1-related ataxia	1	1	Yes (1)	<2	18.0	Motor delay (1)	1	2.0	Gait: 24 Language: 24
ATX-*ITPR1*	1	2	Yes (1)	<2	27.0 / 59.0[φ]	Motor delay (2)	2	5.0 / 1.0[φ]	Gait: 72-108 Language: 24
ATX-*KCNC3*[α]	1	4	No	<2	22.5 [11.2-36.0]	Motor delay (4)	4	2.0 [1.6-2.7]	Gait: 24-36 Language: 24-36
HSP-*SPAST*	1	1	Yes (1)	<2	32.0	Motor delay (1)	1	2.0	Gait: 54 Language: 12
SYNGAP1-related ataxia	1	1	Yes (1)	<2	24.0	Motor delay (1)	1	3.0	Gait: 36 Language:60
ATX/HSP-*KCNA2*[§]	1	1	Yes (1)	<2	51.0	Epilepsy (1)	1	45.0	Normal
ATP1A3-related ataxia	1	3	Yes (1)	1.0 [1.0-][β]	21.0 [17.0-][β]	Paroxysmal ataxia (3)	3	3.0 [2.0-][β]	Normal
Onset 2-10 years									
ATX-*CACNA1A*[§]	1	4	No	7.0 [3.3-9.3]	22.5 [19.8-40.3]	Hemiplegic migraine (4)	4	16.0 [14.3-29.8]	Normal
ATX/HSP-*KCNA2*[§]	2	2	Yes (1)	3.0/ 3.0[φ]	29/ 37[φ]	Epilepsy (1) Spastic gait (1)	2	7.0y/ 5.0y[φ]	Normal
HSP-*KIF1A*	1	1	Yes (1)	5.0	41.0	Gait unsteadiness	1	5.0	Normal
NA	1	1	-	6.0	-	UL dysmetria	1	6.0	Normal
Onset 11-24 years									
ATX-*CACNA1A*[§]	2	4	Yes (1)	14.5 [13.3-18.0]	35.0 [29.8-37.3]	Gait unsteadiness (1) Paroxysmal dystonia (1) Hemiplegic migraine (2)	3	16.5 [13.3-19.0]	Normal
KMT2B-related ataxia	1	1	Yes (1)	22.0	27.0	UL dysmetria	1	23.0	Normal
ATX-T*MEM240*	1	2	UNK	15.0/ 22.0[φ]	76.0/ 43.0[φ]	Gait unsteadiness (2)	2	15.0/ 22.0[φ]	Normal
ATP1A3-related ataxia	1	1	Yes (1)	11.0	44.0	Paroxysmal dystonia	1	15.0	Normal
ATX-*ITPR1*	1	1	Yes	20.0	64.0	UL dysmetria	1	38.0	Normal
SCA2	1	2	No	21.0/ 23.0[φ]	29.0/ 28.0[φ]	UL dysmetria (1) Gait unsteadiness (1)	2	25.0/23.0[φ]	Normal
MJD/SCA3	1	1	No	24.0	33.0	Gait unsteadiness	1	24.0	Normal
SCA7	1	1	No	22.0	23.0	Retinopathy	1	23.0	Normal
NA	2	2	-	14.0/ 23.0[φ]	-	UL dysmetria (1) Gait unsteadiness (1)	2	14.5/ 23.0[φ]	Normal

Legend: AAO – Age at ataxia onset, AO – Age at onset, AD – Age at diagnosis, M – Months, N – Number, UL – Upper limbs, UNK – Unknown, Y – Years, [1] – Adapted from MDS classification, [α] Members of the same family, [§] Members of the same family, [-] * - Mild cognitive impairment, [φ] - Individual data from each patient, [β] - No data from quartile 3

Table 6. Comparison between early-onset and late-onset AD-HCA

	Early-onset AD-HCA	Late-onset AD-HCA	p value
Number of patients	36 (39.6%)	55 (60.4%)	
Male gender	12 (33.3%)	26 (47.3%)	0.201
Age at onset	6.5y [1.0-18.0]	40.5y [34.0-55.0]	**<0.001**
Disease duration at baseline (years)	26.0y [15.5-34.7]	10.0y [5.0-21.7]	**<0.001**
Overall disease duration (years)	28.0y [19.0-38.7]	14.0y [8.2-26.7]	**<0.001**
Time until diagnosis	21.5 [15.0-34.7]	6.0 [1.0-10.0]	**<0.001**
Onset symptoms/signs			
Cerebellar	17 (47.2%)	45 (86.5%)	**<0.001**[a]
Non-cerebellar	19 (52.8%)	7 (13.5%)	
Type of genetic defect			
Oligonucleotide expansion	4 (12.1%)	37 (90.2%)	**<0.001**[b]
Conventional mutation	29 (87.9%)	7(19.4%)	
Clinical severity			
SARA at baseline	14.0 [8.7-15.0]	11.2 [6.7-18.9]	0.682
Disability milestones			
Presence of falls	16 (44.4%)	34 (61.8%)	0.103
Disease duration until falls	7.5y [3.7-27.1]	8.5y [2.7-14.5]	0.453
Use of cane	11 (30.6%)	20 (36.4%)	0.568
Disease duration until cane	19.0y [12.0-44.0]	11.5y [7.5-17.7]	**0.028**
Use of wheelchair	4 (11.1%)	12 (21.8%)	0.190
Disease duration until wheelchair	17.5y [9.2-51.0]	17.5y [8.7-22.5]	0.903

Legend: Y – Years; [a] – Significant association of non-cerebellar onset with early-onset (adjusted standardized residuals value of 4.0), and of cerebellar onset with late-onset (adjusted standardized residuals value of 4.0); [b] - Significant association of conventional mutations with early onset (adjusted standardized residuals value of 6.3) and oligonucleotide expansion with late onset (adjusted standardized residuals value of 6.3).

Isolated cases

Isolated cases refer to patients without family history of neurological disorders, initially investigated for recessive ataxias. Thirteen isolated cases were identified in the cohort, one diagnosed through ataxia multigene panel, the remaining through neuroexome. In eight patients *de novo* pathogenic variants were identified on genes associated with AD inheritance. Implicated genes in *de novo* cases were: *ATP1A3*, *CACNA1A, CTNNB1, KCNA2, KIF1A, KMT2B, SPAST, SYNGAP1*. In the remaining isolated cases the implicated genes were *ITPR1* (two patients), ATP1A3, *CACNA1A, KCNA2* (one patient each).

Article 1 describes a patient with a complex phenotype, where cerebellar ataxia is the predominant feature, who bears a *de novo* pathogenic variant in *KMT2B*. Besides a detailed clinical characterization, functional studies of the identified variant were carried out.

Article 2 concerns a patient, also with a complex phenotype, of a congenital ataxia progressing to spastic-ataxia. This article was written as a reply letter, reinforcing the complex phenotypes associated with *de novo* variants in *SPAST*.

Article 1. Novel KMT2B mutation causes cerebellar ataxia: Expanding the clinical phenotype

Damásio J, Santos M, Samões R, Araújo M, Macedo M, Sardoeira A, Cavaco S, Freitas J, Barros J, Oliveira J, Sequeiros J.
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SHORT REPORT

CLINICAL GENETICS WILEY

Novel *KMT2B* mutation causes cerebellar ataxia: Expanding the clinical phenotype

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Abstract

Hereditary cerebellar ataxias comprise a heterogeneous group of neurodegenerative disorders affecting the cerebellum and/or cerebellar pathways. Next-generation sequencing techniques have contributed substantially to the expansion of ataxia-causing genes, including genes classically described in alternative phenotypes. Herein, we describe a patient with adult-onset cerebellar ataxia, minor dystonia, neuropathy, seizure and ophthalmological pathology, who bears a novel variant in *KMT2B* (NM_014727.2:c.3334 + 1G > A). Bioinformatic analysis suggested this variant completely abolished the splice-site at exon 8/intron 8, which was confirmed through analysis of mRNA extracted from fibroblasts. Exon 8 skipping would ultimately translate as an in-frame deletion at the protein level, corresponding to the loss of 91 aminoacids [p.(Gly1020_Asn1111del)]. So far, *KMT2B* disease causing variants have been described in patients with dystonia or neurodevelopmental delay, with no reports of a cerebellar predominant phenotype. Our findings highlight the possible role of *KMT2B* as a gene involved in hereditary cerebellar ataxias.

KEYWORDS
cerebellar ataxia, dystonia, genetics, *KMT2B*

1 | INTRODUCTION

Hereditary cerebellar ataxias (HCA) comprise a clinical and genetic heterogeneous group of neurodegenerative disorders, affecting the cerebellum and/or cerebellar pathways. Clinically, HCA are characterized by dysarthria, dysmetria and disequilibrium, either in isolation or in combination with other signs. The most recent technologies, particularly next-generation sequencing (NGS), contributed to increase the number of disease-causing genes identified.[1] Furthermore, there has been an expansion of several phenotypes originally recognized as "non-cerebellar", to include cerebellar ataxia (e.g., *SPG7*, *PLA2G6*).[1]

Pathogenic variants in *KMT2B* (OMIM *606834) were described in 2016, in patients with generalized early-onset dystonia (DYT28,OMIM #617284).[2,3] 5 years later, over 100 patients have been reported,[4] with an estimated frequency of 21.5% in a childhood-onset dystonia cohort.[5] Most patients had lower limbs onset, progressing to generalized dystonia in a short period of time.[4–6] Neurodevelopmental delay, in the absence of dystonia, was subsequently described.[4,7–9]

Recently, we identified a novel pathogenic variant in *KMT2B* in a patient from our cohort of HCA, further expanding the spectrum of *KMT2B*-related disorders.

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2 | MATERIAL AND METHODS

2.1 | Subject

The patient participated on a prospective study on HCA, approved by the Ethics Committee, evaluated since 2016 through a protocol (supporting information). Informed consent was obtained for investigation, video and genetic studies.

2.2 | Whole-exome and Sanger sequencing

Whole-exome sequencing (WES) was performed according to manufacturer's recommendations and 92.95% of the targeted regions had a 20× or higher coverage. Data analysis was executed in accordance with Broad Institute's guidelines. Sanger sequencing was used to confirm variants identified by WES and segregation analysis (supporting information).

2.3 | Bioinformatic and gene expression analysis

An in silico analysis to predict potential effect in the donor splice-site, using different bioinformatics tools, was performed (supporting information). A skin punch biopsy was carried out in the patient to obtain fibroblasts; a sample of human dermal fibroblasts from gender-matched control was used for comparison. Total RNA was extracted from cultured fibroblasts, using NZYol (Nzytech/Portugal), followed by purification of the RNA aqueous phase using the RNeasy mini kit (Qiagen/Germany). RNA quantification was performed on NanoDrop 2000 (Thermo Scientific). cDNA was synthesized by reverse transcription-PCR of 1.5 μg total RNA with oligo(dT), using SuperScript III first-strand synthesis system (Invitrogen). The cDNA obtained was amplified by PCR, using a set of primers: the forward primer aligning on exon 6 (5′-GAGAATCAGAGCCCACAGGT-3′) and the reverse primer aligning between exon 11/12 (5′-GAACACCAG CTCGTGGAGTC-3′) of *KMT2B* cDNA. PCR products were purified with Exo/SAP Go (GRiSP/Portugal) and sequenced by Sanger sequencing, using Big Dye Terminator Cycle Sequencing v1.1 (Applied Biosystems) and ABI 3130xl Genetic Analyzer (Applied Biosystems). PCR products were loaded on agarose gel for extraction with Zymoclean Gel DNA Recovery Kit (Zymo Research), and the resulting products sequenced as described above.

3 | RESULTS

3.1 | Clinical characterization

The patient is a 30 years-old daughter of non-consanguineous parents, with unremarkable neonatal period and developmental milestones. At age 8 years, myopia was diagnosed. She completed 12 years of schooling, got a driving license, and worked as a waitress. She had been an anxious adolescent and developed a depressive syndrome, by the time of her first job. At age 22, difficulties in precise tasks were noticed, and 1 year later, her gait became unsteady. When observed (age 25, video S1), a mild scandid dysarthria was noted. Pursuit eye-movements were fragmented and incomplete, on horizontal and vertical planes; saccades were slow and hypometric. She had mild lower limbs spasticity, brisk deep-tendon reflexes, unsustained ankle clonus, and indifferent plantar responses. Mild cervical dystonia was observed, as well as fingers and toes dystonic posturing. Upper and lower limbs dysmetria was clear. While writing, an abnormal posture emerged, with wrist extension and fingers flexion. Gait was mildly broad-based, with difficulties in tandem. She had elongated face, frontal haemangioma, and bulbous nasal tip. By age 26, there was an episode of clonic movements involving the left arm, followed by eye deviation, loss of awareness and tonic posture of left limbs. At 5 years follow-up, lower limbs dysmetria and gait ataxia worsened (SARA score: 15.5), other deficits remaining stable (video S2).

Brain MRI was normal at age 25 (Figure 1(A)-(E)). 5 years later, cerebellar atrophy, affecting mostly the vermis and hemisphere's superior sulci, was present, as well as mild midbrain and bulbar atrophy (Figure 1(F)-(G)). EEGs disclosed a posterior dominant rhythm at 7–8 Hz, irregular and moderately sustained; diffuse theta activity and bilateral bursts of delta activity with maximum on frontal regions, as well as bilateral bursts of delta activity with maximum on posterior regions (Figure 2(A)-(B)). EMG revealed absent sensitive potentials on right superficial peroneal nerve, and reduced amplitude with normal sensitive conduction velocities (SCV) on left superficial peroneal nerve. Bilateral sural and left cubital nerves showed reduced potential amplitudes and normal SCV. Motor conduction studies were normal. Neuropsychological evaluation disclosed significant difficulties in psychomotor speed, semantic/phonemic fluency, and verbal memory. Temporal–spatial orientation, focal attention for auditory information, naming, verbal repetition, visual–spatial perception, visual-construction, visual memory, abstract reasoning, and sequence switching were normal (supporting information). Ophthalmological examination showed a corrected visual acuity of 8/10; optic discs with sharp edges, temporal pallor, and normal retina. On pattern reversal visual-evoked potentials, P100 waves presented bilaterally increased latency (Figure 2(C)); pattern electroretinogram disclosed a reduction of N95: P50 ratio, with normal P50 (Figure 2(D)). Bilateral thinning of retinal nerve fiber layer (RNFL) was identified through optical coherence tomography (Figure 2(E)).

3.2 | Genomic and gene expression analysis

A NGS panel, based in WES, was performed to screen for variants at *loci* related to recessive ataxias, with no disease-causing variants identified. Ataxias caused by repeat expansions have been excluded, and genetic analysis was expanded to 1411 genes associated with neurological diseases: a novel variant, NM_014727.2:c.3334 + 1G > A, was identified in *KMT2B*. Sanger sequencing was performed in the patient and both parents, establishing the variant as de novo.

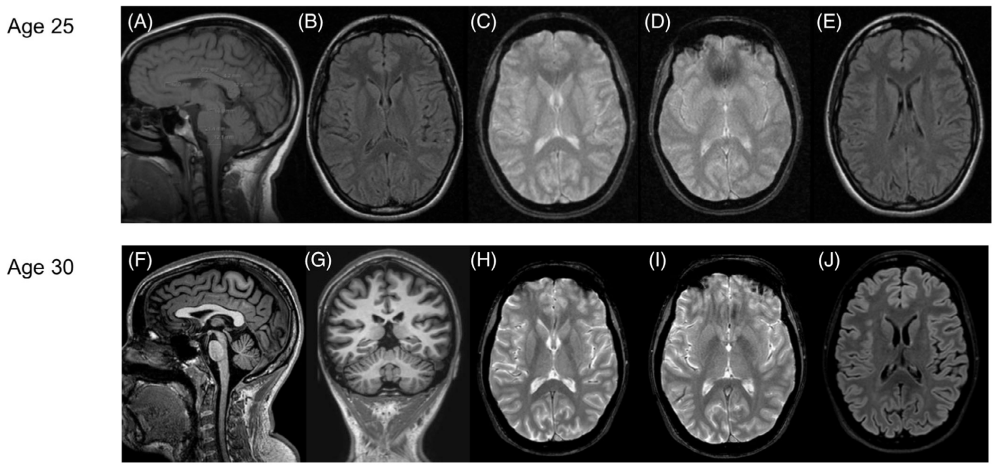

FIGURE 1 Brain MRI (ages 25 and 30). (A) Cerebral hemispheres, brainstem, cerebellum without atrophy; (B),(C),(D),(E) Normal basal ganglia and white matter signal; (F) Vermis, midbrain and bulbar atrophy; (G) Cerebellar hemisphere's superior sulci atrophy; (H),(I),(J) Normal basal ganglia and white matter signal

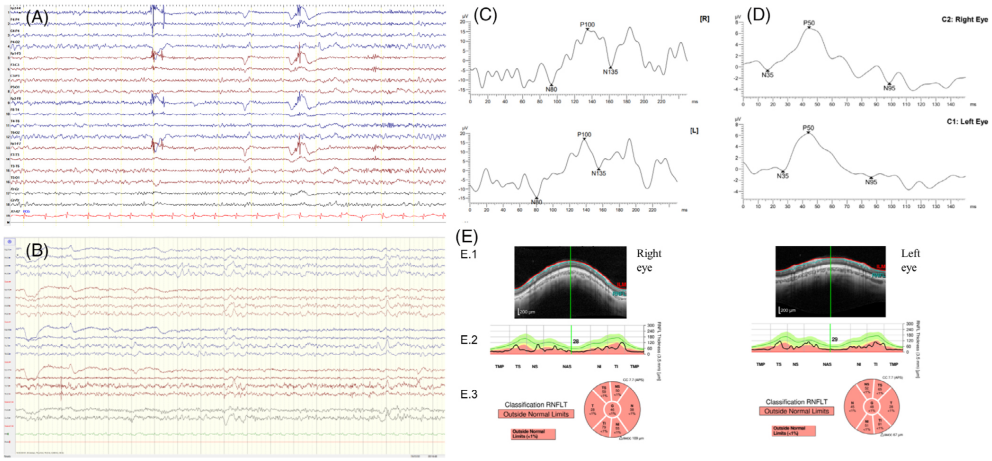

FIGURE 2 EEG and ophthalmological exams. (A) Posterior dominant rhythm at 7–8 Hz, irregular and moderately sustained; (B) Diffuse theta activity and bilateral bursts of delta activity with maximum on posterior regions. (C) Pattern reversal visual-evoked potentials–Bilaterally increased P100 latencies; (D) Pattern electroretinogram–Normal P50 amplitude, reduced N95:P50 ratio; (E1) Optical coherence tomography of the optic disc, with thinning of retinal nerve fiber layer (RNFL); (E2) Graph representing normal RNFL thickness values in green, borderline in yellow and abnormally low in red, black line representing the patient's measurements; (E3) Circle graph representing the patient's RNFL values on red, translating abnormally low parameters (G–global, ILM–inner limiting membrane, L–Left, N/NAS–nasal, NI–nasal inferior, NS–nasal superior, R–right, T/TMP–temporal, TS–temporal superior, TI–temporal inferior) [Colour figure can be viewed at wileyonlinelibrary.com]

To evaluate the potential effect of this variant on splicing, we performed RT-PCR on mRNA extracted from patient's fibroblasts. Sanger sequencing showed the canonical transcript (molecular weight of about 851 bp) and one lacking exon 8 (about 576 bp) (Figure 3(A)-(B)). On control fibroblasts, only the canonical transcript was detected (Figure 3(A)-(B)): the novel splice variant in *KMT2B* lead to exon 8 skipping, resulting in an in-frame deletion at the protein level [p.(Gly1020_Asn1111del)] (Figure 3(C)).

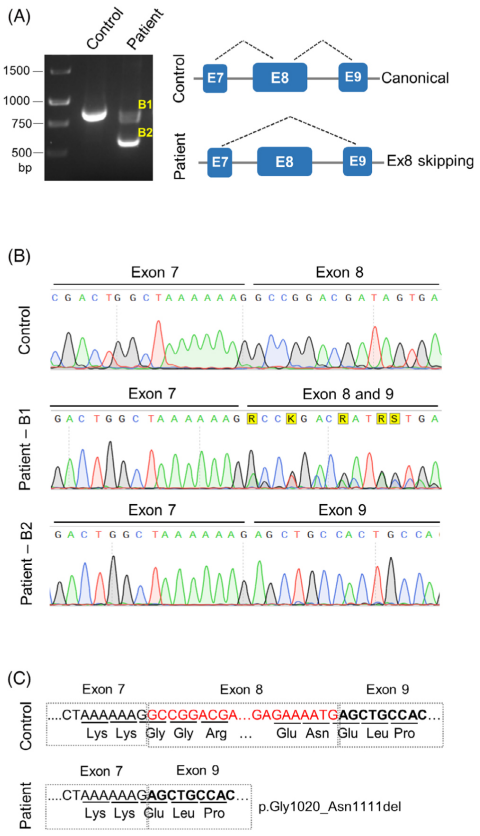

FIGURE 3 Functional analysis of *KMT2B* c.3334 + 1G > A variant affecting a donor splice-site. (A) Agarose gel electrophoresis of the transcripts generated by cDNA amplification of the patient and control, with adjacent schematic representation of the resulting splicing events. B1 band includes both the canonical and the exon 8 skipping transcripts (confirmed by Sanger Sequencing) that migrate almost together (with molecular weight of about 851 bp, as expected for the canonical transcript), probably due to conformational changes, in the patient. B2 band represents the exon 8 skipping transcript with an expected molecular weight of 576 bp. (B) Sanger sequencing electropherogram of gel-purified fragments. (C) Representation of the effect of the splice-site variant for encoded KMT2B protein [Colour figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

We describe a 30 years-old female with cerebellar ataxia since early adulthood, who bears a novel disease-causing variant in *KMT2B*. Until now, *KMT2B*-related disorders included generalized dystonia and neurodevelopmental delay.[2,3,5] Additional signs comprised abnormal eye movements or microcephaly; and less frequently, pyramidal signs, myoclonus, Parkinsonism or epilepsy.[3–6] Non-neurological features encompassed foetal growth restriction, neonatal feeding problems, dysmorphism, short stature, psychiatric, dermatological, and endocrine disorders.[4–6]

Our patient presented some of the recognized traits in *KMT2B*-related disorders, as an elongated face, nasal bulbous tip, abnormal eye movements, and mild psychiatric symptoms. Pyramidal signs and seizures, although less frequent, have also been previously identified.[2–6] Ataxia was described as an additional feature in one patient with generalized dystonia,[4] with no reports of a "cerebellar-prevailing phenotype". Our case presented dystonia, but the cerebellar syndrome clearly overweighed it in terms of severity. These two patients had only in common the fact of being term gestations, with normal neonatal period and psychomotor development. Sensitive neuropathy has not been described thus far, but may be subclinical and under recognized. Ophthalmological defects comprised mostly refractive errors[4] and, in one case, retinal dystrophy.[3] Optic nerve and inner retina dysfunction are first described here. On a repeated MRI, it was possible to document cerebellar/brainstem atrophy and, interestingly, vermis hypoplasia/brainstem asymmetry had already been reported in three, non-ataxic, dystonic patients.[4,5]

KMT2B encodes for a histone lysine methyltransferase, involved in methylation of histone H3, at lysine 4 (H3K4), a relevant epigenetic marker linked to active gene transcription.[3] It is highly expressed in the cerebellum,[3] which should, in theory, be associated with a higher frequency of patients with ataxia.[4,5] Interestingly, *KMT2B* is also fairly expressed in the retina (https://www.proteinatlas.org/ENSG00000272333-KMT2B/tissue) and peripheral nerves (https://gtexportal.org/home/gene/KMT2B), which might explain the retinal pathology and sensory neuropathy in our patient.

Patients with chromosomal microdeletions (involving *KMT2B* and other genes) or truncating variants have, as expected, a more complex phenotype, than those with missense variants.[4] In fact, *KMT2B* haploinsufficiency is the main mutational mechanism associated to dystonia.[2] The novel c.3334 + 1G > A variant identified affects the donor splice-site at exon 8/intron 8. Bioinformatics analysis suggested that it completely abolishes this splice-site, what could result in exon 8 skipping during pre-mRNA maturation, as no other alternative donor splice-site is located nearby. Indeed, we confirmed this by analysis of the mRNA in fibroblasts. Exon 8 skipping may ultimately translate as an in-frame deletion at protein level, with a loss of 91 aminoacids [p.(Gly1020_Asn1111del)]. The extremely low frequency of variants affecting donor/acceptor splice site in *KMT2B* (http://www.ensembl.org/Homo_sapiens/Transcript/ProtVariations?db=core;g=ENSG00000272333;r=19:35717973-35738878;t=ENST00000420124) further corroborates the pathogenicity of the c.3334 + 1G > A variant. To date, only six other *KMT2B* variants affecting splicing have been reported as causing disease: c.3058 + 1G > A (intron 7); c.3528 + 2 T > A (intron 10); c.3642 + 5G > A (intron 11); c.7050-2A > G (intron 29); c.7297 + 1G > A and c.7298-1G > A (both intron 31).[2–4] It is difficult to establish a correlation between this type of variants and a particular phenotype, given their dispersion along the gene and the diversity of possible effects. Furthermore, only one in-frame disease-causing deletion

[c.5405_5428del24, p.(Gln1802_Ala1809del)] has been identified in *KMT2B*.[5] Considering these uncertainties, a gain-of-function may be associated to c.3334 + 1G > A variant. The predicted in-frame deletion at protein level, could give rise to a KMT2B conformational change causing a pathological effect primarily on the cerebellum. Alternatively, we might speculate around the involvement of (yet unidentified) epigenetic factors and/or the contribution of a downstream target of cerebellar KMT2B. One of these might explain the clinical peculiarities of our patient and reported for the first time in this genetic entity.

In conclusion, we contribute further to the characterization of *KMT2B*-related disorders, highlighting the distinctive possibility of a cerebellar presentation.

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CONFLICT OF INTEREST

Joana Damásio acted as consultant for Zambon. The remaining authors have no conflicts of interests.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/cge.14055.

DATA AVAILABILITY STATEMENT

Research data have not been archived at public repository, but are available upon request at CHUPorto.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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Link to online supplementary material and video S1 (in supporting information)

https://onlinelibrary.wiley.com/doi/10.1111/cge.14055

Article 2. Early-onset and severe complex hereditary spastic paraplegia caused by de novo variants in SPAST

Damásio J, Barbot C, Felgueiras R, Brandão AF, Barros J, Oliveira J, Sequeiros J. Mov Disord 2023 May;38(5):910-911. doi: 10.1002/mds.29380.

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LETTERS: PUBLISHED ARTICLES

Early-Onset and Severe Complex Hereditary Spastic Paraplegia Caused by *De Novo* Variants in *SPAST*

online video

We read with interest the manuscript by Mo et al[1] confirming that de novo variants in *SPAST* lead to a severe form of hereditary spastic paraplegia (HSP), with delayed motor milestones.

Impelled by that work, we reevaluated our cohort of 262 patients with ataxia/spastic paraplegia and identified an atypical case with a de novo NM_014946.4:c.1496G>A (p.Arg499His) variant in *SPAST*. This was a 33-year-old woman (mother was 37 and father 45 years old when she was born), first observed at 6 months for head titubation and hypotonia. Sitting occurred at 11 months and first (dysarthric) words by 12 months. On follow-up, upper limb dysmetria and broad base when trying to stand became clear. Assisted gait occurred by age 4 years, but she never walked independently and started using a wheelchair when entering school. At that time, brisk deep tendon reflexes were first noted, slowly progressing to spastic paraplegia. Cognition was normal, completing 12 years of schooling with good performance. Currently (Video S1), there was scandid dysarthria and slow, hypermetric saccades. She had tetraparesis, brisk deep tendon reflexes, and Babinski sign. Upper limb dysmetria

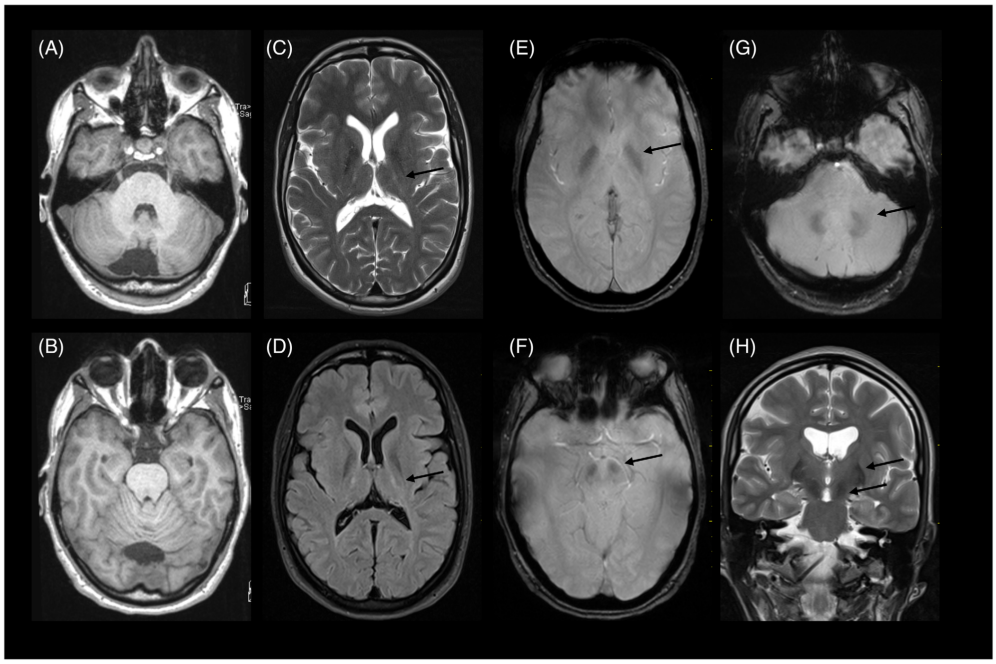

FIG. 1. Brain magnetic resonance imaging (age, 30 years old). (**A**, **B**) T1, axial view: enlarged retrocerebellar space; (**C**, **D**) T2 and fluid attenuated inversion recovery (FLAIR), axial view: hyperintensity of the posterior limbs of the internal capsule (arrows), normal intensity of the remaining parenchyma and no evidence of atrophy; (**E**–**G**) T2*, axial view: hypointensity of the pallida, substantia nigra, and dentate nucleus (arrows); (**H**) T2, turbo spin echo, coronal view: hypointensity of the pallida and substantia nigra (arrows). [Color figure can be viewed at wileyonlinelibrary.com]

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and mild cervical dystonia were also present. Brain magnetic resonance imaging (age 7 years) disclosed T2 white matter hyperintensity at the posterior limb of the internal capsule. Twenty-four years later, T2* hypointensities of the pallida, substantia nigra, and dentate nucleus were also identified (Fig. 1). An exhaustive investigation was performed (Supporting Information Data S1), with genetic diagnosis being achieved by a next-generation sequencing multigene panel based on whole-exome sequencing (WES).

This case consubstantiates the complexity of de novo *SPAST* variants, broadening their phenotype spectrum to include delayed motor milestones, together with early cerebellar signs, fulfilling criteria for nonprogressive congenital ataxia,[2] her initial clinical diagnosis. After a period of slow (although ongoing) motor acquisitions, as seen in nonprogressive congenital ataxia, it was only in the eighth year of life that pyramidal signs emerged. In most series, spastic paraplegia occurred at a much earlier age.[1,3] Further reports will be important to clarify whether this was an anecdotal clinical feature in our patient or if HSP-*SPAST* is being underrecognized in case late-emergent pyramidal signs. Interestingly, despite a severe phenotype, normal imaging has usually been described.[1,3,4] In our patient, magnetic resonance imaging initially suggested a metabolic disorder and, later, brain iron accumulation, posing an additional challenge. The WES-based multigene panel was crucial for diagnosis, after three decades of investigation. Interestingly, the p.Arg499His variant found in our patient was also frequent (16/40 cases) in the cohort reported by Mo et al.[1] Given the genomic context, deamination of a methylated CpG may constitute a suitable explanation for this recurrent de novo variant.

To conclude, we wish to highlight the atypical features and emphasize the observation by Mo et al[1] of de novo HSP-*SPAST* as a complex disorder, with p.Arg499His being the most frequent de novo variant and advanced paternal age a possible risk factor. Use of WES-based panels in clinical practice shortens time to diagnosis, decreases unnecessary investigation, and contributes to phenotypic expansion of neurological disorders.

Ethical Compliance Statement

This study was approved by the ethics committee of Centro Hospitalar Universitário do Porto, and informed consent was obtained from the patient and her parents for case and video publication. The authors confirm that they have read the Journal's position on issues involved in ethical publication and affirm that this work is consistent with those guidelines.

Acknowledgments: We would like to acknowledge the patient and the patient's family.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Reply to: Early-Onset and Severe Complex Hereditary Spastic Paraplegia Caused by De Novo Variants in *SPAST*

We thank Damásio et al for their contribution and agree that their case likely expands the phenotypic spectrum associated with de novo variants in *SPAST*.

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Phenotypic spectrum of polyQ vs. non-repeat expansion ataxias

Of the 74 symptomatic patients with a final genetic diagnosis, 38 (51.4%, 19 families) were confirmed as having a polyQ and 36 patients (48.6%, 21 families) a non-repeat expansion SCA.

Article 3 focuses on the phenotypic spectra of these two types of AD-HCA.

Article 3. Spinocerebellar ataxias: phenotypic spectrum of polyQ versus non-repeat expansion forms.

Moura J, Oliveira J, Santos M, Costa S, Lemos C, Barros J, Sequeiros J, Damásio J.

Submitted

Spinocerebellar ataxias: phenotypic spectrum of polyQ *versus* non-repeat expansion forms

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ABSTRACT

Spinocerebellar ataxias (SCA) are most frequently due to $(CAG)_n$ (coding for polyglutamine, polyQ) expansions and, less so, to expansion of other oligonucleotide repeats (non-polyQ) or other type of variants (non-repeat expansion SCA). In this study we compared polyQ and non-repeat expansion SCA, in a cohort of patients with hereditary ataxia followed at a tertiary hospital. From a prospective study, 88 patients (51 families) with SCA were selected, 74 (40 families) of whom genetically diagnosed. Thirty-eight patients (51.4%, 19 families) were confirmed as having a polyQ (no other repeat-expansions were identified) and 36 (48.6%, 21 families) a non-repeat expansion SCA. Median age-at-onset was 39.5 [30.0-45.5] for polyQ and 7.0 years [1.00-21.50] for non-repeat expansion SCA. PolyQ SCA were associated with cerebellar onset, and non-repeat expansion forms with non-cerebellar onset. Time to diagnosis was longer for non-repeat expansion SCA. The most common polyQ SCA were Machado-Joseph disease (MJD/SCA3) (73.7%) and SCA2 (15.8%); whereas in non-repeat expansion SCA ATX-*CACNA1A* (14.3%), *ATP1A3*-related ataxia, ATX-*ITPR1*, ATX/HSP-*KCNA2,* and ATX-*PRKCG* (9.5% each) predominated. Disease duration (up to inclusion) was significantly higher in non-repeat expansion SCA, but the difference in SARA score was not statistically significant. Cerebellar peduncles and pons atrophy were more common in polyQ ataxias, as was axonal neuropathy.

SCA had a wide range of genetic etiology, age-at-onset and presentation. Proportion of polyQ and non-repeat expansion SCA was similar; the latter had a higher genetic heterogeneity. While polyQ ataxias were typically linked to cerebellar onset in adulthood, non-repeat expansion forms associated with early onset and non-cerebellar presentations.

Keywords: Spinocerebellar Diseases, Hereditary Ataxia, Hereditary Spinocerebellar Degenerations, Trinucleotide Repeat Expansions, Machado-Joseph disease

MAIN TEXT

INTRODUCTION

Spinocerebellar ataxias (SCA) comprise a heterogeneous group of rare autosomal dominant diseases, characterized by ataxia as the cardinal symptom, but showing a wide neurological and extra-neurological expression [1, 2]. Age-at-onset (AO) is commonly between the fourth and sixth decades, with pediatric and later-onset forms being classically considered as less frequent [3-5]. Inter and intra-familial phenotypic diversity increases its diagnostic difficulties [6].

The most frequent SCA worldwide are those caused by CAG repeat expansions, as SCA1 (in *ATXN1*), DRPLA (*ATN1*), SCA2 (*ATXN2*), Machado-Joseph disease (MJD)/ SCA3 (*ATXN3*), SCA6 (*CACNA1A*), SCA7 (*ATXN7),* and SCA17 (*TBP*), usually designated polyglutamine (polyQ) ataxias [7, 8]. As in other polyQ diseases, an inverse correlation is seen between AO and size of the expanded $(CAG)_n$, longer repeats being associated with earlier onset and a more severe phenotype [9, 10]. Expansion of non-CAG repeats and non-repeat expansion SCA (caused by conventional variants as single-nucleotide substitutions or small insertions/deletions) are classically considered rare; nevertheless, its recognition has increased in past years, mostly due to the routine application of next generation sequencing (NGS)-based genetic studies [9, 11, 12]. As opposed to polyQ, non-repeat expansion SCA usually present during childhood, show no anticipation of AO, progress more slowly and may have congenital features (as psychomotor delay) [11, 12]. Studies comparing these two groups of SCA are scarce.

In this report, we compare the clinical and genetic features of polyQ *versus* non-repeat expansion SCA, in a cohort of hereditary cerebellar ataxias (HCA) followed at a tertiary center in the North of Portugal.

PATIENTS AND METHODS

Patient selection

A prospective study on HCA has been conducted at ULSSA, since 2017. Patients with dominant HCA (SCA) were selected from the local ataxia database. From 249 patients (164 families) with HCA, 88 (51 families, 31.1%) had SCA; of these, 74 (40 families, 78.4%) had an established genetic diagnosis, but 11 families were still at different stages of investigation.

All 249 patients were evaluated through a structured protocol (listed in Suppl. Material), comprising a baseline detailed identification of AO of different neurological symptoms/signs (e.g., dysarthria,

dysphagia, limbs' coordination, epilepsy, pyramidal signs, movement disorders), and motor disability milestones (agraphia, falls, gait assistance, confinement to wheelchair). Every twelve months, a questionnaire on new neurological symptoms and disability was applied. The Scale for the Assessment and Rating of Ataxia (SARA) and Inventory of Non-Ataxia Signs (INAS) were evaluated at baseline and on annual examinations.

Definitions

Age-at-onset (AO) was considered as the age at first neurological symptoms, either cerebellar or non-cerebellar. Whenever patients had been prospectively followed since the presymptomatic stage, AO was considered as the age at which the first symptoms were registered during the study. In patients whose symptoms started before, information collected at the study baseline was checked (whenever possible) with two first-degree relatives and clinical records. In case of discordance, due to memory biases or other reasons, information from clinical records was preferred.

Epilepsy was considered only when the participant suffered two or more unprovoked seizures. *Pyramidal signs* were hyperreflexia, spasticity, Hoffman sign, and/or extensor plantar reflex. *Movement disorders* were classified into hypokinetic (Parkinsonism) and hyperkinetic (tremor, dystonia, chorea, myoclonus, tics). *Motor delay* was defined when motor milestones were outside the 95% confidence interval, according to the World Health Organization [13]. *Intellectual disability* was defined as intellectual difficulties, but also problems in conceptual, social, and practical areas of living, according to the Diagnostic and Statistical Manual of Mental Disorders, 5th edition [14]. *Hemiplegic migraine* was considered in presence of migraine with aura, including fully reversible motor weakness and fully reversible visual, sensory, and/ or speech/ language symptoms. *Dysphagia* was registered when occurring repeatedly and leading to bursts of cough. *Psychiatric symptoms* (anxiety, depression, psychosis) were diagnosed as *per* clinical judgement. *Motor disability milestones* included agraphia, falls, use of cane, or confinement to wheelchair. *Agraphia* was considered when the patients could no longer sign their name. *Falls* were registered only if occurring repeatedly. *Loss of independent gait* corresponded to the use of at least one cane; *confinement to wheelchair* meant the permanent use of this aid.

Sample characterization

Patients selected, with SCA, were classified according to the revised nomenclature on Genetic Movement Disorders (except for SCA1, SCA2, MJD/SCA3, SCA6, and SCA7, more readily recognized by their

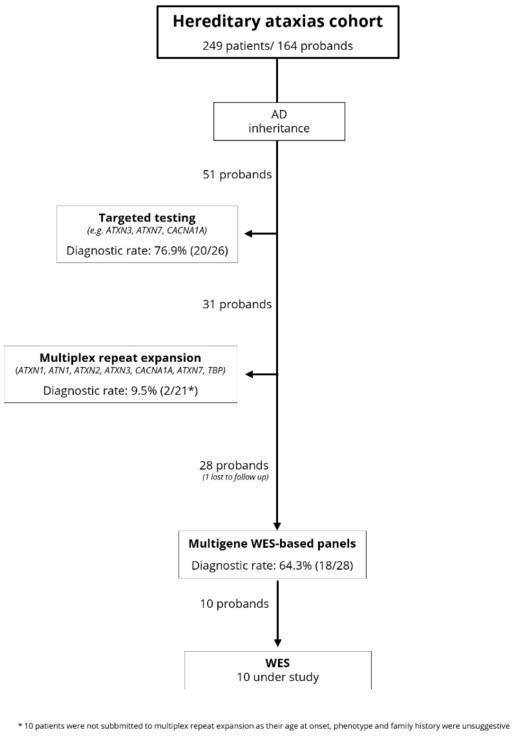
original designations) [15]. For genes not yet associated with an ataxic phenotype by the International Parkinson and Movement Disorder Society Task Force on Classification and Nomenclature of Genetic Movement Disorders, we used the suffix "-related ataxia" after the gene designation. Sex, AO, age at observation, and at genetic diagnosis was recorded. Presenting clinical features, associated neurological symptoms and SARA score were collected. In all patients with disease onset in the first decade of life, metabolic disorders had been excluded *ab initium*. Age, cause and place of death were documented, according to the medical records, if available, or as stated by first degree relatives. Brain MRI was initially performed with 1.5 or 3.0 tesla scanners. Electromyography (EMG) was conducted according to clinical judgement.

Analyses of AO, additional symptoms/ signs, and disability milestones were performed. Brain MRI and EMG were also considered for analysis.

Genetic testing

DNA was collected from peripheral blood and stored at CGPP, IBMC. Following the variants' classification guidelines of American College of Medical Genetics and Genomics[16], a genetic diagnosis was considered as achieved in the presence of one pathogenic/ likely pathogenic variant in heterozygosity, in a gene associated with a dominant phenotype. The *diagnostic yield* (or detection rate) was defined here as the number of patients in whom a genetic diagnosis was established, divided by the total number of patients submitted to that assay (Fig. 1). A proband was, by definition, the first patient to be genetically diagnosed in the family. For the 51 probands, targeted (single-gene) testing was performed in 26, in whom a specific form of SCA was highly suspected (e.g., MJD/SCA3 due to its high prevalence in geographical clusters, SCA7 in the presence of retinopathy, *CACNA1A* expansion in case of a pure cerebellar syndrome, and *CACNA1A* conventional variants in familial hemiplegic migraine) – the diagnostic yield was 76.9% (20/26). In 21 probands (6 undiagnosed after targeted testing, and 25 not submitted to targeted testing – details in Suppl. Material) testing for expansions of the CAG repeat in *ATXN1, ATN1, ATXN2, ATXN3, CACNA1A, ATXN7* and *TBP*, through multiplex polymerase chain reaction (PCR) and fragment analysis was performed – a diagnostic yield of 9.5% (2/21).Ten probands were not submitted to genetic study of polyQ ataxia as their age at onset, clinical presentation and/or family history were highly unsuggestive (details in Suppl. Material). Finally, out of 29 probands, one was lost to follow up and 28 were studied through multigene panels (genes detailed in Suppl. Material) based on whole-exome sequencing (WES) – diagnostic yield of 64.3% (18/28). Ten probands are under WES

study. In affected relatives of probands, diagnosis was by single-gene testing. Variants were considered *de novo* only if both parents had been genotyped and were not carriers.



Statistical analysis

For descriptive statistics, qualitative variables were studied through absolute and relative frequency. Mean with standard deviation or median with interquartile ranges (IQR [Q1-Q3]) were used for quantitative variables, depending on variables following or not the Normal distribution. Group comparisons were performed using the chi-square test for qualitative variables; for small sized samples, the Monte Carlo method was adopted. For quantitative variables, the Student's t (in Normal) or Mann-Whitney tests were used (in non-Normal distribution). The Spearman test was used to assess a possible correlation between number of repeats and AO. A binary logistic regression model was created to evaluate whether every SCA type associated with a particular disability milestone, controlling for disease duration, as in previous studies.[5, 17, 18] A Hosmer-Lemeshow test was used to test fitness of the model

($p<0.05$). Statistical analysis was done using the IBM Statistical Package for the Social Sciences (SPSS), version 27. A P value < 0.05 was considered as statistically significant.

Ethical considerations

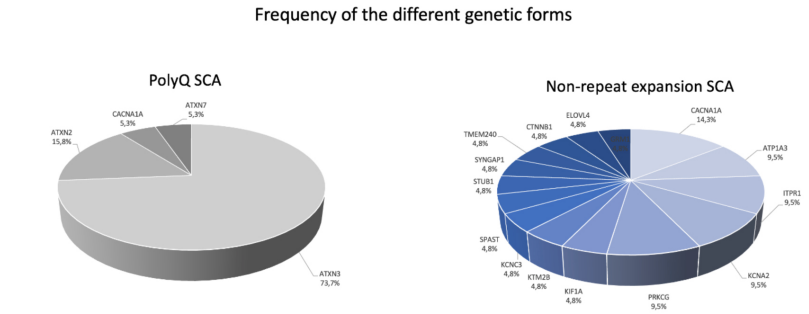
The project was approved by the ULSSA Ethics Committee. Informed consent was obtained in all patients for genetic testing and clinical investigation.

RESULTS

Among the patients with genetic diagnosis, 38 (19 families, 47.5%) had polyQ and 36 (21 families, 52.5%) had non-repeat expansion SCA. Table 1 summarizes the demographic and clinical features between both groups. Of the 11 SCA probands without genetic diagnosis (excluded from the clinical analysis), 10 were being studied through WES and 1 was lost to follow up (studied with PCR and fragment analysis).

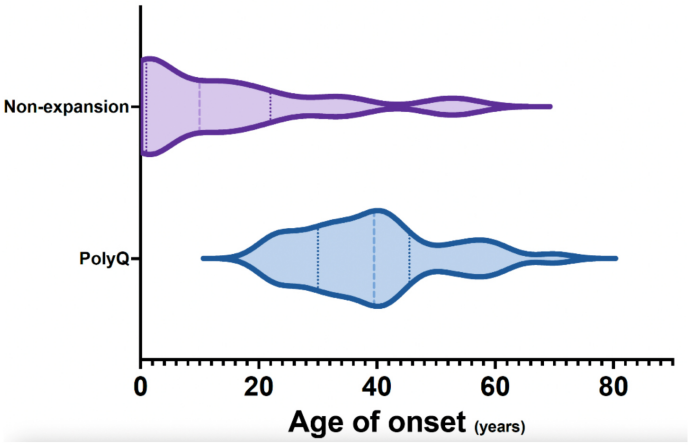
Genetic diagnosis

Diagnosis in polyQ SCA probands was achieved through targeted testing in 17 (89.5%), and multiplex approach in 2 (10.5%). In non-repeat expansion SCA, diagnosis in a proband was obtained by targeted testing in 3 (14.3%) and NGS-based panels in 18 (85.7%). Median time to diagnosis was 4.0 [1.0-8.0] years for polyQ and 25.0 [17.0-33.5] for non-repeat expansion SCA ($p<0.001$). The most common polyQ form was MJD/SCA3, in 14 (73.7%) families; followed by SCA2, in 3 (15.8%); and SCA7 and SCA6, in one (5.3%) family each (Fig. 2). Mean number of repeats in expanded and normal alleles for polyQ is presented in Suppl. Table 1. Genetic diagnoses were more diverse in non-repeat expansion SCA, with pathogenic variants involving 17 genes, the most frequent being ATX-*CACNA1A* (3 families, 14.3%) and *ATP1A3*-related ataxia, ATX-*ITPR1*, ATX/HSP-*KCNA2,* and ATX-*PRKCG* (2 families, 9.5% each) (Fig. 2). *De novo* mutations were seen only in non-repeat expansion forms (8 cases, 38.1% of tested families). Detailed number of patients per families is shown in Suppl. Tables 2 and 3.



Age-at-onset and presenting symptoms

Considering all 74 patients with genetic diagnosis (probands and observed affected relatives, Suppl. Table 2), median AO was 39.5 [30.0-45.5] in polyQ and 7.0 years [1.0-21.5] in non-repeat expansion SCA ($p<0.001$). Patients in the non-repeat expansion group had a wider range of AO, from congenital up to age 55 years (Fig. 3). In MJD/SCA3, there was an inverse correlation between size of the large allele and AO (Spearman rho=-0.47, 95% CI:0.75,-0.05, p=0.027). The same was seen in SCA2, though only marginally significant, probably due to the low number of patients (Spearman rho=-0.67, CI:0.92,-0.04, p=0.055).



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Cerebellar onset was significantly associated with polyQ, and non-cerebellar onset with non-repeat expansion SCA (p<0.001). The most frequent presenting symptom was gait unsteadiness in both groups: 28 (73.7%) in polyQ, and 11 (30.6%) in non-repeat expansion SCA. PolyQ ataxias presented predominantly (35, 92.1%) with cerebellar signs (unsteady gait, diplopia, dysarthria, dysmetria); two patients (4.8%) with MJD/SCA3 had onset with Parkinsonism, and one (2.4%) SCA7 with retinopathy. Non-repeat expansion SCA had a wider range of presenting symptoms, particularly non-cerebellar features: motor delay in congenital forms (8, 22.2%), paroxystic ataxia (3 cases, 8.3%), hemiplegic migraine (6, 16.7%), seizures (2, 5.6%), and other (less frequent), as detailed in Table 1. Precipitating events for disease onset were suspected in 5 (13.1%) polyQ and 11 (30.5%) non-repeat expansion forms; the most common were pregnancy (7, 43.8%) and infection (4, 25.0%).

Disease progression and disability milestones

Median duration from disease onset up to inclusion was 10 [4.8-18.8) years for polyQ and 26.0 [17.0-34.8] for non-repeat expansion SCA (p<0.001), with median prospective follow-up being of 4.0 [2.0-5.0] and 4.5 [2.0-5.0]. Even though disease duration until baseline was significantly higher for non-repeat expansion SCA, there was no significant difference in total SARA score at baseline (13.0 [6.5-22.1] for polyQ and 14.0 [9.0-15.0] for non-repeat expansion). Symptoms throughout disease course also differed between groups (Table 1 and Suppl. Tables 3 and 4): polyQ SCA were more frequently associated with dysphagia (p=0.018), ocular signs (p=0.033), peripheral signs (p<0.001), and psychiatric manifestations (p<0.001); non-repeat expansion SCA displayed more commonly dysarthria (p=0.015), intellectual disability (p<0.001), and epilepsy (p=0.023). There was no significant difference in non-ataxia movement disorders associated; the only two patients with chorea had concurrent Huntington disease. Diplopia (p=0.010), dysphagia (p<0.001), and gait unsteadiness (p<0.001) occurred after a shorter disease duration in polyQ ataxias (Suppl. Table 4). Considering disability, a higher frequency of falls (70.7% *vs*. 38.9%, p=0.006) and confinement to wheelchair (31.7% *vs.* 5.6%, p=0.004) was seen in polyQ-SCA. Disease duration until each disability milestone was similar between groups (Suppl. Table 5), except for assistance in gait (p<0.001) which occurred earlier in polyQ. Five (6.5%) patients died throughout the study course, all with MJD/SCA3, at a median age of 76.0 [69.0-78.0] years. Infection (four respiratory, one sepsis) was the cause of death in all. Three died in the hospital, one in a nursing home, and the other at home.

Complementary exams

Overall, polyQ had a shorter disease duration until the last brain MRI and EMG. Twenty-eight (73.7%) patients with polyQ and 34 (94.4%) with non-repeat expansion forms had information on brain pattern of atrophy on MRI (Table 2); the only significantly different topographies between groups were atrophy of the pons (p=0.027) and cerebellar peduncles (p=0.009), more common in polyQ ataxias. EMG was performed in 21 (55.3%) polyQ and 19 (52.8%) non-repeat expansion SCA patients, the first being associated with sensitive axonal neuropathy (p=0.006).

DISCUSSION

We studied a group of patients with SCA from the North of Portugal. We identified a comparable number of families with polyQ and non-repeat expansion SCA, which was unexpected, as the latter are considered to be responsible for only about 10%-18% of SCA [19, 20]. This could be explained by our cohort having been established at a tertiary referral center, which receives patients from all the northern region of the country, including pediatric neurology clinics (with a higher number of non-repeat expansion SCA). Also, for some time now, the use of NGS has been everyday clinical practice at our center; this has increased the rate of genetic diagnoses in the past years, mostly due to identification of non-repeat expansion forms [11, 12].

Diagnostic strategy and detection rate

A flowchart for our diagnostic strategy in SCA is illustrated in Fig. 1. A final genetic diagnosis was obtained in 78.4%, at the upper limit of the described (31.3%-89.0%) [20-24]. (1) Single-gene testing was used in a selected group of SCA (owing to a strong clinical suspicion or, particularly for MJD/SCA3, its high prevalence in our country), with a detection rate of 76.9%; the remaining probands, followed established diagnostic recommendations [25]. (2) The diagnostic yield of multiplex PCR and fragment analysis for the most frequent SCA was of only 9.5%, lower than in other series (13.8%-70.8%) [21, 23, 26], as most polyQ probands had already been diagnosed through targeted testing. (3) WES-based multigene panels, had an overall yield of 64.3%, concordant with other series reporting 12.1%-64.4% [21-24, 27, 28]. Ten patients are under (4) WES study, and no cases have yet been summitted to (5) whole-genome sequencing (WGS).

Relative frequency of the specific genetic forms

PolyQ were genetically more homogeneous than non-repeat expansion SCA. As in previous studies [7,

29], MJD/SCA3 was, overall, the most frequent form. SCA2 was the second most common polyQ ataxia, replacing dentatorubral-pallidoluysian atrophy (DRPLA), as found earlier in the nation-wide survey [29]. This is most probably explained by the clustering of DRPLA families in the Center and South [30], while our cohort was established in the North. SCA7 and SCA6 were less frequent; both are known to have a national prevalence under 0.10 per 100 000, being also rarer in other European countries [29, 31-34]. As expected [35-37], an inverse correlation of repeat size with AO was observed for MJD/SCA3 and SCA2 (in the remaining polyQ SCA, the low number of patients precluded analysis).

The gene most frequently involved in non-repeat expansion SCA was *CACNA1A*, in line with data from the national survey [29]. *ATP1A3*-related ataxia, ATX-*KCNC3,* and ATX-*ITPR1* had not yet been described by that time; many families had already been identified, but had no definite diagnosis by then. The remaining, ultra-rare, non-repeat expansion forms were present only in one or two families. Relative frequency of specific forms within non-repeat expansion SCA was in accordance with a large international collaborative cohort, where *CACNA1A, PRKCG, AFG3L2, ITPR1, STUB1, SPTBN2*, or *KCNC3* were the most frequent genes [11]. *De novo* pathogenic variants were exclusively identified among non-repeat expansion ataxias (38.1%), all patients with no family history.

Onset age and symptoms

Non-repeat expansion presented earlier than polyQ SCA (7.0 *vs.* 39.5 years), consistent with previous findings. AO in polyQ ataxias is usually between 30 and 50 years of age [4, 5, 36, 38], while in channelopathies and other non-repeat expansion SCA tends to occur sooner [12, 19]. Still, as in other cohorts, we found a wide range of AO in non-repeat expansion SCA, spanning from congenital up to onset in the sixth decade of life [11, 26].

Clinical spectrum differed significantly between groups. In polyQ SCA, cerebellar onset was patent in the majority; whereas in non-repeat expansion SCA, onset with cerebellar and non-cerebellar signs was similarly frequent. Congenital forms and paroxystic episodes (seizures, hemiplegic migraine, ataxia, dystonia) were exclusively seen with non-repeat expansions, as in previous descriptions [11, 19, 34]. The increased occurrence of paroxystic episodes in non-expansion SCA was probably explained by the high frequency of genes (e.g., *CACNA1A, KCNA2, ATP1A3*) coding for ion channels [39-41]. Pathogenic variants in *CACNA1A, KCNA2* and *KMT2B* were associated with epilepsy; seizures are a known feature of the first two [42, 43]. Interestingly, some cases corresponded to channelopathies associated with cognitive and motor delay, in which ataxia has also been described, including *KCNC3, ITPR1, ATP1A3*

and *CTNNB1* [44-48].

Over disease course, polyQ ataxias displayed more frequently ocular disturbances (diplopia and ophthalmoparesis) and peripheral signs (neuropathy and muscle cramps), both known to be associated with MJD/SCA3 and SCA2, the most frequent forms [49, 50]. Depression and anxiety, associated with polyQ SCA, occurred at a higher frequency than in general population [51]. Intellectual disability was exclusive for non-repeat expansion SCA, while dementia was present in both groups, in line with other descriptions [9, 11].

Subsidiary exams

Atrophy of the pons and cerebellar peduncles on MRI was the distinguishing feature between groups, associating with polyQ SCA. Quantitative brain MRI studies have described atrophy in the cerebellar hemispheres, vermis, midbrain, pons, medulla oblongata and cervical spine in polyQ SCA, particularly in MJD/SCA3 [9, 52]. In non-repeat expansion SCA, cerebellar atrophy with no brainstem involvement is common [9, 53, 54], in line with what was observed in our series. Also, the axonal sensitive neuropathy on EMG, associated with polyQ SCA, is consistent with the clinical picture of MJD/SCA3 [55].

Disease progression and disability

At baseline, there was no significant difference between groups in global SARA score, notwithstanding a significantly shorter disease duration in polyQ ataxias suggesting faster disease progression; inclusion of patients with childhood or juvenile onset of polyQ SCA (not observed in our series) would have made this difference even more marked, as these usually have accelerated progression [9]. PolyQ ataxias significantly associated with falls and wheelchair confinement (Table 1), and earlier assistance in gait (Suppl. Table 5) These findings are in line with the fact that non-repeat expansion SCA have slower disease progression, and use of gait assistance devices is less frequent and later [9, 12]. Knowing clinical progression and disability milestones is of the utmost importance, as they are linked to survival [56, 57]. Life expectancy is reduced in polyQ SCA [56]. All our deceased patients had MJD/SCA3; they lived, on average, 5.6 years less than the general population in northern Portugal [58].

Limitations of this study

This is a relatively small sample, from a tertiary hospital receiving complex patients, what prevents generalization of our conclusions. Also, as the study started in 2017, a recall bias of symptoms emerging

earlier cannot be excluded. To overcome this, we collected information from patients and relatives, and checked it with their previous clinical records. Our polyQ group included mainly MJD/SCA3 and SCA2, what may have biased analysis of phenotype. Also, as non-repeat expansion SCA consisted of small groups with variable phenotype, this prevented a more detailed analysis and, possibly, establishing stronger associations. Nevertheless, patients with polyQ and non-repeat expansion SCA were in similar number, reinforcing comparative power between them, our main purpose. Imaging was based on retrospective appraisal of MRI; thus, we were unable to explore patterns specific for each SCA.

CONCLUSIONS

This study defined the major clinical features and progression in polyQ and non-repeat expansion SCA. We highlight the earlier onset, congenital forms, and paroxystic symptoms with non-expansion variants; and adult-onset, cerebellar presentation, ocular and peripheral signs with polyQ. Longer time-to-diagnosis in non-repeat expansion SCA probably occurs due to a combination of factors: early-onset, non-cerebellar presentations, slower progression, lesser severity, and *de novo* variants. Increasing recognition (and widespread use of NGS-based tests, including WGS) will favor a timelier diagnosis. We believe these findings may be of value for future research and impact clinical practice of neurologists, pediatricians, and clinical and laboratory genetics.

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COMPLIANCE WITH ETHICAL STANDARDS

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J. Damásio – Has served as consultant for Abbvie pharmaceuticals.

Ethical approval and informed consent

The authors confirm this work has been approved by institutional review board of ULSSA, and every patient gave written informed consent to participate in the project and for publication of the study's results. The study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki, its later amendments or comparable ethical standards.

Data availability

Additional data supporting the conclusions of this article will be made available by the authors upon reasonable request.

Table 1 – Demographic and clinical data for polyglutamine and non-repeat expansion ataxias

	Polyglutamine ataxias	**Non-repeat expansion ataxias**	**P value**
Number of probands	19 *(47.5%)*	21 (*52.5%*)	
Total number of patients	38 (*51.4%*)	36 (*48.6%*)	
Female gender	18 (*47.4%*)	24 (*66.7%*)	0.107
Age at onset*	39.5 y *[30.0-45.5]*	7.0 y [*1.0-21.5*]	**<0.001**
Age at diagnosis*	41.0 y [*33.8-53.0*]	37.5 y [*24.8-48.3*]	0.1
Time to diagnosis*	4.0 y [*1.0-9.0*]	22.0 y [*15.0-36.3*]	**<0.001**
Disease duration until baseline*	10.0 y [*4.7-18.7*]	26.0 y [*17.0-34.7*]	**<0.001**
Type of onset:			
Cerebellar	35 (*92.1%*)	17 (*47.2%*)	**<0.001**
Non-cerebellar	3 (*7.9%*)	19 (*52.8%*)	
Detailed symptoms at disease onset:			
Cerebellar:			
Gait unsteadiness	28 (*73.7%*)	11 (*30.6%*)	
Upper limb dysmetria	3 (*7.9%*)	3 (*8.3%*)	
Dysarthria	1 (*2.6%*)	-	
Diplopia	3 (*7.9%*)	-	
Non-cerebellar:			
Motor delay	-	8 (*22.2%*)	NA
Hemiplegic migraine	-	6 (*16.7%*)	
Paroxystic ataxia	-	3 (*8.3%*)	
Parkinsonism	2 (*5.3%*)	-	
Seizures	-	2 (*5.6%*)	
Chorea	-	1 (*2.8%*)	
Paroxystic dystonia		1 (*2.8%*)	
Retinopathy	1 (*2.6%*)	-	
Spastic gait	-	1 (*2.8%*)	
Precipitating events for disease onset	5 (*13.1%*)	11 (*30.5%*)	0.051
SARA score at baseline	13.0 [*6.5-22.1*]	14.0 [*9.0-15.0*]	0.785
Symptoms throughout disease course:			
Gait unsteadiness	38 (*100.0%*)	36 (*100.0%*)	0.243
Dysmetria (upper and lower limb)	38 (*100.0%*)	35 (*97.2%*)	0.618
Dysarthria	30 (*78.9%*)	34 (*94.4%*)	**0.015**
Dysphagia	31 (*75.6%*)	17 (*47.2%*)	**0.018**
Ocular symptoms/ signs	35 (*92.1%*)	26 (*72.2%*)	**0.033**
Diplopia	26 (*68.4%*)	4 (*11.1%*)	**<0.001**
Nystagmus	22 (*57.9%*)	13 (*36.1%*)	0.169
Horizontal ophthalmoparesis	24 (*63.2%*)	4 (*11.1%*)	**<0.001**
Vertical ophthalmoparesis	18 (*47.4%*)	4 (*11.1%*)	**0.002**
Hypermetric saccades	22 (*57.9%*)	14 (*38.9%*)	0.254
Hypometric saccades	11 (*28.9%*)	9 (*25.0%*)	1.000
Oculomotor apraxia	2 (*5.3%*)	2 (*5.6%*)	1.000
Pyramidal signs	23 (60.5%)	21 (*58.3%*)	0.848
Intellectual disability	-	11 (*30.6%*)	**<0.001**
Dementia	5 (*13.2%*)	5 (*13.9%*)	1.000
Epilepsy	-	5 (*13.9%*)	**0.023**
Movement disorders	28 (*73.7%*)	24 (*66.7%*)	0.613
Dystonia	27 (*71.1%*)	22 (*61.1%*)	0.463
Tremor	7 (*18.4%*)	4 (*11.1%*)	0.517
Chorea	2 (*5.3%*)**	5 (*13.9%*)	0.255
Parkinsonism	4 (*10.5%*)	1 (*2.8%*)	0.358
Myoclonus	-	3 (*8.3%*)	0.110
Peripheral signs	22 (*57.9%*)	6 (*16.7%*)	**<0.001**
Neuropathy	19 (*50.0%*)	6 (*16.7%*)	**0.003**
Muscle cramps	9 (*23.7%*)	-	**0.002**
Psychiatric comorbidities	29 (*76.3%*)	13 (*37.1%*)	**<0.001**
Depression	26 (*68.4%*)	10 (*28.6%*)	**<0.001**
Anxiety	24 (*63.2%*)	12 (*34.3%*)	**0.019**
Psychosis	0 (*0.0%*)	1 (*2.9%*)	0.479
Disability milestones			
Agraphia	7 (*17.1%*)	2 (*5.6%*)	0.162
Falls	29 (*70.7%*)	14 (*38.9%*)	**0.006**
Unilateral assistance in gait	18 (*43.9%*)	10 (*27.8%*)	0.162
Wheelchair bound	13 (*31.7%*)	2 (*5.6%*)	**0.004**

NA – Not applicable; y - years; *Age expressed in mean and interquartile range [Q1-Q3], ** Two patients with concurrent Huntington disease

Table 2 – Genetic and complementary investigation of polyglutamine and non-repeat expansion ataxias

	Polyglutamine ataxias	Non-repeat expansion ataxias	p value
Number of probands	19 (*47.5%*)	21 (*52.5%*)	
Total number of patients	38 (*51.4%*)	36 (*48.6%*)	
Genetic testing			
Targeted test	17 (*89.5%*)	3 (*14.3%*)	n.a.
Multiplex PCR and fragment analysis	2 (*10.5%*)	-	
Multigene panel (WES-based)	-	18 (85.7%)	
WES	-	-	
De novo variant	0	8 (38.1%)	**<0.001**
Brain MRI	28 (*73.7%*)	34 (*94.4%*)	**0.015**
Disease duration until last exam	6.0 [*3.0-14.0*]	24.0 [*14.0-37.0*]	**<0.001**
Atrophy			
Vermis	22 (*78.6%*)	24 (*70.6%*)	0.566
Cerebellar hemispheres	21 (*75.0%*)	23 (*67.6%*)	0.584
Cerebellar peduncles	9 (*32.1%*)	2 (*5.9%*)	**0.009**
Pons	9 (*32.1%*)	3 (*8.8%*)	**0.027**
Midbrain	5 (*17.9%*)	1 (*3.0%*)	0.085
Medulla	4 (*14.3%*)	1 (*2.9%*)	0.166
Cortex	4 (*14.3%*)	3 (*8.8%*)	0.691
Nerve conduction studies	21 (*55.3%*)	19 (*52.8%*)	1.000
Disease duration until last exam	10.5 y [*6.8-23.0*]	23.0 y [*16.0-37.0*]	**0.010**
Demyelinating neuropathy	0	0	n.a.
Axonal neuropathy	16 (*72.7%*)	5 (*26.3%*)	**0.005**
Sensitive	16 (*72.7%*)	5 (*26.3%*)	**0.005**
Motor	5 (*22.7%*)	2 (*10.5%*)	0.419

Legend: PolyQ – polyglutamine, Y – years, WES – whole-exome sequencing

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FIGURE CAPTIONS

Fig. 1 – Flowchart of genetic diagnostic strategy and diagnostic yield

Fig. 2 – Frequency of the various genetic forms in polyQ and non-repeat expansion SCA

Fig. 3 - Distribution of age-at-onset in polyQ and non-repeat expansion SCA

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Table 1 – Demographic and clinical data of polyglutamine *versus* non-repeat expansion SCA

Table 2 - Genetic and complementary investigation of polyglutamine *versus* non-repeat expansion SCA

SUPPLEMENTARY DATA

Supplementary Material – Methods: clinical protocol, Workflow of genetic testing, screening of ataxias caused by repeats' expansions methodology, NGS based panels methodology, list of genes in multigene panels, whole exome sequencing methodology.

Supplementary Table 1 – Median number of repeats in each allele in patients from the polyQ ataxia group

Supplementary Table 2 – Genetic forms in polyQ and non-repeat expansion SCA

Supplementary Table 3 – Detailed clinical characterization of patients with polyQ ataxias (individual data)

Supplementary Table 4 – Detailed clinical characterization of patients with non-repeat expansion SCA (individual data)

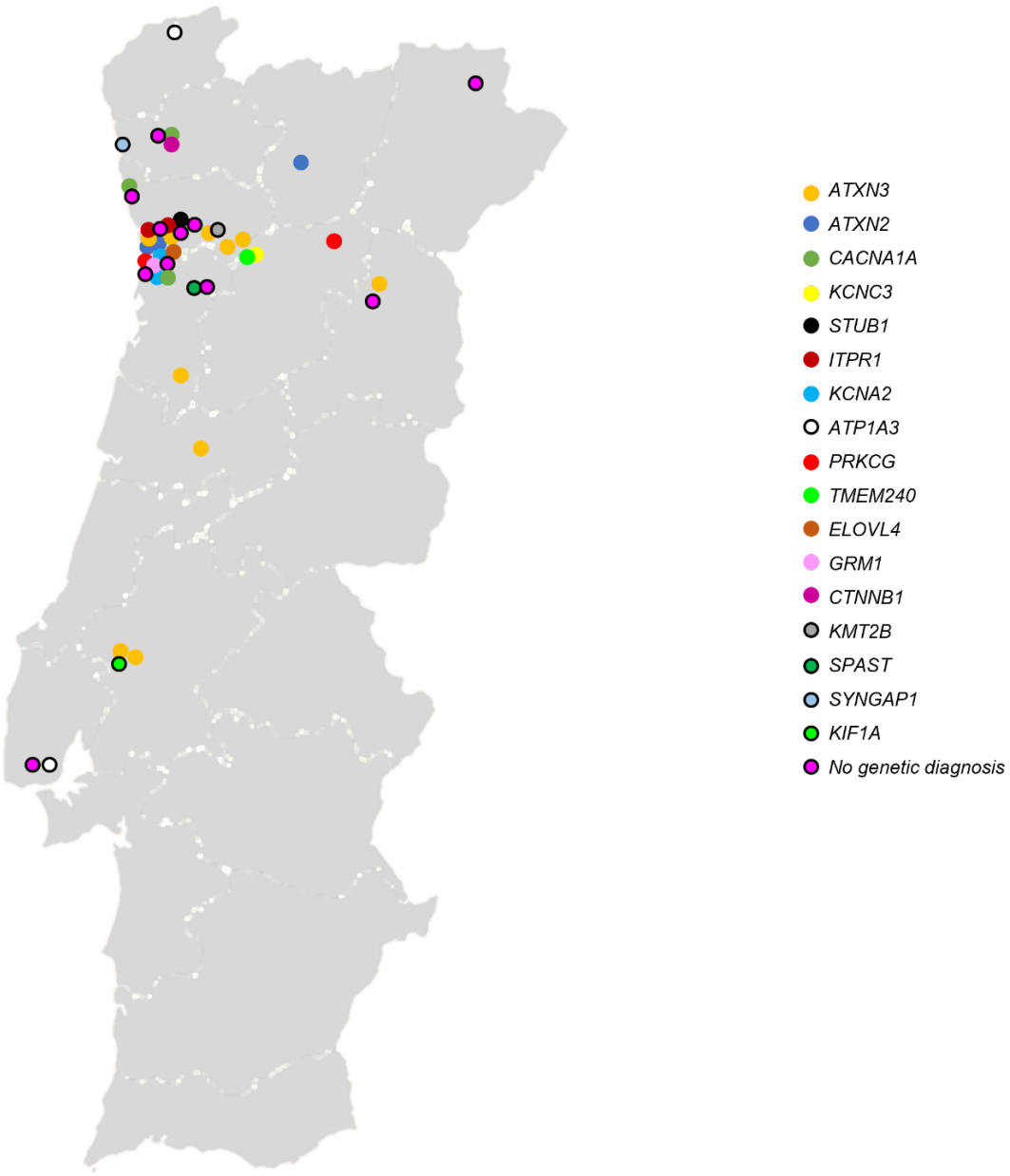
Supplementary Table 5 - Disease duration in years [Q1-Q3] until symptom and disability milestone ultimately reached, according to the type of ataxia

Geographic distribution of the families

Distribution of AD families in mainland Portugal is represented on Figure 9 (families from the archipelagos and oversea not shown). The large majority (35 families, 68.6%) was from the North of the country, followed by the Centre (five families, 9.8%), Alentejo (three families, 5.9%), Azores and Lisbon Metropolitan Area (with two families each, 3.9%). Four families were not Portuguese: three Brazilian (5.9%) and one Syrian (1.9%).

The origin of MJD families was as follows: five from the North, three from Centre, two from Alentejo, two from the Azores, one from Brazil and another from Syria. As for the remaining, all were from the North, with the following exceptions: one with ATX-*PRKCG* and another without a genetic diagnosis from the Centre; one with *ATP1A3*-related ataxia and another without genetic diagnosis from Lisbon Metropolitan Area; one with HSP-*KIF1A* from Alentejo; one with ATX-*CACNA1A* and another with SCA7 from Brazil.

Figure 9: Geographic distribution of AD-HCA families

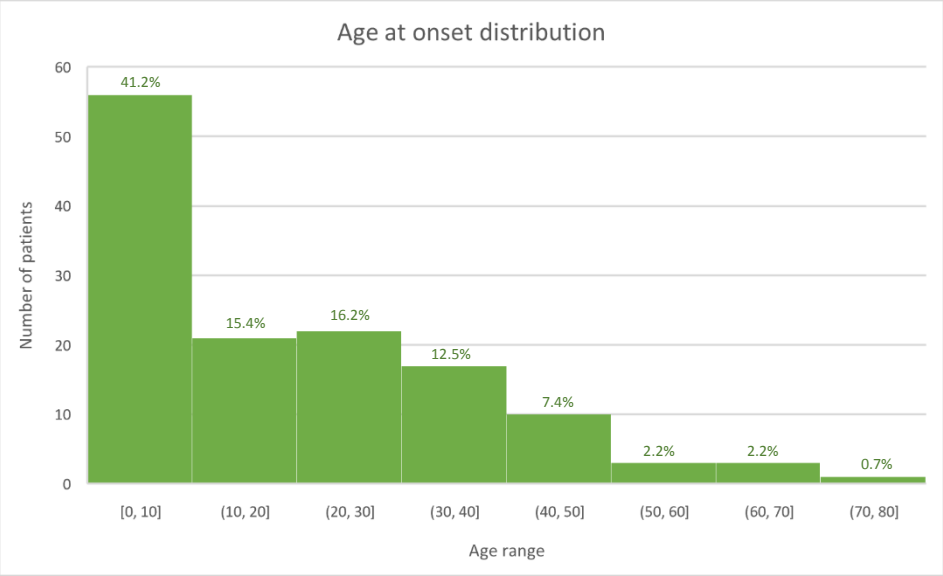


FAMILIES WITH AUTOSOMAL RECESSIVE INHERITANCE

Overall characterization of patients and families

The 91 families with AR inheritance comprised 136 patients, (75 females:61 males). In 64 patients (47.1%) there was no family history of neurological disorders. AO was 15 [4.0-30.75] years (in four patients there was no information). Onset before the age of 10 years comprised the highest number of subjects (56; 41.2%) (Figure 10).

Figure 10: Age at onset distribution in AR-HCA



A non-cerebellar onset was present in most patients (74, 54.4%). Range of non-cerebellar symptoms was wide, the most frequent being cough (29 patients, all with a diallelic *RFC1* expansion). In cerebellar-onset, the most common sign was unsteadiness of gait (49 patients; details in Figures 11 and 12).

Figure 11: Cerebellar symptoms at onset in AR-HCA

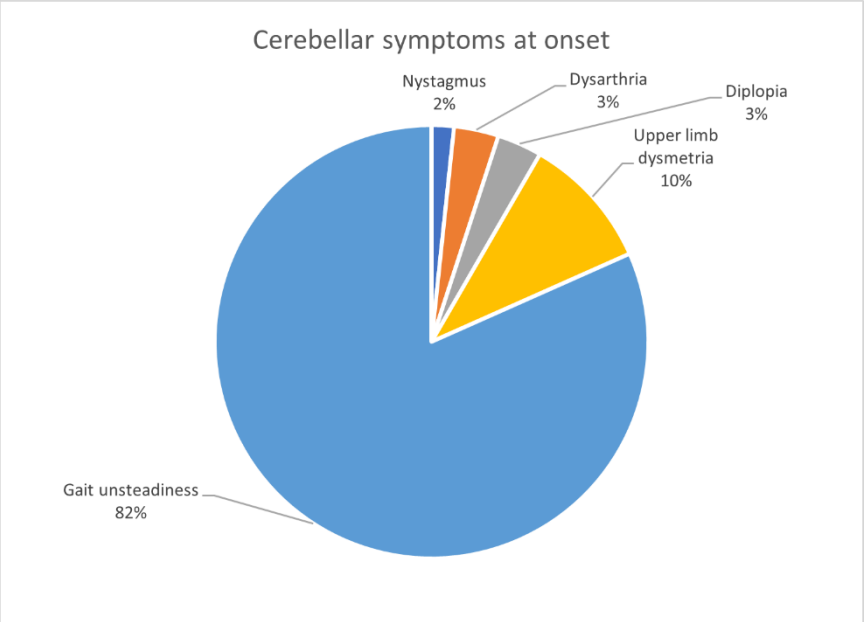
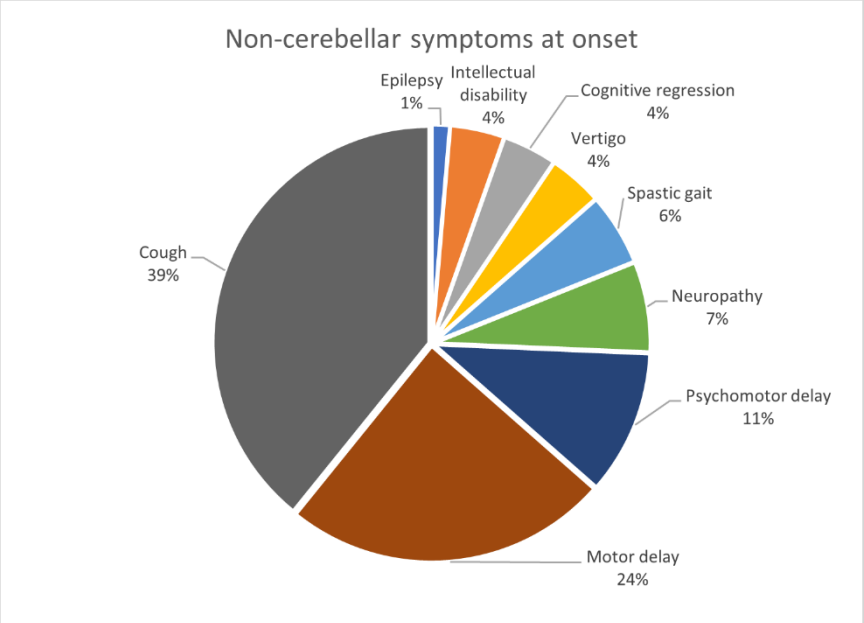


Figure 12: Non-cerebellar symptoms at onset in AR-HCA



Genetic diagnosis

A definite diagnosis was achieved in 71 probands (78.0%): 29 (31.9%) through targeted gene testing, 42 (59.1%) with NGS based panel (37 with ataxia panel and five with neuroexome). Time until genetic diagnosis was of 24.0 [15.0-43.7] years, significantly higher than in dominant ataxias ($p<0.001$). Of the 20 probands who remained without genetic diagnosis, seven had been studied for Friedreich ataxia, nine for ATX-*RFC1*, 20 with multigene WES based panels and eight also with WES.

Nine probands (28 patients in total) with ataxia and spasmodic cough, had been initially classified as AD, due to late onset and evidence or strong suspicion of two consecutive generations affected. They were extensively studied over the years, including with whole exome sequencing (8 probands) or ataxia panel (1 proband); the diagnosis was only obtained after the description of cough in patients with cerebellar ataxia with neuropathy and vestibular areflexia (*ATX-RFC1*). Reanalysis of family trees (with known or suspected consanguinity in the majority) raised the hypothesis of pseudodominant inheritance. Study of *RFC1* confirmed the diagnosis.

Thirty-one genes were involved in AR ataxias (details in Table 7). The most frequent were Friedreich ataxia in 11 (15.5%) and *ATX-RFC1* in 10 (14.1%) probands. ARSACS was diagnosed in eight (11.3%) and ATX-SYNE1 in six (8.5%) probands. Ataxia with oculomotor apraxia was identified in five (7.0%) probands: AOA4 in three, AOA2 in one and AOA1 in another. ATX-*ANO10* was diagnosed in four (5.6%); MxMD-*ATP13A2*, HSP-*KIF1C,* HSP/ATX-*PGN* and HSP-*ZFYVE26* in 2 (2.8%) probands each. The remaining, ultra-rare ataxias, are described in Table 7. Two probands without diagnosis had pure cerebellar ataxia and 18 a complex form (with the additional trait being pyramidal signs, neuropathy, motor/ language delay or deafness – Table 8).

Table 7: Genetic diagnosis of AR-HCA

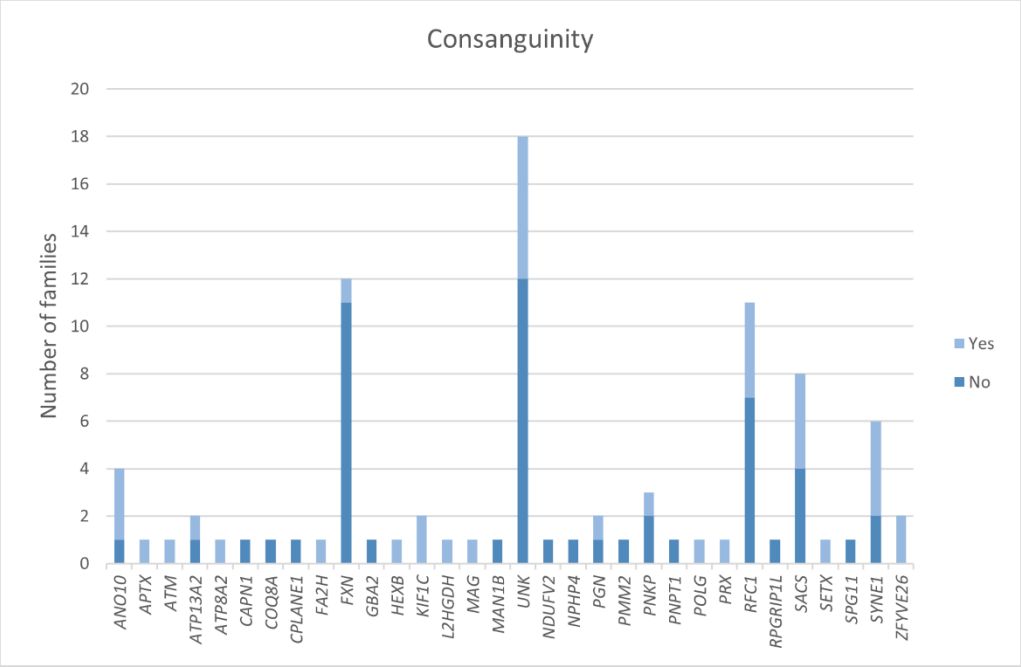
	Number	
	Probands	**Total number of patients**
Congenital ataxias		
Joubert syndrome		
NPHP4-related ataxia	1	1
RPGRIP1L-related ataxia	1	1
CPLANE1-related ataxia	1	1
ATP8A2-related ataxia	1	2
HSP/ATX/NBIA-FA2H	1	1
HSP-*MAG*	1	3
ATX-*PNPT1*	1	1
PRX-related ataxia	1	1
Metabolic ataxias		
Friedreich ataxia	11	15
HSP/ATX-*PGN*	2	2
ATX- *L2HGDH*	1	1
Congenital disorders of glycosilation		
MAN1B1-related ataxia	1	1
ATX-*PMM2*	1	1
Coenzyme Q10 deficiency/ *COQ8A-related ataxia*	1	1
ATX/HSP-*HEXB*	1	1
MxMD-*POLG*	1	1
Mitochondrial complex I deficiency, nuclear type 7/ *NDUFV2*	1	1
DNA repair defects ataxia		
ATX-*ATM*	1	1
Ataxia with oculomotor apraxia		
ATX-*PNKP* (AOA type 4)	3	6
ATX-*SETX* (AOA type 2)	1	6
ATX-*APTX* (AOA type 1)	1	1
Other degenerative ataxias		
ATX-RFC1	10	29
ARSACS/ *SACS*	8	9
ATX-SYNE1	6	8
ATX-*ANO10*	4	4
HSP/ATX-KIF1C	2	3
HSP-*ZFYVE26*	2	2
MxMD-*ATP13A2*	2	2
HSP/ATX-*GBA2*	1	2
HSP-KIAA1840	1	2
HSP/ATX-*CAPN1*	1	1
TOTAL	71	111

Table 8: Phenotypic classification of AR-HCA patients without genetic diagnosis

	Number	
	Probands	**Total number of patients**
Pure ataxia	2	4
Complex cerebellar ataxia		
with pyramidal signs	5	5
with motor or intellectual disability	5	5
with neuropathy	5	7
with dystonia	1	2
with Parkinsonism	1	1
with deafness	1	1
Total	20	25

In 39 (42.9%) families there was known consanguinity. It's distribution according to the genetic diagnosis is depicted in Figure 13.

Figure 13: Consanguinity distribution in AR-HCA, according to gene

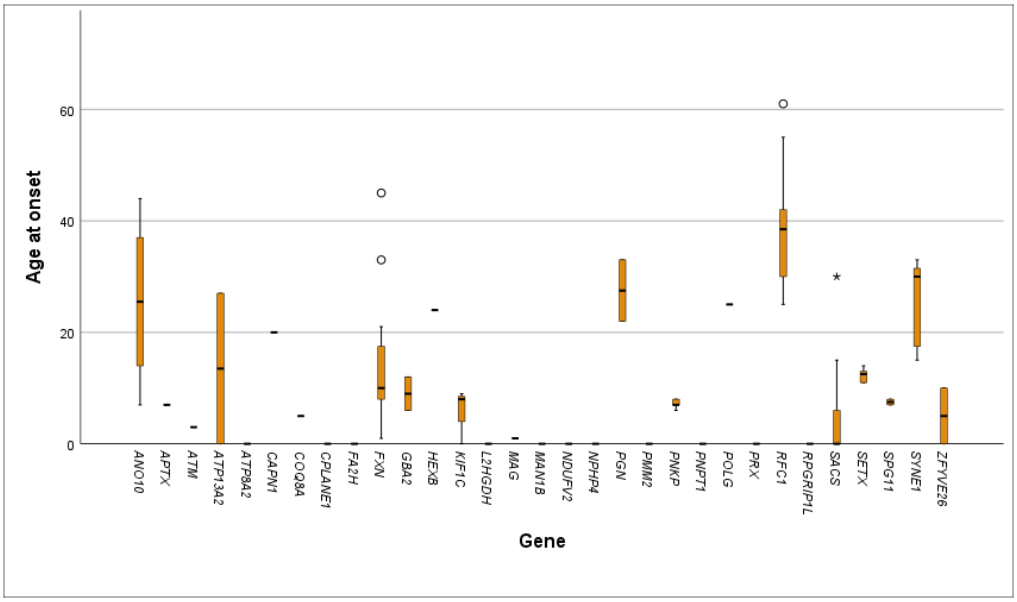


Onset according to genetic diagnosis

Age at onset

All patients with *ATX-RFC1* had late onset (38.5 [30.0-42.0] years). In FA, onset occurred in late childhood (mean of 10.0 years, [8.0-19.0]; one patient had late onset (33 years) and another very-late onset (45 years)). The majority (6) of ARSACS cases had a congenital form, with one having onset in childhood (6 years), one in adolescence (15 years) and another in adulthood (30 years). In ATX-*SYNE1* and ATX-*ANO10*, onset occurred in adulthood (mean of 30.0 years, [16.0-33.0], in the first, and 25.5 [10.5-40.5] in the latter). For AOA, disease emerged later in AOA2 (ATX-*SETX*) than in AOA4 (ATX-*PNKP*), mean of 12.5 years [11.0-13.3] *vs.* 7.0 [6.8-8.0]. AO in the remaining, rarer, ataxias is depicted in Figure 14.

Figure 14: Age at onset by gene in AR-HCA

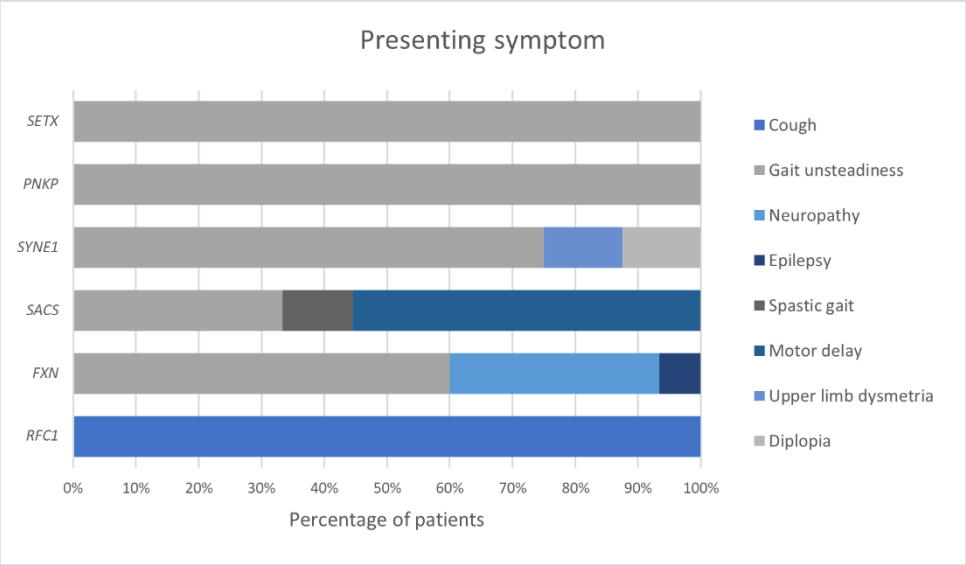


Clinical onset

When considering the first symptom in recessive ataxias with five or more cases per gene (n=73 patients, Figure 15), all AOA2 (ATX-*SETX*) and AOA4 (ATX-*PNKP*) presented with gait unsteadiness, whereas all *ATX-RFC1* presented with cough. In FA, nine (60.0%) presented with gait unsteadiness, five (33.3%) by neuropathy and one (6.7%) by epilepsy. ATX-*SYNE1* presented with gait unsteadiness in six patients (75.0%), upper limb dysmetria or diplopia in one each (12.5%). ARSACS presenting symptoms included motor

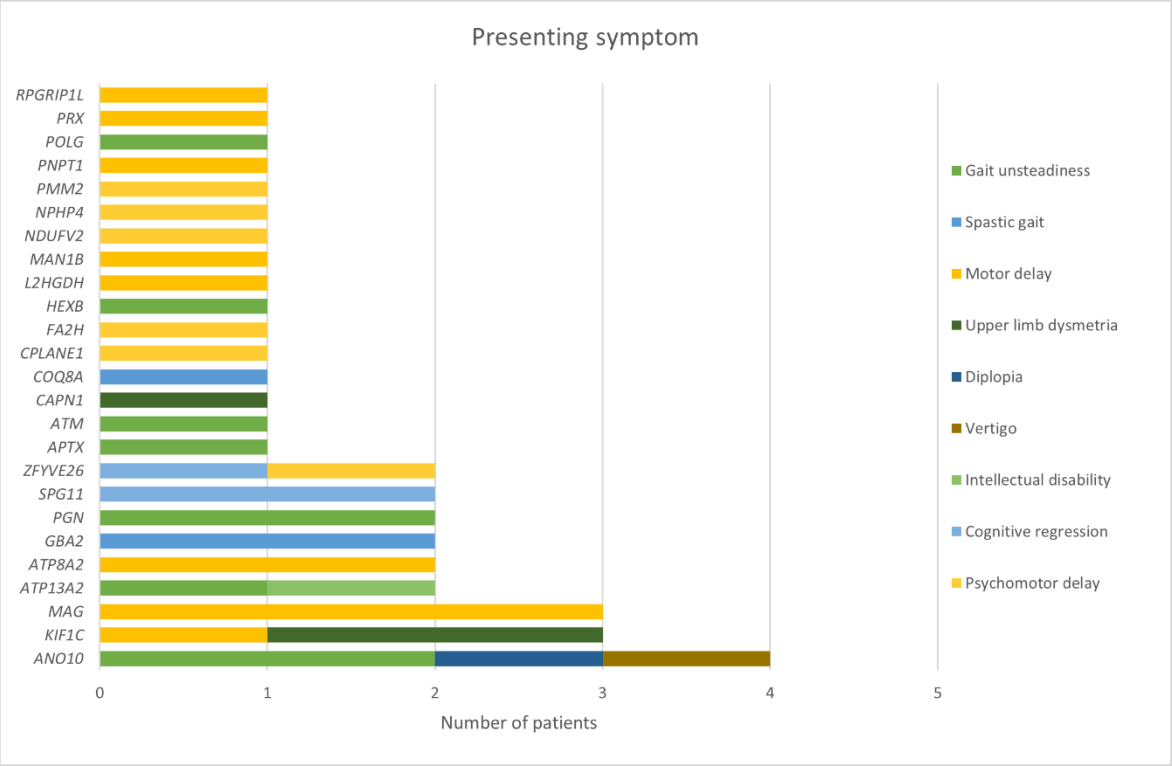
delay in five (55.6%) cases, gait unsteadiness in three (33.3%) and spastic gait in one (11.1%).

Figure 15: Presenting symptom in AR-HCA with 5 or more cases per gene



In ataxias with less than five patients per gene (38 patients, total), onset in ATX-*ANO10* was by gait unsteadiness (2, 50%), diplopia (1, 25%) or vertigo (1, 25%). In HSP/ATX-*KIF1C,* two (66.6%) cases presented with UL dysmetria and one (33.3%) with motor delay. Joubert (*RGGRIP1L*), ATX-*L2HGDH*, HSP-*MAG, ATP8A2, NDUFV2*, *PNPT1* and *PRX*-related ataxias, all presented with motor delay. Onset in congenital disorders of glycosylation (*MAN1B* and *PMM2*), Joubert syndrome (*NPHP4, CPLANE1*) and HSP/ATX/NBIA-*FA2H* consisted on psychomotor delay. HSP/ATX-*PGN*, AOA1, ATX-*ATM*, ATX/HSP-*HEXB* and MxMD-*POLG* presented with gait unsteadiness. The remaining ataxias' presenting symptoms, are depicted on Figure 16.

Figure 16: Presenting symptom in AR-HCA with 5 or less patients included



Phenotypic variation

Congenital ataxias

Of the overall cohort 38 (15.1%) patients had a congenital ataxia, 27 (71.1%) with AR, eight (21.1%) with AD and one (2.6%) with mithocondrial inheritance. Additionally, in two (5.3%) patients the mode inheritance was unknown.

Two siblings, with pathogenic variants in *ATP8A2,* were described in detail in article 4 for having a milder phenotypic presentation, as well as for the laboratory approach to confirm the pathogenicity of the identified variant.

Article 4. Congenital ataxia due to novel variant in ATP8A2.

Damásio J, Santos D, Morais S, Brás J, Guerreiro R, Sardoeira A, Cavaco S, Carrilho I, Barbot C, Barros J, Sequeiros J.
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SHORT REPORT

CLINICAL GENETICS WILEY

Congenital ataxia due to novel variant in *ATP8A2*

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Abstract

Congenital ataxias are a heterogeneous group of disorders characterized by congenital or early-onset ataxia. Here, we describe two siblings with congenital ataxia, who acquired independent gait by age 4 years. After 16 years of follow-up they presented near normal cognition, cerebellar ataxia, mild pyramidal signs, and dystonia. On exome sequencing, a novel homozygous variant (c.1580-18C > G - intron 17) in *ATP8A2* was identified. A new acceptor splice site was predicted by bioinformatics tools, and functionally characterized through a minigene assay. Minigene constructs were generated by PCR-amplification of genomic sequences surrounding the variant of interest and cloning into the pCMVdi vector. Altered splicing was evaluated by expressing these constructs in HEK293T cells. The construct with the c.1580-18C > G homozygous variant produced an aberrant transcript, leading to retention of 17 bp of intron 17, by the use of an alternative acceptor splice site, resulting in a premature stop codon by insertion of four amino acids. These results allowed us to establish this as a disease-causing variant and expand *ATP8A2*-related disorders to include less severe forms of congenital ataxia.

KEYWORDS
ATP8A2, hereditary ataxia, intronic variant, minigene assay

1 | INTRODUCTION

Congenital ataxias (CA) are a clinically and genetically heterogeneous group, characterized by congenital or early-onset ataxia, without evidence of progression or with a tendency to improve. We used whole-exome sequencing (WES), by next-generation sequencing (NGS), to identify disease-causing variants in two siblings with CA. A novel homozygous intronic variant in *ATP8A2*, ((NM_016529.5) c.1580-18C > G; p.(Asp527Alafs*5) - intron 17) was identified.

Joana Damásio and Diana Santos contributed equally to this study.

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ATP8A2 (OMIM # 605870) encodes the ATPase amino-phospholipid transporter class I, type 8A, member 2. It belongs to a subfamily of ATPases involved in lipid flipping, using energy from ATP hydrolysis to transport phospholipids across the cell membrane.[1] Disease-causing variants have been identified in patients with cerebellar ataxia, mental retardation and disequilibrium type 4 (CAMRQ4).[2] More recently, it has been associated with optic atrophy, movement disorders, and a less severe phenotype without encephalopathy.[3-5] Herein, we describe a novel variant and expand *ATP8A2*-related disorders to less severe forms of CA, with near normal cognition and gait acquisition.

2 | MATERIALS AND METHODS

2.1 | Subjects

Two siblings were followed for 16 years, by neurologists dedicated to ataxia. Since 2017, they were part of a prospective study on hereditary ataxias approved by the Ethics Committee (supplementary data S1). Informed consent was obtained for research, video and genetic studies.

2.2 | Whole-exome sequencing and bioinformatics analysis

WES was performed according to manufacturer's recommendations and 92.95% of the targeted regions had a 20× or higher coverage. Data analysis was performed in accordance with Broad Institute's guidelines.[6] An exhaustive in silico analysis to predict potential alterations in donor/acceptor splice-sites was carried out (supplementary data S1). Prioritized variants and reasons for exclusion were given in Supplementary Table S1.

2.3 | Minigene assay

An *ATP8A2* minigene was constructed with an insert of 4260 bp, corresponding to exons 17 to 19 and flanking intronic sequences, amplified with a high-fidelity polymerase from index patient's DNA (Figure 2(A)). PCR fragments were cloned into a pCMVdi vector, through Gibson assembly (Figure 2(B)). A QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies), was used to produce the wild-type (wt) construct. Appropriate primer pairs were designed using the QuikChange Primer Design Program (Agilent Technologies). Constructs were analyzed by Sanger sequencing, to check for the presence of the variant (v) and wt alleles.

2.4 | Variant effect on splicing

A splicing assay was performed, by transfecting 5 μg of each minigene construct (wt and v) into HEK293T cells, using jetPrime transfection reagent (Polyplus); 48 h after transient transfection, total RNA was extracted by a double method using NZYol reagent (Nzytech, Portugal) and RNeasy Mini Kit (Qiagen, Germany). Complementary DNA (cDNA) was synthesized from 5 μg of total RNA, by reverse transcription (RT), using SuperScript III First-Strand Synthesis System (Invitrogen). PCR amplification of the resulting cDNA transcripts from exon 17 to 19 was performed with RANGER DNA polymerase (Bioline, UK). PCR products were analyzed on an agarose gel and Sanger sequenced, to confirm impact on splicing. Amplified products were purified with Exo/SAP Go (Grisp, Portugal), sequenced using a ready reaction kit, and then loaded on an ABI-PRISM 3130 XL automatic sequencer (Applied Biosystems).

3 | RESULTS

The index patient was a 26 years-old son of consanguineous parents (Figure 1(A)), with a delay in developmental milestones, who acquired independent gait by age 4 years. On neurological examination (age 10), he presented scandid dysarthria, gaze-evoked nystagmus on horizontal and vertical testing, hypotonia, hyperreflexia, and flexor plantar responses. There was mild facial and upper limb dystonia, upper and lower limb dysmetria, and ataxic gait. Over time, we observed

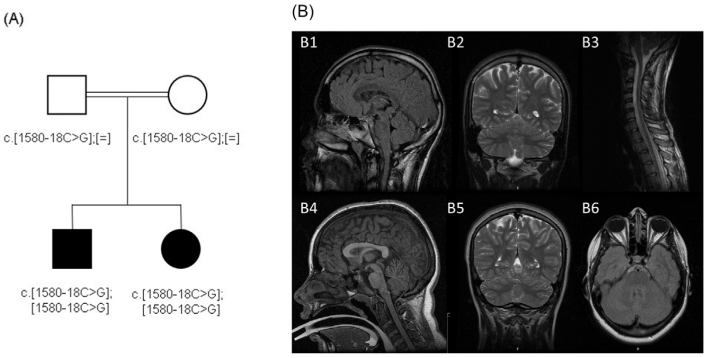

FIGURE 1 (A) Pedigree with variant segregation. (B) Proband MRI: B1, enlarged pineal; B2, normal cerebral/cerebellar parenchyma; B3, normal cervical/dorsal medulla; sister MRI: B4-B6, normal cerebral/cerebellar parenchyma

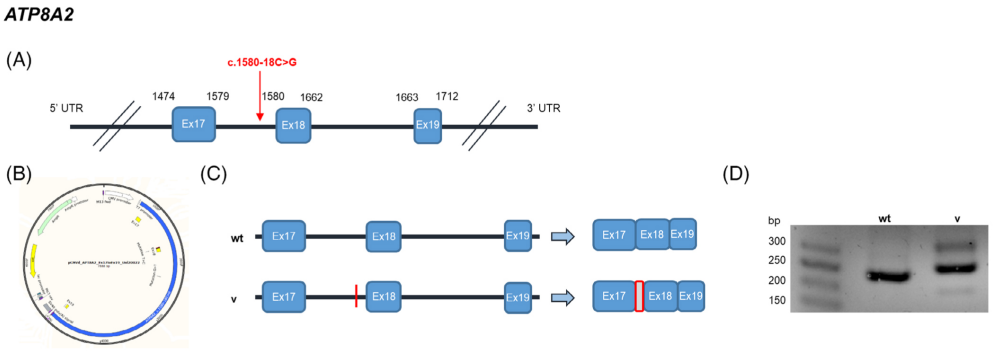

FIGURE 2 Minigene assay. (A) *ATP8A2* schematic representation and variant identified; (B) Map of the plasmid created to obtain a minigene; (C) Scheme showing results, after HEK293T cells transfection and mRNA extraction; (D) PCR amplification of cDNA produced [Colour figure can be viewed at wileyonlinelibrary.com]

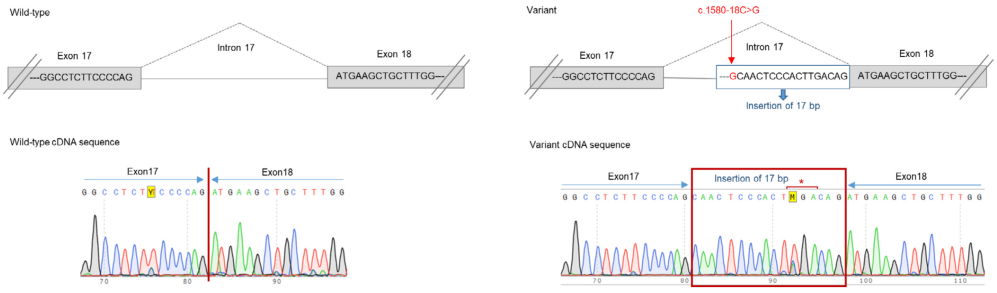

FIGURE 3 Genomic organization and splicing prediction of wild-type (wt) and variant (v) minigene, and electropherograms showing the transcripts generated from both constructs [Colour figure can be viewed at wileyonlinelibrary.com]

progressive gait improvement, and lower limb spasticity emergence (supplementary Video S1). He showed average academic performance and got a technical diploma. At last examination, he scored 14 on Scale for the Assessment and Rating of Ataxia (SARA), was working full time and driving an automatic car. Neuropsychological evaluation (Supplementary Table S2) disclosed deficits in attention/psychomotor velocity, verbal fluency and naming. Visual perception and construction abilities were borderline, with normal oral comprehension, repetition, verbal/visual memory, and executive functions.

His 18-years-old sister also presented delayed milestones. When observed (age 2), she had dysarthric speech, horizontal and vertical gaze-evoked nystagmus, hypotonia, hyperreflexia, and flexor plantar responses. Facial and upper limb dystonia was present, as well as dysmetria, truncal ataxia and wide-base gait (bilateral aids). She acquired independent gait by age 4 (supplementary Video S1) and lower limb spasticity emerged at age 8. Astigmatism was diagnosed in childhood, and while growing up ataxia improved. SARA at last visit was 16.5 (supplementary Video S1). She was at the 12th grade of schooling, with good learning ability. On neuropsychological evaluation (Supplementary Table S2), she scored 100 on verbal WAIS-III and 78 on realization WAIS-III, translating normal cognitive functioning, but taking a longer time in psychomotor/visual construction tasks.

An extensive investigation was conducted in both (supplementary data S1). Brain and medulla MRIs were normal, except for enlarged pineal in the proband (Figure 1(B1-6)). Somatosensory evoked potentials (SSEP) were suggestive of dorsal columns dysfunction.

3.1 | Genetic analysis and functional assay

WES showed both siblings to be homozygous for novel intronic variant on *ATP8A2* ((NM_016529.5) c.1580-18C > G; p.(Asp527Alafs*5) - intron 17 (Figure 2(A)), confirmed by Sanger sequencing. His parents were heterozygous for the variant. The in silico analysis predicted loss of a splicing acceptor-site, creating a new intronic cryptic acceptor splice-site.

To investigate the functional impact of this variant on splicing, a minigene assay was generated (Figure 2(B)). After transfection and cDNA sequencing, the wt minigene produced the expected canonical transcript, while the construct with the novel intronic variant produced a major aberrant transcript (Figure 2(C,D)). The sequence of the RT-PCR product generated from the v minigene retained 17 nucleotides of intron 17, by use of an alternative acceptor splice-site (Figure 3). The effect on the protein would be a change of the reading frame with the insertion of four new amino acids (AlaThrProThr) after codon 526 and introduction of a premature stop codon, p.(Asp527Alafs*5).

4 | DISCUSSION

We describe two siblings with CA, who acquired independent gait and presented steady improvement over the years. WES identified a novel homozygous variant in *ATP8A2* ((NM_016529.5) c.1580-18C > G; p.(Asp527Alafs*5) - intron 17); bioinformatics analysis predicted it would probably affect splicing. Through functional studies, it was possible to establish this variant generated retention of 17 nucleotides of intron 17, causing an aberrant pre-mRNA splicing transcript, by creating a new cryptic acceptor splice-site. This resulted in a reading frameshift with the introduction of a premature stop codon, expected to produce a non-functional truncated protein.

ATP8A2 is highly expressed in the central nervous system, particularly the cerebellum.[3,5] The encoded ATPase amino-phospholipid transporter is determinant in the phospholipid bilayer curvature[7] being involved in vesicle trafficking[8] and regulation of neurite outgrowth.[9] In mouse models, ATP8A2 deficiency induces axonal degeneration and neuronal chromatolysis.[8] Homozygous variant mice present failure to thrive, tremor, anomalous gait and reduced lifespan.[8]

In 2010, Cacciagli et al described a patient with mental retardation, abnormal movements and hypotonia, who had a de novo balanced t(10;13), disrupting the coding sequencing of *ATP8A2*.[3] Onat et al reported a homozygous missense variant in *ATP8A2*, in four members of a family with CAMRQ phenotype, identifying the fourth *locus* associated with this syndrome.[2] Since then, 28 patients were described: 26 with CAMRQ[4,5,10-12] and two with a less severe phenotype,[5] as the patients we report (Supplementary Table S3). Only three patients had independent gait,[2,5] the majority having no ambulation skills.[2,4,5,10-12] A dysarthric, but otherwise normal speech was infrequent in *ATP8A2*-related disorders.[2-5,11,12] Severe cognitive impairment was present in 29 patients,[2-5,11,12] whilst ours had only minor deficits on neuropsychological evaluation. Movement disorders included chorea/choreoatethosis, dystonia and facial dyskinesia.[2,4,10-12] Interestingly, our patients had facial dystonia, but no dyskinesia. Both presented at very young age with hyperreflexia, and later spasticity. In a recent report, hyperreflexia was present in four patients, with no mention to spasticity.[5] Contrarily to what has been recognized, our patients had no deafness or optic atrophy. Normal brain MRIs were described in eight cases,[3,4,11,12] as in ours; non-specific abnormalities have included cortical/cerebellar atrophy, delay in myelination, thin corpus callosum and optic nerves hypoplasia.[2,4,5] Abnormal SSEP, translating dorsal columns dysfunction, were identified in both siblings. Information on SSEP had only been provided in another patient, with a severe CAMRQ4 phenotype, who also presented abnormal recordings.[4] It is most interesting that in all three patients who had SSEP performed, dysfunction of sensory pathways was present at the dorsal columns. Although peripheral axonal degeneration was shown in mouse models, none of the reports mentions peripheral neuropathy.[3,4,12]

In our patients, homozygous for a novel intronic variant, we identified a milder phenotype within *ATP8A2*-related disorders. Although it is predicted the v transcript would be targeted for degradation by nonsense-mediated decay, resulting in a loss-of-function mechanism, a low level of normal *ATP8A2* mRNA may be produced and sufficient to prevent severe disease, as observed in other disorders.[13,14]

Our findings highlight the importance of characterizing intronic variants, to increase molecular diagnostic yield and clarify pathogenic mechanisms. While missense variants usually code for a pathogenic protein by a gain-of-function mechanism, several studies reported that in *ATP8A2*, missense variants may cause lower levels or loss of protein function.[4,5] Notwithstanding, stop-codon and splice-site variants are more likely to act through loss-of-function, by reducing levels of the normal protein.

In conclusion, we hope to contribute to a better characterization of *ATP8A2* phenotypic spectrum. We suggest the inclusion of *ATP8A2* in NGS multigene panels for inherited ataxias, since a wide clinical heterogeneity may be present.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Data have not been archived at public repository, but are available upon request at CHUP.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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Link to online supplementary material and video S1 (in supporting information)

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Late-onset recessive ataxias

Fifty-two (39.1%) individuals had late-onset (median at 38.0 years, [30.0-43.7]; details in Table 9). In 41 (78.8%) a genetic diagnosis was obtained, with the most frequently involved genes being *RFC1* (29 patients), *SYNE1* (4 patients), *FXN* (2 patients) and *ANO10* (2 patients).

Reflecting the large number of patients with expansion in *RFC1*, the most common presenting symptom was cough (29 patients, 55.8%), followed by gait unsteadiness (14, 26.9%), and UL dysmetria (three patients, 5.8%). Onset of gait ataxia was at mean age of 52.5 years ([40.0-60.0]) and time up to genetic diagnosis (when applicable) was of 31.0 [20.0-47.0] years.

Late-onset AR ataxias were significantly associated with oligonucleotide expansions, while the remaining cohort with non-repeat expansion genetic defects (Table 10). Late-onset had a shorter disease duration at baseline, and a lower total SARA. Use of wheelchair was more frequent in early-onset, and disease duration both until use of cane and wheelchair was shorter in early-onset.

Table 9. Late-onset AR-HCA

Disorder*	Families (N)	Subjects (N)	AO (Y)	AD (Y)	First symptom/ sign (N)	Ataxia (N)	AAO (Y)
Known genetic diagnosis							
ATX-RFC1	10	29	40.0 [30.0-42.0]	77.0 [71.0-88.0]	Cough (29)	29	58.0 [50.0-62.5]
ATX-SYNE1	4	4	31.5 [30.0-33.0]	51.0 [45.8-51.8]	Gait unsteadiness (3) UL dysmetria (1)	4	31.5 [30.0-38.3]
ATX-ANO10	2	2	30.0/ 44.0$^{\alpha}$	60.0/ 54.0$^{\alpha}$	Diplopia (1) Gait unsteadiness (1)	2	40.0/ 44.0$^{\alpha}$
Friedreich ataxia	2	2	32.0/ 45.0$^{\alpha}$	32.0/ 65.0$^{\alpha}$	Neuropathy (2)	2	33.0/ 65.0$^{\alpha}$
HSP/ATX-*PGN*	1	1	33.0	67.0	Gait unsteadiness	1	33 .0
MxMD-*ATP13A2*	1	1	27.0	36.0	Gait unsteadiness (1)	1	27.0
MxMD-*POLG*	1	1	25.0	55.0	Gait unsteadiness (1)	1	25.0
ARSACS	1	1	30.0	44.0	Spastic gait (1)	1	35.0
Unknown genetic diagnosis							
	6	11	43.0 [39.0-62.0]	NA	Gait unsteadiness (7) UL dysmetria (2) Vertigo (2)	11	44.0 [39.0-62.0]

Legend: AAO – Age at ataxia onset, AO – Age at onset, AD – Age at diagnosis, M – Months, MRAS - Mitochondrial recessive ataxia syndrome/ N – Number, UL – Upper limbs, Y – Years, *- adapted from MDS classification , $^{\alpha}$ - individual data from each patient, $^{\beta}$ - no data from quartile 3

Table 10. Comparison between late-onset and early-onset AR-HCA

	Late-onset AR	**Early-onset AR**	**p value**
Number of patients	52 (39.1%)	81 (60.9%)	
Male gender	24 (46.2%)	35 (43.2%)	0.739
Age at onset	38.0y [30.0-43.7]	7.0y [0.0-12.5]	**<0.001**
Disease duration at baseline (years)	19.0y [10.0-32.5]	26.0y [18.0-39.5]	**0.003**
Overall disease duration (years)	28.0y [15.0-44.2]	36.0y [23.0-50.0]	**0.043**
Onset symptoms/signs			
Cerebellar	18 (34.6%)	41 (50.6%)	0.070
Non-cerebellar	34 (65.4%)	40 (49.4%)	
Type of genetic defect			
Oligonucleotide expansion	31 (75.6%)	13 (18.8%)	**<0.001**[a]
Conventional mutation	10 (24.4%)	56 (81.2%)	
Clinical severity			
SARA at baseline	11.7 [6.5-16.5]	16.0 [11.0-29.5]	**0.006**
Disability milestones			
Presence of falls	24 (52.2%)	48 (59.3%)	0.439
Disease duration until falls	12.0y [5.0-37.0]	13.0y [9.0-20.0]	0.972
Use of cane	21 (42.9%)	23 (30.7%)	0.165
Disease duration until cane	37.0y [19.5-42.5]	20.0y [9.0-29.0]	**0.002**
Use of wheelchair	11 (22.4%)	32 (40.0%)	**0.040**
Disease duration until wheelchair	40.0y [37.0-47.0]	16.0y [11.2-29.7]	**<0.001**

Legend: Y – Years; [a] - Significant association of oligonucleotide expansion with late onset (adjusted standardized residuals value of 5.9) and of conventional mutations with early onset (adjusted standardized residuals value of 5.9).

The case of Friedreich ataxia

In article 5, we describe a Friedreich ataxia family spanning two successive generations. The youngest generation was composed by four siblings, all affected. Three had the classical phenotype and one a late onset form. The father, observed due to the peculiarity of all his children having FA, had a severe sensory neuropathy, corresponding to a very late onset form.

Article 6 concerns a patient with longstanding Friedreich ataxia, with an extreme phenotype, with blindness and deafness in addition to the typical phenotype.

Article 5. Pseudodominance in Friedreich ataxia impact of high prevalence of carriers and intrafamilial clinical variation.

Malaquias MJ, Oliveira J, Santos M, Brandão AF, Sardoeira A, Sequeiros J, Barros J, ***Damásio J****.*
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Movement Disorders
CLINICAL PRACTICE

CASE SERIES WITH LITERATURE REVIEW

Pseudodominance in Friedreich Ataxia—Impact of High Prevalence of Carriers and Intrafamilial Clinical Variation

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ABSTRACT: Background: Friedreich ataxia (FA) is the most common form of autosomal recessive (AR) ataxia. It is a rare disease, but carriers are frequent (1/100). Pseudodominance in FA has seldomly been reported; it may pose additional challenges for diagnosis.

Cases: A family with two consecutive generations affected by FA is described. The proband and two younger siblings had typical FA, characterized by infantile-onset ataxia, hyporeflexia, Babinski sign, cardiomyopathy, and loss of ambulation in the second decade of life. Another female sibling had delayed-onset (>25 years old), with mild cerebellar and sensitive ataxia since her mid-30s. Their father presented very late-onset FA (>40 years old), with sensitive axonal neuropathy. All five patients had biallelic $(GAA)_n$ expansion in *FXN*. The first three had larger expansions (>800 repeats), while the latter two had one shorter expanded allele (~90 repeats).

Literature Review: Pseudodominant inheritance has been described in 13 neurological disorders. Seven are movement disorders, of which three were associated with high frequency of carriers (FA, Wilson's disease and *PRKN*-related parkinsonism).

Conclusions: Clinicians should be aware of the possibility of pseudodominance when facing an apparent autosomal dominant pedigree, particularly in disorders with high frequency of carriers and variable expression. Otherwise, genetic diagnoses may be delayed.

Friedreich ataxia (FA) (OMIM# 229300) is the commonest autosomal recessive (AR) ataxia in Caucasians, with an estimated prevalence of 0.5–1:100,000.[1,2] Most frequently, it is caused by a biallelic intronic $(GAA)_n$ expansion in *FXN* (OMIM × 606829), resulting in decreased expression of frataxin, a protein involved in mitochondrial iron metabolism.[1] Typical FA starts before age 25, and involves the peripheral nervous system, posterior columns, cerebellum and pyramidal tracts. It is characterized by dysarthria, square wave jerks, muscle weakness, impaired proprioception and vibration, abolished deep tendon reflexes (DTR), Babinski sign and progressive gait and limb ataxia.[1] Non-neurological features include skeletal anomalies (scoliosis, *pes cavus*), neurosensorial deafness, hypertrophic cardiomyopathy, optic atrophy and diabetes.[1] Atypical FA is rare (about 15% of patients) and comprises late-onset FA (LOFA) and very LOFA (vLOFA), when starting after 25 and 40 years of age, respectively. These atypical forms are characterized by milder symptoms, slower progression and paucity of extra-neurological involvement.[3] FA is highly challenging at a genetic level as, (1) in a minority of patients it may be due to a compound heterozygous GAA repeat expansion with a point *mutation*;[1] (2) the range of pathogenic expanded alleles is wide (66–1700 GAAs), with expansions ranging from 44 to 66 being unstable and conferring high risk for disease;[4] and (3) the frequency of heterozygotes in the general population is estimated at 1/100.[1,2,5] Two main clinical consequences emerge from this genotypic variability: (1) inter and intra-

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Keywords: pseudodominance, heterozygous carriers for recessive diseases, late-onset Friedreich ataxia.

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familial phenotypic heterogeneity, in that disease severity correlates partially and inversely with the size of expanded alleles (particularly the shorter one);[5] and (2) a high population frequency of carriers may lead to a pseudodominant transmission, challenging its diagnosis. While the clinical heterogeneity of FA is well known, descriptions of pseudodominance are scarce.[6–11]

Here, we describe a FA family, genetically confirmed, exhibiting phenotypic variability and pseudodominant inheritance, and review pseudodominance in neurological disorders.

Patients and Methods

A Portuguese family of five affected members with FA in two consecutive generations, has been followed at the movement disorders outpatient clinic for 30 years. Information was retrieved from medical records, according to a structured protocol including demographic, clinical data and complementary investigation (MRI, nerve conduction study, echocardiogram, ECG and genetic analysis, when available).

Family Study

Both parents were originally from the same small village (around 500 inhabitants), but there was no known consanguinity (Fig. 1). Three offspring had early-onset and one late-onset FA; the only healthy sibling was found to have been adopted. The fact that all four biological sibs were affected, unusual for an AR condition, prompted our formal examination of both parents. The clinical description and *FXN* genotype of the affected cases are as follows:

Case Series

Case 1 (Patient III-1)

The proband, a male patient, homozygous for a ~ 850 $(GAA)_n$ expansion, had a normal psychomotor development and was otherwise healthy until the age of 5 years, when he started experiencing walking difficulties. Neurologic examination (by age 9) showed absent DTR, bilateral Babinski sign, decreased vibration in the halluces and positive Romberg test. In the

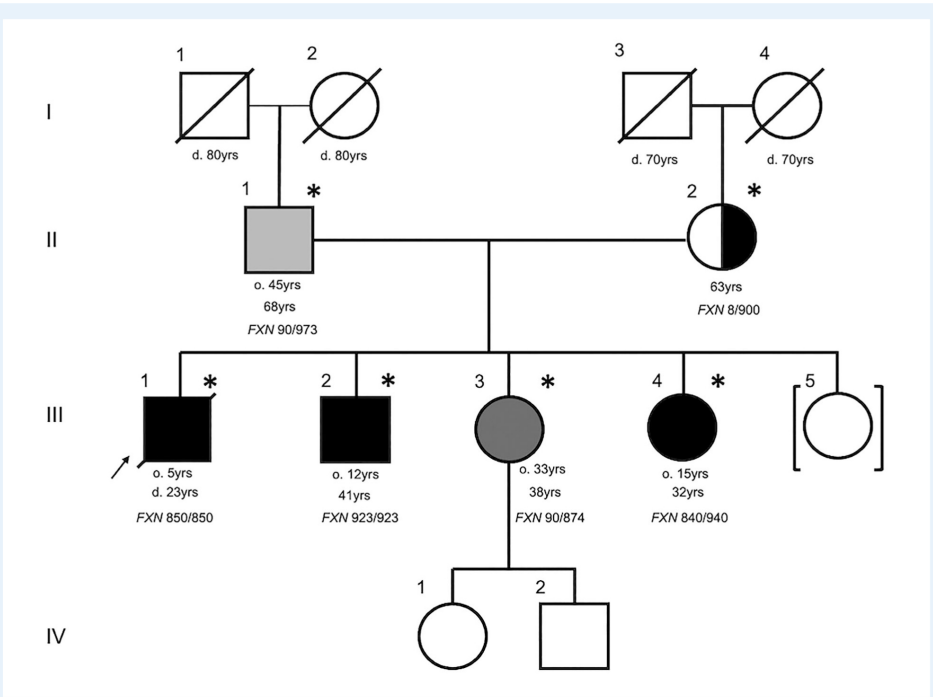

FIG. 1. The family's pedigree shows a pseudo-dominant pattern of transmission. Filled symbols represent affected individuals: black, classical FA; dark gray, LOFA; and light gray, vLOFA; half-filled symbol indicates heterozygous carrier; symbol in bracket means adopted child; and "*" refers to individuals who have been observed and performed genetic analysis of *FXN*. Size of normal and expanded alleles is indicated, together with current age, age-of-onset (o.) and age at death (d.).

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following years, he developed paraparesis, limb dysmetria and cerebellar dysarthria, being on a wheelchair by age 15 years. Scoliosis, *pes cavus*, hypertrophic cardiomyopathy and atrial fibrillation were also present. He died at the age of 23, shortly after a cerebellar embolic stroke.

Case 2 (Patient III-2)

A 41-year-old male sib, homozygous for a ~ 923 $(GAA)_n$ allele, was first observed when 8 years-old. Although asymptomatic, neurologic examination showed absent DTR in lower limbs (LL) and positive Romberg test. Four years later, gait unsteadiness and slurred speech were noticed. These gradually progressed and, by age 20, he was wheelchair-bound. Neurological examination at age 33 years included severe dysarthria, hypotonic-areflexic tetraparesis, bilateral Babinski sign, cervical and hands dystonia, stocking-and-glove hypoesthesia, decreased vibration in the halluces and marked axial and appendicular ataxia, orthostatism being impossible (video 1, segment 1). He also had thoracic scoliosis and Raynaud syndrome. Audiogram and ophthalmologic evaluation ruled out neurosensorial hypoacusis and optic atrophy. Hypertrophic cardiomyopathy was known since pediatric age and, by mid-30s, atrial fibrillation and diabetes were diagnosed.

Case 3 (Patient III-4)

A 32-year-old sister, had a history similar to *III-2*. Her genotype was ~840/~940 GAAs at the *FXN locus*. She was observed while asymptomatic, at 4 years of age: neurologic examination showed only absent DTR in LL. Gait ataxia occurred by age 15, and she was in a wheelchair 5 years later. Cervical/ brain MRI at that time, did not reveal spinal or cerebellar atrophy. On examination, at 27 years of age, she had moderate dysarthria, hypermetric saccades, hypotonia and muscle weakness, with distal atrophy of the hands, Raynaud syndrome and bilateral *pes cavus*; DTR were diminished in the upper limbs (UL) and absent in LL, vibration sense was reduced distally and Babinski sign was bilateral. She presented cervical and hands dystonia, limbs and truncal ataxia, being unable to stand or walk (video 1, segment 2). She had an intra-auricular communication (IAC), surgically corrected in childhood, hypertrophic cardiomyopathy and atrial fibrillation. In her late twenties she suffered a right medial cerebral artery embolic stroke and is currently bedridden.

Case 4 (Patient III-3)

Their 38-year-old sister had a normal examination at age four. Her genotype at *FXN* was ~90/~874. At age 33, she was still asymptomatic, but on neurological evaluation there was a mild cervical dystonia, mild left limbs dysmetria; decreased vibration sense and abolished DTR in LL. Like her sister, she had a congenital IAC, surgically corrected in childhood, with a normal echocardiogram in adulthood and an ECG showing abnormal right ventricular conduction. On MRI there was no cerebellum or cervical atrophy. She currently maintains mild left limbs dysmetria, with abolished LL DTR and diminished vibration distally (video 2).

Case 5 (Patient II-1)

The 68-year-old father (~90/~973 GAAs), had history of alcohol abuse, but was healthy until age 45 years, when he first noticed pain and tingling in the LL, which worsened progressively. He was called for examination at age 65 years, and



Video 1. Typical Friedreich ataxia. **Segment 1.** Patient III-2 is in a wheelchair; we may hear the cerebellar dysarthria and observe head titubation and mild torticollis, distal atrophies of the hands, tetraparesis and upper limbs dysmetria. **Segment 2.** Patient III-4, also in a wheelchair has mildly fragmented ocular pursuit with saccadic intrusions, tetraparesis and upper limbs dysmetria and dysdiadochokinesia. Lower limbs coordination could not be evaluated due to motor deficit. *Pes cavus*, Raynaud phenomenon and hand muscles atrophy are also observed.
Video content can be viewed at https://onlinelibrary.wiley.com/doi/10.1002/mdc3.13694



Video 2. Late-onset Friedreich ataxia. Patient III-3 exhibits mild torticollis to the left and laterocollis to the right, normal ocular movements, mild left limbs dysmetria on finger chasing and mild left dysdiadochokinesia. DTR are normal in the upper limbs and abolished on lower limbs. She has negative Romberg test and normal tandem gait.
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Video 3. Very late-onset Friedreich ataxia. Patient II-1 has normal ocular movements and no upper limbs dysmetria. He has mild upper limbs bradykinesia, left dysmetria on heel–knee test and reduced lower limb DTR. Romberg test is negative, but gait is mildly broad-based.
Video content can be viewed at https://onlinelibrary.wiley.com/doi/10.1002/mdc3.13694

presented diminished DTR, stocking-and-glove hypoesthesia, decreased vibration sense in the halluces, mild left dysmetria on heel-to-knee and mildly broad-based gait (video 3). Nerve conduction studies confirmed a sensitive axonal neuropathy. Myocardiopathy, neurosensorial hypoacusis and diabetes were excluded. Brain and cervical MRI displayed only mild cerebellar atrophy (involving mainly the superior vermis).

Case 6 (Individual II-2)

The 63-year-old mother had a normal neurological examination, and was a carrier of one expanded allele at the *FXN locus* (8/~900 GAAs).

Literature Review

Pseudodominance may result from couples where one partner is homozygote or compound heterozygote and the other harbors (at least) one pathogenic variant in the same gene (and at least one partner is affected) for an AR disease.[12] This may happen (1) with declared or "hidden" consanguinity, or in endogamic populations, often with successive consanguineous mattings (offspring should be homozygotes); or (2) just due to the high frequency of carriers in the population (offspring may be either homozygotes or compound heterozygotes). That should be distinguished from cases when an allele for an AR disease is expressed due to deletion of the other allele (a *hemizygous* state, the equivalent of a female affected by a X-linked disease). Also,

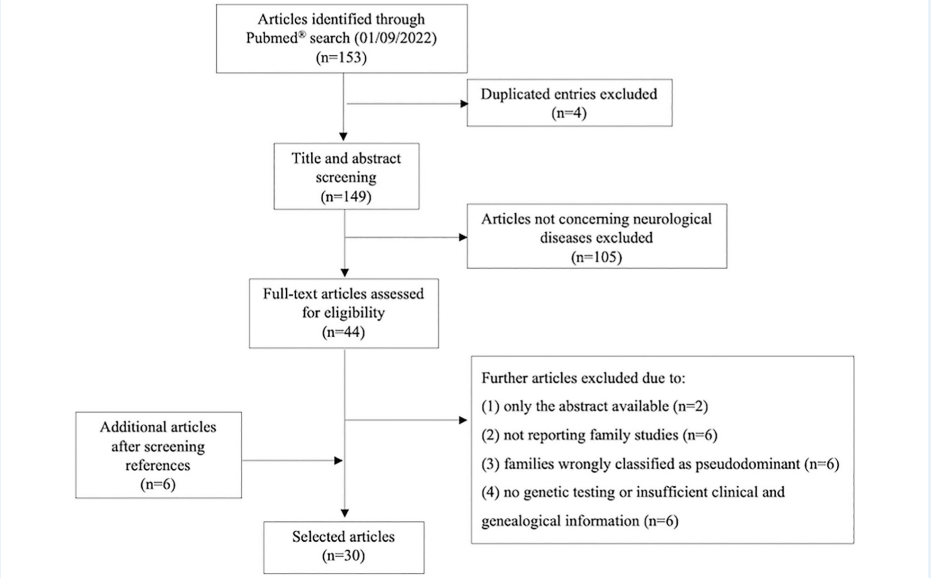

FIG. 2. Flowchart of selected articles for analysis.

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in some diseases, heterozygotes may occasionally show an intermediate (milder and sometimes different) phenotype from the homozygotes,[12] a situation called *manifesting heterozygotes*.

We conducted a PubMed search, without time limit, using the terms: "pseudo-dominance," "pseudo-dominant," "pseudodominance" OR "pseudodominant". All abstracts, written in English, were reviewed and those related to neurological conditions were selected: a total of 44 articles were identified and assessed; 20 were excluded: (1) when only the abstract was available (n = 2); (2) no description of family studies (n = 6); (3) families wrongfully classified as "pseudodominant" (ie, families with AR disorders and homozygotes in one generation and heterozygotes in the other generation) (n = 6); and (4) no genetic testing or insufficient clinical and genealogical information, making pseudodominance questionable (n = 6). Six articles were additionally included, after references screening (Fig. 2). Out of the 30 articles considered, 24 were single or small-series case reports, five were large-scale series and one was a population-based study (supplementary table).

FA, Wilson's disease (WD) and *PRKN*-related Parkinsonism were the diseases more often associated with pseudodominance: eight WD families,[13–15] six FA families,[6–11] and six families with *PRKN*-related Parkinsonism.[16–20] Ten other neurological disorders with pseudodominant inheritance were identified, relying (with few exceptions) on individual family reports: spinal muscular atrophy (five);[21–23] McArdle disease (three);[24–26] ocular motor apraxia type 1 (one) and type 2 (two);[27–29] pseudoxanthoma elasticum with cerebrovascular disease (two);[30,31] and one family each with *PINK1*-related Parkinsonism,[32] neuronal ceroid lipofuscinosis due to *CTSF* variants,[33] Charcot–Marie-Tooth type 2 due to *GDAP1* variants,[34] Charlevoix-Saguenay ataxia,[35] and Brown-Vialetto-Van Laere syndrome (due to *SLC52A3* variants).[36] In FA, WD and *PRKN*-related Parkinsonism there were families in whom pseudodominance was related to a population high frequency of heterozygotes (1/90 in WD; 3.2–4.6% in *PRKN*-related Parkinsonism),[7,10,14,37] while in both FA and *PRKN*-related Parkinsonism there were also reports of consanguinity.[8,11,16,18,20] Of note, analysis of pseudodominance related to *PRKN* may be complex, since single heterozygous variants alone are a risk factor for Parkinson disease.[37] There are no specific studies to evaluate frequency of pseudodominance in families with FA, WD or *PRKN*-related Parkinsonism, but it seems to be a rare event. As for the remaining neurological disorders, prevalence of carriers has rarely been determined. As they are rare (or very rare) in general population, pseudodominance occurred either due to consanguinity or chance homozygote-heterozygote mating.

Discussion

Phenotypic Spectra and GAA Transmission

We report a very interesting family with FA, with no declared consanguinity, in which two consecutive generations were affected and the three clinical forms of FA identified: (1) *typical* FA (*patients III-1*, *III-2* and *III-5*), with infantile-onset ataxia, hyporeflexia, Babinski sign, cardiomyopathy, and loss of ambulation in the second decade; (2) LOFA in *patient III-4*, with mild cerebellar and sensitive ataxia; and (3) vLOFA in *patient II-1*, who developed sensitive neuropathy in middle age, with no extra-neurological involvement. As expected, patients with *typical* FA (and hence severe disease) had two longer expanded alleles (>800 repeats), while both *atypical* patients had one short expansion (~90 repeats), a genotype–phenotype correlation and intra-familial heterogeneity in line with previous reports.[1,2]

The asymptomatic mother carried one expanded allele on *FXN* (~900) and the father one short (~90) and one larger expanded allele (~973 GAAs). There was unstable transmission of the GAA repeat to the offspring (Fig. 1), as the expanded alleles slightly increased or decreased in size, compared to their parents'. Contraction of the maternal expansion occurred on III-1 and III-3 (shorter allele most probably inherited from the father), whereas it expanded further on III-2. The larger expansion from the father contracted upon transmission to III-1 and III-2. In III-4, the paternal or maternal origin of each allele is more difficult to ascertain, though both a maternal and a paternal contraction seem likely. As in other triplet repeat expansion disorders, the expanded GAA in *FXN* has been described as a "dynamic mutation," meaning that during parent-offspring transmission there is instability (changes in size) of the expanded repeat.[5] In autosomal dominant trinucleotide-repeat disorders, the expanded allele tends to increase (creating *genetic anticipation*). On the contrary, in FA further expansion or contraction of expanded alleles is difficult to predict in offspring.[5,6] Two factors have been identified to influence intergenerational variation of the repeat size in FA: (1) parental sex and size of the transmitted allele: paternal alleles tend to contract in offspring, whereas maternal transmission may either lead to further expansion or contraction (with a strong tendency for smaller expansions to expand further and for larger expansions to contract);[5] and (2) genetic status: size of the expanded allele in heterozygotes tends to remain stable throughout generations, which may account for the maintenance of FA expansions in the population.[5] Importantly, not only repeat size and intergenerational instability explains age-of-onset and severity in individual FA patients. Indeed, the role of epigenetic modifiers and $(GAA)_n$ somatic mosaicism across different tissues (heart, pancreas, cerebellum, spinal cord)[38] have been the focus of recent studies.

Pseudodominance

The pattern of pseudodominance in the described family, with two consecutive generations affected and intra-familial heterogeneity, has seldomly been described.[6–11] The clinical impact of such extremely rare pedigrees justifies the importance of this report: the father of this family was only diagnosed after all his offspring were found to have FA. In all seven pseudodominant FA families (including the present report) the affected parent had late or very-late onset, contributing to reproduction and transmission of the disease to the next generation.[6–11] Disease onset was quite variable in the second generations, ranging from 5 to

48 years,[6–11] with two cases being still asymptomatic in their twenties.[9]

The carrier rate of FA in Portugal is 1/106 (similar to other Caucasian populations), prevalence of the disease being 1.0 per 100,000.[2,39] The family here reported originated from a very small and geographically isolated village, in a region where prevalence of FA is the triple of the national mean. Still, we could not be certain whether the pseudodominant pattern was due to the high frequency of carriers or to "hidden" consanguinity. Haplotyping could have helped us to clarify this aspect, but was beyond the scope of the present work. Among the further six families described with FA in two generations, there was also no known consanguinity in three,[7,9,10] although two (like ours) were from the same village.[7,10] This highlights the need for carrier screening and genetic counseling programs for more common recessive diseases, as well as in isolated areas and regions of known higher prevalence.

With this report we wish to raise awareness about (1) atypical FA phenotypes and (2) families with consecutive affected generations in AR disorders (pseudodominance), when there is a high prevalence of carriers or in endogamic populations. Both may delay clinical and genetic diagnosis, and impact on early treatments and clinical management, including proper genetic counseling.

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Author Roles

(1) Research project: A. Conception, B. Organization, C. Execution; (2) Data Analysis: A. Design, B. Execution, C. Review and Critique; (3) Manuscript Preparation: A. Writing of the first draft, B. Review and Critique.

M.J.M.: 1A, 1B, 1C, 2B, 3A.
J.O.: 1C, 2C, 3B.
M.S.: 1A, 2C, 3B.
A.F.B.: 2B, 3B.
A.S.: 2B, 3B.
J.S.: 1A, 2C, 3B.
J.B.: 1A, 2C, 3B.
J.D.: 1A, 1B, 1C, 2A, 2C, 3B.

Disclosures

Ethical Compliance Statement: This study was approved by the ethics committee of Centro Hospitalar Universitário do Porto and informed consent was obtained from family members. The authors confirm that they have read the Journal's position on issues involved in ethical publication and affirm that this work is consistent with those guidelines.

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Supporting Information

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Table 1. Literature review.

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Article 6. Rare occurrence of severe blindness and deafness in Friedreich ataxia: a case report.

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Cerebellum & Ataxias

CASE REPORT Open Access

Rare occurrence of severe blindness and deafness in Friedreich ataxia: a case report

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Abstract

Background: Friedreich ataxia is the most frequent hereditary ataxia worldwide. Subclinical visual and auditory involvement has been recognized in these patients, with co-occurrence of severe blindness and deafness being rare.

Case report: We describe a patient, homozygous for a 873 GAA expansion in the *FXN* gene, whose first symptoms appeared by the age of 8. At 22 years-old he developed sensorineural deafness, and at 26 visual impairment. Deafness had a progressive course over 11 years, until a stage of extreme severity which hindered communication. Visual acuity had a catastrophic deterioration, with blindness 3 years after visual impairment was first noticed. Audiograms documented progressive sensorineural deafness, most striking for low frequencies. Visual evoked potentials disclosed bilaterally increased P100 latency. He passed away at the age of 41 years old, at a stage of extreme disability, blind and deaf, in addition to the complete phenotype of a patient with Friedreich ataxia of more than 30 years duration.

Discussion: Severe vision loss and extreme deafness has been described in very few patients with Friedreich ataxia. Long duration, severe disease and large expanded alleles may account for such an extreme phenotype; nonetheless, the role of factors as modifying genes warrants further investigation in this subset of patients.

Keywords: Friedreich ataxia, Blindness, deafness

Background

Friedreich ataxia (FRDA) is the most frequent hereditary ataxia worldwide. The underlying genetic mechanism is, in the majority of patients, an unstable, pathological GAA expansion in *FXN* gene leading to decreased expression of frataxin, a ubiquitous mitochondrial protein [1]. First symptoms usually appear around puberty, and cardinal neurological features include cerebellar ataxia, sensory loss, pyramidal signs and absent reflexes in lower limbs [2]. Atypical phenotypes, with retained reflexes, late or very late onset have been thoroughly characterized for the last years [3]. The most frequent non-cerebellar symptoms encompass cardiac repolarization abnormalities, cardiomyopathy, scoliosis and urinary system disorders [2, 4]. Optic and auditory involvement may also be present in FRDA, with most patients having only mild or no symptoms at all [2, 4, 5]. Here we report the rare occurrence of blindness and deafness as part of an extreme FRDA phenotype.

Case presentation

A) Methods

This patient was part of a prospective study on hereditary cerebellar ataxias approved by the Institutional Ethics Committee. The patient had been followed at our hospital since 1998; and, from 2016 to 2019, he was

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evaluated through a structured protocol, comprising definition of age-at-onset of the various neurological symptoms/ signs, the Scale for the Assessment and Rating of Ataxia (SARA) and the Inventory of Non-Ataxia signs (INAS). Every 6 months a questionnaire on new neurological symptoms and the INAS was applied, as well as a complete neurological exam, comprising SARA. The missing clinical information was obtained through a detailed review of medical records and video files. Brain MRI was performed in 1.5 T scanner. DNA was collected from peripheral blood of patient and parents, and stored at CGPP-IBMC, i3S.

B) Case report

This was a male patient, homozygous for a 873 GAA expansion in the *FXN* gene, who presented gait instability by age 8 years. Dysarthria and upper limbs dysmetria emerged subsequently, and 4 years after disease onset he was wheelchair-bound. Scoliosis was diagnosed at age 16, and cardiomyopathy detected 1 year later. When 22 years-old he complained of bilateral hypoacusia, progressing to severe deafness over 11 years. By age 26, visual impairment was noticed and rapidly deteriorated: visual acuity decreased to 0.4 in just 1 year, and further decreased to light perception in the following 2 years. At that time, fundoscopic exam showed marked pallor of the optic discs, with sharp edges and normal retina. There was no history of exposure to drugs or toxins that could affect vision or hearing. Diabetes was diagnosed in his early thirties. By the age of 40 he presented scandid dysarthria, blindness, decreased amplitude of horizontal saccades, severe deafness impairing communication, tetraparesis predominantly affecting the lower limbs, absent deep tendon reflexes, absent position sense in the halluces, and truncal and finger-to-nose ataxia, scoring 38 on SARA (video). Even though a neuropsychological assessment could not be performed, there was no evidence of cognitive deterioration. He passed away with 41 years, after severe pneumonia. By the time visual and hearing impairment were identified, a thorough investigation was conducted: haemogram, chemistry, serology and immunology studies were normal. Genetic variants associated with Leber hereditary optic neuropathy (LHON) were excluded. Visual evoked potentials disclosed bilaterally increased latency (right eye: 144 milliseconds, left eye: 148 milliseconds) and decreased amplitudes of P100. Audiograms, performed over a period of 15 years, documented progressive sensorineural deafness, most striking for low frequencies (supplementary figure). On brain MRI (at age 29 years old), there was cerebellar, pons and medulla atrophy, with optic nerves exhibiting normal thickness and signal (Fig. 1).

Discussion

The first description of optic atrophy in FRDA was made by Sjögren, in 1940, in 12% of his patients [2]. Four decades later, Harding identified optic atrophy in 30% of cases: only 5.2% with severely and 13% with mildly reduced visual acuity, the remaining being asymptomatic [2]. Deafness was present in 7.8%, ranging from severe (0.9%) to mild (5.2%) [2].

Across the years, a few studies have demonstrated that subclinical involvement of visual and auditory systems is much more frequent than severe blindness and deafness [5–7]. Reduced visual acuity has been identified in 3.1–13% [4, 6, 8], and increased latency of visual evoked potentials in a much higher percentage of individuals: 34–70% [5, 6, 9]. In a study conducted by Fortuna et al., all the 26 individuals had thinning of the retinal nerve fibre layer on optical coherence tomography, in spite of only five having reduced visual acuity [5]. Involvement of posterior visual pathways was also present in these patients, with significantly higher apparent diffusion coefficients on diffusion-weighted MRI of the optic radiations [5]. Hearing loss of variable severity has been reported in 10–39% [4, 6, 8], with abnormal conduction

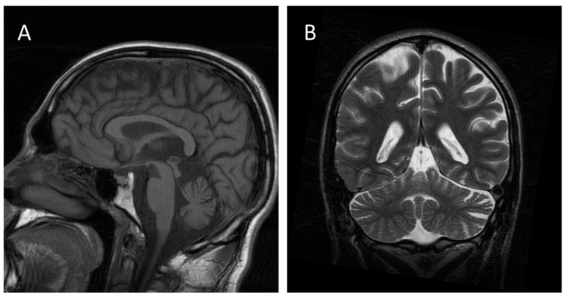

Fig. 1 Brain MRI T1 (**A**) and T2 (**B**) sequences showing atrophy of vermis and cerebellar hemispheres

in central pathways identified in 61–100% [6, 8, 10]. Impaired speech-understanding at levels of everyday background noise has been reported in up to 90% of patients, developing as soon as early-school years [7, 10].

Notwithstanding the relative frequency of subclinical visual and auditory impairment, co-occurrence of severe blindness and deafness are rather uncommon, but may be clustered with diabetes [2]. Critical vision loss resembling LHON has been described in very few patients, most being compound heterozygotes, with large expanded alleles, long duration and advanced disease [5, 11, 12]. The patient here reported had a catastrophic and rapidly progressive optic neuropathy, while hearing loss had a protracted course over 11 years until reaching a stage of critical deafness. Opposed to what has been described he was not a compound heterozygote, but a large expansion and long disease duration could partly account for such a severe phenotype.

Underlying pathophysiological mechanisms are incompletely understood. Current lines of evidence suggest anterior and posterior visual pathways degeneration; whereas in auditory function, the cochlea appears to be spared and central auditory brainstem is affected [5, 7]. A study on a mouse model, specifically addressing retinal ganglion cell death, suggested that optic atrophy was a consequence of increased sensitivity to oxidative stress, due to impaired intra-cellular iron regulation [13]. Frataxin deficiency may impact the respiratory chain, leading to mitochondrial iron deposition and dysfunction with consequent increased cellular susceptibility to oxidative stress [5, 13].

With this report we wish to (1) further contribute to the characterization of optic and auditory involvement in FRDA, (2) stress the need to better understand the underlying mechanisms, as well as their genetic and epigenetic modifying factors (including somatic heterogeneity); and (3) increase awareness for this rare extreme phenotype. Improved healthcare services and longer survival of early-onset patients will probably increase the frequency of these devastating manifestations.

Abbreviations

CGPP: Centro de Genética Preditiva e Preventiva; DNA: Deoxyribonucleic acid; FRDA: Friedreich ataxia; FXN: Frataxin; IBMC: Instituto de Biologia Molecular e Celular; INAS: Inventory of Non-Ataxia signs; MRI: Magnetic Resonance Imaging; SARA: Scale for the Assessment and Rating of Ataxia

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40673-021-00140-6.

Additional file 1: Video Segment 1 The patient presents scandid dysarthria. Throughout the video, both the doctor and his mother have to speak very loudly for him to hear. Mild involuntary head movements, suggestive of titubation, as well as involuntary eyebrow movements, are present. **Segment 2** We observe mild head movements, when initiating horizontal saccades. Also, saccades are slow and with restricted horizontal range. On both video segments, there are involuntary eye movements of a roving nature, probably due to the absence of fixation, as is frequently observed in blind patients.

Additional file 2: Supplementary figure. audiograms documenting progressive sensorineural deafness (b: 2003, c: 2018).

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Authors' contributions

JD – Conception and execution of the project. Writing of the first draft. AS – Organization and execution of the project. Review and critique. MA – Execution of the project. Review and critique. IC – Execution of the project. Review and critique. JS – Conception of the project. Review and critique. JB – Conception of the project. Review and critique. The author(s) read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate

This work is part of a project that has been reviewed and approved by the institutional review board from Centro Hospitalar Universitário do Porto.

Consent for publication

Permission for the publication of this article and associated video was obtained from the patient and his parents, via written informed consent, in compliance with laws regarding patient authorization relating to the use or disclosure of protected health information.

Competing interests

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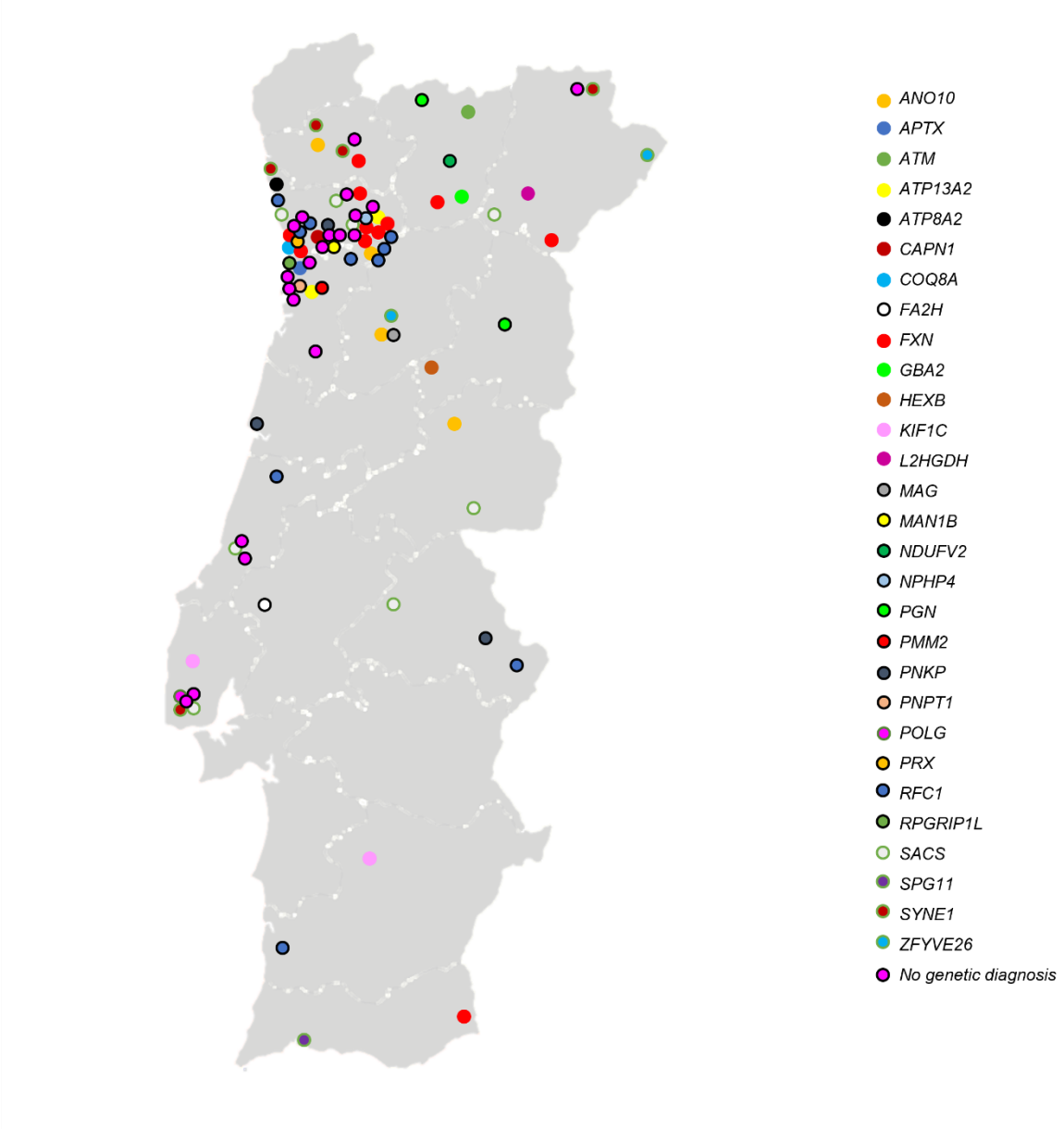
Geographic distribution of the families

Depicted on Figure 17 is the geographic distribution of families with AR inheritance, in mainland Portugal. The majority (58 families, 63.7%) was from the North of the country, followed by the Centre (16 families, 17.6%), Alentejo and Lisbon Metropolitan Area (6 families each, 6.6%), Algarve (2 families, 2.2%) and Azores (1 family, 1.1%). Two additional families were from Cape Verde (1, 1.1%) and Japan (1, 1.1%).

All families with Friedreich ataxia were from the North, except one from the Algarve. Seven ATX-*RFC1* families were also from the North, two from Alentejo and one from the Centre. Four kindreds with ARSACS were from the North, two from the centre, one from Alentejo and one from Lisbon Metropolitan Area. As for the families with ATX-*SYNE1*, four were from the North, one from Lisbon Metropolitan Area and the other from Cape Verde. Two families with ATX-*ANO10* were from the North, the other two from the Centre. Lastly, of the three families with AOA4 one was from the North, one from the Centre and the other from Alentejo. The remaining families were mainly from the North.

Figure 17: Geographic distribution of AR-HCA families

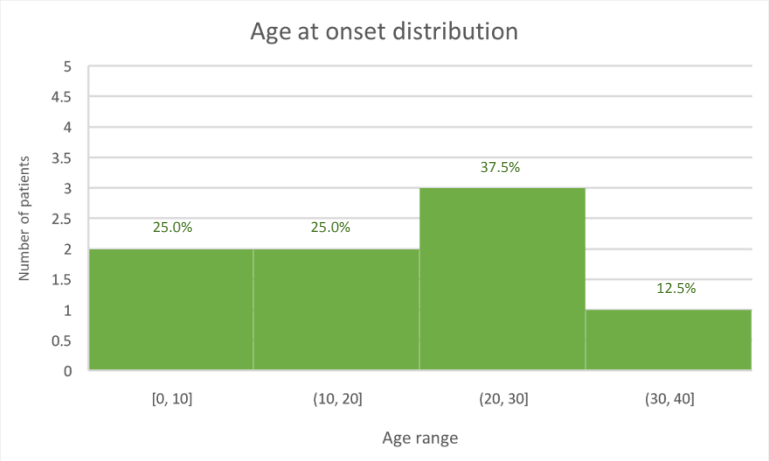


FAMILIES WITH MITOCHONDRIAL INHERITANCE

Overall characterization of patients and families

Eight patients, 4 females:4 males, belonged to five families with mitochondrial inheritance. In three (1.2%) there was no family history of neurological disorders. Mean AO was 18.5 years [7.5-26.3], with all having onset until the fourth decade of life (Figure 18).

Figure 18: Age at onset distribution in mitochondrial inherited HCA



Five (62.5%) patients had cerebellar onset (gait unsteadiness in four and nystagmus in one). Epilepsy was the presentation in two and motor delay in one.

A genetic diagnosis was obtained in all five probands (Table 11): one (20.0%) through targeted testing, four (80.0%) trough NGS based panels (three mitochondrial DNA panel, one neuroexome). Time until diagnosis was of 35.0 [21.0-46.5] years. Two (40.0%) probands had pathogenic variants in *MT-ATP6*, and one (20.0%) each in *MT-CO1*, *MT-CO2* and *MT-TN*.

Table 11: Genetic diagnosis of families with mitochondrial inherited HCA

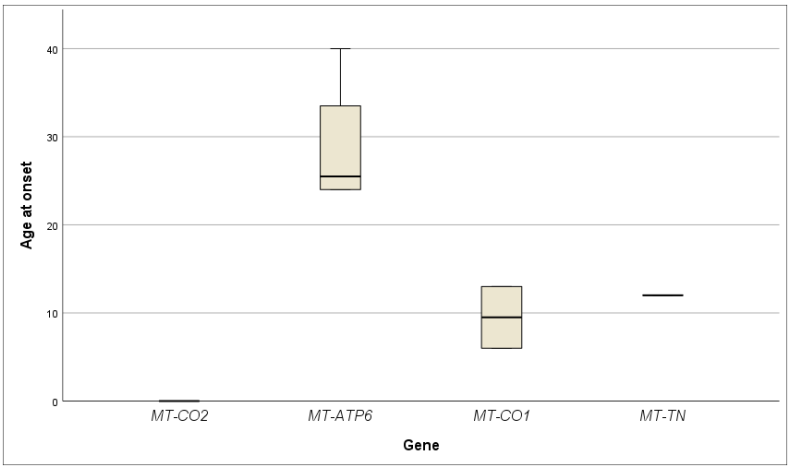
	Number	
	Probands	**Total number of patients**
MT-ATP6	2	4
MT-CO1	1	2
MT-CO2	1	1
MT-TN	1	1
Total	5	8

Onset according to genetic diagnosis

Age at onset

The four patients with variants in *MT-ATP6* had onset at a mean age of 25.5 [24.0-36.8] years. In the two individuals with *MT-CO1*, symptoms onset occurred at 6 and 13 years, and in the one with *MT-TN* at 12 years old. A congenital disorder was present in *MT-CO2* (Figure 19).

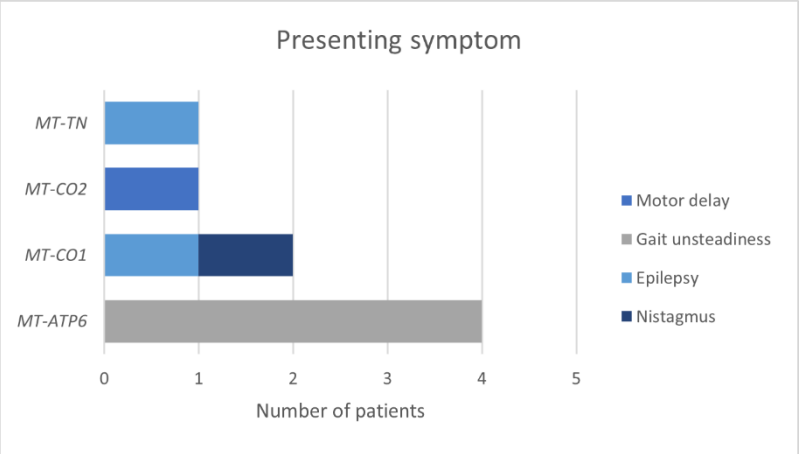
Figure 19: Age at onset by gene in mitochondrial inherited HCA



Clinical onset

All patients with pathogenic variants in *MT-ATP6* had gait unsteadiness at onset. As for *MT-CO1,* one presented with epilepsy and the other with nystagmus. In *MT-CO2* onset was by motor delay and in *MT-TN* by epilepsy (Figure 20).

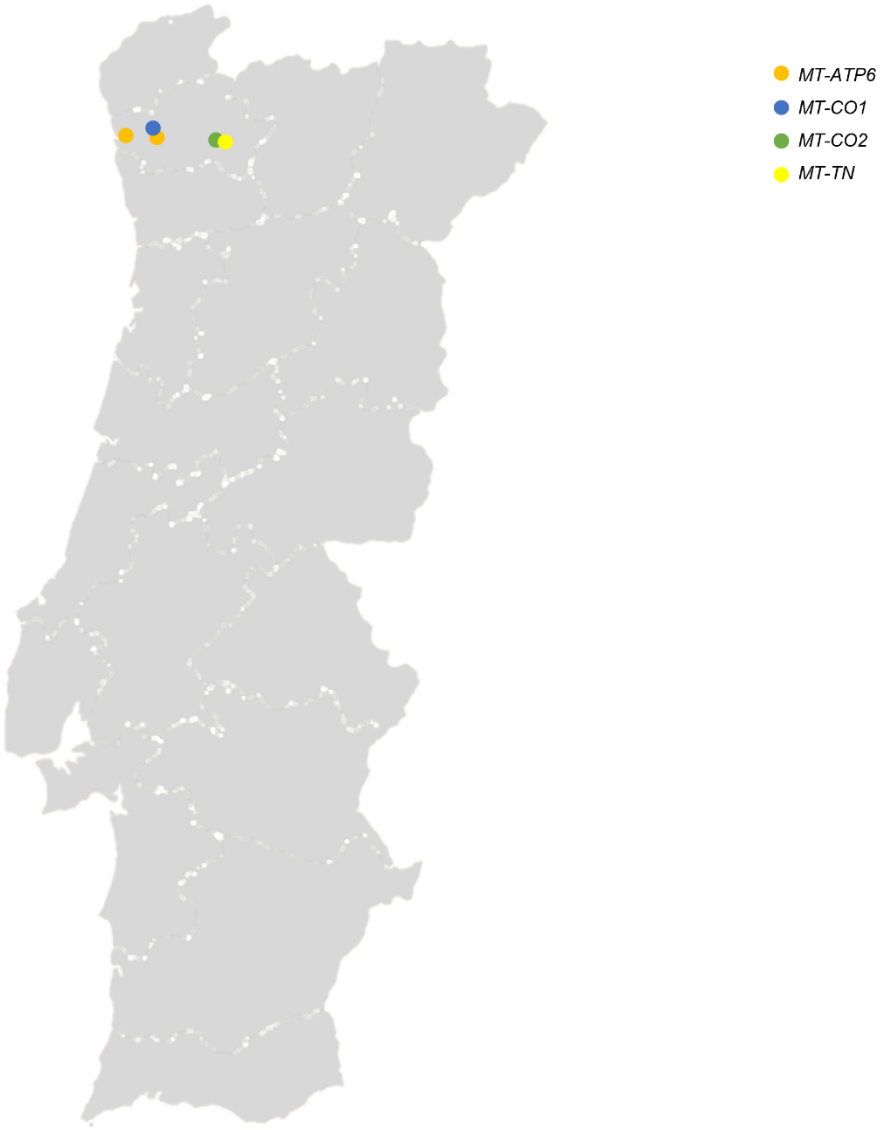
Figure 20: Presenting symptom in mitochondrial inherited HCA



Geographic distribution of the families

Interestingly, all five families were from the North of the country, from Cávado and Vale do Ave regions (Figure 21).

Figure 21: Geographic distribution of families with mitochondrial inherited HCA



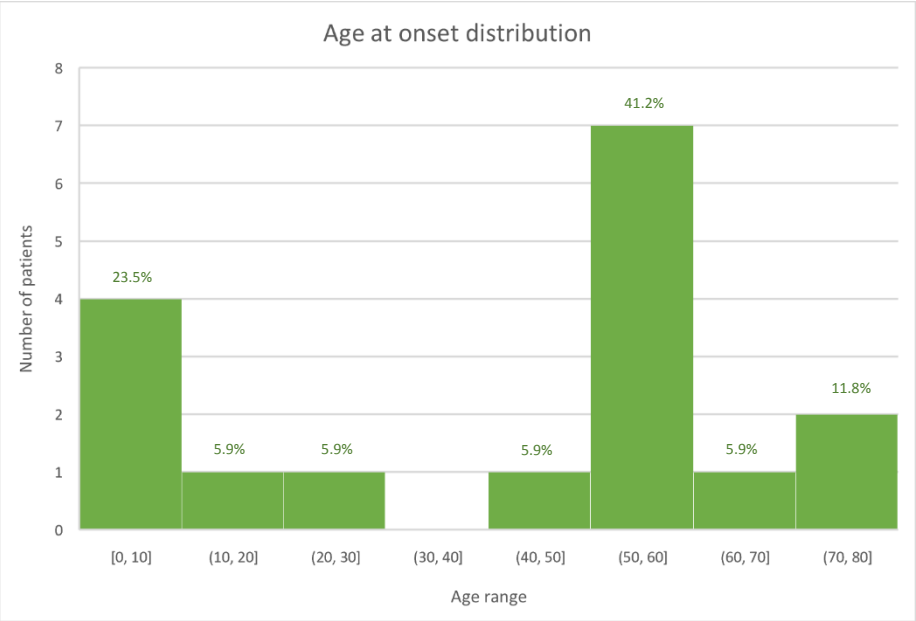
FAMILIES WITH UNDETERMINED MODE OF INHERITANCE

Overall characterization of patients and families

In 17 isolated patients (10 females:7 males), without genetic diagnosis, it was not possible to establish the mode of inheritance. In all, the genetic investigation performed (up to 2022) gave no definite results (details above). In 10 probands, there was no family history of neurological disorders, with parents having died in early adulthood and no other affected relatives available for examination. In seven, there was an unclear family history that could not be confirmed.

Mean AO was 53.0 [12.0-58.5] years, 13 (76.5%) cases having adult-onset. We could observe two modes in the distribution of AO, the first until 10 years of age and the latter from 50-60 years (Figure 22).

Figure 22: Age at onset distribution in families with undetermined mode of inheritance



Eleven (64.7%) patients had cerebellar onset: eight (47.0%) with gait unsteadiness, two (11.8%) with diplopia and one (5.9%) with dysarthria. As for non-cerebellar onset, two (11.8%) had spastic gait, and one (5.9%) each presented with intellectual disability, motor delay, epilepsy or vertigo.

First line genetic testing was the exclusion of Friedreich ataxia in two patients with childhood onset, ATX-*RFC1* in four and of polyglutamine dominant ataxias in seven patients with late onset. Sixteen were submitted to NGS based panel.

Interestingly, the large majority (94.1%) had a complex cerebellar ataxia (Table 12). The most frequently associated features over disease course were pyramidal signs (62.5%), followed by motor or intellectual disability (18.8%). Dystonia, tremor or epilepsy were present in one patient each.

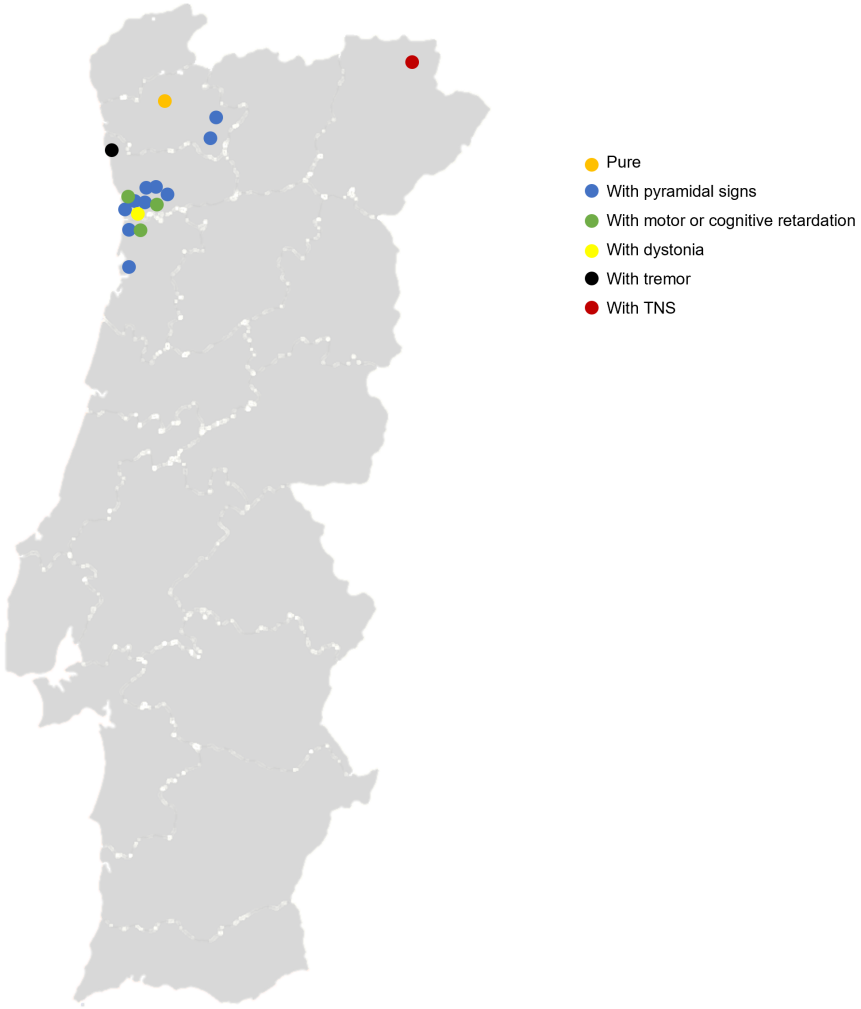
Table 12: Phenotypic classification of patients with undetermined mode of inheritance

	Number Probands
Pure ataxia	1
Complex cerebellar ataxia	
with pyramidal signs	10
with motor or intellectual disability	3
with dystonia	1
with tremor	1
with epilepsy	1
Total	17

Geographic distribution of the families

All 17 families were from the North of the country, particularly Área Metropolitana do Porto (Figure 23).

Figure 23: Geographic distribution of families with undetermined mode of inheritance



B. NON-CEREBELLAR SYMPTOMS AND SIGNS

[Objective 2. Frequency of non-cerebellar symptoms and signs]

[Objective 4. Disease progression]

OVERALL ANALYSIS OF NON-CEREBELLAR SYMPTOMS OR SIGNS

The frequency of non-cerebellar symptoms or signs was determined in the 249 symptomatic patients. Nine individuals (3.6%, 4 females:5 males) had a pure cerebellar ataxia, with mean onset at 53.0 [40.0-59.5] years (Figure 24) and disease duration of 8.0 [3.5-18.5] years (Figure 25): two had SCA2, and one each MJD, SCA6 and ATX-*SYNE1*, while four had no genetic diagnosis.

Forty-six patients (18.5%) had one type of non-cerebellar symptom/ sign, 194 (77.9%) had two or more. Mean age at disease onset in these groups was 24.0 [13.0-42.0] and 22.0 [2.8-37.0] years, respectively (Figure 24); mean disease duration being 25.0 [12.0-31.5] and 26.0 [14.8-39.0] years (Figure 25). Notwithstanding the reduced number of patients with a pure cerebellar ataxia, there was an association with later onset ($p<0.001$) and shorter disease duration ($p=0.06$).

Figure 24: Age at onset (in years) according to the number of non-cerebellar symptoms/signs

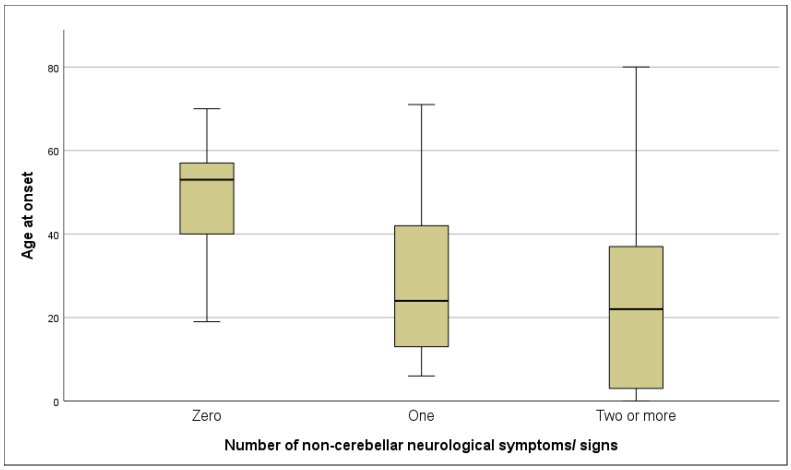
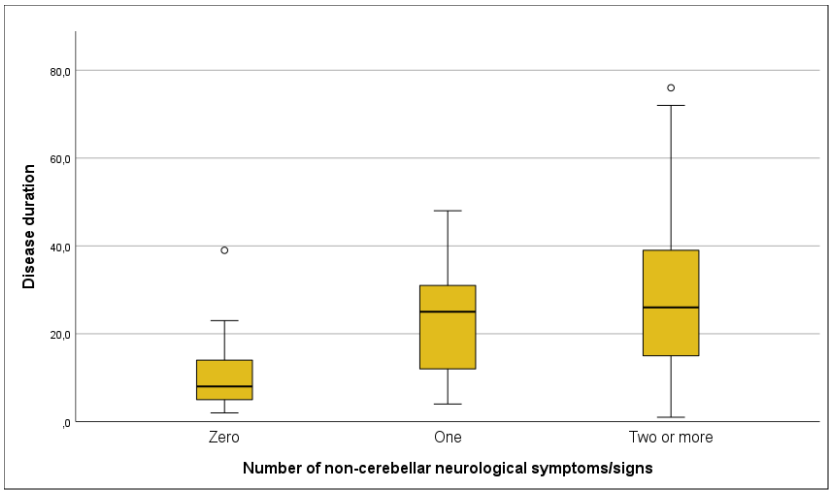


Figure 25: Disease duration (in years) according to the number of non-cerebellar symptoms/signs



Pyramidal signs (148 patients, 59.4%), movement disorders (133 patients, 53.4%) and neuropathy (104 patients, 42.4%) were the most frequently identified non-cerebellar signs. Motor (38 patients, 15.2%) or intellectual (36, 14.5%) disability, cognitive deterioration (34 patients, 13.7%) and epilepsy (13, 5.2%) were only present in patients with two or more non-cerebellar symptoms/ signs.

Pyramidal signs, motor delay and intellectual disability were associated with ARSACS ($p<0.001$), movement disorders with MJD and neuropathy with FA ($p<0.001$) (analysis performed for genes with 5 or more patients). The remaining were not significantly associated with a particular gene.

NON-ATAXIA MOVEMENT DISORDERS

Article 7 includes a detailed description of non-ataxia movement disorders frequency and clinical characterization.

Article 7. Movement disorders in hereditary cerebellar ataxias

Damásio J, Costa S, Moura J, Santos M, Lemos C, Mendes A, Oliveira J, Barros J, Sequeiros J.

In submission

Video is part of ms

Title: Movement disorders in hereditary cerebellar ataxia

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Abstract

Background Hereditary cerebellar ataxia (HCA) is a complex group of disorders, with a wide spectrum of neurological symptoms. Non-ataxia movement disorders (MD) have been increasingly acknowledged in HCA, its frequency varying among HCA.

Objectives To characterize type and frequency of non-ataxia MD in HCA forms. To identify factors associated with MD and analyze its impact on disability.

Methods Prospective cohort established in 2017, with annual visits and a structured protocol. Patients were selected from the study database and their clinical and genetic features analysed.

Results The cohort comprised 252 patients. Machado-Joseph disease (MJD, aka spinocerebellar ataxia type 3, SCA3) and cerebellar ataxia, neuropathy and vestibular areflexia syndrome (ATX-*RFC1*) were the commonest dominant or recessive forms, each with a frequency of 11.5%. MD were present in 133 (52.8%) patients. Dystonia (45.6%) associated with AD inheritance and *ATXN3*; tremor was found in 12.3% and Parkinsonism in 5.6% of patients, the latter associating with higher age-at-onset; myoclonus and tics were seen in 2.8% and 1.2%. Presence of MD associated with higher SARA score at baseline, increased frequency of falls and earlier use of cane.

Discussion Movement disorders, particularly dystonia, were common in our cohort. This highlights the possible role of the cerebellum in (non-ataxia) MD, but also the extra-cerebellar involvement in some HCA. MD associated with higher disease severity and disability, and influenced frequency of falls and earlier use of cane.

BACKGROUND

Hereditary cerebellar ataxias (HCA) involve a complex group of rare neurological diseases, caused by degeneration of the cerebellum, its afferent or efferent connections.[1] Classification of HCA follows mode of inheritance, including autosomal dominant (AD), autosomal recessive (AR), mitochondrial and X-linked forms.[1] Repeat expansions, conventional disease-causing variants (*e.g.*, single-nucleotide substitutions, small insertions/deletions, or copy-number variants), and gene rearrangements have been associated to HCA.[1] Genetic diversity is only partially responsible for their clinical heterogeneity.[2]

Ataxia is the cardinal clinical feature, but a wide spectrum of neurological symptoms is recognized among HCA.[3] Eye movement abnormalities, pyramidal signs, and polyneuropathy are common in the most frequent ataxias, as Machado-Joseph disease (MJD)/ spinocerebellar ataxia 3 (SCA3), SCA2, and Friedreich ataxia (FA).[3-6] Dementia, epilepsy, neuronopathy, optic or auditory involvement are less frequent, but may be important hints in differential diagnosis in some HCA (*e.g.*, epilepsy in DRPLA, neuronopathy in ATX-*RFC1*).[3-5,7] Hypo and hyperkinetic movement disorders (MD) are less well studied, and have been reported with variable frequency in several cohorts.[8-10] The most commonly described in AD-HCA are intention or postural upper-limb (UL) tremor, Parkinsonism, and dystonia.[4,11] MD may emerge with disease progression in classical ataxic phenotypes. Less frequently, MD may be the presenting or predominant clinical feature,[8-11] which may delay genetic diagnosis.

The cerebellum has been increasingly recognized in the pathophysiology of tremor or dystonia, but there is also evidence of degeneration of the basal ganglia and substantia nigra in several HCA.[12] The extent of extracerebellar involvement differs among HCA, and frequency of MD varies accordingly.

We aimed at characterizing type and frequency of MD in a cohort with HCA. Additionally, we sought factors associated with MD, and analysed the impact of MD on disability.

PATIENTS AND METHODS

Study design. A prospective study on HCA was established at our centre from 2017 onwards, with baseline and annual follow-up visits, according to a structured protocol always performed by the same neurologist. At baseline, detailed identification of age-at-onset (AO) of various neurological symptoms/ signs and disability milestones (falls, use of cane, confinement to wheelchair) was performed; new neurological symptoms and disability milestones were investigated annually (Suppl. Material). The Scale for the Assessment and Rating of Ataxia (SARA) and the Inventory of Non-Ataxia signs (INAS) were appraised at baseline and each annual evaluation. Genetic testing followed a pre-established protocol (Suppl. Material); variants were classified according to the guidelines of American College of Medical Genetics and Genomics[13], and a genetic diagnosis considered in case of genotype with pathogenic/ likely pathogenic variant(s), according to the putative mode of inheritance.

Definitions. AO was considered as the age at first neurological symptom, irrespectively of cerebellar or non-cerebellar. In patients followed from a presymptomatic stage, AO was considered as the age at register of the first symptom. When the disease had started before, information at baseline was checked in clinical records and with two first-degree relatives, whenever possible; if discordant, information from clinical records was preferred. MD were classified according to accepted criteria,[14-18] into hypokinetic (Parkinsonism) or hyperkinetic (tremor, dystonia, chorea, myoclonus, tics). As kinetic tremor could be part of the cerebellar syndrome, this was not included in the analysis. Abnormal dystonic posturing (*e.g.*, head, UL, trunk) was registered as *dystonia*. AO of MD was recorded, together with any pharmacological strategies and outcome. Levodopa-equivalent dose was estimated according to the latest recommendations.[19] All patients were classified according to the revised nomenclature on Genetic Movement Disorders,

except for SCA1, SCA2, MJD, SCA6, SCA7, DRPLA, FA and ARSACS, which are more readily recognized by their original designations.[20] In case of genes not yet associated with an ataxic phenotype by the International Parkinson and Movement Disorder Society Task Force on Classification and Nomenclature of Genetic Movement Disorders, we used the suffix "-related ataxia" after the gene name.

Patient selection and sample characterization. Patients with MD were identified from the overall study database, the remaining being considered for comparative analysis. Demographic data, presenting and associated neurological symptoms/ signs were registered. Age at disease onset, and at onset of the various symptoms/ signs and disability milestones was recorded.

Statistical analysis. For descriptive statistics, absolute and relative frequency were used with qualitative variables. Mean (with standard deviation, SD) or median (with quartiles, [Q1-Q3]) were used for quantitative variables, depending on whether data followed or not a Normal distribution, respectively. Group comparisons were performed using the chi-squared test for qualitative variables; in small-size samples, the Monte Carlo method was adopted. For quantitative variables, the Student's T or Mann-Whitney tests were used for Normal or non-Normal distributions. Analyses were performed only for genes with 5 or more patients. A P value < 0.05 was considered statistically significant. Analysis was conducted using the IBM Statistical Package for the Social Sciences (SPSS) v.29.

Ethics. The project has been approved by the hospital Ethics Committee. Informed consent was obtained for clinical investigation and video recordings.

RESULTS

Characterization of the cohort

The overall cohort was composed by 252 patients (249 symptomatic, 3 presymptomatic; 110 males, 142 females), belonging to 164 families. Median AO was 23.0 [6.0-40.0] years;

age at inclusion was 48.0 [33.0-64.0] years; with an average follow-up of 3.0 [1.0-5.0] years. Of the 164 families, 51 (31.1%) had AD, 91 (55.5%) AR, and 5 (3.0%) mitochondrial inheritance; in 17 (10.4%) families, without genetic diagnosis, we could not precisely establish the inheritance mode. A definite genetic diagnosis was obtained in 197 (78.7%) patients, 116 families. The most common AD-HCA were MJD (29 (11.5%) patients, 14 families), ATX/PxMD-*CACNA1A* (11 (4.4%) patients, 4 families), SCA2 (10 (4.0%) patients, 3 families) and ATP1A3- related ataxia and ATX-*KCNC3* (4 (1.6%) patients, 2 and 1 family each). ATX-*RFC1* (29 (11.5%) patients, 10 families), FA (15 (6.0%) patients, 11 families), ARSACS (9 (3.6%) patients, 8 families) and ATX-*SYNE1* (8 (3.2%) patients, 6 families) were the most frequent AR-HCA. All genetic diagnoses were detailed on Suppl. Table 1.

MD were identified in 133 (53.4%) patients: dystonia in 115 (46.2%), tremor in 31 (12.4%), Parkinsonism in 14 (5.6%), chorea in 10 (4.0%), myoclonus in 7 (2.8%), and tics in 3 (1.2%). AO of each MD is shown in Figure 1. Of these, only Parkinsonism was seen as a presenting symptom, in 2 patients. Presence of MD was significantly associated with AD inheritance and *ATXN3*. No significant differences between patients with and without MD were observed for AO, disease duration until baseline, type of onset, or pattern of atrophy on brain MRI (Table 1 and Suppl. Table 2). No patients had been previously treated with dopamine blocking drugs.

Hypokinetic movement disorders

Parkinsonism (n=14) emerged at a mean age of 66.0 [28.8-70.5] years, after 8.0 [1.0-20.5] years of disease duration. Six patients had onset before their fifties, eight in their sixties or later. It was the presenting symptom in two (with MJD), and emerged in the first year of disease in another two. All had bradykinesia and rigidity, six (42.9%) also with rest tremor (Video 1).

Seven patients had AD-HCA (three MJD, one SCA2, one ATX-*PRKCG*, two no disease-causing gene identified), and five AR-HCA (one HSP/ATX-*SPG7*, one HSP-*ZFYVE26*, one MxMD-

ATP13A2, two no gene identified). In two, the mode of inheritance could not be determined (Suppl. Table 3).

Eleven (78.6%) were treated, all with levodopa, two of them needing another dopaminergic agent. With 6.0 [4.0-9.25] years after onset of Parkinsonism, the levodopa equivalent dose (LED) was 400 [300-800] mg. Eight (72.7%) experienced sustained benefit, four of which developed motor fluctuations and two dyskinesia (Video 2). One was submitted to deep brain stimulation of the subthalamic nucleus, with marked improvement (Video 3). Ten performed [^{123}I] FP-CIT SPECT (DaTSCAN), 2.0 [1.0-5.75] years after Parkinsonism onset, with evidence of presynaptic dopaminergic deficit in nine (Figure 2). The only patient with a normal DaTSCAN had shown improvement under levodopa. Of the three patients with no benefit under dopaminergic medication, one did not perform DaTSCAN and the remaining two had an abnormal exam.

When comparing patients with earlier (<50 years) *vs.* later-onset (≥50 years) Parkinsonism, there were no significant differences in clinical features, LED or disability milestones. Two early-onset MJD patients were the only presenting dyskinesia.

Hyperkinetic movement disorders

Dystonia (n=115) occurred at a mean age of 35.0 [18.5-46.2] years, 9.0 [3.7-19.5] years after disease onset. In 57 patients, it was not possible to precisely determine dystonia onset.

Of 56 (48.7%) cases with AD inheritance, 20 had MJD, six SCA2, four ATP1A3-related ataxia, and three ATX/HSP-*KCNA2*. Among 53 (46.1%) patients with AR-HCA, nine had FA, four each ARSACS and ATX-*SYNE1*, and three each showed ataxia with oculomotor apraxia type 4 (AOA4) or ATX-*RFC1*. The remaining, less frequent HCA are described in Suppl. Tables 4-6.

After a mean of 4.0 [2.7-11.2] years of dystonia progression, the vast majority (96 patients, 83.5%) showed segmental dystonia, mostly dystonic posturing of the UL. Less frequent was generalized (9, 7.8%), focal (7, 6.1%) or multifocal (3, 2.6%) dystonia (Video 4).

Dystonia features on the most frequent HCA are listed on Suppl. Tables 4-6.

Tremor (n=31) appeared at a mean age of 43.0 [27.5-67.0] years, after 10.3 [4.5-27.3] years of disease progression. Sixteen (51.6%) patients had AD-HCA (three each with MJD or SCA2, two ATX-*KCNC3*); 12 (38.6%) AR-HCA (three each had ATX-*RFC1* or HSP-*MAG*); and one (3.2%) a mitochondrial ataxia. The remaining AD and AR-HCA are detailed on Suppl. Table 7; two had no determined mode of inheritance.
After mean age of 5.0 [4.0-9.25] years of tremor progression, 29 (93.5%) patients had UL tremor, while one each (3.2%) head or chin tremor. In 18 (62.1%), UL postural tremor was seen, three (10.3%) had rest tremor, and eight (27.6%) rest and postural tremor (Video 5).

Chorea (n=10) was observed, on average, by 25.5 [1.0-56.0] years, after 14 [9.8-29.3] years of disease progression, with a generalized distribution in all (Video 6). Seven patients had a known genetic diagnosis, all with AD inheritance: three *ATP1A3*- related ataxia, two SCA2 who had also Huntington's disease, one each ATX-*PRKCG* and ATX-*STUB1* (Suppl. Table 8).

Myoclonus (n=7) appeared at a mean age of 16.0 [11.0-22.0] years, after 10.0 [5.0-11.0] years of disease duration. Four patients (57.1%) had AD-HCA (two ATP1A3-related ataxia, one *KMT2B*-related ataxia, one without genetic diagnosis); and three (42.9%) showed AR-HCA (one *COQ8A*-related ataxia, one ATX-*ATM*, one with no identified gene) (Suppl. Table 9). Patients with *ATP1A3* and *KMT2B*-related ataxia had multifocal myoclonus, the remaining generalized myoclonus (Video 7). All presented sensitivity to auditory stimuli, but only those with *COQ8A* and *KMT2B*-related ataxia had also tactile sensitivity.

Tics (n=3) were observed only in AR-HCA: one with congenital disorder of glycosylation (CDG), the remaining having no genetic cause identified (Suppl. Table 10). The CDG patient had paediatric-onset tics, while the remaining two had adulthood-onset tics.

Co-occurrence of movement disorders (other than ataxia)

In 35 (26.3%) patients, multiple MD were present. In 25, co-occurrence of two MD was observed; eight showed three and two four MD (Suppl. Table 11). Dystonia occurred most frequently in association with other MD: tremor (12 patients), Parkinsonism (four), and chorea or myoclonus (three each). The remaining associations were observed only in one or two patients, as detailed in Suppl. Table 11.

Co-occurrence of multiple MD was associated with shorter disease duration until baseline visit and AD inheritance, but no other associations were observed (Suppl. Table 12).

Influence of non-ataxia movement disorders in disease progression and disability

There was no significant difference in disease duration until baseline in patients with and without MD, but the first had a higher SARA score at inclusion (Table 2). A higher frequency of falls was observed in patients with MD, despite there being no difference in time until their appearance. (Table 2). The use of cane was observed with a similar frequency in patient with and without MD; but, when needed, it tended to occur significantly sooner on disease course in the first group. Confinement to wheelchair and disease duration before it did not differ between patients with and without MD.

DISCUSSION

In this cohort, MJD and ATX-*RFC1* were the most common HCA, each with a frequency of 11.5%. In line with the results from the Portuguese population-based survey, MJD was the most frequent AD ataxia,[21] while ATX-*RFC1* overpassed FA ataxia among AR forms. *RFC1* expansion was only identified in 2019, and thus not registered in the 2013 survey.[21,22] In fact, 16 patients with no genetic diagnosis at that time were found to have a diallelic expansion in *RFC1*. Still, as the present was a hospital-based study, its results cannot be directly compared with the population survey.

Comparison with other studies

We identified an *overall frequency* of 52.8% of MD in our hospital-based HCA cohort. In previous series, using various methodologies, this ranged from 9.8-54%.[9-11] The coexistence of two or more MD in 26.3% was similar to that described by *Garcia Ruiz et al.* (28.9%).[9] Overall, MD were significantly associated with AD-HCA, particularly MJD, as opposed to the Spanish cohort, where a similar frequency was observed in AR and AD-HCA.[9] This difference could be related to the distinct sample size, prospective design and forms of HCA included.

Dystonia was by far the most frequent MD in our cohort, and associated with early disease onset, dominant inheritance and MJD/SCA3. This is well-known in early-onset MJD since its initial descriptions in Azorean families,[23] and consistently acknowledged henceforth.[8,10,24] Other HCA in which dystonia has been registered include ATX-*TBP*, SCA1, SCA2, ATX-*PRKCG*, and DRPLA.[11,24] In our series it was observed in the majority of HCA, with a frequency (45.6%) higher than previously described (4.0-26.2%).[9-11] A prospective detailed scrutiny of MD possibly allowed identification of subtler forms, as dystonic posturing of the UL, the most frequently observed.

Parkinsonism was less frequent than in the meta-analysis by *Rossi et al.* (5.6% *vs.* 14.0%).[11] We considered it only when in presence of bradykinesia plus rest tremor or rigidity, while some studies include bradykinesia or rigidity *per se*.[8,11] It was seen mostly in HCA classically associated with it, as MJD, SCA2 or HSP/ATX-SPG7,[24,25] but also in others as SCA14 and HSP-*ZFYVE26*. In line with what has been observed in SCA2[26] and MJD families,[24] our patients had later onset of ataxia, with mean AO of Parkinsonism in the 7th decade of life. It was the sole MD occurring prior to ataxia, its prevalence increasing with disease course.[11] Improvement under levodopa or other antiparkinsonian drugs occurred in 72.7%, with motor fluctuations in a quarter of patients, consistent with findings from

other reports.[11,24] Later onset of Parkinsonism, however, cannot exclude a coincidental Parkinson's disease.

Tremor was the second most frequent MD, primarily associating with dystonia (dystonic tremor). Its frequency described varies from 2.4-50.0%, [8-11] the highest estimates including kinetic tremor – this was excluded in our series, possibly resulting in the lower frequency (12.3%) observed.

Chorea (4.0%) was also less frequently registered by us than in the meta-analysis of *Rossi et al.* (13%).[11] It is classically linked to ATX-*TBP*, but also observed in SCA1, SCA2, MJD, ATX-*PRKCG*, and ATX-*STUB1*.[24,27] In our cohort, it associated with AD inheritance, but not with a particular gene; still, as expected, we registered it ATX-*PRKCG* and ATX-*STUB1*, but also in *ATP1A3*-related ataxia. Of note, our SCA2 patients who had chorea, had a concurrent Huntington's disease.

Myoclonus (2.8%) was also less frequent than in *Rossi et al.* (12%).[11] It associated with younger AO, but not with SCA2 or ATX-*PRKCG*, as classically observed.[11,24,28]

Tics have been rarely identified in HCA,[11,24] as occurred also in our series. Tics could be a coincidental disorder, particularly when having paediatric onset; but, when with adult onset (as two in the present cohort), would probably be related to the underlying HCA.

Pathophysiology of MD in HCA

In HCA, MD may stem from cerebellar or extracerebellar involvement, or both. Given the heterogeneity of these diseases, however, a general assumption is not possible. The role of the cerebellum in MD, as tremor, dystonia, and myoclonus, is being increasingly recognized, partially explaining their higher description in presence of cerebellar degeneration. In essential tremor, MRI spectroscopy studies identified a reduction in cerebellar cortical N-acetyl-L-aspartate and total choline,[29] and pathological series evidenced reduced dendritic arbour and loss of Purkinje neurons.[30] Animal models of

dystonia (particularly in rats) revealed increased glutamic acid decarboxylase activity in deep cerebellar nuclei, and improvement of dystonia after cerebellectomy.[31,32] Additionally, abnormal cerebellar firing patterns appear to play a role in the pathophysiology of dystonic motor behaviour in those models.[33] In humans, lesions of the cerebellum or cerebellar pathways (*e.g.*, stroke or tumours) may cause secondary dystonia.[31] Functional MRI studies in acquired and hereditary dystonia have shown atypical cerebellar activation and various anomalies within cerebellar-thalamocortical circuits.[31] Furthermore, transcranial magnetic stimulation of the cerebellum induced transient improvement of cervical dystonia, on a randomized controlled study.[34] As such, the basal ganglia and the cerebellum are likely part of a network that is dysfunctional in dystonia. The cerebellum is also known for its involvement in myoclonus, as cerebellar degeneration may result in reduced inhibition of the motor cortex, acting as a remote effect.[24]

The extent of extra-cerebellar involvement in HCA, particularly of the substantia nigra, pallidum, putamen, caudate and thalamus, also modulates expression of MD. Neuronal loss in basal ganglia is frequently observed in HCA such as MJD, SCA2 or SCA1, with substantia nigra degeneration being common in the first two.[12] Post-mortem studies in SCA2 and MJD by *Schöls et al.* found that severe neuronal loss in the subthalamic nucleus occurred in all patients, except one who displayed Parkinsonian features during his lifetime; therefore, subthalamic degeneration could prevent Parkinsonism in most of these cases.[35] Pathology of the caudate nucleus, relevant in chorea, has been described in SCA1 and ATX-*TBP*.[24] Interestingly, chorea is frequent in DRPLA, where there is degeneration of the pallidum, subthalamic, dentate and red nucleus, but not the caudate.[36]

Neurophysiological and neuropathological studies, beyond the scope of this work, would be of great importance to understand better the pathophysiology of MD in HCA.

Imaging

Cerebellar atrophy has been observed in the absence of ataxia, in "pure" Parkinsonian patients.[11] In our series, no significant difference on brain MRI atrophy patterns was registered in patients with and without MD. Dopaminergic functional imaging disclosed abnormal presynaptic integrity in up to 90% of patients,[11] similarly to our series. Intriguingly, our only patient with a normal DaTSCAN experienced clinical benefit with levodopa, and possibly corresponded to a false-negative exam. Conversely, patients with an abnormal DaTSCAN who had no improvement with dopaminergic therapy may have been undertreated, despite denying the need for an increased dosage.

Disease progression and disability milestones

For similar disease duration, MD associated with a higher SARA score at baseline. Influence of non-ataxia features on SARA has been perceived in a large AR-HCA cohort, with higher scores in the presence of several non-ataxia items (*e.g.*, paresis, cognitive impairment, impaired vibration sense), but not MD.[37] In the Euro-SCA cohort, a higher SARA score was also linked to a higher frequency of non-ataxia symptoms (evaluated by INAS), but not MD in particular.[4] This could be due to a higher overall disability in patients with MD or the influence of MD over the evaluation of the cerebellar function.

When relating disability milestones with coexistent MD, the most significant associations were higher frequency of falls and earlier use of cane. This is in line with the results of *Schöls et al.* showing association of bradykinesia and impaired postural reflexes with loss of unsupported walk.[8]

Limitations

Even though a substantial number of patients was included, this is a genetically heterogeneous sample; analysis per genotype was precluded in 25 disease causing genes, each associated with only one or two patients. Also, as this is a tertiary hospital sample, receiving complex referrals, our conclusions cannot be directly generalized to population-based cohorts. Finally, as the prospective study was started only in 2017, a recall bias

cannot be excluded on AO and at manifestation of MD, when either occurred beforehand. Our strategy consisted on double checking all information with close relatives and clinical records.

Final remarks

With this study we aimed at contributing further into the characterization of MD in HCA, illustrating clinical findings with videos. We highlight the high frequency of dystonia, and the association of MD with motor disability. We believe these findings will be of value for clinical practice, increasing neurologists' awareness towards the recognition and treatment of MD symptoms in HCA.

Figure legends

Figure 1: Distribution of the age at onset of movement disorders

Figure 2: [^{123}I] FP-CIT SPECT from patients with HCA and Parkinsonism. A – SCA2 patient (disease onset: 59 years-old, Parkinsonism: 69 years-old, scan: 70 years-old) with marked and symmetrical decreased [^{123}I] uptake in putamen and caudates; B – ATX-*PRKCG* patient (disease onset: 55 years-old, Parkinsonism: 70 years-old, scan: 74 years-old) with bilaterally reduced I123 uptake at the putamen; C – Unknown genetic diagnosis (disease onset: 30 years-old, Parkinsonism: 75 years-old, scan: 80 years-old), left predominant reduced ^{123}I uptake at the putamen; D – HSP/ATX-*SPG7* (disease onset: 33 years-old, Parkinsonism: 67 years-old, scan: 68 years-old), left reduced ^{123}I uptake at caudate and putamen, right reduced ^{123}I uptake at the putamen; E – HSP-*ZFYVE26* (disease onset: 10 years-old, Parkinsonism: 26 years-old, scan: 28 years-old), bilateral reduced ^{123}I uptake at caudates and absent uptake at putamen; F – HSP/ATX-*ATP13A2* (disease onset: 27 years-old, Parkinsonism: 28 years-old, scan: 37 years-old), left predominant reduced ^{123}I uptake at the putamen.

Tables

Table 1: Summary of clinical characteristics of patients with/ without movement disorders

Table 2: Severity and disability in patients with/ without movement disorders

Supplementary material

A. Patients and Methods: Clinical protocol; Protocol of genetic studies

B. Results: detailed description of dystonia features in the most frequent HCA

Supplementary tables

Supplementary table 1: Genetic diagnosis of the cohort

Supplementary Table 2: Brain MRI findings in patients with/ without movement disorders

Supplementary Table 3: Clinical characteristics of patients with Parkinsonism

Supplementary Table 4: Clinical characteristics of patients with dominant ataxias and dystonia

Supplementary Table 5: Clinical characteristics of patients with recessive ataxias and dystonia

Supplementary Table 6: Clinical characteristics of patients with mitochondrial ataxias or undetermined type and dystonia

Supplementary Table 7: Clinical characteristics of patients with tremor

Supplementary Table 8: Clinical characteristics of patients with chorea

Supplementary Table 9: Clinical characteristics of patients with myoclonus

Supplementary Table 10: Clinical characteristics of patients with tics

Supplementary Table 11: Co-occurrence of two or more movement disorders

Supplementary Table 12: Characteristics of patients with one *vs.* two or more movement disorders

Video legend

Video 1: Video 1 showcases several patients with Parkinsonism. The two first patients have MJD: on the first segment we may observe a lady with facial hypomimia, mild left thumb rest tremor and left sided predominant bradykinesia (observed on finger tapping, hand movements and pronation-supination). The lady on the second segment presents mild cervical dystonia with head tremor and lower-limb predominant rest tremor. She has marked facial hypomimia and left sided predominant bradykinesia. There is difficulty arising from the chair, her gait is slow, with small steps and no arm swing. The third patient, with HSP/ATX-SPG7 exhibits facial hypomimia and left sided predominant bradykinesia. He has a stooped posture, with small-step gait, bilaterally reduced arm swing, and requires multiple steps to turn.

Video 2: On the first segment we may observe the patient with HSP/ATX-*SPG7* and Parkinsonism under levodopa treatment, with a clear improvement on bradykinesia. While walking he has larger steps and requires less steps to turn, but still has bilaterally reduced arm swing. Patient 2, with MJD, was observed under levodopa, has a clear improvement on facial hypomimia and global akinesia, and presents peak-dose dyskinesia mainly affecting the head and hands. Her gait is faster, with larger steps.

Video 3: Again, we observe the patient with MJD and Parkinsonism, now after being submitted to subthalamic nucleus deep brain stimulation, at ON condition. There is an improvement of facial hypomimia and bradykinesia, while still presenting mild cervical dystonia and dystonic posture of the right halux. Rest tremor has completely disappeared. While still requiring two attempts to arise form a chair, and her gait is now faster but has mild unbalance.

Video 4: On video 4 we may observe the expression of dystonia in different patients with

HCA. The first patient, with ATX-*ATM*, presents, at rest, cervical dystonia with mild torticollis to the left and left shoulder elevation and dystonic posturing of the fingers; with arms outstretched fingers dystonia is clearer (also myoclonus is observed). The patient with ATP1A3-related ataxia has a torticollis to the right with laterocollis to the left and dystonic posturing of the fingers. The first patient with HSP/ATX-*GBA2* presents dystonia of the fingers, while the second mild laterocollis to the right and dystonia of the fingers. The young lad with ATX-*LH2GDH* has a segmental dystonia, involving the neck and upper limbs. The first lady with MJD/SCA3 presents dystonia of the face and neck, and with arms outstretched also of the fingers. The gentleman with MJD/SCA3 has dystonic posture of the right hand and postural tremor of the fingers. The second gentleman with MJD/SCA3 presents also dystonic posturing of the fingers, dystonia of the face and mild laterocollis to the right. The first lady with ATX-*SACS* has dystonia of the face, while the second dystonic posture of the fingers.

Video 5: On video 5 we observe four different patients with HCA and chorea. The first with ATX-*PRKCG* has a peri-oral chorea, which observed throughout the video. The second and third patients are two siblings with SCA2 and HD; the lady (at an early stage at the time the video was taken) had a mild chorea of the hands while walking, while the lad present peri-oral choreic movement, motor impersistence (tongue protrusion lasting only 5 seconds) and chorea of the UL and trunk present while testing coordination. The lady with no genetic diagnosis has generalized chorea, present while seated and laying on her back.

Video 6: The first lady, with ATX-*RFC1*, has cervical dystonia with dystonic head tremor, observed throughout the video. The second lady, with ATX-*TMEM240*, treated with 40mg of propranolol has a postural tremor of the hands, distal and

Video 7: Video 7 displays 3 patients with myoclonus. The first, with ATX-*ATM,* has generalized sound-sensitive myoclonus; the second and third, with *ATP1A3*-related ataxia, have distal myoclonus of UL.

Figure 1

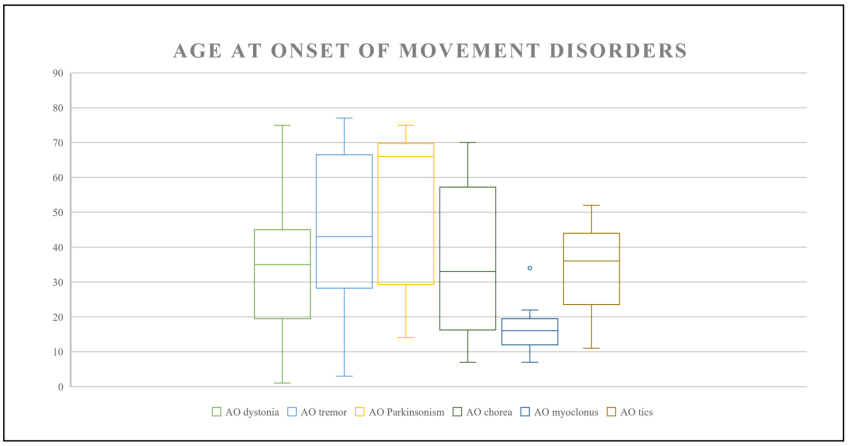

Figure 2

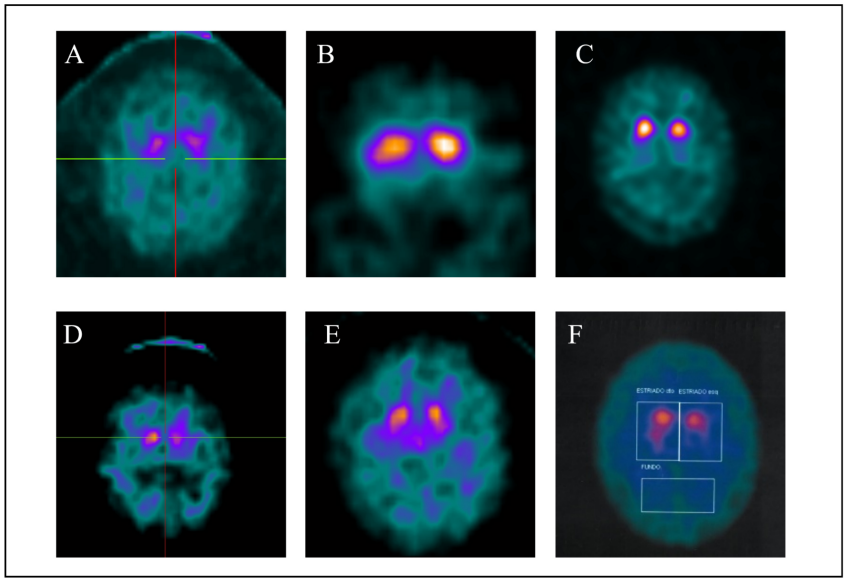

Table 1: Summary of clinical characteristics of patients with/ without movement disorders

	With MD	Without MD	P value
Number of patients	133 (53.4%)	116 (46.6%)	-
Male gender	56 (42.1%)	51 (434.0%)	0.767
Age at onset	22.0y [1.7-36.0]	25.0y [8.0-42.0]	0.091
Onset symptoms/signs			
Cerebellar	79 (59.4%)	59 (51.8%)	0.228
Non-cerebellar	54 (40.6%)	55 (48.2%)	
Mode of inheritance			
Autosomal dominant	61 (45.9%)	27 (23.3%)	**0.003**[a]
Autosomal recessive	61 (45.9%)	75 (64.7%)	
Mitochondrial	3 (2.3%)	5 (4.3%)	
Unknown	8 (6.0%)	9 (7.8%)	
Type of genetic defect			
Oligonucleotide repeat expansion	42 (40.0%)	40 (45.5%)	0.445
Conventional disease-causing variants	63 (60.0%)	48 (54.5%)	
Most frequent genes			
ATXN2	6 (11.1%)	3 (4.5%)	
ATXN3	21 (38.9%)	6 (9.1%)	**<0.001**[b]
FXN	9 (16.7%)	6 (9.1%)	
CACNA1A	2 (3.7%)	9 (13.6%)	
SACS	4 (7.4%)	5 (7.6%)	
SETX	0 (0.0%)	6 (9.1%)	
SYNE1	4 (7.4%)	4 (6.1%)	
PNKP	3 (5.6%)	3 (4.5%)	
RFC1	5 (9.3%)	24 (36.4%)	
Type of movement disorder			
Dystonia	115 (46.2%)	NA	
Tremor	31 (12.4%)	NA	
Parkinsonism	14 (5.6%)	NA	
Chorea	10 (4.0%)	NA	
Myoclonus	7 (2.8%)	NA	
Tics	3 (1.2%)	NA	

Legend: MD – Movement disorders; Y – years.

[a] association of autosomal dominant inheritance with presence of MD (adjusted standardized residuals value of 3.7), with autosomal recessive inheritance and absence of MD (adjusted standardized residuals value of 3.0);

[b] association of *ATXN3* with presence of MD (adjusted standardized residuals value of 3.9), and of *SETX* and *RFC1* with absence of MD (adjusted standardized residuals value of 2.3 and 3.5)

Table 2: Severity and disability in patients with/ without movement disorders

	With MD	Without MD	P value
Disease duration (years) until baseline	20.0y [10.0-31.0]	23.0y [11.0-35.0]	0.251
Clinical severity			
SARA at baseline	14.0 [9.0-20.0]	11.0 [5.2-17.9]	**0.007**
Disability milestones			
Falls	85 (63.9%)	48 (36.1%)	**0.004**
Disease duration until falls	11.5y [5.7-20.0]	11.5y [4.0-30.2]	0.400
Use of cane	46 (35.4%)	37 (34.3%)	0.856
Disease duration until use of cane	14.5 [8.0-21.0]	29.0y [12.0-37.5]	**0.004**
Confinement to wheelchair	41 (30.8%)	23 (20.9%)	0.081
Disease duration until confinement to wheelchair	18.0 [11.0-27.5]	36.0y [12.0-46.0]	0.058

Legend: MD – Movement disorders.

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Spastic ataxia

On article 8 we have performed an analysis of spastic-ataxias, in terms of clinical presentation, symptoms throughout disease course, genetic aetiologies and functional disability.

Article 8. Clinical and genetic panorama of spastic-ataxia

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In submission

Title: Clinical and genetic panorama of spastic ataxia

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ABSTRACT

Background Hereditary cerebellar ataxia (HCA) and spastic paraplegia (HSP) are rare neurological disorders, often seen as extremes of a single clinical spectrum. Spastic ataxia corresponds to the presence of a cerebellar syndrome with overt spasticity, classically associated with a smaller number of genetic causes than HCA.

Objectives To delineate the clinical and genetic features of spastic ataxia patients from an HCA cohort, and compare them with non-spastic HCA.

Methods A prospective cohort of HCA was established in 2017, after a structured protocol and annual visits. Patients with a spasticity grade ≥2 on the modified Ashworth scale were identified, and analysed. HCA patients without spastic ataxia were used for comparison.

Results Fifty-six (22.5%) patients (46 families) with a spastic-ataxia phenotype were identified; overall, these had earlier disease onset and longer disease duration than non-spastic HCA. Recessive inheritance and conventional variants were statistically associated with spastic ataxia and time until genetic diagnosis was significantly higher in this group. Forty-six (82.1%) had a definite genetic diagnosis; 22 causal genes were involved. The most frequent diagnoses were ARSACS (9 patients; 19.6%), and ATX-*SYNE1*, HSP/ATX-*KIF1C* and ATX/HSP-*KCNA2* (3; 6.5% each). Thirty (53.6%) patients with spastic ataxia presented initially with non-cerebellar features, while 26 (46.6%) had cerebellar onset. Overall, the predominant phenotype was upper-limb dysmetria and lower-limb spasticity. After a median disease duration of 28.0 years, 24 (42.9%) still had independent gait. Use of a walking stick occurred by a median disease duration of 35.5 years (24 patients, 45.3%); confinement to wheelchair after a median 40.0 years of progression (20 patients, 35.7%).

Discussion Spastic ataxia was a genetically diverse group, characterized by a longer time to diagnosis, recessive inheritance and more common conventional variants. Earlier disease onset and tendency for non-cerebellar presentation were also observed in this

series.

BACKGROUND

Hereditary cerebellar ataxia (HCA) and spastic paraplegia (HSP) are rare neurological diseases, characterized by degeneration of the cerebellum/ cerebellar pathways or corticospinal tracts.[1] Both are highly heterogeneous groups, from pure ataxia or spastic paraplegia to a mixed presentation or a complex phenotype that may include intellectual disability, dementia, epilepsy, movement disorders or neuropathy.[2] A great diversity of genes and genetic mechanisms has been described, accelerated since the introduction of new genetic diagnostic techniques in clinical practice. Importantly, the widespread use of next-generation sequencing (NGS) based on whole-exome sequencing (WES) disclosed genes classically associated with HSP as the underlying genetic cause of HCA, and vice versa.[1, 3] This genetic overlap in HCA/ HSP and other hereditary movement disorders, led the Task Force for the Nomenclature of Genetic Movement Disorders of the Movement Disorder Society (MDS) to adopt a new classification system; this considers the use of two prefixes followed by the causing gene (e.g., ATX-HSP-*KCNA2*) for combined phenotypes as spastic ataxia.[4-6]

HCA and HSP may be seen as two ends of a clinical continuum,[1] being widely accepted that pyramidal signs are frequent in HCA and cerebellar signs in HSP. Nevertheless, the term spastic ataxia is reserved for the combination of a cerebellar syndrome with overt spasticity; this is linked to a more restricted number of genetic causes.[1, 7, 8] Recessive inheritance is the most frequent amongst spastic ataxia, with autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS), late-onset Friedreich ataxia, HSP/ATX-*PGN* 7 or cerebrotendinous xanthomatosis being the most common.[1, 7, 8] Dominantly inherited spastic ataxia include spinocerebellar ataxia (SCA) type 1, Machado-Joseph disease (MJD, *aka* SCA3) or adult-onset Alexander disease.[1, 7, 8] Other hereditary diseases, such as HSP/ATX-*CAPN1*, HSP/ATX-*UCHL1* or ATX/HSP-*KCNA2*, although rarer, have been increasingly recognized.[4, 9]

The present work aimed at performing a phenotypic and genotypic characterization of a group of patients with spastic ataxia, and compare its clinical features with those of non-spastic HCA patients.

PATIENTS AND METHODS

A prospective study on HCA was begun at our department, in the North of Portugal, in 2017, with annual observations following a structured protocol (previously detailed); in brief, it included a baseline identification of the age-at-onset (AO) of different neurological symptoms/ signs and disability milestones, and an annual questionnaire on new symptoms and disability milestones. The Scale for the Assessment and Rating of Ataxia (SARA),[10] and Inventory of Non-Ataxia Signs (INAS)[11] were applied at all visits, as was also the modified Ashworth scale[12] in presence of *pyramidal signs*. These were defined as occurrence of hyperreflexia in the four limbs, spasticity, or of a Hoffman or Babinski sign. *Spastic ataxia* was diagnosed when in the presence of spasticity ≥2 on the modified Ashworth scale. *Disease onset* was considered as the age of the first cerebellar or non-cerebellar symptom.

Patient selection and sample characterization The overall cohort was composed by 252 individuals (164 families): 249 symptomatic and three asymptomatic carriers (Suppl. Table 1). All patients with spastic ataxia were identified from our HCA database. Demographic data, and presenting and associated neurological symptoms/ signs were gathered. AO of the disease and of different symptoms/ signs, as well as at disability milestones were analysed. Data from patients in the HCA cohort without spastic ataxia were retrieved for comparative analysis.

Genetic testing A proband was, by definition, the first patient genetically diagnosed in the family. DNA was collected from peripheral blood and stored at CGPP, IBMC. A genetic diagnosis was considered only in the presence of pathogenic/ likely pathogenic variant,

according to the guidelines of the American College of Medical Genetics and Genomics.[13]

Patients with conventional variants (single nucleotide substitutions, small insertions/deletions) were compared to those with oligonucleotide (including CAG repeat) expansions.

Statistical analysis Qualitative variables, such as phenotypic traits, were explored using absolute and relative frequencies. Mean (with standard deviation) or median (with quartiles [Q1-Q3]) were used for quantitative variables, depending on whether data followed the Normal distribution. Group comparisons were conducted using the chi-squared test for qualitative variables; for samples of small size, the Monte Carlo method was utilized. The T Student or the Mann-Whitney test were applied for quantitative variables following the Normal or a non-Normal distribution. A linear regression was used to evaluate if an increased baseline SARA score was correlated with spastic ataxia forms, controlling for disease duration. A P value < 0.05 was considered to indicate statistical significance. Analyses were conducted using the IBM Statistical Package for the Social Sciences (SPSS) v.29.[14]

Ethics The ULSSA Ethics Committee previously approved the project (ref. 2017.069). Consent was obtained for clinical investigation, video recording, publication and genetic studies.

RESULTS

Pyramidal signs were identified in 148 (59.4%) of the 249 symptomatic patients (108 of 164 families). A spastic ataxia phenotype (as defined), however, was present in 56 patients (22.5%, 47 families) of our HCA cohort. They had a significantly younger AO, and longer disease duration than the remaining HCA cohort (Table 1). Spastic ataxia was also significantly associated with a *longer time until diagnosis*, as was with *recessive inheritance*

and *conventional variants* (Table 1).

Onset and disease progression in patients with/ without spastic ataxia

There was a tendency for non-cerebellar onset in spastic ataxia, and cerebellar onset in the remaining patients, but without statistical significance. Throughout disease course, dysarthria and cognitive deterioration/ regression significantly associated with spastic ataxia, while diplopia was more common in non-spastic ataxia. Longer disease duration at baseline and higher SARA score were observed in spastic ataxia. When controlling for disease duration, spastic ataxia still associated with a higher SARA score ($p<0.001$). There was no difference in the frequency of dysphagia between patients with spastic ataxia and the remaining cohort, but gastrostomy was more common in the first. Falls and confinement to wheelchair occurred more frequently in patients with spastic ataxia, but with a similar disease duration as in non-spastic HCA (Table 1 and Suppl. Table 2).

Spastic ataxia

Thirty (53.6%) patients presented with non-cerebellar symptoms/ signs, including motor delay (11, 19.6%), spastic gait (9, 16.1%), cognitive regression (3, 5.4%), psychomotor delay and epilepsy (2, 3.6% each), intellectual disability, cough and vertigo (1, 1.8% each). Cerebellar onset (26 patients, 46.4%) comprised gait unsteadiness (20, 35.7%), upper-limb dysmetria (4, 7.1%), and dysarthria and nystagmus (1, 1.8% each). Non-cerebellar onset occurred at a significantly earlier age than cerebellar onset (2.0 [0.0-14.0] years *vs.* 22.0 [7.5-30.0] years, $p=0.001$) (Suppl. Table 3).

Genetic diagnosis In 38 (80.8%) probands, a genetic diagnosis was obtained: nine (23.7%) by targeted single-gene test, and 29 (76.3%) through WES-based multigene panels. All nine (19.1%) probands without definite diagnosis had been studied through WES-based panels and are still undergoing analysis of complete WES. Of the nine affected relatives of probands, one belonged to a family without definite diagnosis and in the remaining

diagnosis was performed by single-gene testing.

Thirty-eight (67.9%) patients (33 families) had a known autosomal recessive disease (Table 2): nine (19.6%) ARSACS; three (6.5%) each ATX-*SYNE1* and HSP/ATX-*KIF1C*; two (4.3%) either *ATP8A2*-related ataxia, ATX-*ANO10, HSP/ATX-PGN, HSP- KIAA1840*, HSP-*ZFYVE26*, HSP/ATX-*GBA2* or MxMD-*ATP13A2*; less frequent genes are detailed in Table 2; consanguinity was known in 21 (63.6%). Eleven (19.6%) patients (11 families) showed dominant inheritance, with ATX/HSP-*KCNA2* identified in three (6.5%), while ATX-*CACNA1A, KMT2B*-related ataxia, MJD/SCA3, HSP-*SPAST* and HSP-*KIF1A* were diagnosed in one (2.2%) patient each. All four (7.1%) cases (four families) with mitochondrial inheritance had ATX-*MT-ATP6*.

Onset according to genetic diagnosis There was no significant association of AO or presenting symptom with mode of inheritance or causal gene (Suppl. Fig. 1; Suppl. Table 4).

Disease progression After 31.0 [18.0-43.0] years of disease progression, including 4.0 [0.0-5.0] years of prospective follow-up, upper-limb dysmetria was seen in all 56 cases, dysarthria in 53 (94.6%), and nystagmus in 22 (39.3%). Lower-limb dysmetria was seen in 54 (not evaluated in two due to marked spasticity), while gait ataxia was present in all 55 where it could be assessed. The pyramidal syndrome was characterized by spasticity and brisk deep tendon reflexes in all; Babinski sign in 44 (78.6%); and Chadock (24, 57.1%) or Oppenheim (six, 14.3%) signs in a smaller number of patients (details by disease/ gene in Suppl. Tables 5-6). In 33 (58.9%) patients, an additional (non-ataxia) movement disorder was present; and, neuropathy was observed in 22 (39.3%). Cognitive deterioration or regression occurred in 13 (23.6%) patients, and epilepsy in five (8.9%) (Table 1; Suppl. Tables 5-6).

Disability milestones and survival time Twenty-four (42.9%) patients still had independent gait after 28.0 [16.0-47.0] years of disease progression; 24 (42.9%) needed a walking stick

at 35.5 [20.3-45.0] years; 20 (35.7%) were wheelchair-confined after 40.0 [28.3-47.0] years of progression; six patients transitioned directly from independent gait to a wheelchair (details per disease gene in Suppl. Table 7).

Death had occurred in four patients: one ATX-*APTX*, at 69 years of age (62 years of progression); one ATX-*RFC1*, at 78 years of age (45 years' progression); one HSP/ATX-*PGN*, at 73 years of age (after 40 years of disease); and one without genetic diagnosis, at 67 years of age (10 years' progression). An infectious cause of death was identified in all; it occurred at home (in two), nursing home or hospital (one each).

DISCUSSION

We describe a group of patients with spastic ataxia, retrieved from a larger hospital-based HCA cohort. As expected, pyramidal signs were common (59.4%) in the global cohort; a spastic-ataxia phenotype was less frequent, but still identified in 22.5% of all HCA patients. Degeneration of cerebellar neurons and/ or spinocerebellar tracts are a hallmark of HCA,[15] while HSP are characterized by axonal degeneration of the longest corticospinal tracts, frequently with impairment of the posterior columns.[16] The number of genes causing either HCA and HSP reflects common cellular mechanisms and pathways, as lipid metabolic, acid metabolic and cytoskeleton/ dendritic intracellular transport pathways.[1, 8, 9] Together with the significant prevalence of pyramidal signs in HCA,[17, 18] this supports the view of a clinical *continuum* between HCA and HSP. Nevertheless, "true" spastic ataxia (as defined here) is less common, as observed in the Portuguese population-based survey[19] and in the present cohort.

Spectrum of genetic aetiology of spastic ataxia

Spastic ataxia associated with younger age at onset and recessive inheritance, which is in line with that observed in other series.[1, 8, 9] Delay in genetic diagnosis was higher in spastic ataxia, conceivably reflecting the difficult investigation of such overlapping phenotypes.

Conventional disease-causing variants were associated with spastic ataxia, while oligonucleotide expansions were the most common in non-spastic HCA.

As expected,[1, 7-9] ARSACS was the most frequent spastic ataxia (19.6%), with classical childhood AO in all,[20] except a single patient with adulthood onset; the triad of ataxia, neuropathy and pyramidal signs[20] was present in all our patients, except one who had no neuropathy. ATX-*MT-ATP6,* the second genetic aetiology in our spastic ataxia series, is classically associated with neuropathy, ataxia and retinitis pigmentosa (NARP);[21] however, a broader phenotypical spectrum may include milder forms with adult-onset SCA or ataxia with spasticity,[22-24] as we observed. Among the next most frequent causes of spastic ataxia, only HSP/ATX-*KIF1C* (also known as SPAX2) has been associated with this phenotype since its first reports.[25, 26 27] ATX-*SYNE1* was originally described as a pure cerebellar ataxia, seldom with pyramidal signs,[27] but its phenotype has broadened to include spastic ataxia and other, more complex, presentations.[28, 29 30] ATX/HSP-*KCNA2*, initially identified in developmental and epileptic encephalopathy,[30] has been increasingly associated with spastic ataxia,[31] as recognized in 2022 by the MDS (mixed phenotype of ataxia/spastic paraplegia);[4] curiously, one of our patients had no epilepsy, and the remaining two had a pharmaco-resistant epilepsy in childhood that was controlled in adolescence. HSP/ATX-*PGN*, the most frequent cause of spastic ataxia in the series of Coutelier et al,[32] had a frequency of 4.3% in our spastic ataxia series, similar to ATX-*ANO10,* HSP- *KIAA1840*, HSP-*ZFYVE26*, HSP/ATX-*GBA2* or HSP/ATX-*CAPN1*. MJD, the most common HCA in Portugal[19] and in our ataxia cohort, corresponded to only 2.2% of spastic ataxia (as its definition was stricter here), much less than described by others (31.7%-44.4%),[18, 33, 34] including a Portuguese cohort.[33] Marked pyramidal signs (including spasticity) are more frequently observed in type 1 MJD[35] , which corresponded to only two out of 29 MJD patients in this cohort. Masking of spasticity by neuropathy is more common in type 3, but tends to occur in most MJD patients with disease progression;[35] that might be another reason for the low frequency of a spastic-ataxia phenotype, as defined in this study. As for remaining

aetiologies, also with a frequency of 2.2%, most have already been associated with spastic ataxia,[4, 6, 36-38] what was now reinforced by our results. We acknowledge the absence of Friedreich ataxia in our series of spastic ataxia. A spastic-ataxia phenotype is usually described in late or very-late onset forms of FA,[1, 8] which corresponded to only two in 15 of our cases. The relatively small number of global FA cases, together with the low frequency of late-onset forms, may explain the absence of a spastic-ataxia phenotype among our FA patients.

Phenotypic scenario of spastic ataxia

Clinical presentations varied, including with a wide range of cerebellar and non-cerebellar symptoms (Table 1). HCA with a spastic-ataxia phenotype tended to start sooner, have a more retracted course, and less cerebellar signs at onset. They were more often inherited as an autosomal recessive, more frequently due to conventional mutations, and have a longer delay up to genetic diagnosis. Clinical features as dysarthria and cognitive deterioration/ regression, were more frequent than in the remaining HCA. With progression, all patients with a spastic-ataxia phenotype eventually presented a predominant cerebellar syndrome in the upper limbs, and predominant spasticity in lower limbs. With a *waving* (ataxic) upper body, and a *restricted* (spastic) lower body, these patients may evoke a mermaid body, as suggested by Paula Coutinho in 2007.[39]

Disability in spastic ataxia

Controlling for disease duration, spastic ataxia associated to a higher SARA score at baseline. This was in line with the findings of Traschütz et al (2023)[40] , in a large cohort of AR-HCA, in which the presence of spasticity was associated with a SARA score 2.2-3.5 points higher than in patients without spasticity. In the group of spastic ataxia, approximately 40% of patients still had independent gait with 28 years' progression. In a large series of AR-HCA, which included spastic ataxia, over 60% of patients with ATX-*ANO10* and HSP/ATX-*PGN* were still ambulatory after 10 years of disease course, while in

ATX-*SYNE1* and ARSACS this was of 40%, at 20 and 26 years of disease.[41] These estimates are in line with those observed in our series.

Limitations of the study

As expected, this is an heterogenous genetic sample, with a low number of patients per causal gene. This precluded a robust genotype-phenotype analysis. Also, as our prospective study started only in 2017, a recall bias about AO and associated symptoms, whenever these started before that year, cannot be excluded; nevertheless, we checked all information with two first-degree relatives and with clinical records. It would now be important to perform a similar study on our patients primarily classified as HSP, among whom some may also have this spastic-ataxia phenotype, and enlarge this sub-cohort of spastic ataxia for prospective studies. Finally, as this is a hospital-based cohort, its results cannot be generalized into population-based settings.

Conclusions

With this study we wanted to contribute further to the characterization of the phenotype of *spastic ataxia* among the HCA. Notwithstanding the number of articles on the topic, cohort analyses are scarce.[33,42] Recognizing that spastic ataxia presents at a younger age, more frequently with non-cerebellar symptoms and is more often associated with recessive inheritance and conventional (non-repeat expansions) mutations, should be of high relevance for all clinicians, and particularly those working in the field of HCA and/or HSP.

Table 1: Clinical features in patients with/ without spastic ataxia

	Spastic ataxia phenotype	Remaining HCA cohort	P value
Total number of patients	56 (22.5%)	193 (77.5%)	
Age at disease onset	8.0y [0-26.0]	27.5y [8.0-41.8]	**<0.001**
Disease duration at first visit	27.0 [16.0-41.0]	20.0 [10.0-31.0]	**0.013**
Overall disease duration (until last visit or death)	36.0y [19.0-48.0]	26.0y [13.2-40.0]	**0.015**
SARA score at baseline	14.7 [12.0-25.7]	12.5 [7.5-18.0]	**0.008**
Mode of inheritance			
Dominant	11 (19.6%)	80 (41.5%)	**0.007**[a]
Recessive	38 (67.9%)	95 (49.2%)	
Mitochondrial	4 (7.1%)	4 (2.1%)	
Undetermined	3 (5.4%)	17 (7.3%)	
Genetic diagnosis	46 (82.1%)	148 (76.7%)	0.386
Time until genetic diagnosis	34.0y [17.0-50.0]	18.0y [7.0-39.0]	**<0.001**
Oligonucleotide expansion	2 (4.3%)	80 (54.1%)	**<0.001**[b]
Conventional mutation	44 (95.7%)	68 (45.9%)	
Type of onset			
Cerebellar	26 (46.4%)	112 (59.6%)	0.081
Non-cerebellar	30 (53.6%)	76 (40.4%)	
Symptom/ signs throughout disease course			
Dysarthria	53 (94.6%)	158 (81.9%)	**0.019**
Upper limb dysmetria	55 (98.2%)	183 (94.8%)	0.276
Gait unsteadiness	55 (98.2%)	186 (96.4%)	0.492
Ocular signs			
diplopia	6 (10.7%)	63 (33.5%)	**<0.001**
nystagmus	22 (39.3%)	88 (46.1%)	0.369
horizontal ophthalmoparesis	10 (17.9%)	42 (22.2%)	0.483
vertical ophthalmoparesis	11 (19.6%)	32 (16.8%)	0.616
hypermetric saccades	21 (46.7%)	71 (42.0%)	0.575
hypometric saccades	9 (20.0%)	33 (19.5%)	0.943
oculomotor apraxia	4 (7.1%)	17 (8.9%)	0.678
Movement disorders (other than ataxia)	33 (58.9%)	100 (51.8%)	0.347
Epilepsy	5 (8.9%)	8 (4.2%)	0.165
Neuropathy	22 (39.3%)	82 (43.4%)	0.586
Motor development delay	13 (23.2%)	25 (13.1%)	0.065
Intellectual disability	12 (21.4%)	24 (12.6%)	0.102
Cognitive deterioration/ regression	13 (23.6%)	21 (11.0%)	**0.017**
Disability milestones			
Anarthria	3 (5.4%)	4 (2.1%)	0.196
Agraphia	9 (16.1%)	21 (11.5%)	0.435
Dysphagia	30 (53.6%)	100 (52.9%)	0.398
Gastrostomy	3 (5.4%)	1 (0.5%)	**0.012**
Presence of falls	39 (69.6%)	95 (50.5%)	**0.012**
Use of cane	24 (45.3%)	59 (31.4%)	0.060
Use of wheelchair	20 (36.4%)	44 (23.0%)	**0.047**

y, years; [a]significant association of autosomal recessive inheritance and spastic ataxia, autosomal dominant inheritance and non-spastic ataxias (adjusted standardized residuals value of 2.5 and 3.0 respectively); [b]significant association of conventional mutation with spastic ataxias, and oligonucleotide expansion and remaining ataxias (adjusted standardized residuals value of 6.0)

Table 2: Genetic diagnosis in families with spastic ataxias

	Total number of patients	Number of probands
Autosomal recessive inheritance		
ARSACS	9	8
HSP/ATX-*KIF1C*	3	2
ATX-*SYNE1*	3	3
ATX-*ANO10*	2	2
ATP8A2-related ataxia	2	1
HSP/ATX-*PGN*	2	2
HSP-*KIAA1840*	2	1
HSP-*ZFYVE26*	2	2
HSP/ATX-*GBA2*	2	1
MxMD-*ATP13A2*	2	2
ATX-*APTX*	1	1
HSP/ATX/NBIA-*FA2H*	1	1
HSP/ATX-*CAPN1*	1	1
ATX-*L2HGDH*	1	1
ATX-*RFC1*	1	1
Genetically undiagnosed	4	4
Autosomal dominant inheritance		
ATX/HSP-*KCNA2*	3	2
MJD	1	1
ATX/PxMD-*CACNA1A*	1	1
KMT2B-related ataxia	1	1
HSP-*KIF1A*	1	1
HSP-*SPAST*	1	1
Genetically undiagnosed	3	1
Mitochondrial inheritance		
ATX-*MT-ATP6*	4	2
Undetermined mode of inheritance	3	3
TOTAL	56	46

Disease names follow the recommendations for nomenclatures of the Task Force of the Society Movement Disorders, except for ARSACS – autosomal recessive ataxia of Charlevoix-Saguenay and MJD – Machado-Joseph disease, which original names were kept, given its wide diffusion and acknowledgement.

ATX – ataxia; HSP – hereditary spastic paraparesis; NBIA – neuronal brain iron accumulation; PxMD – paroxysmal movement disorder.

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Table 1 – Clinical features in patients with/ without spastic ataxia

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SUPPLEMENTARY DATA

Supplementary Table 1 – Genetic diagnoses in the overall cohort

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C. GENOTYPE - PHENOTYPE CORRELATIONS

[Objective 3. Genotype-phenotype correlation]

In 33 patients an analysis on the correlation of a genotype with a particular phenotype was performed, with a particular emphasis on the genetic data.

Article 9 describes a group of patients observed during the national survey, whose diagnosis was recently obtained.

Article 10 reports a family with three siblings affected with a new form of ataxia with oculomotor apraxia associated with pathogenic variants in *MAG.*

Article 9. Molecular Characterization of Portuguese Patients with Hereditary Cerebellar Ataxia.

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Molecular Characterization of Portuguese Patients with Hereditary Cerebellar Ataxia

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Abstract: Hereditary cerebellar ataxia (HCA) comprises a clinical and genetic heterogeneous group of neurodegenerative disorders characterized by incoordination of movement, speech, and unsteady gait. In this study, we performed whole-exome sequencing (WES) in 19 families with HCA and presumed autosomal recessive (AR) inheritance, to identify the causal genes. A phenotypic classification was performed, considering the main clinical syndromes: spastic ataxia, ataxia and neuropathy, ataxia and oculomotor apraxia (AOA), ataxia and dystonia, and ataxia with cognitive impairment. The most frequent causal genes were associated with spastic ataxia (*SACS* and *KIF1C*) and with ataxia and neuropathy or AOA (*PNKP*). We also identified three families with autosomal dominant (AD) forms arising from de novo variants in *KIF1A*, *CACNA1A*, or *ATP1A3*, reinforcing the importance of differential diagnosis (AR vs. AD forms) in families with only one affected member. Moreover, 10 novel causal-variants were identified, and the detrimental effect of two splice-site variants confirmed through functional assays. Finally, by reviewing the molecular mechanisms, we speculated that regulation of cytoskeleton function might be impaired in spastic ataxia, whereas DNA repair is clearly associated with AOA. In conclusion, our study provided a genetic diagnosis for HCA families and proposed common molecular pathways underlying cerebellar neurodegeneration.

Keywords: cerebellar ataxia; recessive ataxia; exome sequencing; molecular mechanisms; de novo variant

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1. Introduction

Hereditary cerebellar ataxias (HCA) comprise a heterogeneous group of neurological disorders. The cardinal cerebellar syndrome of HCA may be present in isolation or

accompanied by a wide constellation of neurological and non-neurological symptoms and signs. All modes of inheritance have been recognized, with the autosomal recessive (AR) forms being the most complex, both from a clinical and genetic point of view [1]. Prevalence of autosomal dominant (AD) HCA ranges from 0.0 to 5.6/100,000, and that of AR-HCA varies from 0.0 to 7.2/100,000 [2]. AD-HCA are generally caused by repeat expansions, with Machado-Joseph Disease/Spinocerebellar ataxia type 3 (MJD/SCA3) being, overall, the most frequent form and SCA2 the second on a global basis, but first in countries such as Cuba or India [2,3]. Friedreich ataxia is the most frequent recessive ataxia, followed by ataxia telangiectasia or ataxia with oculomotor apraxia (AOA), depending on the series [2,3]. The genetic diversity of HCA has greatly increased over the past years, due to the contribution of next generation sequencing (NGS) techniques. With a diagnostic yield of 20–25%, NGS has allowed for the identification of new ataxia-causing genes and redefining genotype–phenotype correlations; however, it has uncovered a large number of variants of unknown clinical significance [4,5].

Despite the diversity of HCA-genes and their ubiquitous expression throughout the brain, genetic defects in HCA predominantly affect the cerebellum, suggesting that cerebellar cells (particularly, Purkinje cells) are more vulnerable. Therefore, the identification of shared molecular pathways between mutated genes is crucial to understand the basis of cerebellar neurodegeneration [6,7]. Many genes function in common pathways, including protein quality control, transcription regulation, and calcium homeostasis in AD-HCA [8]; and DNA repair, mitochondria function, and lipid metabolism in AR-HCA [9]. Based on common pathways identified, it may be possible to develop effective therapeutic strategies, targeting more than one form of HCA [7].

This study focused on 19 Portuguese families with apparent AR-HCA, previously identified during a large population-based survey and studied by targeted testing for the most common genes [10], and now investigated using whole-exome sequencing (WES). The molecular mechanisms potentially responsible for the neurodegeneration associated with the identified HCA causal-genes were reviewed and discussed.

2. Materials and Methods

2.1. Patients and Clinical Study

A national, systematic, population-based survey was conducted in Portugal from 1994 until 2004, aiming at identifying Portuguese families with HCA and hereditary spastic paraplegia (HSP) [10]. Its detailed methodology was previously described [10]; briefly, clinical and family history, neurological evaluation and blood sampling were performed, and genomic DNA extracted. Written informed consent was obtained from participants.

2.2. Genetic Analysis

In the national survey, 250 families (344 patients) with presumed autosomal recessive ataxia were identified. Targeted genetic testing was conducted ab initium, according to the main clinical phenotype; 61 families (83 patients), however, remained without a genetic diagnosis [10]. Pathogenic variants in *PNKP* [11] were later identified in eight families (11 patients), and in *MAG* [12] in one family (3 patients), all with AOA. More recently, expansion of an intronic repeat in *RFC1* [13] was excluded in all families with genomic DNA available who persisted without diagnosis.

From the 61 undiagnosed families, we only had genomic DNA available for 27 families, with a final diagnosis being reached in the 19 here described. We performed whole-exome sequencing (WES) in at least one patient of each family (depending on the availability of genomic DNA). Exome sequencing libraries were prepared using a SureSelect Exome Capture Kit v7 (Agilent), and sequencing was performed on NextSeq550 with 100 bp paired-end reads, according to the manufacturer's instructions. After sequencing, reads were then aligned to the human reference genome hg19/GRCh37 using Burrows-Wheeler Aligner (bwa) v0.7.1 [14] and variants were called using GATK best practices v3.3-0 [15].

Duplicate reads were identified through samblaster v0.1.21 [16]. Variant annotation was performed with snpEff v4.2 [17] and dbNSFP v2.9 [18].

Annotated variants were filtered to include rare variants with a minor allele frequency (MAF) <1% in population databases such as Nucleotide Polymorphism Database (dbSNP; https://www.ncbi.nlm.nih.gov/snp (accessed on 11 February 2022)), Genome Aggregation Database (gnomAD v2.1; https://gnomad.broadinstitute.org (accessed on 11 February 2022)), and the 1000 Genome Project ((http://www.1000genomes.org (accessed on 11 February 2022)). Non-synonymous and splice-site variants, and variants for AR forms were filtered for primary analysis. This analysis was performed using Exomiser v7.2.1 [19], which also allowed variant prioritization based on the human phenotype ontology HP:0001251 (term name:ataxia). Then, we excluded intronic, UTR, intergenic, and synonymous variants, as well as homozygous variants reported in gnomAD. In addition, when de novo variants were suspected, those for AD forms were also filtered and analyzed. The functional predicted impact of missense variants was evaluated using SIFT [20], Mutation Taster [21], Polyphen2 [22], Mutation assessor [23], FATHMM [24], and UMD-Predictor [25]. Analysis of splice-site variants was performed using Splice Site Finder-like [26], MaxEntScan [27], Neural Network SPLICE (NNSPLICE) v0.9 [28], GeneSplicer [29], and Human Splicing Finder (HSF) v3.1 [30]. Variants were classified according to the ACMG/AMP Standards and Guidelines [31]. ClinVar (http://www.ncbi.nlm.nih.gov/clinvar (accessed on 11 February 2022)) and Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php (accessed on 11 February 2022)) were also used for variant classification.

Relevant variants identified by WES were confirmed by Sanger sequencing, which also allowed verifying intrafamilial segregation in several families. For PCR amplifications, we used Ranger Mix (Bioline, London, UK), purified products with Exo/SAP (GRiSP, Porto, Portugal), and performed Sanger sequencing using Big Dye Terminator Cycle Sequencing v1.1 (Applied Biosystems, Foster City, CA, USA) in an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing analysis was carried out using the SeqScape v2.6 software (Applied Biosystems, Foster City, CA, USA).

2.3. Minigene Assays

The *SPG11* and *KIF1C* minigenes constructs were obtained by cloning the exonic and intronic sequences flanking the variants of interest (c.3039-5T > G in *SPG11* and c.1166-2A > G in *KIF1C*) into the pCMVdi vector, kindly provided by Dr Alexandra Moreira [32]. Briefly, *SPG11* exons 16, 17, and 18 with intronic regions or *KIF1C* exons 13, 14, and 15 with intronic regions were PCR amplified from patients' genomic DNA and cloned into the pCMVdi vector, using the Gibson assembly method. Sequences were modified by site-directed mutagenesis to generate the wild-type constructs, using a QuikChange II Kit (Agilent, Santa Clara, CA, USA), according to the manufacturer's protocol.

HEK293T cells were transfected with the minigene constructs using jetPRIME (Polyplus-transfection, Illkirch, France), according to the manufacturer's protocol. RNA was extracted 48 h after transfection, using NZYol (Nzytech, Lisbon, Portugal), as per manufacturer's recommendations, followed by purification of the RNA aqueous phase, using an RNeasy mini kit (Qiagen, Hilden, Germany). RNA quantification was performed on NanoDrop 2000 (ThermoFisher Scientific, Waltham, MA, USA). cDNA was synthesized by reverse transcription-PCR of 2 µg total RNA with oligo (dT), using a SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. The resulting cDNA was amplified by PCR and loaded on agarose gel for extraction with a Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA). The resulting products were sequenced by Sanger sequencing using Big Dye Terminator Cycle Sequencing v1.1 (Applied Biosystems, Foster City, CA, USA) in an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

3. Results

3.1. Genetic and Molecular Characterization

We used WES to identify variants and genes causing HCA in 19 Portuguese families with family history compatible with AR transmission (including 9 with known consanguinity), in a total of 30 individuals: 19 index cases, one affected and 10 non-affected relatives. At least, one relative of seven index cases was available. All relevant variants (rare, pathogenic, likely pathogenic, and/or predicted to be deleterious) were confirmed by Sanger sequencing, and segregation analysis was performed with all available family members. Phenotypic classification considered the main clinical symptom associated with ataxia: spasticity, neuropathy, oculomotor apraxia, dystonia, or cognitive impairment.

Overall, we identified 24 rare nucleotide variants in 13 genes (Table S1, supporting information): *SACS*, *KIF1C*, *ANO10*, *SPG11*, *SYNE1*, and *CACNA1A* were related to spastic ataxia (10/19 families, 52.6%); *KIF1A*, *POLG*, *SETX*, and *PNKP* to ataxia and neuropathy (4/19, 21.1%); *PNKP* to AOA (2/19, 10.5%); *HEXB* and *ATP1A3* to ataxia and dystonia (2/19, 10.5%); and *FA2H* to ataxia with cognitive impairment (1/19, 5.3%). The most frequent genes were those associated with spastic ataxia, ataxia, and neuropathy or AOA: *SACS* (4 families, 21.1%), *KIF1C* (2 families, 10.5%), and *PNKP* (3 families, 15.8%); the remaining genes were identified in single families only.

Variants were classified as pathogenic when having been previously reported as disease-causing (14/24), or by causing an early stop codon (nonsense and frameshift; 8/24) in genes where loss-of-function is a known disease mechanism (Table S2, supporting information). In addition, two variants were predicted to affect splicing by bioinformatics analysis (Table S3, supporting information)—c.3039-5T > G in *SPG11* and c.1166-2A > G in *KIF1C*—both in families with spastic ataxia; their detrimental effect on splicing was confirmed by minigene splicing-assays, concluding that both are expected to result in frameshifts (Figure 1; Figure S1, supporting information).

We also identified two novel missense variants (Figure 2 and Table S3, supporting information), both in heterozygosity, classified as likely pathogenic (2/24): one in *CACNA1A* (c.4996C > G; p.Arg1666Gly; family AR267), at an amino-acid residue where a different missense change has been determined to be pathogenic, located in a well-established functional (transmembrane) domain (Table S4, supporting information) and not found in population databases; and one in *ATP1A3* (c.374T > A; p.Val125Glu; family AR278), a gene with low rate of benign missense variation, not reported in population databases, located in a mutation hotspot within a transmembrane domain (Table S4, supporting information) and predicted to be deleterious. Moreover, the WT residues of human *CACNA1A* (Arg1666) and ATP1A3 (Val125) are conserved across species, while the mutated residues are predicted to change their interactions, probably affecting protein structure (Figure 2). Besides the apparent AR inheritance, the variant in *ATP1A3* was confirmed to occur de novo (absent in the unaffected parents, family AR78). Unfortunately, we did not have parental samples to test for the *CACNA1A* variant (family AR267), but this variant probably occurs de novo, as these are a recurrent cause of *CACNA1A*-related ataxia [33]. In addition, we also identified one de novo variant in *KIF1A* (c.761G > A, described as pathogenic) in a patient with spastic ataxia, and confirmed the unaffected parents and sibling did not carry it (family AR49).

Thus, we reported 10 novel disease-associated variants in nine families (Table S1, supporting information). All variants were absent from population databases or were present only in heterozygosity with very low MAF.

3.2. Clinical Characterization

Beyond the 20 patients tested, we had clinical information (but no DNA sample) from nine additional affected relatives of probands (detailed data Table 1).

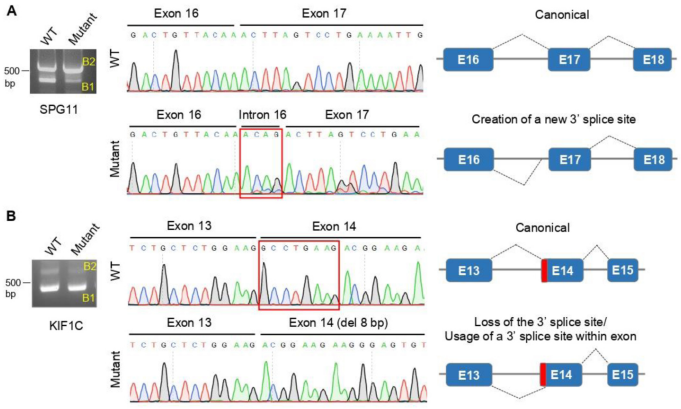

Figure 1. Analysis of *SPG11* (c.3039-5T > G) (**A**) and *KIF1C* (c.1166-2A > G) (**B**) splice-site variants, with minigene splicing assays. Agarose gel electrophoresis of the transcripts generated by the wild-type (WT) and mutant minigene constructs (on the left): B1 bands (<500 bp) represent transcripts affected by the variants; B2 bands (>500 bp) represent amplified transcripts with intron retention, not affected by the variant. Sanger sequencing of the transcripts corresponding to B1 bands (in the middle). Schematic representation of resulting splicing events (on the right): (**A**) *SPG11* variant creates a new 3′ acceptor splice site, resulting in the retention of 4 nucleotides of intron 16; (**B**) *KIF1C* variant abolished the 3′ acceptor splice-site, leading to the usage of a splice site within exon 14 and resulting in the deletion of 8 nucleotides from exon 14. Electropherograms showing the location of the splice-site variants on genomic DNA are shown in Figure S1, supporting information.

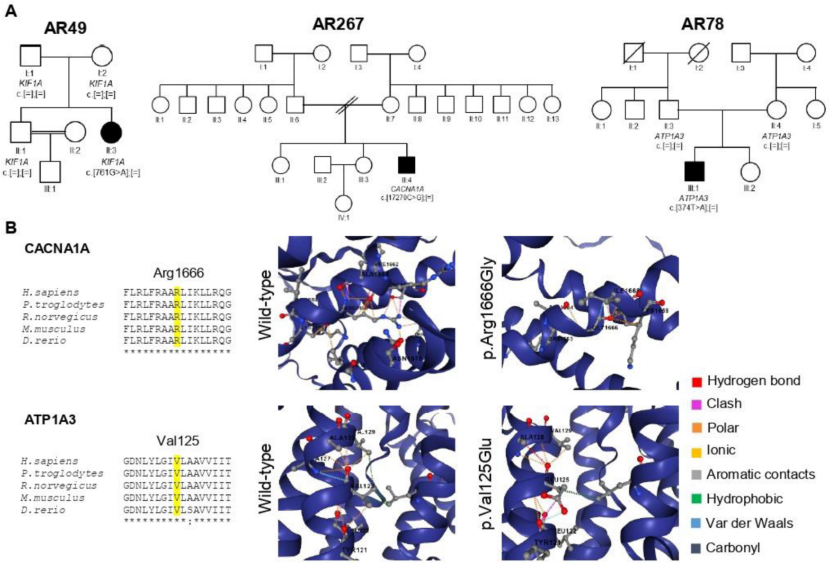

Figure 2. Pedigrees of families harboring de novo variants (**A**), and conservation of the new missense variants and protein models of CACNA1A and ATP1A3 (**B**). (**A**) Black symbols represent individuals

with cerebellar ataxia. (**B**) Sequence alignment of the residues surrounding the mutated residues in CACNA1A and ATP1A3 against other species was performed using the Clustal Omega program. Protein models of CACNA1A and ATP1A3 showing altered residues interactions upon amino acid changes (performed using DynaMut2). The Arg1666 and Val135 residues in CACNA1A and ATP1A3, respectively, are in the center, displaying the various interactions with nearby residues in different colors.

3.2.1. Spastic Ataxia

Spastic ataxia was the largest phenotypic group, comprising 10 families (12 patients). With the exception of AR120 (*SACS*) and AR267 (*CACNA1A*), all patients had onset in the first decade of life. Its presenting symptoms comprised ataxia (AR53 (*SACS*), AR120 (*SACS*), AR103 (*ANO10*), AR109 (*SYNE1*), AR267(*CACNA1A*)), delayed motor milestones (AR77 (*SACS*), AR252 (*SACS*), AR111 (*KIF1C*)), cognitive regression (AR108 (*SPG11*)), and upper limb tremors (AR96(*KIF1C*)); we highlight, due to its rarity, the presence of seizures in AR77 (*SACS*) and the spastic ataxia phenotype in AR267 (*CACNA1A*). Regarding imaging, notably, family AR96 (*KIF1C*) showed basal ganglia hypointensities on T2/FLAIR sequences and AR111 (*KIF1C*) had brainstem atrophy.

3.2.2. Ataxia and Neuropathy

This group included four families (10 patients). Onset was in the first decade in AR49 (*KIF1A*) and AR92 (*PNKP*), second decade in AR4 (*SETX*), and third decade in AR126 (*POLG*). Ataxia was the presenting feature in all families, with neuropathy developing later. It is noteworthy that in AR4 (*SETX*) and AR92 (*PNKP*) none of the patients had oculomotor apraxia. All patients from AR4 presented diplopia and nystagmus, but none from AR92 displayed such features. The patient from AR49 (*KIF1A*) had an extensor plantar response in the absence of spasticity. AR126 had a *POLG*-related ataxia, with the typical optic atrophy, vertical gaze palsy, and epilepsy. One of the members from AR92 died at age 35, with 29 years of disease duration. Imaging was only available in two families (AR4, AR49), all displaying cerebellar atrophy.

3.2.3. Ataxia and Oculomotor Apraxia

Both families (4 patients) had onset in the first decade of life, and disease-causing variants in *PNKP*. Ataxia was the first symptom in all patients. OMA, neuropathy, and obesity were present in all individuals, and dystonia was highly frequent. Cerebellar atrophy was identified on magnetic resonance imaging (MRI); when tested, total proteins/albumin level was decreased, and cholesterol increased. In AR117, death occurred between 37 and 55 years of age, with a mean disease duration of 37 years.

3.2.4. Ataxia and Dystonia/Ataxia and Cognitive Impairment

AR2 (*HEXB*) and AR278 (*ATP1A3*), with one patient each, presented ataxia and dystonia. AR2 (*HEXB*) had adult-onset ataxia, very prominent oromandibular dystonia and muscle wasting, while AR278 (*ATP1A3*) had onset at age 11 years, with paroxysmal lower limbs dystonia induced by walking, and progressive ataxia four years later. Over the disease course, dystonia became permanent and generalized. Both AR2 and AR278 had cerebellar atrophy on MRI.

AR16 (*FA2H*) was the only family with ataxia and cognitive impairment as the major phenotype, in a patient with delayed motor and cognitive milestones, who later developed ataxia and seizures. Cortical and cerebellar atrophy was present on MRI, and increased latency was identified in visual evoked potentials.

Table 1. Clinical characterization of the families.

Family	Ind. Code (Gender)	Tested	Gene	Disease Causing Variants	Consanguinity	Age				First Symptom	Ataxia	OMA	Diplopia	Pyramidal Signs	Peripheral Neuropathy	Dystonia	Cognitive Impairment	Other Symptoms/Signs	Imaging	Others
						Onset	Obs.	Wheelchair	Death											
Spastic ataxia																				
AR53	IV.1 (F)	Yes	*SACS*	c.8148del; c.8148del	Yes	6	53	47	NA	Ataxia	Yes	No	No	Yes	No	No	No		NAv	
AR77	II.3 (M)	Yes	*SACS*	c.1373C >T; c. 5440_5449del	No	<1	31	NA	NA	Delayed motor milestones	Yes	No	No	Yes	Yes	No	No	Seizures 1-16y Nystagmus	MRI: cerebellar atrophy	
AR120	II.1 (F)	Yes	*SACS*	c.3066del; c.2656_2666del	No	15	66	29	NA	Ataxia	Yes	No	No	Yes	Yes	No	No	Nystagmus	NAv	
AR252	II.1 (M)	Yes	*SACS*	c.814C > T; c.814C > T	Not clear	<1	57	54	NA	Delayed motor milestones	Yes	No	No	Yes	Yes	No	No	Kyphoscoliosis	NAv	
AR96	IV.1 (M)	Yes	*KIF1C*	c.393_396del; c.393_396del	Yes	9	52	41	NA	UL tremor	Yes	No	No	Yes	Yes	No	Mild deterioration	Nystagmus	MRI: pallidum, putamen, thalamus hypointensities (T2/FLAIR)	
	IV.2 (F)	Yes				8	50	44	NA	UL tremor	Yes	No	No	Yes	Yes	No	Mild deterioration	Nystagmus	MRI: vermis atrophy	
AR111	III.3 (F)	Yes	*KIF1C*	c.1166-2A > G; c.1166-2A > G	Yes	<1	41	NA	NA	Delayed motor milestones	Yes	No	No	Yes	Yes	No	No	Vertical gaze restriction Mild dysmorphism Pes cavus	CT: brainstem atrophy	
AR103	III.3 (F)	Yes	*ANO10*	c.132dup; c.132dup	Yes	7	40	NA	NA	Ataxia	Yes	No	Yes	Yes	No	No	No	Nystagmus Migraine	MRI: cerebellar atrophy	
AR108	II.3 (F)	Yes	*SPG11*	c.1951C > T; c.3039-5T > G	No	7	43	NA	NA	Cognitive regression	Yes	No	No	Yes	No	No	Yes		MRI: Thin corpus callosum	
	II.2 (M)	No				8	47	NA	NA	Cognitive regression	Yes	No	No	Yes	No	No	Yes		MRI: Chiari type1	
AR109	III.2 (M)	Yes	*SYNE1*	c.17270C > G; c.17270C > G	Not clear	NAv	67	NA	NA	Ataxia	Yes	No	No	Yes	No	No	No		NAv	
AR267	III.4 (M)	Yes	*CACNA1A*	c.4996C > G	No	19	21	NA	NA	Ataxia	Yes	No	No	Yes	No	No	Yes		MRI: cerebellar atrophy	

Table 1. *Cont.*

Family	Ind. Code (Gender)	Tested	Gene	Disease Causing Variants	Consanguinity	Age				First Symptom	Ataxia	OMA	Diplopia	Pyramidal Signs	Peripheral Neuropathy	Dystonia	Cognitive Impairment	Other Symptoms/Signs	Imaging	Others
						Onset	Obs.	Wheelchair	Death											
Ataxia and neuropathy																				
AR4	VI.27 (M)	Yes	*SETX*	c.1514G > A; c.1514G > A	Yes	14	37	NA	NA	Ataxia	Yes	No *	Yes	No	Yes	No		Nystagmus	CT: cerebellar atrophy	
	VI.7 (F)	No				13	49	NA	NA	Ataxia	Yes	No *	Yes	No	Yes	No		Nystagmus	CT: cerebellar atrophy	
	VI.10 (F)	No				11	43	NA	NA	Ataxia	Yes	No *	Yes	No	Yes	No		Nystagmus	CT: cerebellar atrophy	
	VI.19 (F)	No				12	43	NA	NA	Ataxia	Yes	No *	Yes	No	Yes	No		Nystagmus Obesity	CT: cerebellar atrophy	
	VI.20 (F)	No				13	42	NA	NA	Ataxia	Yes	No *	Yes	No	Yes	No	Mild retardation	Nystagmus	CT: cerebellar atrophy	
	VI.31 (F)	No				11	32	NA	NA	Ataxia	Yes	No *	Yes	No	Yes	No		Nystagmus	CT: cerebellar atrophy	
AR49	II.3 (F)	Yes	*KIF1A*	c.761G > A	No	5	26	NA	NA	Ataxia	Yes	No	NAv	Yes	Yes	No	Mild retardation		CT: cortical cerebellar atrophy	
AR92	II.1 (M)	Yes	*PNKP*	c.1123G > T; c.1123G > T	Not clear	6	33	16	35	Ataxia	Yes	No	No	No	Yes	No	No		NAv	
	II.4 (M)	No				7	27	16	NA	Ataxia	Yes	No	No	No	Yes	No	No		NAv	αFP: N
AR126	V.7 (F)	Yes	*POLG*	c.2243G > C; c.2243G > C	Yes	25	41	NA	NA	Ataxia	Yes	No	No	No	Yes	No	Yes	Optic atrophy Vertical gaze palsy Nystagmus Epilepsy	NAv	
Ataxia and oculomotor apraxia																				
AR86	II.2 (F)	Yes	*PNKP*	c.1123G > T; c.1253_1269dup	No	8	17	NA	NA	Ataxia	Yes	Yes	No	No	Yes	No	Yes	Nystagmus Obesity	MRI: cerebellar atrophy (++vermis)	

Table 1. *Cont.*

Family	Ind. Code (Gender)	Tested	Gene	Disease Causing Variants	Consanguinity	Age				First Symptom	Ataxia	OMA	Diplopia	Pyramidal Signs	Peripheral Neuropathy	Dystonia	Cognitive Impairment	Other Symptoms/Signs	Imaging	Others
						Onset	Obs.	Wheelchair	Death											
AR117	II.1 (M)	Yes	*PNKP*	c.1221_1223del; c.1123G > T	Yes	8	32	16	37	Ataxia	Yes	Yes	NAv	No	Yes	Yes (UL)	No	Obesity	NAv	
	II.2 (F)	No				7	35	15	42	Ataxia	Yes	Yes	NAv	No	Yes	Yes (UL)	No	Obesity	MRI: cerebellar atrophy	αFP: ↑ Proteins: ↓ Albumin: ↓ Cholesterol: ↑
	II.3 (F)	No				7	29	15	55	Ataxia	Yes	Yes	NAv	No	Yes	Yes (UL)	No	Obesity	MRI: cerebellar atrophy	Proteins: ↓ Albumin: ↓ Cholesterol: ↑
Ataxia and dystonia																				
AR2	IV.1 (M)	Yes	*HEXB*	c.1514G > A; c.1514G > A	Yes	24	51	NA	NA	Ataxia	Yes	No	No	Yes	No	Yes (OM)	No	Nystagmus Muscle atrophy ↓ vibration hallux	MRI: cerebellar, atrophy	
AR278	III.1 (M)	Yes	*ATP1A3*	c.374T > A	No	11	44	NA	NA	Parox dystonia	Yes	No	No	No	No	Yes	Yes	Supranuclear vertical gaze palsy	MRI: cerebellar atrophy	
Ataxia and cognitive impairment																				
AR16	V.1 (F)	Yes	*FA2H*	c.619_620del; c.619_620del	Yes	<1	14	NA	NA	Delayed motor/cognitive milestones	Yes	No	No	Yes	No	No	Yes	Seizures, Aggressive behavior	MRI: cerebellar atrophy, mild cortical atrophy	VEP: ↑ BEP, SSEP: N

αFP—α feto-protein; BEP—Brainstem evoked potentials; CT—Computed tomography; F—Female; Ind—Individual; M—Male; MRI—Magnetic resonance imaging; NA—Not applicable; Nav—Not available; Obs—Observation; OM—Oromandibular; OMA—Oculomotor apraxia; Parox—paroxysmal; UL—Upper limbs; SSEP—Somatosensory evoked potentials; VEP—Visual evoked potentials; ↓—decreased; ↑—increased * No clear oculomotor apraxia, but all had a poverty of spontaneous saccadic eye movements.

4. Discussion

In this study, we performed WES on 30 individuals from 19 families with (apparently) recessive cerebellar ataxia, aiming at providing a conclusive genetic diagnosis for them. These families had been identified during our national population survey in Portugal [10], but remained for many years without a molecular diagnosis, after testing the most common genes. More recently, new causal-genes were identified in undiagnosed families, including *PNKP* in eight families with AOA4 [11], *MAG* in one family with AOA and neuropathy [12], and *DAB1* in three AD families with SCA37 [34].

In this report, pathogenic and likely-pathogenic variants in several genes were identified and clearly associated with HCA (Table 1 and Table S1, supporting information). Division of the cohort into phenotypic subgroups had been performed during the original survey, and we retained this classification for a better genotype–phenotype characterization, and for clinician guidance in everyday practice. All the causal genes now identified had previously been described within their respective phenotypic subgroup [35–38]. The most frequent were *SACS*, *KIF1C*, and *PNKP*. To note, Friedreich ataxia, AOA (AOA1 and 2) and L-2-hydroxyglutaric aciduria had been previously screened and found to be the most prevalent types of AR-HCA [10]; *KIF1C* and *PNKP* were described only a few years later to be the causative genes for SPAX2 and AOA4 [11,39]. Present data reinforce *PNKP*-related ataxia as one of the most prevalent AR-HCA in Portugal [11]. Both *PNKP* and *SETX* were universally associated with neuropathy, but not with AOA; the reason why AR4 had not been previously tested for *SETX*. This has been described in the meantime [40]. Oculomotor apraxia, thus, should now be regarded as a clinical sign with high specificity, but less sensitivity for AOA2 and AOA4. Additionally, we identified four novel disease-variants in *SACS* and two in *KIF1C*, broadening the genetic spectrum of ARSACS and SPAX2.

We also provided functional data to validate the detrimental effect of two new splice-site variants (Figure 1) that would have been otherwise classified as variants of unknown clinical significance. Using minigene assays, we inferred that *SPG11* c.3039-5T > G variant creates a new 3′ acceptor splice site, while *KIF1C* c.1166-2A > G abolished the canonical 3′ acceptor slice site. Both were predicted to modify the open reading-frame, resulting in a premature stop codon.

4.1. Heterozygous De Novo Variants

All families analyzed had been classified as AR-HCA, based either on the presence of affected relatives in the same generation with unaffected parents; absence of other affected relatives; or consanguinity. Nevertheless, de novo variants in heterozygosity were identified (or presumed) in three families with only one affected member (Figure 2), highlighting that various modes of inheritance should considered when analyzing sequencing data in such cases. We identified one previously reported de novo missense variant in *KIF1A* [41], and two novel variants in *CACNA1A* and *ATP1A3 were* classified as likely pathogenic. *KIF1A* has been associated with AR and de novo AD diseases [42]. Patients with de novo *KIF1A*-related variants have shown a complex phenotype, including developmental delay, ataxia, spastic paraplegia, and neuropathy, with onset in the first year of life [41,43,44]. Our patient from family AR49 had had cerebellar ataxia since age 5 years, no spasticity, but an extensor plantar response, and neuropathy. Age of onset and combination of neurological syndromes were similar to those observed in Friedreich ataxia, but this was not the case in other cases previously described [43,44]. Nevertheless, our patient raises the hypothesis that heterozygous variants in *KIF1A* should be considered in 'Friedreich ataxia-like' patients.

Opposed to *KIF1A*, *CACNA1A* and *ATP1A3* are known to cause only autosomal dominant disorders [45–48]. CAG repeats in the last exon of the longest isoform of *CACNA1A* cause SCA6, and missense mutations affecting the calcium channel-encoding sequence cause progressive cerebellar ataxia, familial hemiplegic migraine (FHM1), and episodic ataxia type 2 (EA2) [49]. A congenital form of cerebellar ataxia with cognitive impairment has also been recently described [50]. A spastic ataxic phenotype, as observed in family AR267, has only been described once [35], probably being a less frequent presentation in

CACNA1A-related disorders. Interestingly, *CACNA1A* variants were one of the most prevalent causes of AD-HCA, after SCA3 and dentatorubral-pallidoluysian atrophy (DRPLA), in our population-based survey [10]. Variants in *ATP1A3* produce a wide spectrum of AD neurological and psychiatric disorders, ranging from infantile to adult onset. Cerebellar ataxia is classically present in CAPOS syndrome (cerebellar ataxia, areflexia, *pes cavus*, optic atrophy, and sensorineural hearing loss), but atypical phenotypes with ataxia (either relapsing, of acute onset or slowly progressive) have been increasingly recognized [51]. The proband of AR278 had paroxysmal dystonia induced by walking, resembling exercise-induced dyskinesia (PED), and, later, slowly progressive ataxia and generalized dystonia. A phenotype similar to PED has been reported in a family with four affected individuals, two of which also display cerebellar ataxia [52].

4.2. Molecular Mechanisms

HCA can be caused either by loss-of-function, gain-of function, or a dominant-negative effect, in a multitude of apparently unrelated genes. Generally, AR-HCA is associated with loss-of-function variants, whereas AD-HCA can be caused by a combination of gain and/or loss of function [7]. Our study highlights the diversity of cerebellar ataxia-related genes and phenotypes, possibly reflecting different disease mechanisms (Table S2, supporting information). A pivotal question that remains unanswered is why the Purkinje cells are particularly affected [6,7]. Most genes/proteins identified in our study do not show elevated cerebellar expression, compared to other brain regions (Table S5, supporting information), as per RNA and protein data from the Human Protein Atlas, and in agreement with current literature [7]. We reviewed the molecular mechanisms underlying the main clinical phenotypes (Table S2, supporting information) and discuss potential common disease-pathways in AR-HCA below.

4.2.1. Spastic Ataxia

Spasticity and pyramidal signs are hallmarks of several cerebellar ataxias [53,54]. As demonstrated by the diversity of genes causing spastic ataxia in our study, there is not a particular molecular pathway underlying this phenotype. Nevertheless, we identified one molecular mechanism that may be shared between a set of genes: (1) *SACS*, *KIF1C*, *SPG11*, and *SYNE1* interact with the cytoskeleton and may function to ensure proper vesicle/organelle trafficking and/or neuronal structure.

SACS encodes the large protein sacsin, the function of which has not yet been clearly established. The presence of both ubiquitin-like (UBL) and DNaJ domain (implicated in chaperon-mediated folding process), and a potential role in the degradation of aberrant ataxin-1, suggest that sacsin may integrate the ubiquitin–proteasome system and Hsp70 chaperone function [55]. Moreover, studies in *SACS* knockout mice, revealed that sacsin regulates the neurofilament cytoskeleton and mitochondria dynamics [56,57]. Most *SACS* variants lead to the complete loss of sacsin, although some missense variants are associated with low levels of the protein [58,59]. Since most variants are upstream of the C-terminal DnaJ domain, it is speculated that ARSACS is associated with loss of chaperone function [60]. Concordantly, we identified known and novel variants in an unspecific region or within the sacsin-repeating region with homology with Hsp90 (Table S4, supporting information); all upstream of the DNaJ domain.

Kinesin family member 1C (*KIF1C*) is a ubiquitously expressed motor protein involved in both anterograde transport of vesicles and retrograde transport from Golgi to the ER [61]. Few reports have analyzed the impact of *KIF1C* variants, but it was suggested that variants in the motor domain may affect microtubule binding and impair ATP hydrolysis, while truncating variants lead to reduced protein levels and may impair cargo binding [62]. Therefore, truncating variants, such as the ones identified in our study, are probably associated with a loss-of-function mechanism.

SPG11 encodes spatacsin, a large protein involved with neuronal axonal growth, intracellular cargo trafficking, and lysosome function. SPG11 variants span the entire

spatacsin protein [63]. Functional studies of truncating variants indicated that spatacsin loss-of-function is at the basis of neurodegeneration, as evidenced by reduced expression of spatacsin [64]; reduction in the anterograde vesicle trafficking, indicative of impaired axonal transport [65]; reduction in axonal complexity and neurite outgrowth [65]; or the presence of abnormal lysosomes [64]. Given the nature of the variants we identified, it is plausible that they also cause loss of spatacsin function.

SYNE1 is a large gene that encodes the synaptic nuclear envelope protein 1 (SYNE1), also known as nuclear envelope spectrin 1 (nesprin 1). This protein mediates the link from the nuclear envelope to the actin cytoskeleton (LINC complex), being important for nuclear migration [66,67]. Most *SYNE1* variants associated with ARCA1 are nonsense or frameshift, and span the giant nesprin-1 isoform, but exclude the C-terminal Klarsicht-ANC-Syne-homology (KASH) [68]. Thus, ARCA1 may be caused by defective synaptic transmission through synaptic vesicles trafficking impairment and/or cerebellar dendrites defects; however, this remains to be confirmed [69]. Nevertheless, many missense variants were also identified in ARCA1, including one identified by us, within a spectrin repeat domain.

In addition, variants in *ANO10*, which encodes a putative calcium-activated chloride channel, named anoctamin-10 (ANO10), are associated with SCAR10/ARCA3 [70], with decreased coenzyme Q10 (CoQ10) levels. One study showed that truncating variants cause a reduction in *ANO10* mRNA expression in patient's skin fibroblasts [71], but the pathogenic mechanism of *ANO10* variants remains unexplored.

4.2.2. Ataxia and Neuropathy

Peripheral neuropathy is a common feature associated to HCAs; but, as far as we know, this combination has not been linked with specific disease pathways. In this study, we found three genes (*POLG*, *SETX*, and *PNKP*) with various molecular functions, causing recessive ataxia and neuropathy.

POLG encodes the catalytic subunit of the mitochondrial DNA polymerase gamma protein (POLG), which functions in the replication and repair of mtDNA [72]. Functional studies showed that missense variants cause decreased activity, DNA binding and processivity of the polymerase, and reduction of functional mtDNA [73,74].The *POLG* variant identified in our study (p.Trp748Ser) is within the C-terminal domain of the polymerase, an important domain for its activity [75]. We can, therefore, speculate that it causes reduction or loss of POLG function.

SETX and *PNKP* are discussed in the following sub-section.

4.2.3. Ataxia and Oculomotor Apraxia

AOA has been associated with the DNA damage repair pathway. Ataxia telangiectasia, AOA1, AOA2, AOA4, *XRCC1*-AOA, and spinocerebellar ataxia with axonal neuropathy 1 are all caused by genes with a role in DNA repair [9]. We identified variants in *SETX* and *PNKP*, causing AOA2 and AOA4, respectively. Interestingly, in one family with pathogenic variants in *SETX* and one in *PNKP*, oculomotor apraxia was not present. Both had been phenotypically classified as 'ataxia with neuropathy', due to severe neuropathy. This possible occurrence has been previously described and is reinforced in this study.

SETX encodes a large nuclear protein termed senataxin, with an RNA/DNA helicase domain. Senataxin is involved in RNA metabolism, DNA maintenance, and damage response. Several types of biallelic variants in *SETX* were identified in AOA2 [76–78], probably causing a loss-of-function phenotype. In addition, dominant missense variants in *SETX* cause amyotrophic lateral sclerosis (ALS) with juvenile onset (ALS4) [79], suggesting that gain-of-function leads to ALS4. Altered gene expression and mRNA processing, and increased susceptibility to oxidative DNA damage, have all been associated with *SETX* variants [79–82]. There have been no functional studies of the SETX p.Gly2047Cys variant [83], but as it lies within the DNA/RNA helicase domain, it may cause a reduction or loss of senataxin function.

Variants in *PNKP*, encoding the nuclear polynucleotide kinase 3′ phosphatase protein (*PNKP*), result in a range of AR disorders. The enzymatic activity of *PNKP* is involved in the repair of both DNA double strand-break (DSB) and single-strand break (SSB); impaired function can cause neurodevelopmental dysfunction (microcephaly, seizures, and developmental delay (MCSZ)) [84] and neurodegeneration (AOA4 and Charcot-Marie-Tooth disease (CMTD)) [11,36]. Few functional studies have described the effects of *PKNP* variants. Nevertheless, most variants seem to cause reduced stability and levels of *PNKP*, and reduced DNA phosphatase activity in MCSZ or reduced kinase activity in neurodegenerative diseases. These ultimately lead to reduced DNA repair [85,86]. Moreover, several types of variants were identified in *PNKP*; most are in the kinase domain, particularly in neurodegenerative diseases [11,87], as shown in this study.

4.2.4. Ataxia and Cognitive Impairment or Dystonia

Additionally, other neurological signs may be present in AR-HCA, such as dystonia, and cognitive impairment. In our study, we identified a known variant in *HEXB* in one family with Sandhoff disease, and a new variant in *FA2H* causing ataxia with cognitive impairment.

Sandhoff disease is caused by variants in *HEXB* encoding the β subunit of the enzyme hexosaminidase (HEXB) [88]. HEXB is a lysosomal hydrolase that catalyzes the degradation of GM2 ganglioside. The spectrum of *HEXB* variants is wide, but most reduce GM2 hydrolysis, resulting in the accumulation of GM2 ganglioside [89,90]. Moreover, it was reported that several variants, including missense, lead to reduced mRNA expression and affect HEXB protein structure [91–93]. Particularly, the p.Arg505Gln variant, located in the catalytic domain, was reported to disturb the biochemical properties and cause loss of activity of HEXB and accumulation of GM2 ganglioside [90,94].

FA2H encodes the fatty acid 2-hydroxylase (FA2H) protein, which is involved in the synthesis of 2-hydroxy fatty acid galactolipids, the most abundant lipids in the myelin sheath [95]. Pathogenic variants in *FA2H* are speculated to cause neurodegeneration through a loss-of-function mechanism, since there is evidence of decreased hydroxylation of myelin lipids [96]. Moreover, a missense variant causing neurodegeneration with brain iron accumulation, led to reduced FA2H protein expression [97]. Since the *FA2H* variant we identified is predicted to cause a frameshift, it is also possible it leads to loss-of-function.

5. Conclusions

Our study provided the molecular diagnosis of 19 families with various types of HCA through WES, expanding the genetic and phenotypic spectrum of HCA. Most of the mutated genes caused a spastic ataxia phenotype, but *PNKP* associated with either ataxia and neuropathy or AOA was also amongst the most frequent causal-genes. We highlight three families with de novo variants in *KIF1A*, *CACNA1A*, and *ATP1A3*, despite having been initially classified has AR. These results evidence the importance of performing a differential diagnosis (AR vs. AD forms) in the absence of affected relatives of the proband. All the remaining families were associated with AR-HCA related genes. In addition, we provided a review on potential common mechanisms underlying neurodegeneration in AR-HCA, namely cytoskeleton function in spastic ataxia, and DNA repair in AOA. Translation of genetic findings into a better understanding of HCA mechanisms may help the development of effective therapies [6,7].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cells11060981/s1, Figure S1: Electropherogram with the position of the SPG11 (c.3039-5T>G); Table S1: Pathogenic and likely pathogenic variants identified by WES; Table S2: Molecular functions and disease mechanisms associated with recessive cerebellar ataxia genes and encoded proteins, grouped by main clinical phenotype [11,36,38,39,55–57,61,64,65,67,69–71,73,74,76,77,79,84,85,90,95,96,98–109]; Table S3: Pathogenic splice site variants and likely pathogenic missense variants, and pathogenicity predictions; Table S4: Protein domain location of variants identified;

Table S5: RNA and protein expression data obtained from the Human Protein Atlas for genes and respective proteins identified in this study [110].

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Institutional Review Board Statement: The reanalysis of the families here studied was approved by the Ethics Committee of Centro Hospitalar Universitário do Porto; approval code 2017-069(063-DEFI-063-CES); last approval date 2017.

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Article 10. Novel MAG Variant Causes Cerebellar Ataxia with Oculomotor Apraxia: Molecular Basis and Expanded Clinical Phenotype

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Novel *MAG* Variant Causes Cerebellar Ataxia with Oculomotor Apraxia: Molecular Basis and Expanded Clinical Phenotype

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Abstract: Homozygous variants in *MAG*, encoding myelin-associated glycoprotein (MAG), have been associated with complicated forms of hereditary spastic paraplegia (HSP). MAG is a glycoprotein member of the immunoglobulin superfamily, expressed by myelination cells. In this study, we identified a novel homozygous missense variant in *MAG* (c.124T>C; p.Cys42Arg) in a Portuguese family with early-onset autosomal recessive cerebellar ataxia with neuropathy and oculomotor apraxia. We used homozygosity mapping and exome sequencing to identify the *MAG* variant, and cellular studies to confirm its detrimental effect. Our results showed that this variant reduces protein stability and impairs the post-translational processing (N-linked glycosylation) and subcellular localization of MAG, thereby associating a loss of protein function with the phenotype. Therefore, *MAG* variants should be considered in the diagnosis of hereditary cerebellar ataxia with oculomotor apraxia, in addition to spastic paraplegia.

Keywords: cerebellar ataxia; myelin-associated glycoprotein; exome sequencing

1. Introduction

Hereditary cerebellar ataxias (HCAs) are a heterogeneous group of neurodegenerative disorders, characterized by motor incoordination and unsteady gait, often associated with speech and eye movement disturbances [1,2]. Autosomal recessive cerebellar ataxias are generally associated with peripheral sensorimotor neuropathy and non-neurological features. The most common of these is Friedreich ataxia, but recent advances in genetics, particularly the use of next-generating sequencing (NGS) technologies, have expanded the number of causative genes to more than 50 [3–5]. Additionally, HCAs share clinical features and disease mechanisms with hereditary spastic paraplegias (HSPs), with several ataxia-related genes found to cause HSPs and vice versa [6,7].

Homozygous variants in *MAG*, encoding the myelin-associated glycoprotein (MAG), have been associated with a complex neurological syndrome, including spastic paraplegia type 75 (SPG75),

progressive neuropathy, ataxia and prominent sensorial dysfunction [8–11]. MAG, also known as Siglec-4a (sialic acid-binding immunoglobulin (Ig)-type lectin), is a transmembrane glycoprotein expressed by myelination cells, including oligodendrocytes and Schwann cells [12,13]. MAG is important for the maintenance of axon-glia attachment [14] and has a dual role in the inhibition of neurite outgrowth and axonal regeneration [15–17] and in neuroprotection from axonal injury [18,19].

In this study, we report a novel homozygous missense variant in *MAG* (c.124T>C; p.Cys42Arg) in a Portuguese family with early-onset autosomal recessive ataxia with neuropathy and oculomotor apraxia, identified by exome sequencing. Cell studies confirmed that the variant reduces protein stability, and impairs the post-translational processing and subcellular localization of MAG. Our findings thus suggest that this variant causes protein loss-of-function and associate *MAG* variants with ataxia with oculomotor apraxia (AOA).

2. Experimental Section

2.1. Genetic Analysis

A consanguineous Portuguese family affected by early-onset ataxia with oculomotor apraxia (AOA) was identified during a national systematic population-based survey (1994–2004) aiming to identify Portuguese families with HCA and HSP [20]. This survey included clinical history, family information, neurological evaluation and blood collection. Samples were collected after receipt of written informed consent from participants. In this study, we used only previously collected DNA samples. Variants in genes associated with Friedreich ataxia and the AOA phenotype (*APTX*, *SETX*, *PIK3R5* and *PNKP* genes) were excluded [20,21]; recently, the expansion of an intronic repeat in *RFC1* [22] was also excluded.

Therefore, we performed whole-genome genotyping in two affected individuals using Illumina Infinium technology to identify the presence of large regions of homozygosity (>1 Mb). The samples were genotyped using the HumanOmniExpress-24v1-0_a BeadChip according to the manufacturer's instructions, and data were visualized using the GenomeStudio Data Analysis Software (Illumina, San Diego, CA, USA).

We also performed exome sequencing in the two affected individuals. Genomic DNA was prepared according to Illumina's TruSeq Sample Preparation v.3, and exome capture was performed using Illumina's TruSeq Exome Enrichment, according to the manufacturer's instructions. Sequencing was performed on an Illumina HiSeq2500 with 100-bp paired-end reads. We performed sequence alignment and variant calling against the reference human genome (UCSC Human Genome Browser hg19) by using the Burrows-Wheeler Aligner [23] and the Genome Analysis Toolkit [24,25]. Prior to variant calling, PCR duplicates were removed with the Picard software. Given the apparent autosomal-recessive inheritance and consanguinity, we focused the analysis on homozygous variants located in loss of heterozygosity (LOH) regions. We filtered variants present in those regions using Exomiser v7.2.1 [26] with the following parameters: minor allele frequency (MAF) < 2%, autosomal recessive inheritance pattern, and human phenotype ontology HP:0001251 (term name: ataxia). Then, we excluded intronic, UTR, intergenic and synonymous variants and variants found in homozygosity in the Genome Aggregation Database (gnomAD; https://gnomad.broadinstitute.org). The functional predicted impact of variants was evaluated using the SIFT, PolyPhen-2, MutationTaster and CADD v1.5 software.

We also used Sanger sequencing to confirm variants identified by exome sequencing and verified intrafamilial segregation. We performed PCR amplifications, using Ranger Mix (Bioline, London, UK) and purified products with Exo/SAP (GRiSP, Porto, Portugal), then performed Sanger sequencing using Big Dye Terminator Cycle Sequencing v1.1 (Applied Biosystems, Foster City, CA, USA) and an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing analysis was carried out using the Seqscape v2.6 software (Applied Biosystems, Foster City, CA, USA).

2.2. Antibodies

Primary antibodies: mouse monoclonal anti-EGFP antibody (MAB1765, Abnova, Taipei City, Taiwan), mouse monoclonal anti-GFP antibody (600-301-215, Rockland, Limerick, PA, USA), mouse monoclonal anti-GM130 antibody (610822, BD Biosciences, San Jose, CA, USA), and rabbit polyclonal anti-calnexin antibody (ADI-SPA-860, Enzo Life Sciences, Farmingdale, NY, USA). Secondary antibodies: horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (sc-2005, Santa Cruz Biotechnology, Heidelberg, Germany), HRP-conjugated goat anti-rabbit IgG (401393, Calbiochem, EDM Millipore, Darmstadt, Germany,), goat anti-mouse IgG Alexa Fluor®568 conjugate (A-11004, ThermoFisher Scientific, Waltham, MA, USA), and goat anti-rabbit IgG Alexa Fluor®568 conjugate (A-11011, ThermoFisher Scientific, Waltham, MA, USA).

2.3. Expression Vectors

Human MAG cDNA was amplified from the pME18-MAG plasmid, kindly provided by Dr. Hisashi Arase [27], using the following primers: Forward 5′-GATCCTCGAGATGATATTCCTCACGGCACTG-3′ and reverse 5′- CGAGGAATTCTCTTGACCCGGATTTCAGC-3′. The purified PCR product was cloned into the pEGFP-N1 plasmid (Clontech, Mountain View, CA, USA) by restriction enzyme digestion (with XhoI and EcoRI, ThermoFisher Scientific, Waltham, MA, USA) and ligation with T4 DNA ligase (New England Biolabs, Ipswich, MA, USA). This plasmid was modified by site-directed mutagenesis, using the QuikChange II Kit (Agilent, Santa Clara, CA, USA) to produce disease-associated MAG plasmids. The following primers were used to introduce the C42R and S133R variants: Forward 5′-GCGTCTCCATCCCCCGCCGCTTTGACTTC-3′ and reverse 5′-GAAGTCAAAGCGGCGGGGGATGGAGACGC-3′ and forward 5′- CTTCTCAGAGCACAGGGTCCTGGATATCGTC-3′ and reverse 5′-GACGATATCCAGGACCCTGTGCTCTGAGAAG-3′, respectively.

2.4. Cell Culture and Transfection

HEK293T cells (kindly provided by Elsa Logarinho, IBMC/i3S, Porto) were grown in DMEM high glucose GlutaMAX™ supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Gibco, ThermoFisher Scientific, Waltham, MA, USA), at 37 °C, in a humidified 5% CO2 atmosphere. Cells were transiently transfected with each plasmid using jetPRIME (Polyplus-transfection, Illkirch, France) or Fugene®HD (Promega, Madison, WI, USA), according to the manufacturer's protocol.

In order to inhibit protein synthesis, cells were treated at 24 h post transfection with cycloheximide (100 µg/mL, Calbiochem, EDM Millipore, Darmstadt, Germany) and collected after 1, 3, 5, 7 or 24 h of incubation.

To inhibit/enhance different proteolytic pathways, cells were treated at 24 h post-transfection with the following drugs for 18 h: The proteasome inhibitor MG132 (5 µM, Calbiochem, EMD Millipore, Darmstadt, Germany), autophagy inducer rapamycin (200 mM, Calbiochem, EMD Millipore, Darmstadt, Germany), endoplasmic reticulum-associated protein degradation (ERAD) inhibitor EerI (10 µM, Calbiochem, EDM Millipore, Darmstadt, Germany) or control vehicle DMSO (Sigma-Aldrich, St. Louis, MO, USA), and the lysosomotropic agent NH4Cl (15 mM, Sigma-Aldrich, St. Louis, MO, USA) or control vehicle DMEM (Gibco, ThermoFisher Scientific, Waltham, MA, USA).

2.5. Real-Time PCR

Total RNA was isolated from transfected cells, using NZYol (Nzytech, Lisbon, Portugal) as per the manufacturer's recommendations, followed by the purification of the RNA aqueous phase, using the RNeasy mini kit (Qiagen, Hilden, Germany). RNA quantification was performed on NanoDrop 2000 (ThermoFisher Scientific, Waltham, MA, USA). cDNA was synthesized by the reverse transcription-PCR of 3 µg total RNA with oligo(dT), using the SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's

protocol. Templates were diluted 1000-fold and used with primers specific for human *MAG* (forward 5′-GATTATGATTTCAGCCTCC-3′′ and reverse 5′-CTATTGAAGTACCAGACAC-3′) and human β-actin (forward 5′-GCACTCTTCCAGCCTTCCTTC-3′ and reverse 5′-GTGATCTCCTTCTGCATCCTGTC-3′), along with the PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Foster City, CA, USA). All reactions were performed in triplicate and replicated three times, using the Applied Biosystems 7500 Fast Real-Time PCR (Applied Biosystems, Foster City, CA, USA).

2.6. Deglycosylation Assay

Cells transfected with each plasmid for 48 h were collected in ice-cold 1× PBS with cOmplete™ Protease Inhibitor Cocktail (Roche, Basel, Switzerland) and centrifuged at 500 *g* for 3 min. The supernatant was discarded and the pellet resuspended in glycoprotein denaturing buffer and heated at 100 °C for 10 min. Then, GlycoBuffer 3 with EndoH enzyme (New England Biolabs, Ipswich, MA, USA) was added, and the mixture was incubated at 37 °C for 3 h. The reaction was stopped by adding Laemmli buffer. Samples were further analyzed by Western blotting.

2.7. Western Blot Analysis

Cells expressing target proteins were collected in RIPA buffer (150 mM NaCl, 1.0% IGEPAL®CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0; Sigma-Aldrich, St. Louis, MO, USA) supplemented with cOmplete Protease Inhibitor Cocktail (Roche, Basel, Switzerland), except for deglycosylation and co-immunoprecipitation assays, and then sonicated. Total protein concentrations were measured with the Pierce BCA protein assay kit (ThermoFisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Samples (30 µg of total protein) were separated by SDS-PAGE and electrophoretically transferred onto PVDF membranes (Merck-Millipore, Darmstadt, Germany), using a wet electroblotting system (Bio-Rad, Hercules, CA, USA). These were stained with Ponceau S staining in order to assess equal gel loading and total protein quantification [28]. Then, membranes were blocked in 5% non-fat dry milk in TBS-T for 1 h, at room temperature (RT), and subsequently incubated with specific primary antibodies, as indicated (diluted in 3% non-fat dry milk in TBS-T), overnight at 4 °C. The membranes were washed with TBS-T and then incubated with HRP-conjugated secondary antibody for 1 h at RT. Following three washes with TBS-T, detection was achieved using WesternBrightTM Sirius (Advansta, San Jose, CA, USA), and chemiluminescence was detected with a ChemicDocTM XRS+ Imaging System (Bio-Rad, Hercules, CA, USA). Protein bands were quantified using the Image LabTM 5.2.1 Software (Bio-Rad, Hercules, CA, USA). Quantitative comparisons between the samples of each experiment were always performed on the same blot. When necessary, membranes were stripped by incubation in stripping solution (62.5 mM Tris-HCl, pH 6.7; 2% SDS; 100 mM beta-mercaptoethanol) at 50 °C for 30 min, with gentle agitation.

2.8. Co-Immunoprecipitation

Cells transfected with each plasmid for 48 h were collected in lysis buffer (50 mM Tris-HCl, pH 8.0; 120 mM NaCl; 0.5% Triton X-100), supplemented with the Pierce Protease and Phosphatase Inhibitor tablets (ThermoFisher Scientific, Waltham, MA, USA), and sonicated. DynabeadsTM M-280 Sheep Anti-Mouse IgG (ThermoFisher Scientific, Waltham, MA, USA) were washed in 3% BSA/1× PBS. GFP antibody was coupled to Dynabeads (1 µg antibody/50 µL beads), by incubating with rotation overnight at 4 °C. Cell lysates were precleared with 5 µL Dynabeads for 1 h at 4 °C and then incubated with antibody-Dynabeads with rotation overnight at 4 °C. The immunoprecipitates were washed in 3% BSA/1× PBS and then in PBS, and further transferred to a clean tube. The proteins were eluted, by boiling in 1× Laemmli buffer at 90 °C for 10 min.

2.9. Immunofluorescence

Cells expressing target proteins were fixed using 4% paraformaldehyde/4% sucrose for 20 min and permeabilized with 0.3% triton X-100 for 20 min. After washing with PBS, cells were blocked in 3% BSA/PBS for 45 min and then further incubated with the primary antibodies overnight at 4 °C. Then, cells were incubated with the secondary antibodies for 1 h at RT, washed with PBS and stained with Hoechst 33342 (ThermoFisher Scientific, Waltham, MA, USA) for 5 min to label the nucleus. Preparations were mounted with ProLong™ Gold Antifade Mountant (ThermoFisher Scientific, Waltham, MA, USA) and visualized using an epifluorescence Zeiss Axio Imager Z1 microscope equipped with an Axiocam MR3.0 camera and Axiovision 4.7 software (Carl Zeiss, Oberkochen, Germany).

2.10. Statistical Analysis

Analyses of data were performed using the IBM SPSS Statistics 25.0 software (IBM, Armonk, NY, USA). All quantitative data are expressed as mean ± standard deviation (SD) of at least three independent experiments. Statistical significance analysis was conducted using one-way ANOVA with Tukey's post-hoc test; the level of statistical significance was set at $p < 0.05$.

3. Results

3.1. Identification of a Novel MAG Variant in Ataxia Patients

The family studied here (Figure 1A) was identified during the national population-based survey (1994–2004), aiming to identify families with HCA and HSP [20]. In a first phase, variants in genes associated with the AOA phenotype and prevalent forms of recessive ataxia were excluded. Then, we performed whole-genome genotyping and exome sequencing (patients II.1 and II.3). We found 15 LOH regions shared by both patients (Table S1). Then, we extracted the variants present in those regions and, after filtering, identified four homozygous variants in four genes (*MAG*, *LGI4*, *PLEKHG2* and *PLCE1*) (Table S2). Only *MAG* had been associated with a clinical phenotype similar to that of the patients, as it is known to cause HSP that commonly overlaps with HCA [7]. Moreover, only the variant in *MAG* (NM_002361: c.124T>C; p.Cys42Arg; 4) was not reported in population databases, including gnomAD and the Nucleotide Polymorphism Database (dbSNP; https://www.ncbi.nlm.nih.gov/snp). We confirmed that both affected siblings carried the homozygous variant in *MAG* by Sanger sequencing (Figure 1B) and verified that the variant was present in heterozygosity in the non-affected mother (DNA from father not available), supporting intrafamilial segregation. In silico analysis with several pathogenicity prediction programs anticipated the amino acid substitution (p.Cys42Arg) to be deleterious (SIFT score = 0; Mutation Taster score = 1; Polyphen-2 score = 1; CADD_phred = 27.9). Moreover, the Cys42 residue of human MAG is conserved across different species (Figure 1C).

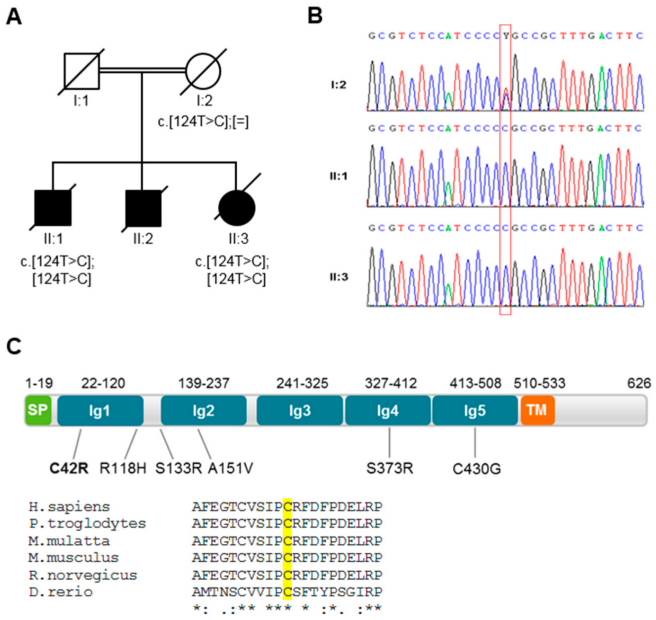

Figure 1. A family pedigree and schematic representation of the myelin-associated glycoprotein (MAG) protein. (**A**) A pedigree with segregation analysis of the new *MAG* variant (c.124T>C; p.Cys42Arg). Black symbols represent individuals affected by cerebellar ataxia. (**B**) An electropherogram with the position of the c.124C>T variant boxed. (**C**) A schematic representation of the MAG protein (NP_002352.1) with the location of the variant found in this study (in bold) and other variants previously associated with different clinical phenotypes. A sequence alignment of the residues surrounding Cys42 of human MAG against other species is also shown and was performed using the Clustal Omega program.

3.2. Clinical Features of the Patients Carrying the MAG Variant

The main clinical features and available medical history of the affected members from this family are summarized in Table 1. Briefly, all three patients presented ataxia as the first sign of disease at one year of age, had a very slow disease progression, despite severe motor handicap, and had longer survival. A delay in gait acquisition was present in all, with independent gait being achieved only after the age of three years. Early in the disease course, they presented upper limb dysmetria and mild intention tremor. Over the years, oculomotor apraxia emerged, and there was a progressive clinical deterioration, with severe ataxia, arreflexic tetraparesis, distal muscle wasting and extremely atrophic hands/feet. They all presented adult-onset optic atrophy. There was no evidence of cognitive impairment, though no formal neuropsychological assessment was performed. All three were wheelchair-bound between the third and fourth decades of life. In adulthood, the most prominent sign was a severe peripheral neuropathy. In addition to cerebellar atrophy on MRI, patient II:1 had spontaneous hyperintensity of the dentate nuclei, on T2-weighted images. EMG of the three siblings showed axonal neuropathy. Serum levels of alpha-fetoprotein, albumin and cholesterol were normal. The patients died after longstanding disabilities and long disease durations.

Table 1. The clinical, biochemical and imaging features of the patients carrying the *MAG* variant.

	II:1	II:2	II:3
Gender	Male	Male	Female
Age of onset (years)	1	1	1
Last examination (years)	59	56	54
Age of death (years)	63	56	7th decade
First sign	Ataxia	Ataxia	Ataxia
Prominent sign	Neuropathy	Neuropathy	Neuropathy
OA	+	++	++
Wheelchair use (years)	20	21	30
Dystonia	-	-	-
Cognitive impairment	-	-	-
Motor deficit	+++	+++	+++
Spasticity	-	-	-
Pyramidal signs	-	-	-
Limb edema	-	-	-
Scoliosis	-	-	-
Pes cavus	+	+	+
Optic atrophy	+	+	+
Obesity	-	-	-
MRI findings	Cerebellar atrophy. Dentate nuclei hyperintensity on T2 sequences	Cerebellar atrophy	Cerebellar atrophy
EMG findings	Axonal neuropathy	Axonal neuropathy	Axonal neuropathy
VEP	Optical nerve damage	Optical nerve damage	Optical nerve damage
Alpha-fetoprotein levels	N	N	N
Albumin levels	N	N	N
Cholesterol levels	N	N	N

+, present; ++, moderate; +++, severe; -, absent; OA, oculomotor apraxia; N, normal levels; EMG, electromyography; VEP, visual evoked potentials.

3.3. Cellular Characterization of the New MAG Variant

To understand the consequences of the p.Cys42Arg variant on the biochemical features of MAG, we generated constructs that direct the expression of wild-type MAG (MAG-WT) or MAG carrying the p.Cys42Arg variant (MAG-C42R) or the p.Ser133Arg variant (MAG-S133R), previously described in [8], in fusion with a C-terminal EGFP-tag. Constructs, including the control construct, were transfected into HEK293T cells. Immunoblotting analysis revealed that MAG-C42R had significantly reduced protein levels when compared with MAG-WT (Figure 2A, about a 67% decrease; $p = 0.017$). MAG-S133R had similar expression to MAG-C42R (about a 60% decrease compared with MAG-WT; $p = 0.029$).

Moreover, while MAG-WT appeared as an apparent double band, indicating the presence of different MAG glycosylated forms, both MAG variants appeared as a single band, suggesting that the variants affected the post-translational processing of MAG. The incubation of cell lysates with EndoH showed that MAG-WT contained both high mannose N-linked glycans, which are removed by EndoH, and complex N-linked glycans (Figure 2B). MAG-C42R and MAG-S133R only contained high mannose N-linked glycans, as indicated by the downward shift after EndoH treatment (Figure 2B). The presence of high mannose glycans and absence of complex glycans indicate the endoplasmic reticulum (ER) retention of immature glycoproteins that have not trafficked to the Golgi [29].

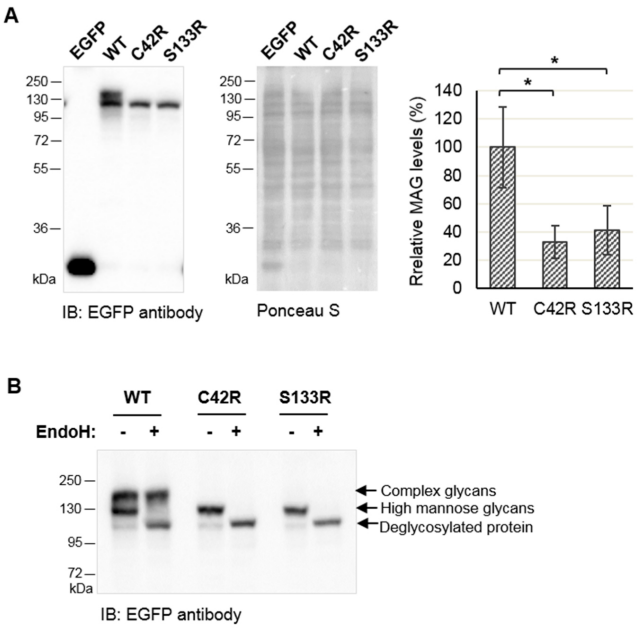

Figure 2. The MAG-C42R variant results in reduced protein expression and impairs glycosylation. (**A**) Analysis of protein expression of EGFP-tagged MAG clones in HEK293T cells by immunoblotting with anti-EGFP antibody. Ponceau S staining was used as the loading control. Quantification data (graph) are presented as the mean ± SD of three independent experiments; * $p < 0.05$, compared with MAG-WT (one-way ANOVA/Tukey). (**B**) Cell lysates were incubated with EndoH, which cleaves high mannose N-linked glycans but not complex N-linked glycans, and subjected to immunoblotting with anti-EGFP antibody. The original blot is presented in Figure S1.

The retention of MAG-S133R in the ER has been previously reported [8]. Therefore, we performed fluorescence microscopy to analyze the MAG-C42R distribution, and showed that it is very similar to that of MAG-S133R (Figure 3A). Cells labelled with an ER marker (calnexin) or a cis-Golgi marker (GM130) showed that MAG-C42R is located on the ER, while MAG-WT is predominantly found in the Golgi but is also scattered throughout the cell (Figure 3A). The immunoprecipitation of EGFP-tagged MAG proteins also demonstrated that only MAG-WT co-immunoprecipitated with GM130 (Figure 3B). On the other hand, calnexin was found in the immunoprecipitates of both MAG-WT and the MAG variants, but in higher levels in the latter (Figure 3B).

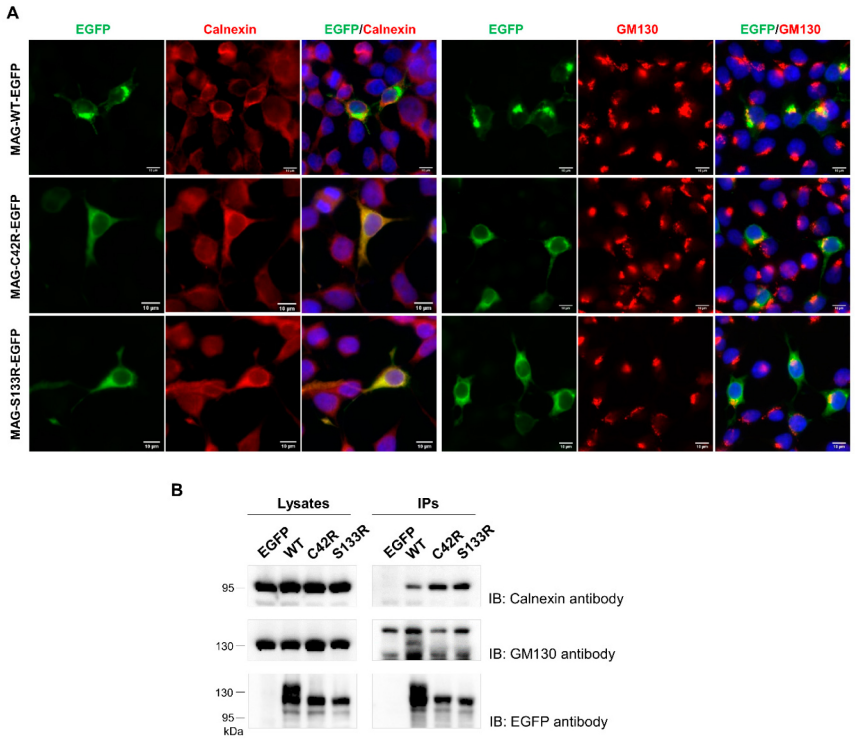

Figure 3. MAG-C42R has an altered subcellular localization. (**A**) The localization of EGFP-tagged MAG clones in HEK293T cells. EGFP was directly visualized. The presence of MAG in the endoplasmic reticulum and Golgi complex was analyzed by probing cells with anti-calnexin and anti-GM130 antibodies, respectively, both detected with Alexa Fluor 568-conjugated secondary antibodies (red). DNA was stained with Hoechst 33342 (blue). Photographs were acquired using a Zeiss Axio Imager Z1 microscope. Bars: 10 µm. (**B**) The co-immunoprecipitation of EGFP-tagged MAG clones with endogenous calnexin and GM130 in HEK293T cells. Cell lysates were immunoprecipitated with anti-GFP antibody. The immunoprecipitates were subjected to immunoblotting with anti-GM130 antibody, then stripped and reprobed with anti-calnexin antibody, followed by stripping and reprobing with anti-EGFP antibody. The same methodology was applied to whole cell lysates. Calnexin and GM130 immunoblotting figures have different exposure times (original blots are presented in Figure S2).

Given the low protein levels of MAG-C42R and to determine if the variant affects protein stability, we analyzed the half-life of MAG using a cyclohexamide chase assay. Our results showed that MAG-C42R had a higher degradation rate compared with MAG-WT (Figure 4). While the half-life of MAG-C42R was of about 3 h, MAG-WT levels were relatively stable (only decreased about 25%) after 24 h of cyclohexamide treatment (Figure 4A). Therefore, we treated cells with different drugs to identify the proteolytic pathway(s) responsible for MAG-C42R degradation. The treatment of cells with the proteasome inhibitor MG132 resulted in a significant increase in both MAG-WT and MAG-C42R protein levels (Figure 4B, $p = 0.032$ and $p < 0.001$, respectively). Even though the increase was substantially higher for MAG-C42R (an increase of about 250%, in contrast with the 40% increase for MAG-WT). The inhibition of autophagy nor ERAD—promoted by NH4Cl or EerI, respectively—nor the induction of autophagy by rapamycin significantly altered the levels of MAG-WT or MAG-C42R

(Figure 4B). These results show that MAG-C42R is preferentially targeted to the proteasome for degradation, since only MG132 was capable of restoring protein levels. Of note, quantitative real-time PCR showed no significant reduction in MAG-C42R mRNA (Figure S3), confirming that decreased levels of MAG-C42R are not the result of a reduction in or instability of the mRNA.

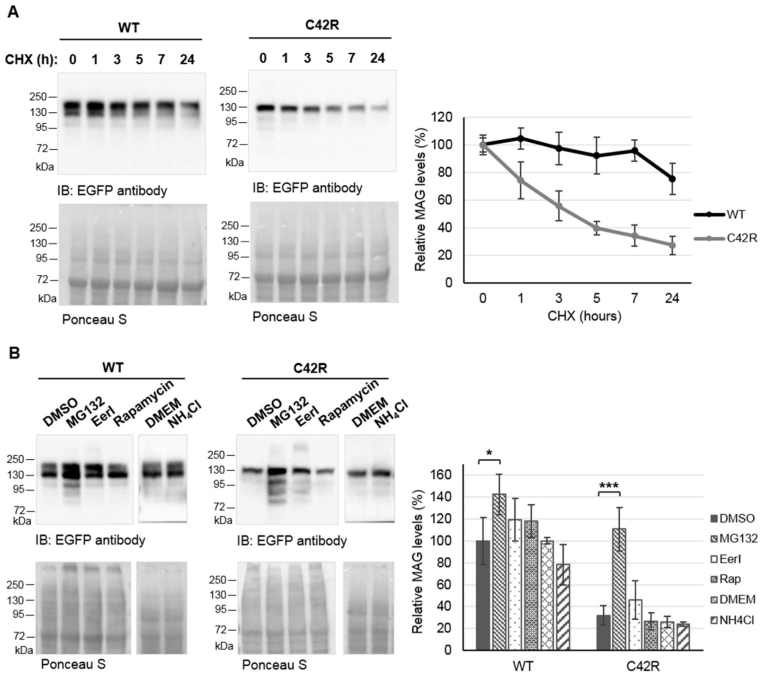

Figure 4. MAG-C42R impairs protein stability and is degraded by the proteasome. Cells expressing EGFP-tagged MAG clones were incubated with cycloheximide (CHX) for the indicated periods of time (**A**) or with different drugs for 18 h (**B**). Membranes were stained with Ponceau S for total protein quantification (whole blots are presented in Figure S4) and then cut and probed with anti-EGFP antibody. Quantification data are presented as the mean ± SEM (**A**) or mean ± SD (**B**) of three independent experiments; * $p < 0.05$, *** $p < 0.001$ (one-way ANOVA/Tukey).

4. Discussion

We identified a novel missense variant in *MAG* (c.124T>C; p.Cys42Arg) in homozygosity, in a consanguineous family presenting with cerebellar ataxia. The variant was identified using whole-genome genotyping and exome-sequencing, and functional studies in cells confirmed its detrimental effect on the MAG protein. Moreover, we showed that the biochemical and cellular characteristics of the MAG-C42R variant were very similar to those reported for a MAG-S133R variant [8].

Prominent cerebellar signs and oculomotor apraxia had prompted a clinical diagnosis of AOA, but genetic testing excluded pathogenic variants in known AOA genes [20,21]. Oculomotor apraxia is here described for the first time in association with *MAG* variants, broadening its clinical phenotype. In adulthood, the main clinical picture was of AOA with severe peripheral neuropathy and no pyramidal signs. As opposed to other AOA types [21,30], patients had no movement disorders, no suggestive biochemical anomalies, and all had optic neuropathy. When comparing with previously identified

patients with *MAG* variants [8–11], the absence of pyramidal signs was a clear-cut difference. This could be due to the severe neuropathy obscuring pyramidal signs at clinical observation, though pyramidal tract degeneration could not be excluded. Additionally, none of the three patients showed apparent cognitive impairment. Nonetheless, there are some features overlapping with the previously reported *MAG*-related phenotype: delay of gait, cerebellar ataxia, peripheral neuropathy, optical nerve atrophy and cerebellar atrophy on MRI [8–11]. Moreover, all previous patients but one [9] presented their first symptoms very early in life (from their first days to around 3 years of age), and all had severe motor deficits, being wheelchair-bound or needing support for walking early in life.

During our analysis, we also found homozygous variants in the genes *LGI4*, *PLEKHG2* and *PLCE1* (Table S2), in addition to *MAG*; however, these genes have been associated with clinical features distinct to those of the patients of this study. Variants in *LGI4* cause arthrogryposis multiplex congenital; variants in *PLEKHG2* cause mental retardation, dystonia and microcephaly; and those in *PLEC1* have been shown to be involved in a renal disease (nephrotic syndrome). Variants in these genes are all described in population databases, except the one identified in *MAG*. Therefore, *MAG* was the main candidate gene in our study, as it is known to cause HSP that commonly overlaps with HCA [7].

The discovery of several genes causing both cerebellar and pyramidal signs has contributed to the understanding that HCA and HSP share many phenotypic but also pathophysiological features [6,7]. In addition, genes commonly associated with HSP, such as SPG7, were found to cause a predominant cerebellar ataxia phenotype in some patients lacking pyramidal signs [31,32]. NGS has been playing a major role in the discovery of new disease-causing genes and the broadening of the phenotypic spectrum of known ones, highlighting its power in the molecular diagnosis of these pathologies.

MAG is a component of myelin found in oligodendrocytes in the central nervous system and Schwann cells in the peripheral nervous system, important for the formation and maintenance of myelinated axons [12]. Thus, it is not surprising that some patients had white matter hyperintense lesions in T2 sequences, probably of a demyelinating nature, identified as soon as age 4 years [8–10]. Nonetheless, the two siblings described by Novarino and colleagues had normal brain MRI, which could be due to the younger age at the time of the exam [11]. None of our patients had white matter lesions, but one had a bilaterally increased signal at the dentate nucleus, in T2 images (not shown). Due to its high iron content, the dentate nuclei usually appears hypointense in T2; hyperintensity is described in a variety of acquired and genetic disorders, such as multiple sclerosis, Leigh syndrome or glutaric aciduria type 1 [33]. Over the past years, it has also been described in degenerative diseases, such as SCA48 and SPG7 [34,35].

Interestingly, the process of myelination is not affected in MAG-deficient mice, which show subtle morphological abnormalities in myelin. It seems that MAG is not critical for myelin formation, but is needed for the maintenance of myelinated axons [36,37].

Structurally, MAG is a type I transmembrane protein, consisting of an N-terminal extracellular region with five disulfide-bonded Ig-like domains, a single transmembrane domain and a short cytoplasmic region (Figure 1C). The first Ig-like domain (Ig1), where the MAG-C42R variant is located, has a V-type Ig fold like other Siglec family members, is conserved across sialoproteins, and is critical for sialic acid binding and interaction with axons. Moreover, Cys42 and Cys100 are predicted to form a disulphide bridge [13,38]; thereby, the replacement of Cys42 will abolish the cysteine bridge (Figure S5), probably altering the structure of the protein and thus disturbing its stability [39].

Accordingly, our results demonstrated that the MAG-C42R variant affects the protein's stability, post-translational modification (glycosylation) and subcellular distribution. Cyclohexamide assays demonstrated that MAG-C42R undergoes rapid degradation, when compared to MAG-WT, which was relatively stable (Figure 4A). Moreover, we showed that this premature degradation that causes lower levels of MAG-C42R protein (Figure 2A) is mainly due to proteasome-dependent degradation (Figure 4B). However, the mutated protein was still expressed at moderate levels when overexpressed in cells, raising the question if the endogenous protein could be fully degraded by the proteasome in patients' cells. Indeed, other authors reported similar results for overexpressed MAG-S133R variant [8].

Moreover, they also demonstrated that MAG is absent in the myelin sheath from sural nerve biopsies. Unfortunately, we did not have access to patients´ cells or tissues to confirm this.

MAG is a heavily glycosylated protein and contains eight N-linked glycosylation sites scattered along the extracellular region [40]. The initial modification of glycans is accomplished in the ER; properly folded glycoproteins then move to the Golgi to generate the complex glycans found in mature glycoproteins. It is thought that N-linked glycans provide highly hydrophilic groups that are important for proper folding and the modulation of protein conformation [29,41]. Our study demonstrated that while MAG-WT contained both high mannose N-linked glycans and complex N-linked glycans, MAG-C42R contained only high mannose N-linked glycans that are sensitive to EndoH treatment (Figure 2B). The absence of complex glycans suggests that MAG-C42R is retained in the ER and does not traffic to the Golgi for further modification. Thus, our results indicate that MAG-C42R stays in the ER for further degradation, as it is not properly folded. In line with this, we showed that both MAG-C42R and MAG-S133R are present in the ER and interact with calnexin (Figure 3), which participates in the folding of glycoproteins and prevents the export of improperly folded proteins from the ER [29,41]. Thus, it is not surprising that calnexin also binds to MAG-WT, though apparently with lower affinity than to MAG-C42R or MAG-S133R. Moreover, we also showed that MAG-WT traffics to the Golgi complex, where it associates with GM130 (Figure 3), a cis-Golgi matrix protein. Additionally, we did not find evidence of MAG-C42R degradation by the ERAD, as the treatment of cells with the EerI drug (which associates with VCP to inhibit ERAD) did not significantly change the levels of the protein.

In summary, we identified and characterized a novel variant in *MAG* and expanded the clinical phenotype associated with its disease-causing variants. Therefore, *MAG* should be considered in the diagnosis of recessive ataxia with oculomotor apraxia. The low expression and premature degradation of the mutant protein supports a loss-of-function phenotype.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/9/4/1212/s1, Figure S1: Original blot referring to main Figure 2B, Figure S2: Original blots referring to main Figure 3B, Figure S3: Quantitative analysis of MAG mRNA levels at 48h after transfection by real time-PCR, Figure S4: Original blots stained with Ponceau S referring to main Figure 4A,B, Figure S5: Visualization of the amino acids Cys42 (left figure, in green) and Arg52 (right figure, in red) on the protein structure of MAG, as seen on the HOPE report, Table S1: Genome studio results showing loss of heterozygosity regions common to patients II.1 and II.3, Table S2: List of homozygous variants located in loss of heterozygosity (LOH) regions.

Author Contributions: Conceptualization: M.S., J.B., I.A. and R.G.; Acquisition and analysis of data: M.S., J.D., C.K.-R., C.B., J.S., J.B., I.A. and R.G.; Writing—original draft: M.S., J.D., J.B., I.A., R.G.; Writing—review and editing: all authors. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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V. GENERAL DISCUSSION

V. GENERAL DISCUSSION

This study was based on the continuous recruitment and follow up of patients with HCA. The pool of ataxia patients identified during the national survey (original period from 1994 until 2004)[79] was complemented by a large group of patients followed at our outpatient clinic (from 2017 to 2022). There was a high inclusion of cases in the first year, both from the outpatient clinic and the national survey. Enrolment of new patients in the following years was much lower; it consisted of patients from the national survey whose updated contacts could only be obtained at a later period, of new referrals (new families) and from the observation of affected relatives (previously known families). Referrals were made by family doctors, neurologists or colleagues from other medical specialties (either from ULLSA, other hospitals from SNS or private practice).

The COVID19 pandemic had a moderate impact on the study. For two months, there were only telephone appointments, either for first or follow-up visits. Ambulatory in-person observations were restarted in early May 2020, but solely for new patients or urgent situations. From August 2020 onwards, regular in-person appointments became possible, but many patients still preferred telephone calls. It was only from March 2021 onwards that follow-up telephone calls became the exception. For new cases, the pandemic had a minor impact, but several follow-up visits were delayed to one year (instead of the usual six months) during that period. Still, as HCA are slowly progressive diseases (varying from 0.6 to 2.1 SARA points/ year),[236,241] we believe that overall analysis of disease progression was not significantly compromised.

A. APPROACH TO A PATIENT WITH HEREDITARY CEREBELLAR ATAXIA

HCA are usually diagnosed in patients complaining initially of gait or balance problems. Early in disease course, we may only identify subtle cerebellar signs, progressing over time to scandid dysarthria, ocular signs (saccadic intrusions, nystagmus, hypo/ hypermetric saccades), lower and/ or upper limb dysmetria, and ataxic gait. In addition, other neurological signs may or may not be present. Genetic investigation should always start by inquiring about family history and drawing a (at least) three generations' pedigree, questioning known or hidden consanguinity, and observing the largest possible number of affected relatives. The better the clinical picture of each member is confirmed, and the

higher number of relatives are examined, the more likely we are to achieve a definite diagnosis.

Of note, an isolated patient does not always imply an acquired (sporadic) cerebellar ataxia (which should be excluded *ab initium*, whenever suspected). It occurs more frequently in AR-HCA, but is also possible with *de novo* cases and age-dependent penetrance (late-onset) HCA (usually AD). Isolated cases were identified in 35.8% of the overall cohort: 25.4% in AR ataxias, 5.2% in AD ataxias,1.2% in mitochondrial ataxias and 4.0% in ataxias with uncertain mode of heredity. In AD forms, 61.5% of isolated cases corresponded to *de novo* pathogenic variants, while in the remaining 38.5% the progenitors could not be studied, precluding any further conclusions. Prevalence of isolated cases of suspected HCA has been estimated at 1.1-8.4:100,000,[100] but we could not find any other series analysing by mode of inheritance, as we present .

As the most frequent genetic aetiology for AD-HCA is a repeat expansion, which is not usually detected by routine short-read exome sequencing, it is recommended to test for the most frequent repeat expansions as the first approach.[259] Multigene panels (ataxia or neuroexome, 2nd and 3rd tier respectively) or analysis of WES (4th tier) should be performed afterwards, in “expansion-negative” patients.[259] For AR-HCA there are no such formal recommendations. We believe that expansions (either in FA or ATX-*RFC1*) should be tested at first, according to the AO and phenotype, proceeding afterwards into WES based panels. Whenever the presentation if discordant with FA or ATX-*RFC1,* WES based panels can be performed as the first genetic diagnostic procedure.

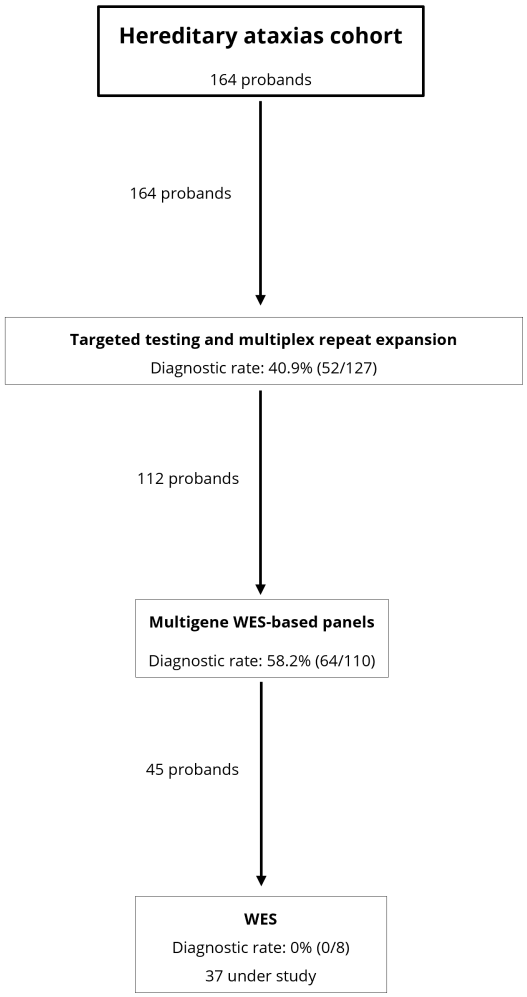
Diagnostic yield of the genetic investigation strategy

A final genetic diagnosis was obtained in 116 (70.7%) probands/ families (Figure 26). When combining single gene-testing (performed in all types of inheritance) with *1st-tier* approach (multiplex with PCR and fragment analysis for the most frequent AD-HCA) the diagnostic yield was of 40.9% (52/127), in line with other series, where it varied from 13.8% to 70.9%.[260-262]

Of the 112 probands who remained without genetic diagnosis, one was lost to follow up and another had only been studied through single-gene. Multigene WES-based panels had a diagnostic yield of 58.2% (64/110), in line with the reported (21.7%-64.4%) in similar series.[260-262] The diagnostic yield of WES in HCA varies from 21%-64%;[263-265] in the present cohort it was performed in eight probands, with no definite diagnosis, while 37 are under study.

Overall, the success of our diagnostic approach (70.7%) was at the upper limit of the reported in recent series using a similar diagnostic approach, where it varied from 31.3% to 77.2%.[260-262,266] As multigene panels, performed at a given time point, may miss some newly described genes, it has been proposed that either analysis of WES or a clinical exome should be performed as the 2nd tier, immediately after testing for repeat expansions.[259] Higher cost and the likely higher number of VUS, however, would come as a disadvantage to that strategy, particularly in settings where functional essays are not readily available.

Figure 26: Flowchart of genetic diagnostic strategy and diagnostic yield



Relative frequency found among the various forms of HCA

As expected, MJD was the most frequent AD-HCA (31.9%), confirming the result of the national survey.[79] Relative frequency of ATX-*CACNA1A* (12.1%) and SCA2 (11.1%) was similar to that found in the survey[79]. DRPLA, the second most common AD-HCA on a national basis[53], was not present in our cohort, as those families live in the centre and

south of the country. Also, reflecting the use of NGS, more ataxias due to conventional mutations were identified in this cohort.

In AR-HCA, ATX-*RFC1* was now the most frequent (21.3%), overpassing Friedreich ataxia (11.0%). A recent national collaborative retrospective study, however, recognized 67 patients with biallelic expansion in *RFC1*,[267] (below the 103 cases of Friedreich identified in the national survey).[79] Having different timings and methodologies, those samples cannot be directly compared. AOA was the third most frequent (9.5% overall, with AOA2 and AOA4 in 4.4% each, and AOA1 in 0.74%), followed by ARSACS (6.6%) and ATX-*SYNE1* (5.9%). ATX-*SYNE1* was not described in the results of the national survey,[79] as it had been only recently identified by then.[268]

Mitochondrial ataxias showed a relative frequency of 3.2%, with *MT-ATP6* being the gene most frequently associated. Though the low number of cases limits robust conclusions, we would highlight that only one had a complex multisystemic phenotype. “SCA-like” phenotypes, the majority in our series, have been increasingly recognized, particularly in association with *MT-ATP6* (5.3% in a large German HCA cohort).[223] As NGS multigene panels are periodically updated, now including mtDNA analysis, and can be revisited for newly found causal genes (if WES-based), this frequency may increase in the future.

The percentage of families without genetic diagnosis was of 29.3%, while in similar cohorts it varied from 22.8%-71.2%.[260-262,266] The lower percentage of undiagnosed families in relation to other cohorts may be explained by: a) more genes included in NGS panels at the time of our study and b) more recent testing for the *RFC1* expansion. Still, the recent introduction of *FGF14* (SCA27B) in our diagnostic list, is likely to decrease further the number of families without genetic diagnosis, as this is being shown to be a frequent cause of late-onset ataxia.[103,187,188,269]

With the general availability of NGS, increasingly affordable and efficient, clinicians might be tempted to perform it earlier in the diagnostic path of an ataxic patient. Nevertheless, this should not be perceived as a shortcut, as a precise delineation of phenotype and mode of inheritance is crucial to guide the analysis and interpret its results.

HOW CLINICAL PRESENTATION MAY GUIDE GENETIC TESTING

We recognize the low number of patients associated with some genes, particularly in ultra-rare ataxias, may limit discussion on this topic. Yet, when analysing onset either by cerebellar or non-cerebellar symptoms/ signs, AD ataxias were significantly associated

with a cerebellar onset (70.5%), while AR forms were mainly associated with non-cerebellar presentations (55.8%) (p=0.002). This reflected the high prevalence of MJD and SCA2 among the first, which present with gait unsteadiness in the vast majority,[25,270] and of ATX-*RFC1* and ARSACS (and other rarer ataxias) in the latter, where a variety on non-cerebellar presenting symptoms occurs.[113,214,217,236]

Unsteadiness of gait was the most frequent presentation in the general cohort (42.1%), irrespectively of mode of inheritance, whereas the remaining cerebellar presenting symptoms had a much lower frequency (UL dysmetria: 6.3%; diplopia: 2.8%; dysarthria: 1.6%). In the majority of ataxias presenting with cerebellar symptoms, particularly in polyQ forms, gait unbalance is observed at onset, preceding speech and hands coordination problems (Article 3).[5,270] Gait unsteadiness, however, is not particularly associated with any specific genetic form.[5] Still, in our present series of MJD patients it had a lower frequency than the described by Coutinho (74% *vs.* 92.4%),[25] possibly biased by our much lower number of MJD patients (29 *vs.* 144).

Considering non-cerebellar presentations, **spasmodic cough** was the most frequent, exclusively present in patients harbouring biallelic *RFC1* expansions, preceding ataxia in several years. This group of families with cough as a presenting feature, had been noted well and first reported by Coutinho, in 2006.[215] It is a peculiar form of cough, occurring by bursts, not accompanied by respiratory symptoms, which did not respond to standard treatment and could lead to cyanosis and collapse.[215] Families often referred they could this way identify onset of the disease in relatives. Cough had a frequency of 100% in ours, but varied from 55.5 to 97.0% in other cohorts with $(AAGGG)_{exp}$/ $(AAGGG)_{exp}$ in *RFC1* (the repeat motif identified in all our cases).[214,267,271,272] Interestingly, cough appears to be less frequent (14.3%) in Japanese patients with $(ACAGG)_{exp}$/ $(ACAGG)_{exp}$.[273]

Paroxystic events (hemiplegic migraine, epilepsy, vertigo, episodic ataxia or dystonia) were strongly associated with dominant ataxias (p=0.006) and conventional mutations (p=0.002) (Article 3). Due to the low number of patients, analysis per gene was not performed. Still, in line with what is classically recognized,[274] hemiplegic migraine was present at onset in all patients with ATX-*CACNA1A*. Interestingly, in an international cohort of non-repeat expansion AD-HCA,[105] epilepsy was also common in ATX-*CACNA1A*. Episodic ataxia was present in up to 46%[103,189] in the recently described ATX-*FGF14*/SCA27B; we are now starting to test for this intronic expansion in *FGF14* in all our unexplained cases.

Psychomotor delay (presence of both intellectual disability and motor delay) was observed only in AR ataxias, in congenital forms and others (e.g., HSP- *ZFYVE26* ,

HSP/ATX/NBIA-*FA2H*), where it has been classically pointed as a distinctive feature.[198,275] **Motor delay** or **intellectual disability** *per se* were observed both in AR and non-repeat expansion AD ataxias; the latter has been increasingly recognized over the past years (Article 3).[105]

B. PHENOTYPIC VARIATION

AGE AT ONSET

Several factors modulate AO in HCA, as detailed in Introduction. AD-HCA usually present in adulthood,[148,149] while AR-HCA tend to have a paediatric onset.[195,243] This is largely influenced by the high relative frequency of polyQ forms among AD and of FA in AR ataxias. Nevertheless, current use of NGS and identification of the *RFC1* expansion came to change that view, particularly as more adult-onset AR ataxias (such as HSP/ATX-*PGN,* MxMD-*POLG,* ATX-*ANO10* and *ATX-RFC1*) are being recognized.[236]

Mean AO for AD-HCA was 30 years, while it was 15 years for AR-HCA, in our cohort; this reflects the weight of the most commonly diagnosed forms within each group. We were particularly interested in analysing cases with “unusual” phenotype (including AO), in either group. Curiously, most of those patients with early-onset AD or late-onset AR presented with non-cerebellar symptoms at onset.

Early-onset AD ataxias

An early-onset occurred in 39.6% of AD-HCA, with most (58.3%) having onset until the age of 10 years. Non-repeat expansion ataxias corresponded to the majority (87.9%), with the most frequent genes being *CACNA1A, KCNC3* and *ATP1A3* (Article 3).

In the group of **ATX-*CACNA1A*** patients*,* 80% had onset below age 25 years, though none in the first year of life. Thus, we had no patients presenting with epilepsy or intellectual disability, usually associated with very early onset (<1 year-old).[105] We believe that, at our centre, these patients may be followed by the epilepsy team, not being referred to the ataxia clinic. All our infantile and juvenile patients with ATX-*CACNA1A* presented with hemiplegic migraine (except one with paroxysmal dystonia); cerebellar signs emerged later.

ATX-*KCNC3* presented as congenital ataxia in all patients, a percentage higher than the 27% recently described,[105] possibly due to our low number of patients. In line with other reports,[105] all exhibited intellectual disability.

All our patients with ***ATP1A3*** pathogenic variants had early onset with paroxysmal symptoms. *ATP1A3* has been associated with different phenotypes, but with the large majority of patients having at least one type of paroxysmal event throughout their lifetime.[276] Progressive ataxia, present in all patients from our series, has been reported in up to 54% of all cases, with CAPOS syndrome, alternating hemiplegia of childhood 2 and other phenotypes.[276,277]

ATX-*ITPR1* patients had all early onset. Two distinct forms have been associated with ATX-*ITPR1*: congenital (also known as SCA29) and adult-onset (SCA15). Adult-onset cases have an earlier onset than classically reported for AD ataxias.[278] Opposed to what is recognized, we had more congenital than early cases, but their low number overall precludes any conclusions. From a phenotypic point of view our patients were similar to the described.[105]

All three patients with **ATX/HSP-*KCNA2*** presented before the age of 25. Pathogenic variants in *KCNA2* were initially described in developmental and epileptic encephalopathies, associated or not with ataxia.[279,280] In our two cases who had epilepsy, this was initially refractory, but becoming controlled over the years. None had encephalopathy, but there was intellectual disability in all.

Some of our congenital cases had a severe phenotype, with intellectual disability, spasticity and additional movement disorders. The genes involved were *CTNNB1, SYNGAP1* and *SPAST*, in one patient each. ***CTNNB1*** has been associated with several phenotypes, including various hereditary cancers; the phenotype exhibited by our patient would fit in “neurodevelopmental disorder with spastic diplegia and visual defects” (OMIM #615075), as the patient had delayed milestones, pyramidal signs, visual defects and ataxia (this one present in up to 35.7% of cases).[281] ***SYNGAP1*** has been described in “intellectual developmental disorder” (OMIM #612621), with our patient having psychomotor delay and ataxia. Ataxia was initially considered a rare feature, but may actually be present in up to 50.9% cases.[282] Detailed discussion on the case associated with ***SPAST*** follows in the next section (also in Article 2).

HSP-*KIF1A* and ATX-*TMEM240* are recognized as having onset from paediatric up to juvenile age.[283,284] Our patient with **HSP-*KIF1A*** had a spastic-ataxia with normal developmental milestones, and not the classically described congenital ataxia phenotype (Article 9).[285,286] The **ATX-*TMEM240*** family was one of the first acknowledged as having

normal cognition,[287,288] but a cerebellar cognitive-affective syndrome seems to be more frequent.[289]

PolyQ ataxias corresponded to the forms presenting at a later age (from 21 to 24 years) and included MJD, SCA2 and SCA7. In all there was a known family history of ataxia, with anticipation of AO being observed. With the exception of retinopathy in SCA7, a cerebellar onset was present in the remaining patients.

Of note, 12 (33.3%) of our patients with early AD-HCA were isolated cases, eight (27.6%) corresponding to *de novo* pathogenic variants (Article 3). Those patients, having early onset and no family history, had all been initially studied for AR ataxias or metabolic disorders. Causal variants in genes associated with AD-HCA in cases with suspected AR inheritance had a frequency of 18.7% in a previous report.[290] As could be expected, time to diagnosis in the subgroup with onset before age 25 years was longer than in the overall series of AD-HCA (21.5[15.0-37.4] *vs.* 6.0 [1.0-10.0] years). Following the recommendation by some authors of analysing of WES or clinical exome immediately after exclusion of repeat expansions[259] would have shorten diagnostic delay in such cases.

Late-onset AR ataxias

Late-onset occurred in 39.1% of our cases of AR-HCA, with a median age of 38.0 [30.0-43.7] years. The genes most frequently involved were *RFC1, SYNE1, FXN* and *ANO10*. The majority of patients had a non-cerebellar onset.

In those harbouring biallelic expansions in ***RFC1***, onset occurred by age 40.0 [30.0-42.0] years, with paroxysmal cough attacks preceding ataxia, by a mean of 18 years (age at ataxia onset: 58.0 [50.0-62.5] years). Having a similar methodology as in other studies, ataxia onset was similar (6th decade),[109,214,236] while the interval between cough and ataxia was shorter (18 *vs.* 30 years). As our sample was smaller, the observed difference could be related to its size, memory bias or could actually reflect a later age of cough onset (modulated by other, unexplored, factors).

Late-onset occurred in 50.0% of **ATX-*ANO10*** and **ATX-*SYNE1*** patients. Besides gait unsteadiness, presenting symptoms in late-onset ATX-*ANO10* included diplopia, while in ATX-*SYNE1* UL dysmetria. ATX-*ANO10* onset has been described as varying 27-53 years,[220,236] slightly higher than we observed in the overall cohort. ATX-*SYNE1* patients from our series were similar to those originally described in Québec, with no congenital

neuromuscular cases.[291] On the large European AR cohort, including both ATX-*ANO10* and ATX-*SYNE1*, patients with the first had a slightly later onset than the latter, though both in adulthood.[236]

One out of two patients with **HSP/ATX-*PGN*** had late-onset, both with spastic-ataxia phenotype (Article 8). SPG7 is usually an adult-onset disorder (as in its original descriptions[292]), even when in the presence of ataxia.[208]

Only two (13.3%) **FA** had late-onset, a slightly lower frequency than the 16.6-28.2% described.[71,246] Both presented with neuropathy, without pronounced scoliosis or cardiomyopathy. In other late-onset series, neuropathy varied from 47-100%, pronounced scoliosis 1.9-14.0% and cardiomyopathy 0-11.1%.[71,194,246]

The spastic-ataxia phenotype of **MxMD-*ATP13A2*** has been reported has having onset in young adults,[293,294] as happened in our cohort (Article 8). This spastic-ataxia presentation tends to have a later onset, when compared to the several phenotypes associated with ***ATP13A2***.[293]

Overall analysis of age at onset groups

Late onset in our study was associated with repeat-expansion ataxias, mainly due to polyQ forms in AD-HCA (84.7%) and *RFC1* intronic expansion in AR-HCA (70.7%). On the opposite side, early onset was more frequent in non-repeat expansion ataxias, both in AD-HCA (87.9% caused by non-repeat expansions) and AR-HCA (81.2% non-repeat expansion genetic defects, the remaining corresponding to the intronic *FXN* expansion in FA). As the pathogenic basis of polyQ expansions *vs.* intronic *RFC1* expansion differs widely, we cannot be certain on the underlying reason for both being associated with a later AO. Non-repeat expansion ataxias may present with a wide interval of AO, from congenital to late-onset forms, with the majority presenting early in life, as observed.[271,295]

In AD-HCA, even though early-onset forms had a significantly longer disease duration at baseline, this was not associated with a higher SARA score (early-onset: 14.0 [8.7-15.0] *vs.* late-onset: 11.2 [6.7-18.9] points). This is probably related to the faster disease progression in the late-onset polyQ forms (1.49 in SCA2 and 1.56 SARA points/year in MJD),[241] *vs.* non-repeat expansion early-onset forms. Long-term disease progression of non-repeat expansion AD-HCA has not been subject of detailed investigation, but our results suggest it may be slower than in the remaining forms (Article 3).

In AR-HCA, late-onset forms, with shorter disease duration associated with lower SARA score (early-onset: 16.0 [11.0-29.5] *vs.* late-onset: 11.7 [6.5-16.5] points). In early-onset forms from other cohorts, ARSACS and HSP/ATX-*PGN* had a slow progression (0.6 point/year), while AOA2 and FA a faster progression (1.14 and 1.49 point/year, respectively).[113,236] On late-onset AR-forms, ATX-*RFC1* has been described as having intermediate progression (0.9 point/year), while ATX-*SYNE1* a slower progression (0.55 point/per year).[113,236]

The high genetic heterogeneity precluded a subgroup analysis per gene, due to the low number of patients in each group.

Age at onset and phenotypic variation in Friedreich ataxia

Marked intra-familial variability of AO has been reported in FA, particularly in the presence of two affected generations.[8,296-301] This was observed in one family from our series, where the father and one affected daughter had an atypical onset, at 45 and 33 years old, while the remaining patients had typical FA (Article 5).[8] In ours, as in those described in the literature, the affected parent carried one large and one shorter expansion and had late onset, while the asymptomatic parent carried only one expanded allele.[8,296-301] The presence of no symptoms or only mild ones contributed to transmission of the disease to their offspring. The majority of patients in the second generation had typical FA, more severe than in the previous one, the exception being those who inherited a shorter expansion.[8,296-301]

Uncommon manifestations, as blindness and deafness[302-305], have been observed in certain FA patients with early onset and large expanded alleles.[67,246] Despite the relatively common occurrence of subclinical visual and auditory impairment,[305,306] the simultaneous presence of severe blindness and deafness is rare, although it may be associated with diabetes, as occurred in one patient from our cohort (Article 6).[67,302] These severe phenotypes, which increase disability in patients with an already debilitating condition, still require additional investigation of the underlying mechanisms.

UNCOMMON PHENOTYPES

The boundaries of classical phenotype-genotype correlations have been fading, since the widespread use of multigene panels and WES, with broadening of the phenotypic

spectrum in many diseases. NGS has greatly increased our diagnostic rate, but additional challenges appeared. In the setting of an unusual or even new phenotype for a given gene, functional studies are essential when bioinformatic predictive tools are inconclusive. If segregating studies are not possible, as in small families or isolated patients, functional studies assume a greater relevance, particularly in case of an atypical phenotype. The balance between not giving a diagnosis without a high degree of certainty and neglecting genetic findings that may give a final diagnosis to a family is a difficult one, but of the utmost importance. Clinicians should create a synergy together with basic scientists, from which all, particularly patients and their families, would benefit.

Uncommon phenotypes found in our series often implied a new phenotype-genotype correlation, sometimes leading to the expansion of a known phenotypic spectrum. Some clinical presentations, e.g., ataxia associated to ***KMT2B*** or AOA with variants in ***MAG***, had not been previously recognised, or were only briefly reported in a small number of patients. Additionally, increased clinical severity with novel variants in ***SPAST***, a milder phenotype in ***ATP8A2***, and a spastic-ataxia phenotype in ***CACNA1A*** extended the phenotype known to be associated with those genes.

During the national survey on hereditary ataxias and spastic paraplegia, 21 families (41 patients) with AOA were identified, that eventually led to the description of three new genetic entities:[84-86] six families (13 patients) with AOA1, three families (11 patients) with AOA2 and eight families (11 patients) with AOA4.[79,84] In a consanguineous family with AOA, who remained without diagnosis after exhaustive studies, studies based on whole exome sequencing allowed the identification of pathogenic variants, in homozygosity, in ***MAG*** (Article 10).[307] Functional studies then confirmed the variants' detrimental effect on MAG protein expression.[307] Pathogenic variants in *MAG* had been described in association with early-onset complex spastic paraplegia (SPG75) and with ataxia, neuropathy, optic atrophy and cognitive impairment.[308,309] Oculomotor apraxia was first reported in this family.[307] As the ones previously identified, these patients also had an early onset ataxia with peripheral neuropathy and optic atrophy. Interestingly, impaired ocular movements had already been recognized, with nystagmus, saccadic smooth pursuit, saccadic dysmetria and square-wave jerks.[308,310,311] We speculate whether oculomotor apraxia might have been present in the remaining families but had been overlooked. Oculomotor apraxia may be missed in a classical neurological examination, being more readily recognized when patients are observed in their natural context.[82] Also, the previously reported patients with pathogenic variants in MAG were observed earlier in the disease course when oculomotor apraxia might not be as evident. In addition, contrary to previous descriptions, patients from this family had no cognitive impairment or

pyramidal signs. As they had congenital onset and were observed only in the 6th decade of life, pyramidal signs could have been muddled by the severe neuropathy (as is seen in Friedreich or ARSACS), and not have a true absence of pyramidal tract involvement. This family thus provided evidence for new inclusion of *MAG* in multigene panels for AOA.

KMT2B was first associated with neurological disorders in 2016, in patients with generalized early-onset dystonia.[312] Rarer forms, with neurodevelopmental delay in the absence of dystonia, were more recently described.[313] In that same report, one case had also cerebellar ataxia as a minor trait, a feature not highlighted by the authors. A *de novo* and novel variant in *KMT2B* was identified in one of our patients with cerebellar ataxia starting in early adulthood, progressing to a complex phenotype with seizures, ophthalmoplegia, pyramidal signs, minor dystonia and neuropathy (Article 1).[314] Bioinformatic analysis suggested it abolished the splice site at exon8/intron8, resulting in exon8 skipping during pre-mRNA maturation, which was confirmed by analysis of mRNA in fibroblasts.[314] *KMT2B* is highly expressed in the cerebellum,[315] which could, in theory, be associated with a higher frequency of ataxia, but also highlights the possible role of the cerebellum in the pathophysiology of dystonia. As haploinsufficiency is the main mutational mechanism linked to *KMT2B*-related dystonia, we speculate whether a gain of function could be associated with the newly identified variant, as the predicted in-frame deletion at the protein level could lead to a conformational change, with higher pathological effect in the cerebellum. The low frequency of patients with *KMT2B*-related ataxia could be due to the rarity of this underlying pathogenic variant.

De novo pathogenic variants in ***SPAST*** were recently associated with complex spastic paraplegia, in contrast to the inherited variants typically described in families with pure spastic paraplegia.[316,317] In two recent large series[210,211], patients with *de novo* variants in *SPAST* have been detailed characterized. They present a complex disorder, with motor delay, early-onset progressive spastic paraplegia, movement disorders, intellectual disability and epilepsy.[316,317] One of our patients with congenital ataxia, studied through neuroexome, had a *de novo* variant in *SPAST* (that had already been associated with spastic paraplegia, but not with ataxia) (Article 2).[318] This patient, who had delayed milestones, showed continuous acquisitions (as occurs in congenital ataxias) until the age of seven years, when pyramidal signs first emerged.[318] In patients with a complex phenotype, pyramidal signs had been recorded at a much earlier age.[316,317] Most had normal brain MRI, while our patient presented hyperintensity of the posterior limbs of internal capsule at an early age and, later, hypointensity of the pallida, substantia nigra and dentate nucleus.[318] These findings posed an additional challenge, as they were initially suggestive of a metabolic disorder and, later on, of neuronal brain iron

accumulation (that had not been previously described in association with this gene). The underlying reasons why the same variant may lead either to a pure spastic paraplegia, or a complex phenotype, depending on being inherited or *de novo*, remain unknown. Severe phenotypes tend to be associated with *de novo* variants due to reduced genetic fitness.[319] In addition, the influence of (unknown) noncoding variants on disease expression may also contribute to the observed phenotypic variation.[316] Our report provided justification for the inclusion of *SPAST* in multigene ataxia panels.

We identified a novel intronic variant in ***ATP8A2*** in two siblings with congenital ataxia, born of consanguineous parents (Article 4).[193] Both patients had delayed milestones, but showed progressive acquisitions throughout life, with average school performance, and the oldest sib working full time. While our manuscript[193] was in preparation, a series of five patients was published, in which three also had a mild phenotype.[320] Functional studies allowed us to determine that the identified variant led to retention of 17 nucleotides in intron 17, causing the introduction of a premature stop codon and producing a non-functional truncated protein.[193] We theorized that a low level of normal ATP8A2 mRNA could still be produced, sufficient to prevent a severe disorder.[193]

Expansion of the $(CAG)_n$ in the last exon of ***CACNA1A*** causes SCA6, while missense or frameshift mutations affecting the calcium channel-encoding sequence may cause a wide range of neurological phenotypes, including progressive cerebellar ataxia, familial hemiplegic migraine, episodic ataxia type 2, epilepsy and intellectual disability.[5,321] In one patient presenting with spastic ataxia in early adulthood, we identified a novel pathogenic variant in *CACNA1A* (Article 9).[285] Spasticity had only been reported in two patients with *CACNA1A*-related disorders, appearing to be a rare phenotypic expression, even in the more severe, congenital, phenotypes.[290,321] In a large series of non-polyQ ataxias, pyramidal signs were seen in 12.1% of patients, but including only milder signs, as brisk deep tendon reflexes or the Hoffman sign.[105] Spasticity, however, was not mentioned; in that cohort, pyramidal signs associated with longer disease duration. *CACNA1A* thus appears to be yet another gene involved in the spectrum HCA/HSP, even if more frequently with mild pyramidal signs rather than true spasticity.

GENETIC DEFECT INFLUENCE ON THE PHENOTYPE OF AD CEREBELLAR ATAXIAS

The number of AD-HCA known to be caused by single-nucleotide substitutions, small insertions/deletions or copy number variants (non-repeat expansion ataxias) has greatly increased over the past years.[105,259] The classical view that these correspond to a

minority[295] will possibly change in the near future, as new causal genes are being uncovered. In our present cohort, there was already a similar proportion of patients with polyQ and non-repeat expansion ataxias (Article 3). In addition to evidence of newly found genes and the increasing usage of NGS, we believe this relatively high prevalence of non-repeat expansion ataxias could also be due to our cohort being established at a tertiary referral centre, receiving patients from all the North region, including paediatric neurology clinics.

Non-repeat expansion ataxias presented at an earlier mean age (7.0 years), than polyQ ataxias (39.5 years), irrespective of other demographic characteristics. This was in line with previous reports, as AO tends to be earlier (including some congenital forms) in channelopathies and other non-repeat expansion ataxias,[105,295] while more frequent between the 4th and 6th decades of life in ataxias associated with polyQ.[322] Nevertheless, it is important to note that, in ours and other studies, non-repeat expansion ataxias presented a wide range of onset age, varying from congenital up to late-onset forms.[105,295]

Non-repeat expansion ataxias show a higher genetic heterogeneity than those due to repeat expansions. Relative frequency among polyQ ataxias was consistent with the results from the national survey[79], with the exception of DRPLA (as discussed above).[215] Some patients who had no genetic diagnosis by then, were later diagnosed as a non-repeat expansion form. Seventeen genes were involved in non-polyQ AD-HCA, the most frequent being *CACNA1A*, followed by *ATP1A3, ITPR1, KCNA* and *KCNC3.* The remaining 10 forms were identified in only one proband each. In other series, the genes most frequently involved, after *CACNA1A,* were *PRKCG* and *AFG3L2*,[105,290,295]; as numbers are small, the difference may be fortuitous. *De novo* pathogenic variants were exclusively identified in non-polyQ ataxias, in patients with no known family history, who had been initially studied for recessive ataxias (as discussed above).

Overall, clinical phenotype differed between polyQ and other ataxias. In our study, there was clearly a frequent cerebellar onset in the first group, while cerebellar and non-cerebellar presentations showed similar frequency in the latter. Patients with polyQ ataxias displayed ocular symptoms/ signs (diplopia, ophthalmoparesis) and neuropathy more frequently than those with non-repeat expansion forms; both are known to have a high frequency in various polyQ ataxias.[323,324] Paroxystic episodes (either in the form of seizures, hemiplegic migraine, episodic ataxia or dystonia) and congenital forms were exclusively identified in non-repeat expansion ataxias, in agreement with other publications.[105,290,295] This is likely associated with the high frequency of genes coding for ion channels (e.g., *CACNA1A, KCNA2, ATP1A3*), historically associated with episodic

disorders and, more recently, neurodevelopmental conditions.[325] As for ataxia, it is well recognized that ion channels causing human disorders are expressed in the central nervous system, particularly in the cerebellum. Purkinje cells are especially susceptible to impaired function, due to a high autonomous *firing rate*, which requires a synchronized action of numerous ion channels.[131] Channel dysfunction would lead to neuronal impairment and subsequent degeneration.[131,325]

On EMG, an axonal and sensitive neuropathy was more frequent in polyQ ataxias, in line with clinical observations. As for brain MRI, the only distinctive feature was atrophy of the pons and cerebellar peduncles in polyQ forms. Quantitative brain MRI studies have shown atrophy in the cerebellar hemispheres, vermis, midbrain, pons, medulla oblongata and cervical spine in polyQ ataxias, particularly in MJD.[326] Cerebellar atrophy was also seen in non-repeat expansion ataxias; however, as this group is genetically more heterogeneous, it has been difficult to perform a comparative analysis due to scarcity of cases of each individual form. We cannot explain the difference observed in atrophy of the pons and cerebellar peduncles; a future protocolled analysis of the atrophy pattern would be of great interest.

Notwithstanding its tendency for earlier onset, non-repeat expansion ataxias had a slower progression. Overall, polyQ forms had a more severe course than the remaining, being significantly associated with falls and confinement to wheelchair, with unilateral assistance in gait occurring earlier than in non-repeat expansion forms. Even though such a group comparison has not been reported before, this is in line with findings from large cohorts in polyQ and non-repeat expansion ataxias.[236,241]

C. NON-CEREBELLAR SYMPTOMS AND SIGNS

Cerebellar ataxia is, by definition, the most prominent clinical feature in HCA, reflecting degeneration of the cerebellum and cerebellar tracts. Still, in a large number of HCA, there is evidence of dysfunction of other neuronal populations as cranial nerves nucleus, basal ganglia, substantia nigra, pyramidal tracts and peripheral nerves.[327] According to the extent of extracerebellar involvement, frequency of non-cerebellar symptoms varies among the different HCA forms.

In our cohort, only 3.6% had a pure cerebellar ataxia, corresponding to patients with later onset and shorter disease duration. The remaining had at least one non-cerebellar

symptom or sign, with the majority having two or more. Frequency of pure cerebellar ataxias was similar to that described in the large AR-HCA European cohort (4.0%),[113] and lower than in EUROSCA (in AD-HCA), where there were no patients with a pure form.[148] Presence of two or more non-ataxia symptoms was dependent on disease duration, as also described by *Schmitz-Hubsch et al.* (2008).[148]

Pyramidal signs, non-ataxia movement disorders and neuropathy were the non-cerebellar signs most frequently identified. Pyramidal signs were associated with ARSACS, non-ataxia movement disorders with MJD and neuropathy with FA, in line with other reports.[148,217,246]

MOVEMENT DISORDERS (OTHER THAN ATAXIA)

Non-ataxia movement disorders (both hypo and hyperkinetic) have been reported with variable frequency in several HCA cohorts.[151,328,329] In AD-HCA, the most frequently described were intention or postural upper limb tremor (3.8%), Parkinsonism (2.6%) and dystonia (0.9%).[330] Movement disorders may emerge along disease progression, or, less frequently, be the presenting predominant feature.[151,328-330] In the last case, genetic diagnosis may be delayed, for the difficulty in recognizing less typical phenotypes.

Non-ataxia movement disorders were present in approximately half (52.8%) of our patients (Article 7). Different studies, using variable methodologies, identified a frequency of 9.8% up to 54%.[328-330] In our cohort, movement disorders were significantly associated with AD-HCA (67.0% in AD-HCA *vs.* 33.0% in AR-HCA), particularly with MJD. In another study, a similar frequency was described among AR and AD ataxias.[328] This difference could be related to the distinct sample size, study design, population factors or others.

Dystonia was the most frequent movement disorder in our cohort, with a frequency of 45.6%, higher than in others (where it varied from 4% to 26.2%).[328-330] It was associated to early disease onset (22.0 [3.0-34.0] years *vs.* 25.0 [8.0-42.0]), dominant inheritance (48.7% *vs.* 23.9%) and MJD (39.2% *vs.* 10.1%). Presence of dystonia in early-onset MJD patients has been recognized since its early descriptions[25,27,30,36] and reproduced across the years.[151,329,331] Its frequency was higher than in other series, with a possible explanation for this discrepancy being a more detailed observation of movement disorders in our study, allowing the identification subtle forms of dystonia (as dystonic posturing of the hands). Still, our results are not completely unexpected, as there is evidence of basal ganglia pathology in the most frequent HCA (e.g., MJD, SCA2) and the role of the

cerebellum in the pathophysiology of dystonia has been increasingly acknowledged.[332,333]

Tremor was the second most frequent movement disorder in our series (12.3%), and was reported in 2.4-50.0% of other HCA cohorts.[151,328-330] The series with highest frequency included intention tremor,[151,330] which was excluded from our analysis (for being part of the cerebellar syndrome).

Frequency of **Parkinsonism** (5.6%) in our cohort was lower than described (14.0%).[330] We only considered it when in the presence of bradykinesia and rigidity, while others included patients with only one Parkinsonian sign (e.g. bradykinesia or rigidity).[151,330] Parkinsonism was present in HCA that had been classically associated with it (as MJD, SCA2 or HSP/ATX-*PGN*)[331,334], but also in SCA14, HSP-*ZFYVE26* and MxMD-*ATP13A2*. It was either the presenting symptom or emerged in the first year of disease in 28.6%; overall mean AO being in the 7th decade of life. Comparison between early *vs.* late-onset Parkinsonism was limited by the low number of patients. In MJD and SCA2, severe degeneration of the *substantia nigra* is well known; still, Parkinsonism is a rare motor feature.[331,335] The *post-mortem* studies by *Schöls et al.* (2015) showed that besides degeneration of the *substantia nigra*, severe neuronal loss in the subthalamic nucleus occurred in all MJD and SCA2 patients, except one who displayed Parkinsonism during his lifetime.[335] Thus, degeneration of the subthalamic nucleus would prevent manifestation of Parkinsonism in the large majority of cases.[335] Dopaminergic functional imaging showed presynaptic involvement in 90% of our patients, in line with what was observed in other series.[330] In patients having late-onset Parkinsonism, we questioned whether it could be a coincidental Parkinson disease; the answer would rely on pathological studies (to which we had no access).

Frequency of *chorea* (4.0%) and *myoclonus* (2.8%) was lower than reported by *Rossi et al*. (13% and 12%, respectively) (2014).[330] **Chorea** is classically linked to SCA17, but has been described also in SCA1, SCA2, MJD, SCA14 and SCA48.[331,336] In our cohort, it was related with dominant inheritance (80.0% *vs.* 33.5%), but not with any particular gene. Still, as expected, it was observed in SCA2, SCA14 and SCA48 patients, but also in all members of a family with *ATP1A3*-related ataxia. **Myoclonus** was linked to younger disease onset, but not with SCA2 or SCA14 as classically observed.[330,331,337] Interestingly, in the present cohort, all patients with myoclonus exhibited a conventional mutation. **Tics** have rarely been observed in HCA,[330,331] as seen also in this series. Tics could be a coincidental disorder, particularly with paediatric onset; when onset during adulthood (as in two cases without genetic diagnosis from the present series), they could probably be related to the underlying disorder.

When analysing *disability milestones*, the coexistence of movement disorders was associated with a higher frequency of falls and shorter disease duration until the use of cane. These results were in line with the described by *Schöls et al.* (2000), with bradykinesia and impaired postural reflexes being associated with loss of unsupported walk.[151]

SPASTIC ATAXIA

Pyramidal signs are frequently observed in HCA, with several authors considering hereditary spastic paraplegia (HSP) and HCA as two ends of the same spectrum.[338] However, s*pastic-ataxia* is a designation restricted to the combination of a cerebellar syndrome with overt spasticity, and classically connected with a limited number of genetic diseases, usually with recessive inheritance. [122,338,339]

Spastic-ataxia corresponded to 22.5% of our cases of HCA and were related with younger AO [8.0[0-26] years *vs.* 27.5 [8.0-41.8] years] and with recessive inheritance (67.9% vs. 49.2%) (Article 8), in line with what is seen in other series.[100,338-340] Time until genetic diagnosis was greater in the presence of spastic-ataxia (34.0 [17.0-50.0] years *vs.* 18.0 [7.0-39.0] years), reflecting the complex investigation of overlapping phenotypes. Conventional mutations were more frequent in spastic ataxias (95.7% *vs.* 45.9%) and oligonucleotide expansions in non-spastic HCA (54.1% *vs.* 4.3%).

As expected, ARSACS was the most frequent form of spastic ataxia (19.6%), with a classical presentation in all, except one case with late-onset. ARSACS presents usually in childhood, and late-onset forms are rare.[341] ATX-*MT-ATP6* (7.1%)*,* the second genetic aetiology in this series, is classically related with Leigh syndrome, but a wider phenotypic spectrum has been described, including milder forms with late-onset SCA or ataxia with spasticity.[223,342,343] As for the third genetic causes of spastic ataxias in our series (HSP/ATX-*KIF1C,* ATX-*SYNE1* and ATX/HSP-*KCNA2,* each with a frequency of 6.5%), only HSP/ATX-*KIF1C* (also known as SPAX2) has been linked to this phenotype since its first descriptions.[344,345] ATX-*SYNE1* was originally described in pure recessive cerebellar ataxia, rarely with pyramidal signs;[268] application of NGS led to its clinical redefinition, to include spastic ataxia and other, more complex, presentations.[218,346] ATX/HSP-*KCNA2* was first observed in developmental and epileptic encephalopathies,[347] but has been increasingly found with spastic ataxia,[348] as recognized by the 2022 MDS classification as a mixed ataxia/spastic paraplegia.[140] HSP/ATX-*PGN*, the most frequent cause of spastic-ataxia in some studies,[349] had a frequency of 4.3% in ours, comparable to ATX-*ANO10,*

ATP8A2-related ataxia, HSP/ATX-*GBA2*, HSP/ATX-*CAPN1, HSP- KIAA1840* or HSP-*ZFYVE26*. The remaining genes causing a spastic ataxia phenotype (*APTX*, *FA2H*, *CAPN1*, *CACNA1A, KMT2B, KIF1A, SPAST* and *ATXN3*) in this cohort, had a frequency of 1.8%, some in need of confirmation of its association with spastic-ataxia in larger series.

Approximately 40% of all our HCA patients with spastic ataxia still had independent gait, after 2.5 decades of disease progression. Median disease duration until use of a walking stick was of 35.5 [20.3-45.0] and until use of wheelchair of 40.0 [28.3-47.0].

D. Limitations of this study

Families' identification and follow up

Our main difficulty occurred in patients who were just observed once during the survey, as in 56.5% the addresses and phone numbers were outdated, and their referring family doctors or neurologists, if known, could not be reached (for being retired in the majority of cases). For those patients, the information available was just that collected at the time of the survey and no follow-up data could be obtained.

The duration of this study (2017-2022) encompassed a consecutive inclusion of new patients over this timeframe and their prospective annual observation. This approach led to a heterogeneous follow-up, which constrained a more general applicability of our conclusions. Still, as these are rare disorders, we believe the continuous patients' inclusion was important for a more detailed clinical characterization.

Subjectivity and other difficulties in determining age at onset

Determining AO in disorders with an insidious character and slow progression, can be very challenging. In addition, the majority presented with unsteadiness, which may be underestimated at first. As so, there may be a recall bias on disease onset and of associated symptoms, mainly whenever these occurred before beginning of the study. Among those who were followed at our clinic before onset of their first symptoms, we observed some inconsistency between the information provided at inclusion and their clinical records. Frequently, patients perceived onset of disease as having occurred later

than documented in medical files. In those cases, the information from clinical records was considered. In cases of missing information from the files, or when patients were not being followed at the clinic, we could not double-check the information. Memory biases may affect data interpretation, but are common in studies with a (complete or partial) retrospective nature.

GENETIC INVESTIGATION AND DIAGNOSIS

At the time this study was closed, 56 patients without a definite diagnosis were still at different stages of genetic study, which might have biased the final results.

Altogether, there were 53 different genes implicated in 116 probands genetically diagnosed; 48 genes were involved in less than five probands each (35 of which in just one). This significant genetic heterogeneity precluded a more robust analysis of the phenotype-genotype correlation.

COMPARISON WITH OTHER SERIES

As there is large variation of the relative frequency of HCA among different populations,[89] comparison with other series has to be cautiously read. First, epidemiological studies are scarce, with the largest number of series concerning the relative frequency in hospital-based cohorts. We have performed a comparison between our data and the Portuguese nation-wide study, as a reflection, but acknowledging the different methodologies preclude a definite comparison. Second, as the relative frequency of individual types of HCA varies, some of the observed differences in AO, first symptoms or disease progression, might be just a reflection of this. We have always tried to compare data on the same type of ataxias, within different series, but cannot exclude this bias influence on our conclusions.

VI. CONCLUSIONS

VI. CONCLUSIONS

With this project, we performed a clinical and genetic characterization of a large cohort of patients with HCA. Inclusion of patients mostly from the North, but also other regions of the country, provided an updated perspective of the most frequent aetiologies in an hospital-based environment.

FREQUENCY AND MOLECULAR DIAGNOSIS OF HCA

. We gathered at ULS Santo António a large prospective cohort of HCA, from which we derived genetic studies and selected patients for additional projects on non-cerebellar symptoms and signs.

. Two disorders emerged as the most frequent, both repeat expansion ataxias: MJD and *ATX-RFC1*.

. This reinforces the message that the most frequent HCA (both dominant and recessive) are still not amenable to genetic diagnosis through WES-based multigene panels, which may be erroneously perceived by clinicians as a universal solution for the diagnosis of HCA. However, this is expected to change in the near future.

. After exclusion of repeat expansion ataxias, given their relative frequency among HCA, NGS proved of high importance, allowing the detection of rare and ultra-rare forms that otherwise would remain without a definite diagnosis for many years.

SYMPTOMS AT ONSET

. Globally, onset by cerebellar signs was more frequent than non-cerebellar onset (54.8% *vs.* 43.3%), with gait unsteadiness being the commonest presenting symptom.

. In AD-HCA this tendency was even clearer (70.5% *vs.* 29.5%), but in AR-HCA the opposite was seen (44.8% *vs.* 55.2%), with non-cerebellar onset overcoming a cerebellar presentation.

. A wide range of non-cerebellar symptoms was observed in AR-HCA, with motor delay and cough being the most frequent.

. Except for cough in *ATX-RFC1*, no specific non-cerebellar presentation was associated with a particular genetic diagnosis in AR-HCA.

NON-CEREBELLAR SYMPTOMS AND SIGNS OVER DISEASE COURSE

. Pure cerebellar ataxias were uncommon (3.6%), with patients with the longest disease progression having more non-cerebellar symptoms.

. The most frequently identified were pyramidal signs (59.4%), movement disorders (51.8%) and neuropathy (41.8%).

. The presence of movement disorders, other than ataxia, associated with dominant inheritance and MJD.

. Pyramidal signs, motor delay and intellectual disability were associated with ARSACS, and neuropathy with FRDA.

. Spastic ataxias were less common than ataxia with pyramidal signs.

. Typically, spastic ataxias had AR inheritance, earlier onset and a longer time until definite genetic diagnosis.

. Twenty-two genes were involved in spastic ataxia phenotype.

. The most frequent spastic ataxia was ARSACS, followed by HSP/ATX-*KIF1C*, ATX-*SYNE1 and ATX/HSP-KCNA2*

. We identified new genes for spastic ataxia, needing further confirmation in larger cohorts.

DISEASE PROGRESSION

. PolyQ AD-HCA appear to have a faster disease progression than non-repeat expansion forms, as we observed a similar SARA score notwithstanding a significantly shorter disease duration until baseline.

. PolyQ AD-HCA significantly associated with falls and wheelchair confinement.

. In the global cohort, non-ataxia movement disorders associated with a faster disease progression. For a similar disease duration, patients with movement disorders (other than ataxia) showed higher SARA score. Also, a higher frequency of falls was registered,

together with a shorter disease duration up to the use of a cane, in the ones with non-ataxia movement disorders.

GENOTYPE-PHENOTYPE CORRELATIONS

. For classical ataxias (as FA), we contributed with further recognition of their intrafamilial variability.

. Pseudodominant inheritance was observed in FA and ATX-*RFC1*.

. Non-ataxia movement disorders are a frequent expression of AD-HCA (particularly in MJD).

. Non-repeat expansion AD-HCA, showed more often early onset, congenital forms and paroxystic symptoms.

. Late-onset, cerebellar presentation, ocular and peripheral signs were more frequent in polyQ SCA.

. We showed that AOA may be also be caused by pathogenic variants in *MAG*.

. *KMT2B*, a gene classically associated with generalized dystonia, may be implicated in adult-onset HCA.

. We strengthened the finding that *de novo* variants in *SPAST* are frequently associated with complex phenotypes, including motor delay and spastic-ataxia.

. *ATP8A2*, a gene for years recognized as involved in a severe phenotype with quadrupedal gait, may also be involved in a form of congenital ataxia, in which, despite delayed milestones and cerebellar syndrome, patients may have an independent adult life.

IMPLICATIONS FOR FUTURE STUDIES

We established an ongoing cohort of HCA, regularly following approximately 200 living patients *per* year (mean inclusion of 20 new individuals yearly), with a prospective detailed phenotypic characterization. Providing our patients with the best care, by establishing our site as a reference centre for ataxia is a major objective of our team.

Off-label treatment offer to HCA patients, as with riluzole, is being performed for several years. Our centre was selected for two large multicentre clinical trials on MJD (but one has been cancelled and the other postponed).

A project of a clinical trial on neuromodulation in HCA is now being planned for 2025-2026.

Our future projects will continue to integrate clinical and basic research, in a close collaboration with other national neurological departments, i3S, ICBAS and, most recently, the University of Bordeaux.

We initiated a national, multicentre network on ATX-*FGF14* and plan to get initial results by the end of 2025.

We intend to start multicentric studies on the influence of pregnancy on HCA and on the prevalence of dysautonomia in HCA.

We integrate other national or international (Global Ataxia Initiative, Non-polyQ SCA) clinical studies, especially for ultra-rare ataxias, where gathering information from various centres would be highly valuable.

We are particularly interested in maintaining functional studies on promising VUS, either through mRNA studies in patients' fibroblast (as in the patient with *KMT2B* pathogenic variant*)* or minigene essay (as in the two siblings with *ATP8A2* pathogenic variants).

Additionally, we aim to delve deeper into the study of HCA patients with pathogenic variants in heterozygosity, in genes associated with AR inheritance. Analysis of intronic variants and runs of homozygosity would be of high importance.

Finally, we hope to contribute further to knowledge on (genetic and environmental) modifier factors, as determinants of AO and disease progression, in classical and newly described HCA.

VII. REFERENCES

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VIII. APPENDICES

VIII. APPENDICES

APPENDIX 1

Protocolo de avaliação inicial

Pseudónimo_____

Hereditariedade_____

Diagnóstico_____

Data da avaliação____/____/_____
Local de avaliação_____

1. Identificação do doente

a. Nome_____
b. DN ____/____/_____
c. Naturalidade_____
d. Residência/ Código Postal_____
e. Anos escolaridade__
f. Lateralidade Dextro__ Canhoto__
g. Situação laboral
   Empregado__ Reformado__ Idade__
   Estudante__
   Desempregado__ Inválido__ Idade__
h. Profissão _____
i. Estado civil
   Casado/União de facto__ Solteiro__ Divorciado__
   Viúvo__

2. Antecedentes Pessoais

a. Diabetes__ Idade__
b. HTA__ Idade__
c. Dislipidemia__ Idade__
d. Hábitos tabágicos__ Idade__ Quantificação_____
e. Hábitos etílicos__ Idade__ Quantificação_____
f. Outros__ Quais?_____ Idade__

3. Identificação da família

a. Nome mãe_____
   Naturalidade família materna_____
   Naturalidade ancestral mais remoto afectado _____
b. Nome pai_____
   Naturalidade família paterna_____
   Naturalidade ancestral mais remoto afectado _____
c. Consanguinidade Sim__ Não__
   Origem dos antepassados em comum_____
d. Outros familiares afectados Sim__ Não__
e. Se sim, quais e idade primeiros sintomas
   Pai__ Idade__
   Mãe__ Idade__
   Irmãos__ Quantos___ Idades_____
   Avó paterna__ Idade__
   Avô paterno__ Idade__
   Avó materna__ Idade__

Avô materno __		Idade__
Tios paternos__	Quantos___	Idades_____
Tios maternos__	Quantos___	Idades_____
Filhos__	Quantos___	Idades_____
Netos__	Quantos___	Idades_____
Sobrinhos	Quantos___	Idades_____
Primos paternos__	Quantos___	Idades_____
Primos maternos__	Quantos___	Idades_____

f. Árvore familiar:

4. Sintomas iniciais

a. Idade de aparecimento dos primeiros sintomas____

b. Tipo de primeiros sintomas

Cerebelosos__ Não cerebelosos__

c. Caracterização dos primeiros sintomas

Cerebelosos	*Não cerebelosos*
disartria__	atraso psicomotor__ cognitivo__ motor__
diplopia__	deterioração cognitiva__
desequilíbrio na marcha __	epilepsia__ focal __ generalizada__
quedas__	sind piramidal
descoordenação dos MS__	PNP
caligrafia__	oftalmoparesia__ supra-nuclear__ extrínseca__
hipotonia__	tremor__ repouso__ postura/ acção__
outros__ quais?_____	parkinsonismo__
	coreia__
	distonia__ focal__ segmentar__ hemi__ generliz__
	mioclonias__ focal__ segmentar__ generliz__
	marcha espástica
	alterações de esfíncteres__
	sintomatologia psiquiátrica__ qual?_____
	sonhos vividos__
	outros__ quais?

5. Sintomas subsequentes

a. Quando os sintomas iniciais não foram cerebelosos, qual a idade de início dos **sintomas cerebelosos**?____

b. Quando os sintomas iniciais não foram cerebelosos, quais foram os **primeiros sintomas cerebelosos**

disartria__
diplopia__
desequilíbrio na marcha __
quedas__
descoordenação dos MS__
caligrafia__
hipotonia__
outros__ quais?_____

c. O doente tem outros **sintomas cerebelosos** para além dos anteriores? Sim__ Não__

d. Se sim identifique quais e assinale com que idade surgiram

disartria__	Idade____
diplopia__	Idade____
desequilíbrio na marcha __	Idade____
quedas__	Idade____
descoordenação dos MS__	Idade____
caligrafia__	Idade____
hipotonia__	Idade____
outros__ quais?_____	Idade____

e. Quando os sintomas iniciais foram cerebelosos, o doente tem **sintomas não cerebelosos**? Sim__Não_
f. Com que idade surgiram os primeiros **sintomas não cerebelosos**?__
g. Quais foram os primeiros **sintomas não cerebelosos**?

atraso psicomotor__ cognitivo__ motor__
deterioração cognitiva__
epilepsia__ focal __ generalizada__
sind piramidal
PNP
oftalmoparesia__ supra-nuclear__ extrínseca__
tremor__ repouso__ postura/ acção__
parkinsonismo__
coreia__
distonia__ focal__ segmentar__ hemi__ generliz__
mioclonias__ focal__ segmentar__ generliz__
marcha espástica
alterações de esfíncteres__
sintomatologia psiquiátrica__ qual?_____
sonhos vividos__
outros__ quais?

h. O doente tem outros sintomas **não cerebelosos** para além dos anteriores? Sim__ Não__
i. Se sim identifique quais e com que idade surgiram

atraso psicomotor__ cognitivo__ motor__
deterioração cognitiva__
epilepsia__ focal __ generalizada__
sind piramidal
PNP
oftalmoparesia__ supra-nuclear__ extrínseca__
tremor__ repouso__ postura/ acção__
parkinsonismo__
coreia__
distonia__ focal__ segmentar__ hemi__ generliz__
mioclonias__ focal__ segmentar__ generliz__
marcha espástica
alterações de esfíncteres__
sintomatologia psiquiátrica__ qual?_____
sonhos vividos__
outros__ quais?

j. Nas ataxias congénitas assinale a idade de aquisição de
    i. Controlo cefálico__
    ii. Controlo tronco (sentar)__
    iii. Aquisição marcha__

k. Assinale as aplicáveis e com que idade surgiram
anartria__ Idade__

incapacidade de assinar__ Idade__
disfagia__ Idade__
perda de marcha autónoma__ Idade__
perda de capacidade de marcha__ Idade__

6. Medicação habitual

Nome comercial/ dose/ posologia	Data início	Motivo

7. Realização de fisioterapia Sim__ Não__
   a. Se respondeu sim à pergunta anterior
      i. Frequência semanal das sessões
         1. <2 vezes por semana__
         2. 2 a 4 vezes por semana__
         3. >4 vezes por semana__
      ii. Periodicidade das sessões
         1. <2 meses por ano__
         2. 2 a 6 meses por ano__
         3. 6 a 12 meses por ano__

8. Realização de terapia da fala Sim__ Não__
   a. Se respondeu sim à pergunta anterior
      i. Frequência semanal das sessões
         1. <2 vezes por semana__
         2. 2 a 4 vezes por semana__
         3. >4 vezes por semana__
      ii. Periodicidade das sessões
         1. <2 meses por ano__
         2. 2 a 6 meses por ano__
         3. 6 a 12 meses por ano__

9. Exames complementares de diagnóstico

	Data	Resultado
RMN		

Testes genéticos		
EMG		
EEG		
Avaliaçao Neuropsicologica		
Outros		

10. Exemplo da caligrafia

O jovem craque Belga falhou vinte partidas de xadrez

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11. Escalas de avaliação clínica
    a. SARA
    b. INAS

APPENDIX 2

Protocolo de avaliação subsequente de doentes

Pseudónimo_____
Data da avaliação__/__/___
Local de avaliação_____

a. Situação laboral
   i. Empregado__
   ii. Desempregado__
   iii. Reformado__ Idade__
   iv. Inválido__ Idade__
b. Profissão _____
c. Estado civil
   i. Casado/União de facto__
   ii. Solteiro__
   iii. Divorciado__
   iv. Viúvo__
d. Óbito
   i. Data
   ii. Causa de morte
      1. Infecção__
      2. Acidente vascular cerebral__
      3. Traumatismo__
      4. Suicídio__
      5. Neoplasia__
      6. Outra (e qual?)__
   iii. Local de falecimento
      1. Domicílio__
      2. Lar__
      3. Hospital__
      4. Desconhecido__
   iv. Autópsia Sim__ Não__

2. Desde a última avaliação surgiu, de novo algum dos seguintes?
   a. Sintomas cerebelosos? Sim__ Não__
   b. Se sim assinale quais e com que idade surgiram

disartria__
diplopia__
desequilíbrio na marcha __
quedas__
descoordenação dos MS__
caligrafia__
hipotonia__
outros__ quais?_____

   c. Sintomas não cerebelosos? Sim__ Não__
   d. Se sim identifique quais e com que idade surgiram

atraso psicomotor__ cognitivo__ motor__
deterioração cognitiva__
epilepsia__ focal __ generalizada__
'sind piramidal
'PNP
oftalmoparesia__ supra-nuclear__ extrínseca__
:tremor__ repouso__ postura/ acção__
parkinsonismo__
coreia__
distonia__ focal__ segmentar__ hemi__ generliz__
ımioclonias__ focal__ segmentar__ generliz__
ımarcha espástica
alterações de esfíncteres__
sintomatologia psiquiátrica__ qual?_____
sonhos vividos__
outros__ quais?

e. Assinale as aplicáveis e com que idade surgiram

anartria__ ıdade__
incapacidade de assinar__ ıdade__
disfagia__ ıdade__
perda de marcha autónoma__ ıdade__
perda de capacidade de marcha__ ıdade__

3. Medicação habitual

Nome comercial e dose	ata início	lotivo

4. Realização de fisioterapia Sim__ Não__
   a. Se respondeu sim à pergunta anterior
      i. Frequência semanal das sessões
         1. <2 vezes por semana__
         2. 2 a 4 vezes por semana__
         3. >4 vezes por semana__
      ii. Periodicidade das sessões
         1. <2 meses por ano__
         2. 2 a 6 meses por ano__
         3. 6 a 12 meses por ano__

5. Realização de terapia da fala Sim__ Não__
   a. Se respondeu sim à pergunta anterior
      i. Frequência semanal das sessões
         1. <2 vezes por semana__
         2. 2 a 4 vezes por semana__
         3. >4 vezes por semana__
      ii. Periodicidade das sessões

1. <2 meses por ano__
2. 2 a 6 meses por ano__
3. 6 a 12 meses por ano__

6. Exames complementares de diagnóstico

	ata	esultado
RMN		
Testes genéticos		
Outros		

7. Exemplo da caligrafia

O jovem craque Belga falhou vinte partidas de xadrez

8. Escalas de avaliação clínica
    a. SARA
    b. INAS

APPENDIX 3

Protocolo de vídeo (opcional)

c. Doente sentado:
    i. visão global do doente (movimento lento da camara da cabeça até aos pés e movimento inverso), durante 15 segundos
    ii. conversa com o doente durante 20 segundos, repetição de algumas frases: "nem mas em meio mas", "o rato roeu a rolha da garraFA do rei da Rússia", "patacaaaaa"
    iii. com os braços em repouso sobre as coxas durante 30 segundos (10 segundos camara a captar a face e as mãos do doente, 10 segundos camara a captar a face e as mãos do doente com contagem decrescente de 20-10, *zoom* de 10 segundos para as mãos do doente)
    iv. com os braços estendidos durante 30 segundos (10 segundos camara a captar a face e as mãos do doente, 10 segundos camara a captar a face e as mãos do doente com contagem decrescente de 20-10, 10 segundos zoom para as mãos do doente)
    v. prova dedo-nariz-dedo, durante 10 segundos, cada mão
    vi. movimentos alternados das mãos sobre as coxas, durante 10 segundos, cada mão
    vii. *finger tapping* de cada mão alternadamente, durante 10 segundos, cada mão
d. Doente deitado
    i. realização de prova calcanhar-joelho, durante 20 segundos, cada perna
e. Doente em pé
    i. estação de pé
    ii. marcha realizando um percurso de 4-5 metros três vezes, por forma a fazer duas inversões de marcha (tempo variável, estimado 30-45 seg)
    iii. marcha pé-ante-pé

APPENDIX 4

Autorização n.º 4506/ 2017

Joana Catarina Damásio Correia dos Santos notificou à Comissão Nacional de Protecção de Dados (CNPD) um tratamento de dados pessoais com a finalidade de realizar um Estudo Clínico sem Intervenção, denominado Ataxias hereditárias - Sintomas cerebelosos, não cerebelosos e incapacidade .

A investigação é multicêntrica, decorrendo, em Portugal, nos centros de investigação identificados na notificação.

O participante é identificado por um código especificamente criado para este estudo, constituído de modo a não permitir a imediata identificação do titular dos dados; designadamente, não são utilizados códigos que coincidam com os números de identificação, iniciais do nome, data de nascimento, número de telefone, ou resultem de uma composição simples desse tipo de dados. A chave da codificação só é conhecida do(s) investigador(es).

É recolhido o consentimento expresso do participante ou do seu representante legal.

A informação é recolhida diretamente do titular e indiretamente do processo clínico.

As eventuais transmissões de informação são efetuadas por referência ao código do participante, sendo, nessa medida, anónimas para o destinatário.

A CNPD já se pronunciou na Deliberação n.º 1704/2015 sobre o enquadramento legal, os fundamentos de legitimidade, os princípios aplicáveis para o correto cumprimento da Lei n.º 67/98, de 26 de outubro, alterada pela Lei n.º 103/2015, de 24 de agosto, doravante LPD, bem como sobre as condições e limites aplicáveis ao tratamento de dados efetuados para a finalidade de investigação clínica.

No caso em apreço, o tratamento objeto da notificação enquadra-se no âmbito daquela deliberação e o responsável declara expressamente que cumpre os limites e condições aplicáveis por força da LPD e da Lei n.º 21/2014, de 16 de abril, alterada pela Lei n.º 73/2015, de 27 de junho – Lei da Investigação Clínica –, explicitados na Deliberação n.º 1704/2015.

O fundamento de legitimidade é o consentimento do titular.

A informação tratada é recolhida de forma lícita, para finalidade determinada, explícita e legitima e não é excessiva – cf. alíneas a), b) e c) do n.º 1 do artigo 5.º da LPD.

Assim, nos termos das disposições conjugadas do n.º 2 do artigo 7.º, da alínea a) do n.º 1 do artigo 28.º e do artigo 30.º da LPD, bem como do n.º 3 do artigo 1.º e do n.º 9 do artigo 16.º ambos da Lei de Investigação Clínica, com as condições e limites explicitados na Deliberação da CNPD n.º 1704/2015, que aqui se dão por reproduzidos, autoriza-se o presente tratamento de dados pessoais nos seguintes termos:

Responsável – Joana Catarina Damásio Correia dos Santos

Finalidade – Estudo Clínico sem Intervenção, denominado Ataxias hereditárias - Sintomas cerebelosos, não cerebelosos e incapacidade

Categoria de dados pessoais tratados – Código do participante; idade/data de nascimento; género; composição do agregado familiar sem identificação dos membros; dados da história clínica; dados dados de exame físico; dados de meios complementares de diagnóstico; medicação prévia concomitante; genéticos; relativos à atividade profissional com conexão com a Investigação

Exercício do direito de acesso – Através dos investigadores, por escrito

Comunicações, interconexões e fluxos transfronteiriços de dados pessoais identificáveis no destinatário – Não existem

Prazo máximo de conservação dos dados – A chave que produziu o código que permite a identificação indireta do titular dos dados deve ser eliminada 5 anos após o fim do estudo.

Da LPD e da Lei de Investigação Clínica, nos termos e condições fixados na presente Autorização e desenvolvidos na Deliberação da CNPD n.º 1704/2015, resultam obrigações que o responsável tem de cumprir. Destas deve dar conhecimento a todos os que intervenham no tratamento de dados pessoais.

Lisboa, 20-04-2017

A Presidente

Filipa Calvão

centro hospitalar
do Porto

Hospital Santo António | Hospital Maria Pia | Maternidade Júlio Dinis | Hospital Joaquim Urbano

Largo Prof. Abel Salazar
4099-001 PORTO
www.hgsa.pt

Exma. Sra. Dra. Joana Damásio
Serviço de Neurologia

ASSUNTO: Trabalho Académico – Doutoramento - **"Ataxias Hereditárias - Sintomas cerebelosos, não cerebelosos e incapacidade"** - N/ REF.ª 2017.069(063-DEFI /063-CES)

O Conselho de Administração do CHP autoriza a realização do estudo acima mencionado, a realizar no Serviço de Neurologia desta Instituição e tendo como Investigador Principal a Dra. Joana Damásio.
O estudo foi previamente analisado pela Comissão de Ética para a Saúde, pelo Gabinete Coordenador de Investigação, pela Direção do Departamento de Ensino, Formação e Investigação do CHP e pelo Presidente do Conselho de Administração, tendo obtido parecer favorável.

Cumprimentos,

CONSELHO DE ADMINISTRAÇÃO
07/06/2017
7 JUN. 2017
Dr. PAULO BARBOSA — Presidente
Dr.ª ÉLIA GOMES — Vogal Executiva
Prof. Doutor JOSÉ BARROS — Diretor Clínico
Dr. RUI PEDROSO — Vogal Executivo
Enf.º EDUARDO ALVES — Enfermeiro Diretor

* Em todas as eventuais comunicações posteriores sobre este estudo é indispensável indicar a nossa ref.ª.

centro hospitalar do Porto

Hospital de Santo António Maternidade Júlio Dinis Hospital Maria Pia

Largo Professor Abel Salazar
4099-001 PORTO
www.hgsa.pt

APRECIAÇÃO E PARECER PARA A REALIZAÇÃO DE **TRABALHO ACADÉMICO - DOUTORAMENTO**

Título: **"Ataxias Hereditárias - Sintomas cerebelosos, não cerebelosos e incapacidade"**		Ref.ª: **2017.069(063-DEFI /063-CES)**
		Investigador: **Dra. Joana Damásio** **Serviço de Neurologia**

DIREÇÃO DE ENFERMAGEM:	**PRESIDENTE DO CONSELHO DE ADMINISTRAÇÃO:**
☒ **NÃO SE APLICA**	☒ **PARECER FAVORÁVEL**
☐ **PARECER FAVORÁVEL**	☐ **PARECER NÃO FAVORÁVEL**
☐ **PARECER NÃO FAVORÁVEL**	
Data:	Data: 06/06/17 Dr. PAULO BARBOSA Presidente do Conselho de Administração do CHP

Em conformidade. Pode ser autorizado

Prof.ª Doutora Luísa Lobato
Diretora do DEFI
30/05/2017

Luísa Lobato
Diretora do DEFI

centro hospitalar
do Porto

Hospital de Santo António Maternidade Júlio Dinis Hospital Maria Pia

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COMISSÃO DE ÉTICA PARA A SAÚDE

APRECIAÇÃO E VOTAÇÃO DO PARECER

Deliberação	Data: 17.5.2017	Órgão: Reunião Plenária

Título: **"Ataxias Hereditárias - Sintomas cerebelosos, não cerebelosos e incapacidade"**		Ref.ª: **2017.069(063-DEFI /063-CES)**
Protocolo/Versão: **TA - DT**	Promotor: **o(a) próprio(a)**	Investigador: **Dra. Joana Damásio Correia dos Santos – Médica Neurologista, Serviço de Neurologia, CHP. Aluna de Doutoramento em Ciências Médicas, ICBAS.**

A Comissão de Ética para a Saúde – CES do CHP, ao abrigo do disposto no Decreto-Lei n.º 97/95, de 10 de Maio, em reunião realizada nesta data, apreciou a fundamentação do relator sobre o pedido de parecer para a realização de **TA - DT** acima referenciado:

Ouvido o Relator, o processo foi votado pelos Membros da CES presentes:

Presidente: Dr.ª Luisa Bernardo
Vice-Presidente: Dr.ª Paulina Aguiar

Dr.ª Fernanda Manuela, Enf.ª Paula Duarte, Prof.ª Doutora Carla Teixeira, Prof.ª Doutora Maria Manuel Araújo Jorge, Dr. Gonçalo Senhorães Senra.
Resultado da votação:

PARECER FAVORÁVEL

PARECER FAVORÁVEL

A deliberação foi aprovada por unanimidade.

Pelo que se submete à consideração superior.

06/06/017

Dr. SEVERO TORRES
Assessor do Presidente do Conselho de Administração

Data 17.5.2017

A Presidente da CES

Dr.ª Luisa Bernardo

Imp.10/2009

01/6/017

Cerebellar and non-cerebellar symptoms in hereditary ataxias

Joana Catarina Damásio Correia dos Santos

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

