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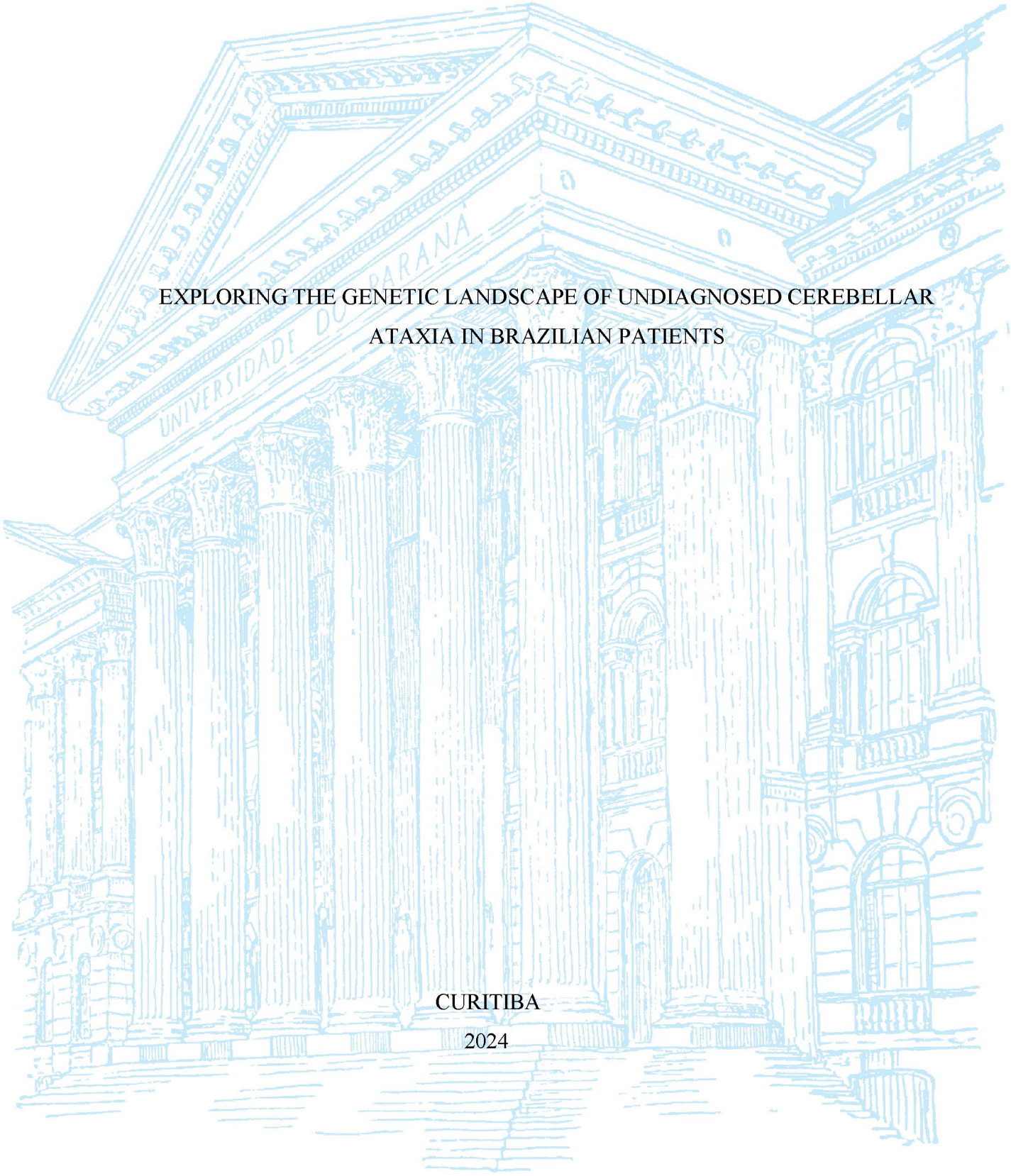

EXPLORING THE GENETIC LANDSCAPE OF UNDIAGNOSED CEREBELLAR ATAXIA IN BRAZILIAN PATIENTS

CURITIBA

2024

LUIZ EDUARDO NOVIS DE FARIAS

EXPLORING THE GENETIC LANDSCAPE OF UNDIAGNOSED CEREBELLAR ATAXIA IN BRAZILIAN PATIENTS

Tese apresentada ao curso de Pós-Graduação em Medicina Interna e Ciências da Saúde, Setor de Ciências da Saúde, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Medicina Interna e Ciências da Saúde.

Orientador: Prof. Dr. Helio Afonso Ghizoni Teive
Coorientador: Prof. Dra. Mariana Spitz

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No dia dezenove de dezembro de dois mil e vinte e tres às 08:00 horas, na sala Virtual do PPGMICS - UFPR: https://tinyurl.com/ms24ftb8, Auditório da Neurologia - 4º andar CHC/UFPR (Veiculada pela plataforma Microsoft TEAMS), foram instaladas as atividades pertinentes ao rito de defesa de tese do doutorando **LUIZ EDUARDO NOVIS DE FARIAS**, intitulada**: "EXPLORING THE GENETIC LANDSCAPE OF UNDIAGNOSED CEREBELLAR ATAXIA IN BRAZILIAN PATIENTS".**, sob orientação do Prof. Dr. HÉLIO AFONSO GHIZONI TEIVE. A Banca Examinadora, designada pelo Colegiado do Programa de Pós-Graduação MEDICINA INTERNA E CIÊNCIAS DA SAÚDE da Universidade Federal do Paraná, foi constituída pelos seguintes Membros: HÉLIO AFONSO GHIZONI TEIVE (UNIVERSIDADE FEDERAL DO PARANÁ), GUSTAVO LEITE FRANKLIN (UNIVERSIDADE FEDERAL DO PARANÁ), RENATO PUPPI MUNHOZ (UNIVERSITY OF TORONTO - CANADA), SALMO RASKIN (FACULDADE EVANGÉLICA MACKENZIE DO PARANÁ - FEMPAR), SIMONE CARREIRO VIEIRA KARUTA (FACULDADES PEQUENO PRÍNCIPE). A presidência iniciou os ritos definidos pelo Colegiado do Programa e, após exarados os pareceres dos membros do comitê examinador e da respectiva contra argumentação, ocorreu a leitura do parecer final da banca examinadora, que decidiu pela APROVAÇÃO. Este resultado deverá ser homologado pelo Colegiado do programa, mediante o atendimento de todas as indicações e correções solicitadas pela banca dentro dos prazos regimentais definidos pelo programa. A outorga de título de doutor está condicionada ao atendimento de todos os requisitos e prazos determinados no regimento do Programa de Pós-Graduação. Nada mais havendo a tratar a presidência deu por encerrada a sessão, da qual eu, HÉLIO AFONSO GHIZONI TEIVE, lavrei a presente ata, que vai assinada por mim e pelos demais membros da Comissão Examinadora.

Curitiba, 19 de Dezembro de 2023.

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HÉLIO AFONSO GHIZONI TEIVE
Presidente da Banca Examinadora

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação MEDICINA INTERNA E CIÊNCIAS DA SAÚDE da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **LUIZ EDUARDO NOVIS DE FARIAS** intitulada: **"EXPLORING THE GENETIC LANDSCAPE OF UNDIAGNOSED CEREBELLAR ATAXIA IN BRAZILIAN PATIENTS".**, sob orientação do Prof. Dr. HÉLIO AFONSO GHIZONI TEIVE, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

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I dedicate this present thesis to my family, my beloved son Guilherme, my wife Ester Cury, my grandmother Marta Maria de Faro Novis, and my two great inspirations: my mother Ana Luiza de Faro Novis and my grandfather Sérgio Augusto Pereira Novis. Without all of you, this journey would have been much more challenging.

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Lastly, I would like to thank God for all the opportunities and blessings that have come my way and for the chance to complete my Ph.D.

"Saber o certo, fazer o possível."

Sérgio Augusto Pereira Novis

RESUMO

Este projeto de pesquisa teve como propósito avaliar o perfil genotípico de pacientes portadores de ataxia cerebelar hereditária (AH) que não haviam obtido diagnóstico molecular após extensa investigação clínica. Para alcançar esse objetivo, recrutamos um total de 87 pacientes, provenientes de 76 famílias, com AH, cujo diagnóstico permanecia indefinido, provenientes de três diferentes centros especializados em ataxia situados em distintas regiões do Brasil. Esses centros são o Hospital das Clínicas da Universidade Federal do Paraná, Curitiba-PR, com 58 pacientes; o Hospital Universitário Pedro Ernesto, Rio de Janeiro-RJ, com 21 pacientes; e o Hospital Estadual do Amazonas, Amazonas-AM, com 8 pacientes. Os pacientes incluídos nesta pesquisa foram submetidos a uma investigação minuciosa em busca de causas secundárias e etiologias genéticas mais prevalentes, como SCA 1, 2, 3, 6, 7, 10 e Ataxia de Friedreich. Amostras de DNA obtidas desses pacientes foram posteriormente submetidas a um rastreio em busca de expansões bialélicas não-canônicas do tipo AAGGG no gene *RFC1*. Para aqueles que não apresentaram essa mutação, foi realizado um sequenciamento completo do exoma (SCE), com análise adicional por meio do ExpansionHunter. Os resultados obtidos revelaram que duas famílias receberam diagnóstico positivo para mutações no gene *RFC1*. Das 74 famílias submetidas ao SCE, 10 apresentaram mutações definitivamente patogênicas, enquanto 12 apresentaram mutações provavelmente patogênicas, representando, respectivamente, 13,5% e 16% da coorte total. A investigação genética realizada neste estudo com pacientes portadores de AH sem diagnóstico revelou um diagnóstico definitivo ou provável em 32% dos casos. Por outro lado, trinta pacientes não apresentaram nenhuma variante suspeita na investigação (40,5%), enquanto vinte e dois pacientes (30%) apresentavam pelo menos uma variante de significado incerto (VUS). Em um segundo estudo, 42 pacientes dos casos não resolvidos foram selecionados para investigar a presença de expansão intrônica GAA no gene *FGF14*, uma mutação recentemente descrita. A esse grupo original, acrescentaram-se mais 51 pacientes recrutados do Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto, em São Paulo-SP, seguindo os mesmos critérios de inclusão da coorte original. Entre os 93 pacientes incluídos no estudo, oito deles (9%) apresentaram uma expansão $(GAA)_{>250}$. Esses dados evidenciam que as expansões recentemente descritas nos genes *RFC1* e *FGF14* são frequentes na população de pacientes brasileiros com AH sem diagnóstico molecular. Além disso, sugerem que o SCE é uma ferramenta útil para ser utilizada nesses casos, podendo, inclusive, ser considerada como uma abordagem de primeira linha na investigação de AH.

Palavras-chave: Ataxia Cerebelar Hereditária; Sequenciamento Completo do Exoma; ExpansionHunter; Sequenciamento de Nova Geração; Replication Factor Complex subunit 1 (RFC1); Fibroblast Growth Factor 14 (FGF14)

ABSTRACT

This research project aimed to assess the genotypic profile of patients with hereditary cerebellar ataxia (HA) who had not received a molecular diagnosis despite extensive clinical investigation. To achieve this goal, a total of 87 patients from 76 families with undiagnosed HA were recruited from three different specialized ataxia centers located in various regions of Brazil. These centers were the Hospital das Clínicas da Universidade Federal do Paraná in Curitiba-PR, with 58 patients; the Hospital Universitário Pedro Ernesto in Rio de Janeiro-RJ, with 21 patients; and the Hospital Estadual do Amazonas in Amazonas-AM, with 8 patients. The patients included in this study underwent thorough investigations to explore secondary causes and more prevalent genetic etiologies, such as SCA 1, 2, 3, 6, 7, 10, and Friedreich's Ataxia. DNA samples collected from these patients were subsequently sent to screening for non-canonical biallelic AAGGG repeat expansions in the *RFC1* gene. For those who did not have this mutation, whole-exome sequencing (WES) was performed, with additional analysis using ExpansionHunter. The results revealed that two families received a positive diagnosis for *RFC1* gene mutations. Out of the 74 families subjected to WES, 10 had definitively pathogenic mutations, while 12 had mutations that were likely pathogenic, representing 13.5% and 16% of the total cohort, respectively. The genetic investigation conducted in this study with patients suffering from undiagnosed HA provided a definitive or probable diagnosis in 32% of cases. Conversely, thirty patients showed no suspicious variants in the investigation (40.5%), while twenty-two patients (30%) presented at least one variant of uncertain significance (VUS). In a follow-up study, 42 patients from the unresolved cases were selected to investigate the presence of intronic GAA repeat expansions in the *FGF14* gene, a mutation recently described. An additional 51 patients recruited from the Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto in São Paulo-SP, following the same inclusion criteria as the original cohort, were included. Among the 93 patients included in the study, eight of them (9%) exhibited an expansion $(GAA)_{>250}$. These data highlight the prevalence of the recently described expansions in the *RFC1* and *FGF14* genes in the Brazilian population of patients with undiagnosed HA. Furthermore, they suggest that WES is a valuable tool to be used in these cases, possibly even as a first-tier diagnostic approach in the investigation of HA.

Key-words: Hereditary cerebellar ataxia; Whole Exome Sequencing; ExpansionHunter; Next Generation Sequencing; Replication Factor Complex subunit 1 (*RFC1*); Fibroblast Growth Factor 14 (*FGF14*)

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LIST OF ABBREVIATIONS

ABraOM	Brazilian Genomic Variability Project
AD	Autosomal Dominant
ADCA	Autosomal Dominant Cerebellar Ataxia
ADL	Adrenoleukodystrophy
AOA	Ataxia with Oculomotor Apraxia
AOO	Age of Onset
AR	Autosomal Recessive
AR (italic)	Androgen receptor
ARCA	Autosomal Recessive Cerebellar Ataxia
ARSACS	Autosomal Recessive Spastic Ataxia of Charlevoix-Saguenay
ASAT	Ataxia with Sideroblastic Anemia
ATN1	Atrophin-1
ATXN	Ataxin
BAM	Binary Alignment Map
CA	Cerebellar Ataxia
CACNA1G	Voltage-Gated Calcium Channel Subunit Alpha1 G
CNV	Copy Number Variations
CT	Computed Tomography

DRPLA	Dentatorubral-pallidoluysian atrophy
EA	Episodic ataxia
FXTAS	Fragile X-Associated Tremor/Ataxia Syndrome
FGF14	Fibroblast Growth Factor 14
FA	Friedreich's Ataxia
FA2H	Fatty Acid 2-Hydroxylase
GnomAD	Genome Aggregation Database
HA	Hereditary Ataxia
HGMD	Human Gene Mutation Database
HEXA	Hexosaminidase A
HPO	Human Phenotype Ontology
HTT	Huntingtin
ICF	Informed Consent Form
Indels	Insertions or Deletions
LRS	Long-Read Sequencing
LOF	Loss-of-Function
LR-PCR	Long-Range Polymerase Chain Reaction
MA	Mitochondrial Ataxias
MERFF	Myoclonic Epilepsy with Ragged Red Fibers
Mit	Mitochondrial Genetic Disorder

MRI	Magnetic Resonance Imaging
mtDNA	Mitochondrial DNA
NARP	Neuropathy, Ataxia, and Retinitis Pigmentosa
NGS	Next-Generation Sequencing
NOP56	Nucleolar Protein 56
NPC1	Niemann-Pick C1
OMIM	Online Mendelian Inheritance in Man
PCR	Polymerase Chain Reaction
PDYN	Prodynorphin
PMM2	Phosphomannomutase 2
PNKP	Polynucleotide Kinase Phosphatase
PPP2R2B	Protein Phosphatase 2 Regulatory Subunit B Beta
PRDX3	Peroxiredoxin 3
PS	Pathogenic Strong
PP	Pathogenic Support
REVEL	Rare Exome Variant Ensemble Learner
RFC-1	Replication Factor Complex subunit 1
RP-PCR	Repeat-Primed Polymerase Chain Reaction
SCA	Spinocerebellar Ataxia
SNV	Single Nucleotide Variant

SPG11	Spastic Paraplegia type 11
SPG7	Spastic Paraplegia type 7
STUB1	STIP1 Homology and U-Box Containing Protein 1
SYNE1	Spectrin Repeat Containing Nuclear Envelope Protein 1
TBP	TATA-Box Binding Protein
TSP	Targeted Sequencing Panels
UniProt	Universal Protein Resource
VCF	Variant Call Format
VUS	Variant of Uncertain Significance
WES	Whole-Exome Sequencing
WGS	Whole-Genome Sequencing
XL	X-linked inheritance
XLCA	X-Linked Cerebellar Ataxias

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Exploring the Genetic Landscape of Undiagnosed Cerebellar Ataxia in Brazilian Patients

1- INTRODUCTION

1.1 General Overview

Cerebellar ataxia is a syndrome characterized by a multitude of signs and symptoms, including gait ataxia (tandem gait), dysarthria (scanning speech), titubation, hypotonia, the presence of pendular reflexes, rebound phenomenon, abnormal eye movements (nystagmus, saccadic and pursuit movement disorders), kinetic and intentional tremors, and affective cognitive cerebellar syndrome (HARDING, 1983; SCHMAHMANN; SHERMAN, 1998).

Cerebellar ataxias (CAs) can be categorized as primary, which include heredodegenerative, non-hereditary degenerative ataxias, and secondary, or acquired ataxias, resulting from impairment of the cerebellum and its connections due to diseases such as multiple sclerosis, tumors, cerebrovascular disease, drugs, infections, paraneoplastic syndromes, endocrine diseases, and autoimmune diseases (HARDING, 1983).

Among primary cerebellar ataxias, hereditary ataxias (HAs) are the most significant from clinical and epidemiological perspectives. HAs can be classified based on genetic inheritance into autosomal recessive HAs, autosomal dominant HAs, X-linked ataxias, and mitochondrial HAs, and they exhibit considerable genotypic and phenotypic heterogeneity (WITEK; HAWKINS; HALL, 2021).

1.1.1 Autosomal Dominant Cerebellar Ataxias

The prevalence of HAs, including autosomal recessive, dominant, and sporadic forms, has been inadequately studied, with reported rates varying from 1 case to 17.8 cases per 100,000 people in different published studies (RUANO; MELO; SILVA; COUTINHO, 2014).

Autosomal dominant cerebellar ataxias, represented by Spinocerebellar Ataxias (SCAs), comprise a heterogeneous group of HAs that affect successive generations, with 50% of descendants inheriting the mutation. SCAs have a progressive clinical course, typically starting between ages 30 and 50, although cases with onset before 20

years and after 60 years have been reported (SCHÖLS; AMOIRIDIS; BÜTTNER; PRZUNTEK *et al.*, 1997; SCHÖLS; BAUER; SCHMIDT; SCHULTE *et al.*, 2004).

SCAs are characterized by various neurological symptoms, including gait and limb ataxia, dysarthria, cerebellar and supranuclear oculomotor abnormalities, retinopathy, optic atrophy, spasticity, movement disorders, peripheral neuropathy, sphincter disorders, cognitive impairments, and epilepsy (DURR, 2010; TEIVE; MUNHOZ; ASHIZAWA, 2012). Presently, around 50 subtypes of SCAs have been described (COARELLI; COUTELIER; DURR, 2023; PELLERIN; DANZI; WILKE; RENAUD *et al.*, 2023).

International studies have been conducted to assess the prevalence of Spinocerebellar Ataxias (SCAs) worldwide. The prevalence has been reported as 3/100,000 cases in the Netherlands (VAN DE WARREMBURG; SINKE; VERSCHUUREN-BEMELMANS, 2003), 4.2/100,000 in southern Norway (ERICHSEN; KOHT; STRAY-PEDERSEN; ABDELNOOR *et al.*, 2009), and 5.6 cases per 100,000 inhabitants in Portugal (COUTINHO; RUANO; LOUREIRO; CRUZ *et al.*, 2013). Notably, Spinocerebellar Ataxia type 3 (SCA3 or Machado-Joseph disease) has been the most frequently identified mutation in these studies.

In Brazil, SCA3 is the most prevalent among the diseases, followed by SCA2 and SCA10, particularly in the southern region of the country (TEIVE; MEIRA; CAMARGO; MUNHOZ, 2019). However, it is important to acknowledge that Brazilian studies have primarily focused on the South and Southeast regions (DE CASTILHOS; FURTADO; GHENO; SCHAEFFER *et al.*, 2014; LOPES-CENDES; TEIVE; CALCAGNOTTO; DA COSTA *et al.*, 1997; NASCIMENTO; RODRIGUES; PELLOSO; CAMARGO *et al.*, 2019). Further investigations are required in other geographical areas to accurately estimate the actual prevalence of each SCA subtype across the nation.

Recently, Pellerin *et al.* (2023) described a dominantly inherited deep intronic GAA repeat expansion in the *FGF14* gene as a significant cause of autosomal dominant late-onset cerebellar ataxia (LOCA). The study was conducted in a population with European and South-Asian ancestry, making the prevalence in other populations unknown. It is noteworthy that, although a family history is common, sporadic cases were not uncommon, accounting for 31% of cases. In line with this study, Hengel *et al.*

(2023) showed that SCA27B was responsible for 16% of the diagnosis of SCA in a large german cohort.

1.1.2 Autosomal Recessive Cerebellar Ataxia

Autosomal recessive cerebellar ataxias (ARCAs), akin to SCAs, represent a diverse group of hereditary ataxias characterized by slow and progressive cerebellar degeneration and its associated afferent and efferent pathways. However, ARCA often involves the extra-cerebellar domains, including the peripheral nervous system, extrapyramidal pathways, oculomotor motility, cognition, and others. Furthermore, extra-neurological manifestations such as cardiopathies, deafness, retinopathies, bone deformities, and endocrine disorders are frequently observed in ARCA. This stands in contrast to SCAs, where the clinical presentation is predominantly pure cerebellar ataxia. Clinically, ARCA typically manifests before the age of 40, but onset may occur later. Unlike SCAs, ARCA often does not follow a pattern of successive generations and may even present sporadically (DRAGAŠEVIĆ-MIŠKOVIĆ; STANKOVIĆ; MILOVANOVIĆ; KOSTIĆ, 2022; DURR, 2010; PALAU; ESPINÓS, 2006; WITEK; HAWKINS; HALL, 2021).

Western studies have shown that Friedreich's ataxia (FA) is the most common and prevalent recessive cerebellar ataxia globally, accounting for approximately 25% of all recessive spinocerebellar ataxias (RUANO; MELO; SILVA; COUTINHO, 2014). Following FA, Replication Factor Complex subunit 1 (*RFC1)*-ataxia, Spastic Paraplegia type 7 (*SPG7*), *SYNE-1*-related ataxia and Autosomal Recessive Spastic Ataxia of Charlevoix-Saguenay (ARSACS) are also notable causes (TRASCHÜTZ; ADARMES-GOMEZ; ANHEIM; BAETS *et al.*, 2023). In a big cohort of patients from South America, FA accounted for 57% of the diagnosis, followed by *RFC1*- ataxia (8%) and Ataxia-Telangiectasia (7%) (GAMA; BRAGA-NETO; RANGEL; GODEIRO *et al.*, 2022).

Recently, Cortese *et al.* (2019) described a biallelic pentanucleotide non-reference AAGGG intronic expansion in the *RFC1* gene as a causative factor for Cerebellar Ataxia with Neuropathy and Vestibular Areflexia Syndrome (CANVAS), which has been identified as a frequent cause of recessive ataxia with adult onset. Prevalence studies of *RFC1*- ataxia in undiagnosed patients with adult-onset cerebellar ataxia have reported frequencies ranging from 3.2% to 22%. It's worth noting that the

prevalence of *RFC1*-related disorder appears to increase significantly when sensory neuronopathy and bilateral vestibular areflexia are present (ABOUD SYRIANI; WONG; ANDANI; DE GUSMAO *et al.*, 2020; BARGHIGIANI; DE MICHELE; TESSA; FICO *et al.*, 2022) . Although the South American cohort had 8 Brazilian ataxia centers involved, the prevalence of *RFC1* -ataxia strictly in the Brazilian territory is unknown (GAMA; BRAGA-NETO; RANGEL; GODEIRO *et al.*, 2022).

1.1.3 X-Linked Cerebellar Ataxias

X-Linked Cerebellar Ataxias (XLCA) are caused by pathogenic variants or genomic imbalances on the X chromosome. These diseases are exceedingly rare, except for Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS). Among other more frequent etiologies are Ataxia with Sideroblastic Anemia (ASAT), X-Linked Mental Retardation Syndrome - Christianson Subtype (MRXSCH), Adrenoleukodystrophy (ADL), and Pelizaeus-Merzbacher Disease. The mode of transmission is maternal, affecting individuals of the male sex (MANTO; GANDINI; FEIL; STRUPP, 2020; WITEK; HAWKINS; HALL, 2021; ZANNI; BERTINI, 2018).

1.1.4 Mitochondrial Ataxias

Mitochondrial Ataxias (MA) are rare causes of cerebellar ataxia resulting from variants in mitochondrial DNA (mtDNA). Among the primary representatives of this group are MERFF (Myoclonic Epilepsy with Ragged Red Fibers), NARP (Neuropathy, Ataxia, and Retinitis Pigmentosa), and Kearns-Sayre Syndrome. The phenotype is typically broad, extending beyond the Central Nervous System, with patients possibly manifesting cardiomyopathy, deafness, retinopathy, short stature, among other symptoms. Transmission is maternal, and it can manifest in both sexes (DA POZZO; CARDAIOLI; MALFATTI; GALLUS *et al.*, 2009; FINSTERER, 2009).

1.1.5 Episodic Ataxias and other types of ataxia

Episodic ataxias (EAs) represent a group of rare hereditary ataxias characterized by episodes of ataxia, often accompanied by symptoms such as vertigo, nausea, vomiting, dysarthria, and diplopia. These episodes typically last from minutes to hours and can occur spontaneously or be triggered by factors like stress, exercise, alcohol, or

caffeine. Several subtypes of EAs exist, with the most common being EA1 and EA2 (JEN; WAN, 2018; WITEK; HAWKINS; HALL, 2021). EA1 is caused by mutations in the KCNA1 gene, which encodes a potassium channel, while EA2 is associated with mutations in the CACNA1A gene, responsible for encoding a calcium channel. The diagnosis of EA is primarily based on clinical findings, with genetic testing serving to confirm the diagnosis. Treatment is primarily symptomatic, and in some cases, 4-aminopyridine and acetazolamide have been found to be effective in reducing the frequency and severity of attacks (FEIL; BREMOVA; MUTH; SCHNIEPP *et al.*, 2016; MUTH; TEUFEL; SCHÖLS; SYNOFZIK *et al.*, 2021).

Numerous other types of ataxias exist, including gluten ataxia, alcohol-induced cerebellar degeneration, ataxia associated with mitochondrial diseases, among others (TEIVE; ASHIZAWA, 2015).The diagnostic workup for these ataxias involves a comprehensive evaluation comprising a detailed medical history, physical examination, and laboratory tests, including genetic testing when deemed appropriate. Treatment strategies are contingent upon the underlying cause and may encompass medications, dietary modifications, and physical therapy (BARSOTTINI; ALBUQUERQUE; BRAGA-NETO; PEDROSO, 2014).

1.2 Molecular Diagnosis in HA - Review of the Literature

Traditionally, the diagnostic investigation of patients with HA relied on genetic testing utilizing the polymerase chain reaction (PCR) technique. This approach is founded on in vitro amplification of specific DNA regions, allowing relatively rapid detection of trinucleotide expansions, which constitute the substrate for the most common hereditary ataxias globally, including those in Brazil. Nonetheless, on average, more than two-thirds of patients remained undiagnosed following screening for the most prevalent mutations within a specific region (GAMA; BRAGA-NETO; RANGEL; GODEIRO *et al.*, 2022; RUANO; MELO; SILVA; COUTINHO, 2014; TEIVE; MEIRA; CAMARGO; MUNHOZ, 2019; TRASCHÜTZ; ADARMES-GOMEZ; ANHEIM; BAETS *et al.*, 2023). Subsequent investigation would necessitate gene-by-gene analysis, a process deemed expensive and time-consuming. Consequently, many patients remained undiagnosed for extended periods, thereby restricting access to genetic counseling and prenatal diagnosis (PYLE; SMERTENKO; BARGIELA; GRIFFIN *et al.*, 2015). Importantly, these studies were conducted at different times,

with varying genetic resources and patient cohorts, potentially introducing selection bias that could either underestimate or overestimate certain results.

Presently, with advancements in molecular genetic techniques, next-generation sequencing (NGS) has emerged as the primary diagnostic tool for cerebellar ataxia in many centers worldwide (REXACH; LEE; MARTINEZ-AGOSTO; NÉMETH *et al.*, 2019). NGS offers an accuracy rate ranging from 30% to 50% in the molecular diagnosis of patients with a broad spectrum of ataxia-related phenotypes (NGO; REXACH; LEE; PETTY *et al.*, 2020; SUN; JOHNSON; NELAKUDITI; GUIDUGLI *et al.*, 2019). This approach has led to the discovery of numerous genetic loci and genes on different chromosomes, facilitating the diagnosis of these patients, the identification of novel ataxia-associated genes, and the expansion of the genotypic and phenotypic landscape of hereditary ataxias (NÉMETH; KWASNIEWSKA; LISE; PAROLIN SCHNEKENBERG *et al.*, 2013). However, it is well-established that younger patients (<25 years) and those with a history of consanguinity have a higher likelihood of receiving a molecular diagnosis (COUTELIER; HAMMER; STEVANIN; MONIN *et al.*, 2018; COUTELIER; STEVANIN; BRICE, 2015).

1.2.1 – Next-Generation Sequencing

In comparison to the Sanger method, which is regarded as the gold standard for genetic sequencing, Next-Generation Sequencing (NGS) has the capability to simultaneously and massively sequence multiple genes, offering a substantial volume of accurate and swift information. In contrast, the Sanger method, while reliable, sequences genes one at a time, resulting in a slower and more costly investigative process (HUI, 2014).

In practical terms, NGS can be employed in three primary modes:

1. Targeted Sequencing Panels (TSP), considered the most cost-effective approach, involve the analysis of a limited number of genes located in coding regions (exons).
2. Whole-Exome Sequencing (WES) entails the analysis of all coding regions of the human genome, encompassing approximately 85% of all pathogenic mutations.
3. Whole-Genome Sequencing (WGS), although the most expensive method, has the capacity to detect mutations in both coding (exons) and non-coding (introns)

regions, as well as variations in copy number (XUE; ANKALA; WILCOX; HEGDE, 2015).

A recent Brazilian study, conducted by Da Graça and colleagues (2022), was conducted in parallel with this study. In this investigation, a cohort of 76 patients with undiagnosed adult-onset cerebellar ataxia underwent WES. The study revealed that WES identified pathogenic and likely pathogenic variants in 35% of the cohort, with variants in *SACS* and *SPG7* being the most frequently observed. Notably, WES yielded more favorable results in patients presenting with spasticity associated with ataxia.

While NGS is now considered the premier diagnostic tool for hereditary ataxias, only WES is more readily available in clinical practice, with WGS still primarily limited to research applications. It is important to note that WES does have certain limitations that merit acknowledgment:

1. Limited effectiveness in diagnosing nucleotide replications, mutations in GC-rich regions, variants in mitochondrial DNA, structural variations of DNA, and mutations in non-coding regions (intronic mutations).
2. Potential for incidental and undesired detection of genetic mutations predisposing to cancer, Alzheimer's, or other degenerative diseases.
3. Generation of a large number of variants, necessitating the sequencing of family members to "filter" variants of uncertain significance, which can reduce specificity and increase the cost of the procedure.
4. Challenges in establishing a genotypic-phenotypic relationship for a new or non-associated variant in the context of ataxia through bioinformatics processing, a task that can be highly complex.
5. Technical issues such as reading errors, coverage gaps, and insufficient depth, with the latter being the most common problem associated with the loss of variant detection, which may compromise results (HUI, 2014; NÉMETH; KWASNIEWSKA; LISE; PAROLIN SCHNEKENBERG *et al.*, 2013; PENA; JIANG; SCHOCH; SPILLMANN *et al.*, 2018; PYLE; SMERTENKO; BARGIELA; GRIFFIN *et al.*, 2015).

However, some of the aforementioned issues have been addressed with the development of new methods and algorithms. An example is the challenge of detecting trinucleotide expanded alleles, which constitute the most prevalent cause of hereditary

ataxias. Recent methods like ExpansionHunter, exSTRa, STRetch, and TREDPARSE can identify expanded alleles using WES and WGS data, offering high accuracy Nevertheless, it is important to note that these methods have not yet been validated for clinical practice and are primarily utilized in a research context (DASHNOW; LEK; PHIPSON; HALMAN *et al.*, 2018; DOLZHENKO; DESHPANDE; SCHLESINGER; KRUSCHE *et al.*, 2019; DOLZHENKO; VAN VUGT; SHAW; BEKRITSKY *et al.*, 2017; TANG; KIRKNESS; LIPPERT; BIGGS *et al.*, 2017; TANKARD; BENNETT; DEGORSKI; DELATYCKI *et al.*, 2018).

The information presented above underscores the challenges frequently encountered in neurological clinical practice when assessing patients with hereditary ataxias. The substantial genotypic and phenotypic heterogeneity in this patient population contributes to a significant diagnostic gap. Consequently, it is of utmost importance to investigate patients without identified mutations after screening for the most prevalent ataxias in Brazil. This approach aims to identify potential alternative genetic etiologies within our territory, along with their distribution, prevalence, and the genotypic-phenotypic correlations. This study seeks to utilize whole exome sequencing with Expansion Hunter and screening for recently described repeat expansions, such as *RFC1*-related ataxia and *FGF14*-related ataxia, to comprehensively assess the genotype spectrum observed in Brazilian patients with unsolved hereditary ataxias.

1.3 Justification of the study

The profound diversity in genotypic and phenotypic characteristics presents a formidable diagnostic hurdle in the realm of cerebellar ataxias for clinical neurologists. Over two-thirds of patients grappling with HA endure prolonged, exhaustive investigations that span years. This protracted quest for answers is exacerbated by the uncertain genetic landscape within Brazilian borders, primarily due to the limited accessibility of NGS technologies in Brazil.

2 - OBJECTIVES

2.1 General Objective

The principal aim of this study is to identify the genetic profiles of Brazilian patients who have undergone comprehensive diagnostic evaluations without successfully determining the molecular etiology.

2.2 Specific objectives

1. Understand the current role in clinical practice of NGS technologies in the diagnosis of HA.

2. Establish the diagnostic yield of WES in the Brazilian population with undiagnosed cerebellar ataxia and correlate these findings with previous national and international studies.

3. Correlate the genotypic findings with the phenotype presented by the patient, potentially expanding the genotypic-phenotypic spectrum of HAs.

4. Explore the utility of Expansion Hunter for the molecular diagnosis of Brazilian patients with undiagnosed cerebellar ataxia.

5. Define the prevalence of recently described diseases, such as *RFC-1*-related ataxia and GAA-*FGF-14*-related ataxia, in the Brazilian population with undiagnosed cerebellar ataxia.

6. Identify the most frequent genes involved in the etiology of undiagnosed cerebellar ataxia ataxia, correlating them with previous national and international studies.

3 – MATERIAL AND METHODS

3.1 Ethical Approval

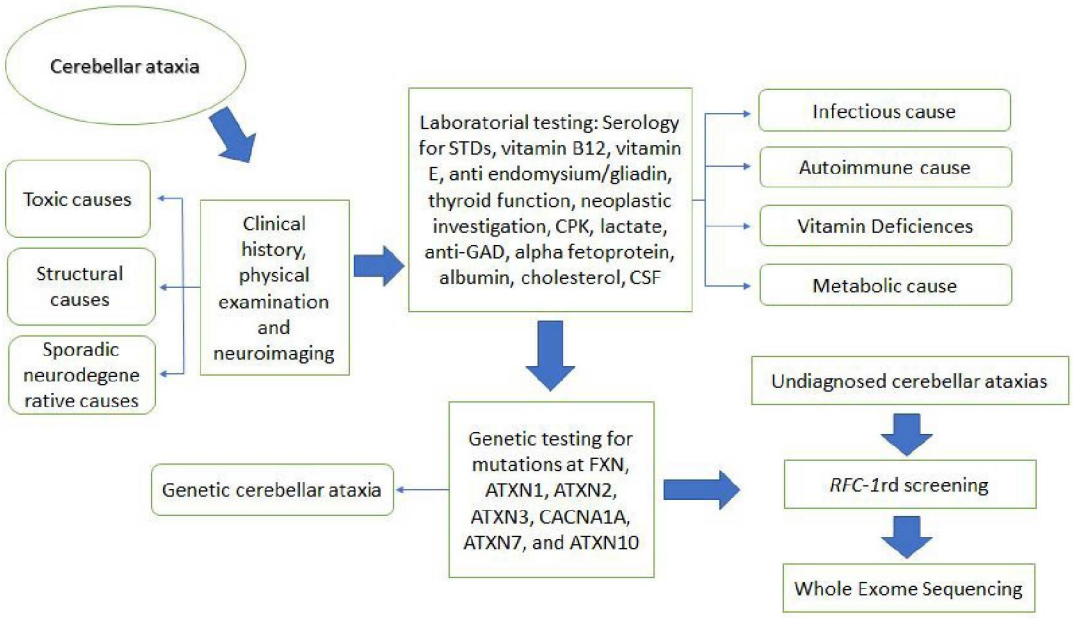
The institutional review board of the Complexo Hospital de Clínicas, Federal University of Paraná (CHC/UFPR) approved this research project (CAAE nº 06128812.0.0000.0096, final amendment; Appendix 1). Furthermore, all patients were thoroughly informed about the study's objectives and implications, and they provided their explicit consent by signing the Informed Consent Form (ICF – Appendix 2).

3.2 Patient recruitment

Biological samples were collected from all patients with sporadic or familial cerebellar ataxia between the period of June 2019 to June 2022. All samples were collected at the university centers involved in this study (Hospital das Clinicas da Universidade Federal do Paraná, Hospital Universitário Pedro Ernesto-RJ and Hospital

Estadual do Amazonas). Patients with progressive cerebellar ataxia without a diagnosis after an extensive investigation for secondary/acquired causes, negative for SCA 1, 2, 3, 6, 7 and 10 panel and genetic testing for Friedreich's ataxia, were included (Figure 1 – retrieved from manuscript 2). The presence of laboratory or imaging exams suggestive of a secondary cause or incomplete clinical and genetic investigation was used as exclusion criteria. Patients of all age groups, with different phenotypes, regardless of family history or the availability of relatives for segregation analysis, were included. In-depth phenotypic data were collected from patients' medical records or, when possible, patient reassessment.

Figure 1: Investigation work-flow for common genetic and secondary causes of cerebellar ataxia



STDs – sexually transmitted diseases ; CPK creatine-phosphokinase; anti-GAD: anti-glutamic acid decarboxylase; CSF: cerebrospinal fluid; *RFC-1*rd – Replication Factor C subunit-1 related disorders; *ATXN*- Ataxin; *CACNA1A*: calcium voltage-gated channel subunit alpha1 A.

3.3 DNA Extraction and Preliminary Genetic Analysis

Genomic DNA was obtained from peripheral blood lymphocytes using the Quick-DNA Miniprep Plus Kit (Zymo Research) performed at a collaborating laboratory located in Curitiba-PR (GENETIKA). Samples with negative results for all

applied tests (figure 1) were referred to The National Hospital for Neurology and Neurosurgery, Queen Square, London, for testing for biallelic expansion of the *RFC1* gene in recessive or sporadic cases, and complete exome sequencing in all samples with negative investigation for *RFC1*. Only patients without diagnosis after WES investigation were selected for GAA-*FGF14* repeat expansion screening.

3.4 Genetic analysis for biallelic expansion of the *RFC1* gene

All patients recruited in this study were submitted for this analysis. Flanking polymerase chain reaction (PCR) and repeat-primed PCRs were performed to detect the pathogenic AAGGG repeat expansion and non-pathogenic AAAGG or AAAAG repeat expansions in *RFC1*, as previously described (CORTESE; SIMONE; SULLIVAN; VANDROVCOVA *et al.*, 2019). Positive cases were defined as samples showing no PCR amplifiable product on flanking PCR, negative repeat primed PCR for (AAAGG)exp and (AAAAG)exp configurations, and the presence of a sawtooth pattern on repeat primed PCR for the pathogenic AAGGG repeat expansion. If enough DNA were available, Southern blotting was performed in positive cases to confirm the presence and measure the size of the AAGGG repeat expansions, according to our previously described protocol.

3.5 NGS raw data collection

WES was performed at Macrogen Inc (Amsterdam, Netherlands). 1ug of DNA were used for sequencing in Illumina's NovaSeq 6000 platform using Agilent SureSelect Human All Exon V6 for library kit, achieving a mean coverage and depth of 50x on target of V6 bait.

3.5.1 Alignment and variant calling

After sequencing, BCL file conversion and demultiplexing was process inside Illumina instrument using the sample sheet file. Demultiplexed data was processed using GATK 4.1.4.0 best practices guideline from FastQ to variant calling (DEPRISTO; BANKS; POPLIN; GARIMELLA *et al.*, 2011). BAM files were aligned against GRCh38-alt-masked reference genome using Burrows-Wheeler Aligner BWA-MEM with indel realignment and duplicate removal (ROBINSON; HAWKINS; SANTANA-

CRUZ; ADKINS *et al.*, 2017). Later, SNVs and indel variants were called and stored in VCF files (Variant Call Format).

3.5.2 Variant annotation and filtering

Variants were annotated with Annovar (v. 2019Oct24) and with some selected databases such as: ClinVar, UniProt and HGMD public for known clinical significance of variants; ABraOM SABE-WGS-1171 (hg38) and gnomAD v2.1.1 for population frequency; Human Phenotype Ontology (HPO), OMIM and UNIPROT diseases for disease information and gene association; and REVEL for pathogenicity score prediction (WANG; LI; HAKONARSON, 2010).

Annotated variants were separated in 4 different Excel spreadsheets: exonic selected genes variants; intronic selected genes variants; ACMG 73 genes variants; all other exonic and intronic variants.

We have selected a list of 441 genes for analysis, based on Sun *et al* (2019), which provided a molecular diagnosis rate of 52%.

Variant filtering started in the exonic selected gene spreadsheet. If there was an exonic variant in a recessive gene without a second hit, the same gene was filtered in the intronic selected gene spreadsheet. If there was no suspected variant (VUS or pathogenic) in the main list, a more intensive search was performed in the general variant spreadsheet.

This second search in the general list prioritized variants according to following arguments: Alle Frequency (AF) > 0.3; it was a rare (<=1 %) PVS1 type variant; it was a rare (<=0.1%) SNVs or indel variant. All variants without OMIM, UniProt or The Human Phenotype Ontology (HPO) disease association or located in genes that did not match the phenotype were discarded from classification analysis. If a recessive variant was found in this list, all filters were removed in the aim to find the second hit. Other online tools such as DECIPHER and STRING were used to help manual prioritization of variants (FIRTH; RICHARDS; BEVAN; CLAYTON *et al.*, 2009; SZKLARCZYK; GABLE; LYON; JUNGE *et al.*, 2019).

3.5.3 ExpansionHunter analysis

We used Expansion Hunter (v5.0.0) to find expansions of sequence repeats (DOLZHENKO; VAN VUGT; SHAW; BEKRITSKY *et al.*, 2017). Briefly, we used patients exome BAM files that were mapped to human reference genome hg38, along with the variant catalog of genes of interest. Output VCF files, containing lengths of repeat units, were used to filter for variants that pass the normal or pathogenic thresholds of repeat units (Supplementary Table 1). REViewer v0.2.7 was applied to visualize aligned reads in tandem repeat regions (DOLZHENKO; DESHPANDE; SCHLESINGER; KRUSCHE *et al.*, 2019). We did not add *ATXN8* in our analysis since it was not covered in our exome sequence data.

3.5.4 Variant classification

Suspected variants were classified using ClinGen Variant Curation SOP Version 2.0 (January 2021) recommendations with some rule's adjustments following the examples listed in the guide (CLINGEN, 2022). Furthermore, the rules PS3 following Odds Path and PP4 for functional evidence from patient-derived material was used following its specific guidelines (BRNICH; ABOU TAYOUN; COUCH; CUTTING *et al.*, 2019).

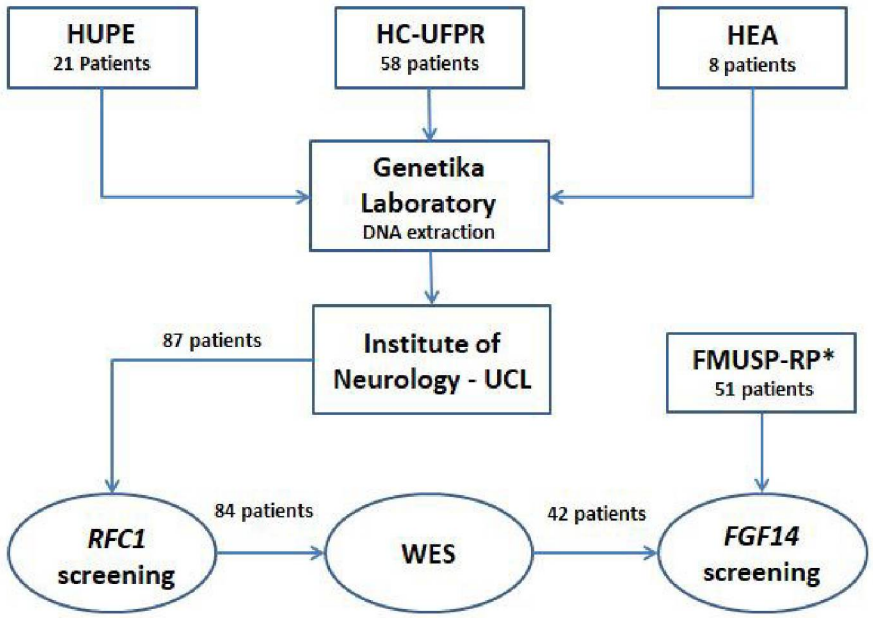
We have adopted a structured approach to variant classification and diagnosis. Definitive diagnoses will exclusively comprise variants with a pathogenic classification. Conversely, variants categorized as likely pathogenic will be designated as candidates or probable diagnoses. For patients harboring compound heterozygous variants, in which at least one variant carries an uncertain classification (referred to as a Variant of Uncertain Significance or VUS), their diagnostic status will remain uncertain. In the context of repeat expansion disorders, we establish that threshold for a definitive diagnosis will be met only when the repeat expansion size reaches full penetrance. Conversely, repeat expansions falling within the spectrum of incomplete penetrance will be considered as possible diagnoses.

3.6 Genetic screening for GAA-*FGF14*-related ataxia

3.6.1 Patient recruitment for GAA-*FGF14*-related ataxia screening

Ninety-three index patients were recruited for this analysis. Forty-two patients from our original cohort without a definitive or probable diagnosis after investigation with WES, Expansion Hunter and *RFC1* biallelic repeat expansion screening were selected (6 patients from Rio de Janeiro, 4 patients from Amazonas, 32 patients from Curitiba). Fifty-one patients from a different collaborator ataxia center (Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto, São Paulo, Brazil) were added to our cohort for this analysis, as part of our collaborative research, to increase the chance of identify this particular mutation in Brazilian population and establish its prevalence. The patients added to the original cohort presented with unsolved neurodegenerative ataxia with onset after the age of 20 years. All patients had previously undergone comprehensive investigation to exclude acquired and genetic causes, which included screening for SCA1, SCA2, SCA3, SCA6, SCA7, SCA10, *RFC1*-related ataxia, and Friedreich ataxia. WES was not performed in the 51 patients added to this study. The genetic testing workflow and patients included on this thesis are summarized in Figure 2.

Figure 2 – Genetic Testing workflow and Patient Enrolled in This Thesis



HUPE:Hospital Universitário Pedro Ernesto; HC-UFPR: Hospital das Clínicas da Universidade Federal do Paraná; HEA: Hospital Estadual do Amazonas; UCL: University College of London; FMUSP-RP:

Faculdade de Medicina da Universidade de São Paulo – Ribeirão-Preto; *RFC1*: Replication Factor Complex subunit 1; WES: Whole Exome Sequencing; *FGF14*: Fibroblast Growth Factor 14.

*Patients from FMUSP-RP were included as part of a collaborative effort to identify *FGF14* mutation in Brazilian population

3.6.2 Genetic analysis for GAA-*FGF-14* repeat expansion

The *FGF14* repeat locus was genotyped in a three-step approach described previously (BONNET; PELLERIN; ROTH; CLÉMENT *et al.*, 2023). In the first step, the intronic *FGF14* GAA repeat locus was amplified by fluorescent long-range PCR (fLR-PCR). Patients with expanded alleles were submitted to a bidirectional RP-PCR to ascertain the presence of GAA expansion at the repeat locus. The presence of characteristic saw-toothed products indicated the presence of a GAA repeat expansion at the *FGF14* repeat locus. Expansions of at least 250 GAA repeat units were considered pathogenic (PELLERIN; DANZI; WILKE; RENAUD *et al.*, 2023; RAFEHI; READ; SZMULEWICZ; DAVIES *et al.*, 2023). For samples with inconclusive results, a gel electrophoresis of long-range PCR products and Sanger sequencing was performed to determine the sequence and repeat motif of the *FGF14* locus.

3.7 Statistical Analysis

All analyses were conducted using the statistical package IBM SPSS Statistics, version 28.0. Data are expressed as absolute frequency and percentage for categorical variables. Quantitative variables are described as mean, standard deviation or median, minimum, and maximum depending on their distribution. Shapiro-Wilk test was used to assess the normality of continuous variables. For group comparisons of quantitative variables, the nonparametric Kruskal-Wallis and post-hoc Dunn test were used. Fisher's exact test and Pearson's chi-square test were used to evaluate categorical variables. Statistical significance was defined as a p-value < 0.05. Bonferroni adjustment was used for correction due to multiple comparisons.

4 - RESULTS AND DISCUSSION

4.1. Scientific article format

In this thesis, we have chosen to present our results and discussion in the format of three scientific articles. In the first manuscript, we conducted a literature review focusing on next-generation sequencing technologies, their advantages, disadvantages, and their application in the context of autosomal dominant cerebellar ataxias, which are the most prevalent in our population (NASCIMENTO; RODRIGUES; PELLOSO; CAMARGO *et al.*, 2019; TEIVE; MEIRA; CAMARGO; MUNHOZ, 2019). The primary objective of this initial manuscript is to comprehend and substantiate the rationale behind employing WES in this specific patient cohort.

In the second manuscript, we selected 87 patients from three different ataxia centers in Brazil (Hospital da Clínicas da Universidade Federal do Paraná in Curitiba-PR, Hospital Universitário Pedro Ernesto in Rio de Janeiro-RJ, and Hospital Estadual do Amazonas in Amazonas-AM). All patients underwent a comprehensive investigation to ascertain the cause of cerebellar ataxia, with secondary and more prevalent genetic causes ruled out (Figure 1 of manuscript 2). These patients underwent additional genetic investigation, including screening for *RFC1*-ataxia and WES with ExpansionHunter. The aim of this study is to unravel the genotype of this specific patient population and validate the utility of this tool in the Brazilian population with undiagnosed cerebellar ataxia.

In the third manuscript, we continued the etiological investigation of cerebellar ataxia in patients where no variants were initially identified (42 patients of the manuscript 2). These individuals underwent a reassessment for intronic GAA repeat expansion in the *FGF14* gene (also known as spinocerebellar ataxia type 27B, or SCA27B), recently recognized as a common cause of late-onset cerebellar ataxia. We added 51 patients from another Brazilian ataxia center (Clinical Hospital of the Faculty of Medicine of Ribeirão Preto, São Paulo, Brazil) to this cohort to understand the prevalence of this novel mutation in a large cohort of undiagnosed cerebellar ataxia patients. All the added patients had previously undergone an extensive etiological investigation, as described in Figure 1.

4.2- Manuscript 1

Title:

Evidence and practices of the use of next generation sequencing in patients with undiagnosed autosomal dominant cerebellar ataxia: a review.

Evidências e práticas do uso do sequenciamento de nova geração em pacientes com ataxia cerebelar autossômica dominante não diagnosticada: uma revisão.

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Conflict of interest: There is no conflict of interest to declare.

ABSTRACT:

Autosomal dominant cerebellar ataxias (ADCA) are heterogeneous diseases with a highly variable phenotype and genotype. They can be divided into Episodic Ataxia and Spinocerebellar Ataxia (SCA), the latter is considered the prototype of the ADCA. Most of the ADCA are caused by polyglutamines expansions, mainly SCA 1, 2, 3, 6, 7, 17 and DRPLA. However 30% of patients remain undiagnosed after testing for these most common SCA. Recently, several studies have been demonstrating that the new generation of sequencing methods are useful tools for diagnosing these patients. This review focus on searching the evidence on the literature, its usefulness in clinical practice and future perspectives.

Keywords: next generation sequencing, autosomal dominant cerebellar ataxias, spinocerebellar ataxias.

RESUMO:

As ataxias cerebelares autossômicas dominantes (ADCA) são doenças heterogêneas com fenótipo e genótipo altamente variáveis. Eles podem ser divididos em Ataxia Episódica e Ataxia Espinocerebelar (SCA), sendo este último considerado o protótipo do ADCA. A maior parte do ADCA é causada por expansões de poliglutaminas, principalmente SCA 1, 2, 3, 6, 7, 17 e DRPLA. No entanto, 30% dos pacientes permanecem sem diagnóstico após o teste para essas SCA mais comuns. Recentemente, vários estudos têm demonstrado que a nova geração de métodos de seqüenciamento são ferramentas úteis para o diagnóstico desses pacientes. Esta revisão se concentra na revisão sistemática da literatura, na sua utilidade na prática clínica e em perspectivas futuras.

Palavras-chave: sequenciamento de nova geração, ataxias cerebelares autossômicas dominantes, ataxias espinocerebelares.

4.2.1-Introduction

Autosomal dominant cerebellar ataxias (ADCA) comprise a group of inherited cerebellar ataxias that are clinically and genetically heterogeneous (DURR, 2010). They can be caused by several mechanisms, such as expansion of Short Tandem Repeats (STR), mainly trinucleotide repeat expansions (TRE), and less commonly, single nucleotide variation and short insertions and deletions (indels); The most studied mechanism related to TRE is the one caused by expanded polyglutamines. These proteins have a pathological gain of function with a subsequent neuronal toxic effect. In these cases there may be an anticipation phenomenon, which is characterized by increasingly early onset of symptoms as the disease is transmitted from one generation to the next. Clinically ADCA are progressive neurodegenerative diseases that share cerebellar ataxia as the core symptom, associated with progressive cerebellar atrophy. However, other brain regions, such as the brainstem, basal ganglia, spinal cord, and peripheral nerves, may also be involved. Within this group, we will herein emphasize

the spinocerebellar ataxias (SCA), often used as a synonym for autosomal dominant ataxias.

The SCA has a wide range of neurological symptoms, including gait and appendicular ataxia, dysarthria, oculomotor abnormalities of cerebellar and supranuclear origin, retinopathy, optic atrophy, spasticity, extrapyramidal, peripheral neuropathy, sphincter disorders, cognitive changes, and epilepsy (DURR, 2010; SCHÖLS; AMOIRIDIS; BÜTTNER; PRZUNTEK *et al.*, 1997; SCHÖLS; BAUER; SCHMIDT; SCHULTE *et al.*, 2004). Clinical diagnosis is challenging due to large phenotypic and genotypic variability. To facilitate clinical evaluation, Harding et al. suggested a classification into 3 subtypes: ADCA type 1, characterized by cerebellar ataxia, optic atrophy, ophthalmoplegia, extrapyramidal symptoms, pyramidal signs, peripheral neuropathy, amyotrophy and dementia; ADCA type 2, when CA is associated with retinal degeneration; ADCA type 3, composed of "pure" cerebellar ataxias (HARDING, 1982) [**table 1**]. Currently the classification of SCA is based on the identified mutation/expansion, also known as clinical-genetic classification (JUVONEN; HIETALA; KAIRISTO; SAVONTAUS, 2005). Forty eight SCA subtypes have been described to date (GENIS; ORTEGA-CUBERO; SAN NICOLÁS; CORRAL *et al.*, 2018; GENNARINO; PALMER; MCDONELL; WANG *et al.*, 2018; SULLIVAN; YAU; O'CONNOR; HOULDEN, 2019) and this number tends to grow in the following years, thanks to the availability of new DNA sequencing techniques. **Table 2**, adapted from a review from Sullivan et al. (2019) , shows the main clinical characteristics of each SCA subtype.

In this review, we will address the challenges in the diagnosis of spinocerebellar ataxias, the recent diagnostic tools that are helpful when we have a patient with negative DNA test (herein called "negative ataxias") and future perspectives in the field.

4.2.2. Epidemiological context

The prevalence of hereditary ataxias in general has been little studied. A meta-analysis by Ruano et al. (2014), which included 22 studies from 16 countries with more than 14500 patients, showed that the average prevalence of ADCA is 2.7/100,000. However, it is worth mentioning that this prevalence is variable in different regions. International studies have been conducted to assess the prevalence of ADCA around the

world. A prevalence of 3/100,000 cases was found in the Netherlands, 4.2/100,000 in Southern Norway and 5.6/100,000 in Portugal (COUTINHO; RUANO; LOUREIRO; CRUZ et al., 2013; ERICHSEN; KOHT; STRAY-PEDERSEN; ABDELNOOR et al., 2009; VAN DE WARREMBURG; SINKE; VERSCHUUREN-BEMELMANS, 2003). In all studies spinocerebellar ataxia type 3 (SCA3 or Machado-Joseph disease) is the most commonly mutation found.

In Brazil it is believed that the great epidemiological variability is due to the founder effect of different geographical regions (ALONSO; MARTÍNEZ-RUANO; DE BIASE; MADER *et al.*, 2007; BRUSCO; GELLERA; CAGNOLI; SALUTO *et al.*, 2004; COUTINHO; RUANO; LOUREIRO; CRUZ *et al.*, 2013; STOREY; DU SART; SHAW; LORENTZOS *et al.*, 2000; VELÁZQUEZ-PÉREZ; SANTOS; GARCÍA; PANEQUE *et al.*, 2001). Worldwide several epidemiological studies have demonstrated a higher frequency of SCA 1 in countries such as Italy and India; SCA 2 in Mexico, Cuba, India and Canada; SCA 6 in Australia and Canada; SCA 8 in Finland, and DRPLA in Japan. In Brazil, the main subtype found is SCA3, and cases of SCA 1, 2, 6, 7 and 10 occur less frequently; other types are considered to be very rare. Jardim *et al.* (2001) conducted a research on ADCA in Southern Brazil evaluating 66 cases of SCA. The authors concluded that the proportion of cases of SCA 3 was very high, suggesting an Azorean founding effect. The frequency of SCA 3 in the region was 1.8/100,000, versus 0.2/100,000 for other forms of autosomal dominant ataxia. Cintra *et al.* (2014) found an even higher prevalence in the region of São Paulo, 5/100,000, considered to date the highest prevalence of SCA 3 found in Brazil. In a study with 104 families with SCA, Teive *et al.* (TEIVE; MUNHOZ; ARRUDA; LOPES-CENDES *et al.*, 2012) found a high prevalence for SCA 3 (72.46%) followed by SCA 10 (11.6%). Also, a more recent study from the same group investigated 213 families from Southern region of Brazil, and once again found that the most frequent subtype was SCA3 (45.7%), followed by SCA10 (18.3%) and Other SCAs (13.3%). Approximately 23% of all patients had negative genetic testing for the most common SCAs (NASCIMENTO; RODRIGUES; PELLOSO; CAMARGO *et al.*, 2019). **Table 3** shows the frequencies of SCA in the Brazilian territory.

4.2.3. Molecular Diagnostic challenges in ADCA

The diagnostic investigation of patients with ADCA involves PCR technique (polymerase chain reaction), and is based on the in vitro amplification of specific regions of the DNA, allowing the detection of nucleotide expansions, which are the substrate for the most common ADCA worldwide (BRUSCO; GELLERA; CAGNOLI; SALUTO *et al.*, 2004; DE CASTILHOS; FURTADO; GHENO; SCHAEFFER *et al.*, 2014; RUANO; MELO; SILVA; COUTINHO, 2014; VAN DE WARREMBURG; SINKE; VERSCHUUREN-BEMELMANS, 2003). Approximately 30% of the patients investigated for ADCA by the conventional method (PCR) have negative results.
The absence of a diagnosis can be very frustrating for both the patient and the physician. Obtaining a diagnosis can be an important factor of psychological impact, prognosis, genetic counseling and family diagnosis. In addition, it may be essential for the development of specific treatments based on a better understanding of the mutation (SUBRAMONY; FILLA, 2001).

When initial DNA investigation fails, the next step would be to conduct a gene-to-gene search, which is considered a time-consuming and expensive method. But nowadays, with the fantastic advance in the development of molecular genetic techniques, with next-generation sequencing (NGS) technology, it is possible to carry out sequencing of several genes simultaneously, saving time and costs. Each NGS technique has its advantages and disadvantages, which must be weighed to choose the ideal method for the diagnosis.

4.2.4. Genetic technology evolution: solution or additional problems?

In 1977, Frederick Sanger and colleagues developed a DNA sequencing method based on chain-termination inhibitors. In this method, a DNA template is replicated using a primer and a DNA polymerase that incorporates dideoxynucleotides in the sequence synthesis, causing its early termination. After multiple reactions, DNA fragments of different lengths are formed and can be read by an automated apparatus, providing DNA sequencing (SANGER; NICKLEN; COULSON, 1977).

Compared to Sanger sequencing, considered a gold standard for genetic sequencing, NGS is capable of sequencing several genes (or DNA templates) simultaneously, providing a large amount of information in an accurate and fast way, whereas Sanger sequencing, despite reliable, can sequence only one gene at a time, making investigation time-consuming and costly (HUI, 2014).

When one suspects of SCA and performs a DNA test, such as WES, about 64% of the diagnoses made by this method are from mutations traditionally known to be responsible for causing hereditary ataxias, while 30% are from newly discovered genes and 6% from genes that were not typically considered to cause ataxia (GALATOLO; TESSA; FILLA; SANTORELLI, 2018).

In practical terms, NGS can be employed in three ways: (1) targeted sequencing panels (TSP), considered the most cost-effective approach, involving the analysis of a restricted number of genes in coding regions (exons); (2) whole exome sequencing (WES), where there is analysis of all coding regions of the human genome, site of about 85% of all pathogenic variants; and (3) whole genome sequencing (WGS), considered to be the most expensive method yet capable of detecting mutations in coding (exons) and noncoding (introns) regions, as well as copy number variations (CNV) (HUI, 2014; XUE; ANKALA; WILCOX; HEGDE, 2015).

The excess information provided by these methods can also be a trap. Sometimes variants detected in WES may not necessarily be related to the patient's disease, representing incidental and/or non-specific findings. The latter, also known as variants of uncertain significance – VUS, represent variants of a gene found in genetic testing without a known functional or health consequence to the proband. The former represents pathogenic mutations related to other diseases not related to the investigated ataxia, such as the identification of a mutation in the BRCA gene 1 related to breast and ovarian cancer, but not to ataxia. In these cases, It is important to explain to patients and obtain a consent form, before starting the genetic test, on the possible risks associated with the incidental findings of genes predisposing to other potentially serious diseases (BAMSHAD; NG; BIGHAM; TABOR *et al.*, 2011; MARELLI; GUISSART; HUBSCH; RENAUD *et al.*, 2016). In this context, it is important to highlight the need of gathering clinical data in order to determine the most likely types of SCA to be

investigated in a specific patient (JUVONEN; HIETALA; KAIRISTO; SAVONTAUS, 2005).

Other limitations of WES are: failure to effectively identify nucleotide repeat expansions, as well as mutations in GC-rich regions, mitochondrial DNA variants and copy number variations (CNV). They are also subject to sequential reading errors and technical problems such as insufficient depth and coverage (HUI, 2014).
Thus the current recommendations are to search for nucleotide repeat expansions most associated with SCA by the PCR technique initially, taking into account the phenotype and epidemiological contexts. However, after ruling out this as a cause, another 70 genes associated with different forms of ataxias may be involved (BAMSHAD; NG; BIGHAM; TABOR *et al.*, 2011). Therefore, if the initial results are negative, alternative methods for diagnosis, such as NGS, should be considered.

4.2.5. The Use of Next Generation Sequencing: Evidence of Literature

Recent studies have shown encouraging results of the use of NGS to confirm diagnosis in patients with hereditary ataxia. Pyle *et al.* (2015) found pathogenic variants in 41% of patients without diagnosis in 22 families, using the WES method. Efficacy was similar between patients with early onset (<20 years) and late onset (> 20 years). Although there was criticism of this study (VAN DE WARRENBURG; SCHOUTEN; DE BOT; VERMEER *et al.*, 2016), it revealed the potential impact of WES in patients with hereditary ataxias at any age.

In contrast, Nemeth *et al.* (2013) showed that the TSP method identified 18% of cases in a similar cohort. Larger sequencing of the genome is the likely explanation for the superior results of the study by Pyle *et al.* (2015), since it allowed the detection of mutations in genes that, although known to cause ataxia, are not considered "ataxia genes" and therefore not usually included in the gene panels.
Meanwhile, Fogel *et al.* (2014) used WES and identified a percentage similar to that found from Nemeth, with 21% of cases identified (16/76) in patients with late-onset cerebellar ataxia, predominantly sporadic.

In a study with 412 patients with a negative molecular diagnosis of ataxia, Coutelier *et al.* (2017) performed TSP combined with PCR, finding relevant genetic variants in 14.3% of the cases. The same group carried out another study with 319 patients with cerebellar ataxia, with no history compatible with autosomal dominant pattern and undiagnosed, using WES (COUTELIER; HAMMER; STEVANIN; MONIN *et al.*, 2018). Relevant genetic variants were identified in 28.5% of the cases (22.6% with definitive diagnosis and 6% with a possible pathogenic variant). In this cohort, younger patients (<25 years) with a history of consanguinity were associated with better chances of diagnosis, which had been previously demonstrated (IQBAL; RYDNING; WEDDING; KOHT *et al.*, 2017; OHBA; OSAKA; IAI; YAMASHITA *et al.*, 2013; SAWYER; SCHWARTZENTRUBER; BEAULIEU; DYMENT *et al.*, 2014).

These studies have demonstrated that NGS technologies play a crucial role in the diagnosis confirmation of ADCA, leading not only to a decrease in the time of diagnosis, but also in the correlation of the genotypic-phenotypic spectrum, a source of discovery of new genes that cause ataxia, whether unpublished (WANG; YANG; XIA; HU *et al.*, 2010) or not previously associated with ataxia (VAN DE WARRENBURG; SCHOUTEN; DE BOT; VERMEER *et al.*, 2016). It is important to point out that the studies have heterogeneous populations, and the comparison between them may be statistically inappropriate.

4.2.6. Strengths and Pitfalls of Whole exome sequencing and Target sequencing panel

Target Sequencing Panels (TSP) are considered a faster and cheaper method when compared to WES, the former representing a useful tool to test for mutations outside the exons, decreasing VUS and incidental findings, which are important limitations of WES. In addition, it provides more concise information, which can be complemented with confirmatory methods, such as Sanger's sequencing, which fills any data gaps unread by TSP (HUI, 2014). The great limitation of TSP method is the need to formulate a genetic panel compatible with the phenotype and family history presented by the patient, which depends exclusively on previously reported clinical findings for the selection of genes, which may allow the escape of more rare genes, linked to atypical presentations or new mutations (FOGEL; LEE; DEIGNAN; STROM *et al.*, 2014). In addition, new TSP designs are needed as new genes are described.

On the other hand, in WES there is a broad genetic evaluation, without the need for previous clinical information. This allows the discovery of novel genotypic-phenotypic associations, extension of the phenotypic spectrum of a particular gene or recognition of very rare diseases or new mutations (VAN DE WARRENBURG; SCHOUTEN; DE BOT; VERMEER *et al.*, 2016). In addition, it can detect about 100-fold more genes compared to the mean detected by diagnostic panels (100-200 genes). It is an excellent tool for patients with hereditary ataxia, considering the great phenotypic and genotype heterogeneity of these patients (HADJIVASSILIOU; MARTINDALE; SHANMUGARAJAH; GRÜNEWALD *et al.*, 2017). Another advantage of WES is the possibility of reanalysis of the previously obtained data, as new genes are discovered and disseminated in the scientific community, enabling a retrospective diagnosis , reducing time and cost compared to TSP.

Among the problems related to WES are: (1) poorly effective for the diagnosis of nucleotide replications, mutations in GC-rich regions, variants in mitochondrial DNA, structural variations of DNA, mutations in non-coding regions (intronic mutations) ; (2) incidental and undesired finding of genetic mutations predisposing to cancer, Alzheimer's or other degenerative diseases; (3) generation of large number of variants, which requires the sequencing of family members to "filter" variants of uncertain meaning, reducing specificity and increasing the cost of the procedure; (4) to establish a genotypic-phenotypic relationship of a new or non-associated variant prior to ataxia through bioinformatics processing, which may be highly complex; (5) technical problems, such as reading errors, coverage and insufficient depth - the most commonly problem associated with loss of variant detection - may compromise results (HUI, 2014; NÉMETH; KWASNIEWSKA; LISE; PAROLIN SCHNEKENBERG *et al.*, 2013; PENA; JIANG; SCHOCH; SPILLMANN *et al.*, 2018; PYLE; SMERTENKO; BARGIELA; GRIFFIN *et al.*, 2015).

Whole Genome Sequencing (WGS) is a method that was restricted to research centers, however, now it becomes each day more used in the routine of genetic laboratories worldwide. It is known that WGS has a much broader coverage of coding regions compared to WES, as well as covering non-coding regions. However, the amount of information generated may require a lot of time for analysis, considered

highly complex, and the cost is much higher than WES. Also, WGS have the same limitation of WES and TPS in detecting repeat expansions (LELIEVELD; SPIELMANN; MUNDLOS; VELTMAN *et al.*, 2015). **Table 4** summarizes the main advantages and disadvantages of the NGS methods.

It is important to note that although some studies points WGS to be a cost-effective approach (SAWYER; SCHWARTZENTRUBER; BEAULIEU; DYMENT *et al.*, 2014), it is still an expensive and unavailable method for most patients in Brazil.

4.2.7. Short Tandem Repeat Expansions and NGS: Solutions

As mentioned throughout the text, NGS methods are not suitable for STR identification. This is due to the fact that currently available methods perform short readings (about 150bp per reading) and the STR expansions responsible for SCA, with few exceptions, usually have expansions that go beyond this limit.

In order to solve this problem, in recent years analysis methods have been developed, such as ExpansionHunter, exSTRa, STRetch and TREDPARSE, which applied together with NGS, are capable of detecting STR expansions where the expanded allele size is greater than the length of standard short-read sequencing reads (DASHNOW; LEK; PHIPSON; HALMAN *et al.*, 2018; DOLZHENKO; VAN VUGT; SHAW; BEKRITSKY *et al.*, 2017; TANG; KIRKNESS; LIPPERT; BIGGS *et al.*, 2017; TANKARD; BENNETT; DEGORSKI; DELATYCKI *et al.*, 2018).

Previously there were other detection methods for STR, such as HipSTR and LobSTR, but both have the limitation of detecting only STR alleles with repeat lengths smaller than the read length employed in the sequencing. All methods except exSTRa perform better when are applied to a WGS platform, preferably PCR free, where library preparation protocols yield the best data to allow repeat expansion detection, although platforms such as WES provide enough data to detect expansions in STR loci.
Dashnow *et al* (2018) demonstrated the use of STRetch in four patients without diagnosis after screening for most common expansions (SCA1-3, SCA 6, SCA 12, SCA 17 and DRPLA) and use of WGS screening for SNV. STRetch was capable of identify a SCA 8 expansion in one patient, confirmed by PCR. Tankard *et al* (2018), compared all

four methods in different NGS platforms for detection of expansions of tandem repeat, and showed that all of them performed with a good sensitivity and specificity (> 87% and >97%, respectively, when the methods were applied with WGS PCR free platform) and none of them were better than the other, suggesting that the use of all existing methods could be advantageous, improving the accuracy of the results (DE LEEUW; GARNIER; KROON; HORLINGS *et al.*, 2019). Each of these alternative methods has its own technical advantages and disadvantages that goes beyond the scope of this review and must be seen elsewhere.

Although these techniques can detect novel repeat expansions, all of them rely on a priori knowledge of STR loci to be examined, that can be assembled by using annotation of STRs from Tandem Repeats Finder results. Hence, de novo mutation can't be detected by these techniques yet. Furthermore, some STR loci are poorly captured due to their extreme GC content, such as repeat expansions alleles underlying FRAXA (FMR1), FRAXE (FRM2) and FTDALS1 (C9orf72). Despite these limitations, several authors recommend the implementation of these methods in routine screening with NGS (BRAGA-NETO; PEDROSO; FURTADO; GHENO *et al.*, 2017).

It's important to remember that gold-standard techniques for diagnosis of STR expansion, such as Southern blots and TP-PCR (Tripled Primed PCR) shouldn't be abandoned. The new NGS techniques are considered screening methods, requiring validation with gold-standard methods. Southern Blot or TP-PCR are still the most accurate method for detecting STR expansions and the size of the expanded allele, including whether there are interruptions, which has prognostic implications for age of onset, disease progression and outcome (BRAGA-NETO; PEDROSO; FURTADO; GHENO *et al.*, 2017; DE LEEUW; GARNIER; KROON; HORLINGS *et al.*, 2019).

4.2.8. Long read sequencing: A future not so distant

Sometimes even after extensive investigation with NGS short-read technologies the diagnose remains unknown. This is particularly true in cases with complex expanded alleles (BRAGA-NETO; PEDROSO; FURTADO; GHENO *et al.*, 2017; LOPES-CENDES; TEIVE; CALCAGNOTTO; DA COSTA *et al.*, 1997), where the repeat may be interrupted multiple times. In this case, long read sequencing could be useful. These technologies, such as PacBio and Nanopore sequencing are gaining notoriety and

interest by bioinformatics. Readings can reach tens of thousands in comparison with few hundreds in short-readings NGS. Rather than estimating an STR expansion, the LRS will capture the entire expanded allele in a read fragment, providing more accurate information about that expansion. While encouraging, LRS is still considered very expensive (about 10x more compared to conventional NGS methods) and is therefore not cost effective for routine use (DE LEEUW; GARNIER; KROON; HORLINGS *et al.*, 2019). However, this should change soon when LRS will be a valuable tool for the diagnosis of Mendelian diseases such as ADCA.

4.2.9. Conclusion

Hereditary ataxias are a complex group of diseases from a clinical and genetic point of view. About 30% of patients with ADHA remain undiagnosed after an initial investigation into the most common gene variants. Guidelines for the investigation of SCA recommend that the initial investigation be done according to the phenotypic characteristics and family history, which may favor one type of SCA compared to others (DE SILVA; GREENFIELD; COOK; BONNEY *et al.*, 2019; GASSER; FINSTERER; BAETS; VAN BROECKHOVEN *et al.*, 2010).

Although there are limitations, studies have shown that the use of NGS may be useful in the investigation of patients with undiagnosed ataxias. The most common mutations related to SCA are due to the expansion of nucleotides, which is a limiting factor in NGS technologies. However, in the context of a negative molecular diagnosis of ataxias, several other molecular variants such as deletions, missense, nonsense and splice mutations (SCA 5, 11, 13, 14, 15/16 and 27), mutations in non-coding regions (SCA 8,10 and 12) or mutations associated with other diseases such as spastic paraplegia, recessive ataxia, and channelopathy, may be responsible. In these cases, and NGS have proven effective in accelerating the diagnostic process.

Furthermore, new techniques for detections of STR expansions with NGS, such as exSTRa, STRetch, ExpansionHunter and TREDPARSE, are proving to be valuable tools in diagnosing STR related diseases, which includes SCA (BAHLO; BENNETT; DEGORSKI; TANKARD *et al.*, 2018; DE LEEUW; GARNIER; KROON; HORLINGS *et al.*, 2019). Long read sequencing it's another promising diagnostic

method for mendelian diseases, but it's not widely available and it's too expensive for routine use in clinical practice. The unbridled evolution of neurogenetic research may answer many current questions soon enough.

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Table 1. Autossomal dominant cerebellar ataxia clinical classification.

ADCA 1	SCA 1-4, 8, 12-14, 15, 17-22, 25, 27, 28, 31, 32, 34-37, 38, 42-44, 46-48, DRPLA, DNMT1
ADCA 2	SCA 7
ADCA 3	SCA 5, 6, 10, 11, 23, 26, 30, 37, 41, 45

Source: adapted from Sullivan et al.[6]

ADCA: autossomal dominant cerebellar ataxia; SCA: spinocerebellar ataxia; DRPLA: dentatorubral-pallidoluysian atrophy; DNMT1: DNA methyltransferase.

Table 2. Phenotype characteristics of each spinocerebellar ataxia.

Associated clinical features	Genetic subtypes
Peripheral neuropathy	1, 2, 3, 4, 18, 25, 38, 43, 46
Pyramidal signs	1, 3, 7, 8, 10, 14, 15, 17, 35, 40, 43
Dystonia	3, 14, 17, 20, 35
Myoclonus	14
Parkinsonism	2, 3, 10, 14, 17, 19/22, 21
Tremor	12, 15, 27
Chorea	17, 27, DRPLA*
Cognitive impairment	2, 8, 13, 17, 19/22, 21, 36, 44, 48, DRPLA
Psychiatric symptoms	2, 17, 48
Ophthalmoplegia	2, 3, 28, 40
Visual impairment	7
Face/tongue fasciculation	36
Ichthyosiform plaques	34
Seizures	10, 19/22, ATN 1**
Narcolepsy	DNMT1***
Hearing loss	31, 36, DNMT1

Source: adapted from Sullivan et al.[6]

*dentatorubral-pallidoluysian atrophy; **atrophin-1; ***DNA methyltransferase.

Table 3. Prevalence of spinocerebellar ataxias across the world.

Country	n	SCA1	SCA2	SCA3	SCA6	SCA7	SCA8	SCA 10	SCA 12	SCA14	SCA 17	DRPLA	und.	References
Mexico	108	ND	45,4	12	ND	7,4	ND	13,9	ND	NR	2,8	ND	18	Alonso et al.[15]
Portugal	199	ND	2,5	80,5	<1	1,25	1	ND	ND	<1	<1	8,5	26,5*	Coutinho et al.[12]
Cuba	177	ND	86,8	1,2	ND	ND	NP	NP	NR	NP	ND	ND	12	Velázquez et al. 2009[16]
Italy	225	21	24	<1	<1	<1	<1	ND	ND	NP	<1	<1	41	Brusco et al.[14]
Australia	88	16	6	12	17	2	NP	NP	NR	NP	NP	ND	41	Storey et al.[13]
China	85	4,7	5,9	48,2	ND	ND	NP	NP	NR	NP	NP	ND	41,2	Tang et al.[17]
Japan (Honshu)	101	ND	5,9	33,7	5,9	NP	NP	NP	NR	NP	NP	19,8	?	Watanabe et al.[18]
Finland	49	4	2	ND	2	12	18	ND	ND	NP	2	ND	61	Juvonen et al.[19]
Germany	77	9	10%	42	22	NP	NP	NP	NR	NP	NP	NP	17	Schöls et al.[2]
Norway	48	<1	<1	<1	ND	NP	NP	NP	NR	NP	NP	NP	92	Erichsen et al.[11]
India	77	15,6	24,7	2,6	ND	2,6	ND	NP	6,5	NP	NP	ND	48	Srivastava et al.[20]

Results are displayed in percent. ND: not detected; NP not performed; und.: undetermined; *of 174 undiagnosed patients, only 87.3% (152 patients) underwent the adopted genetic test, resulting in 26.48% of patients with unidentified mutations.

Table 4. Prevalence of spinocerebellar ataxia in Brazil.

Reference	n	SCA1	SCA2	SCA3	SCA6	SCA7	SCA 8	SCA10	SCA12	SCA17	DRPLA	und.
Silveira[28]	67	5%	NP	55%	NP	NP	NP	NP	NP	NP	2%	61,20%
Lopes-Cendes[29]	54	6%	9%	44%	NP	NP	NP	NP	NP	NP	NP	40%
Jardim[21]	52	ND	ND	92%	ND	2%	*	NP	NP	NP	ND	6%
Trott[30]	114	ND	4,40%	84,20%	1,80%	ND	NP	1,80%	NP	ND	ND	6%
Freund[31]	115	ND	5,20%	21,70%	0,80%	2,60%	NP	NP	NP	NP	NP	69,50%
Teive[23]	104	2,90%	7,20%	72,50%	ND	4,30%	NP	11,60%	NP	NP	NP	33,70%
Cintra[22]	150	6%	3%	81%	1,50%	7%	0,80%	0,80%	NP	NP	NP	12,70%
Castilhos[25]	359	5,20%	7,80%	59,60%	1,40%	5,60%	NP	3,30%	ND	ND	ND	18,10%
Teive[26]	460	4.3%	6.5%	45.7%	0.6%	1.8%	NP	18.3%	NP	NP	NP	22.8%
Braga-Neto[27]	487	4,30%	11,50%	53,60%	1,20%	4,50%	NP	2,20%	0,20%	ND	0,20%	22.3%

und.: undetermined.

Table 5. Main mutations found with next-generation sequencing technology.

Authors	Genes mutations
Coutelier et al.[44]	CACNA1A (16 cases); Del. ITPR1(11 cases); SPG 7 (9 cases), AFG3L2 (7 cases)
Hadjivassiliou et al.[43]	CACNA1A (11 cases), PRKCG (5 cases),
Coutelier et al.[45]	SPG 7 (14 cases); SACS (8 cases); SEXT
Németh et al.[41]	SEXT (2 cases), TTBK2 (1 case), PRKCG
Fogel et al.[42]	SYNE 1 (3 cases), SPG 7 (2 cases)
Pyle et al.[40]	SPG 7 (3 cases), SACS (3 cases),

Figure 1. Whole exome sequencing [34,35,36,39,42,45,47,50,51].

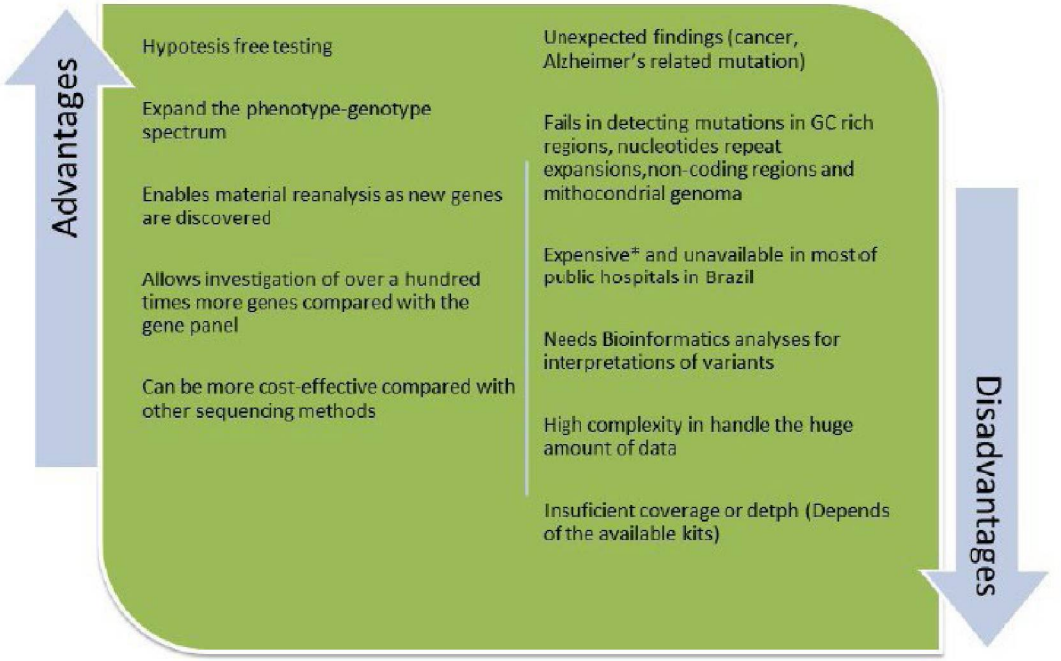


Figure 2. Target sequencing panel [34,35,36,39,42,45,47,50,51].

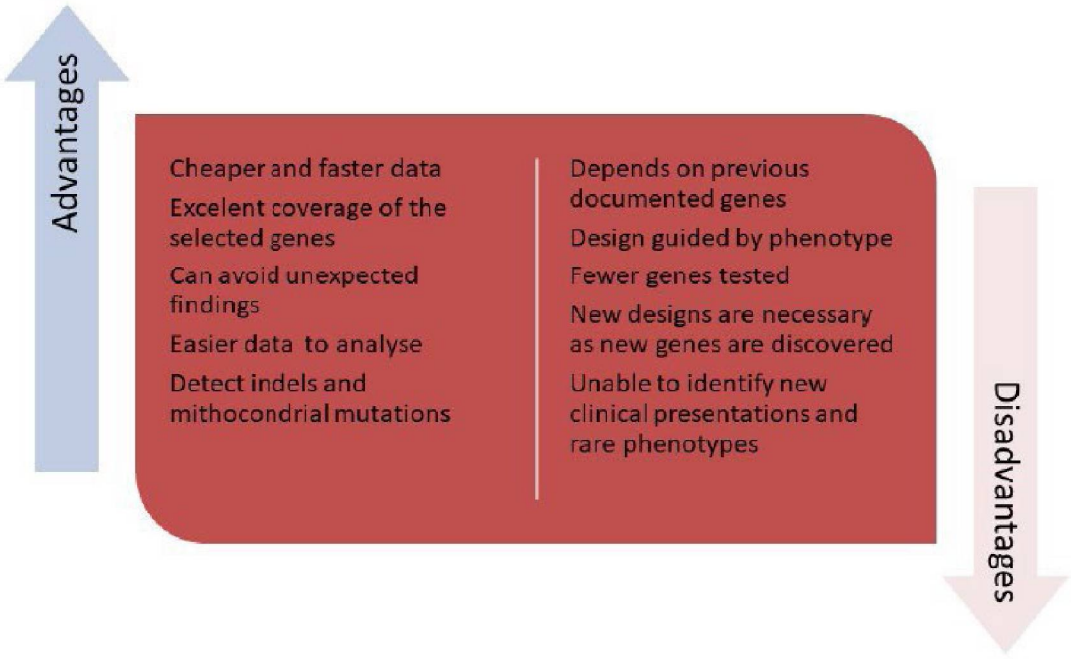
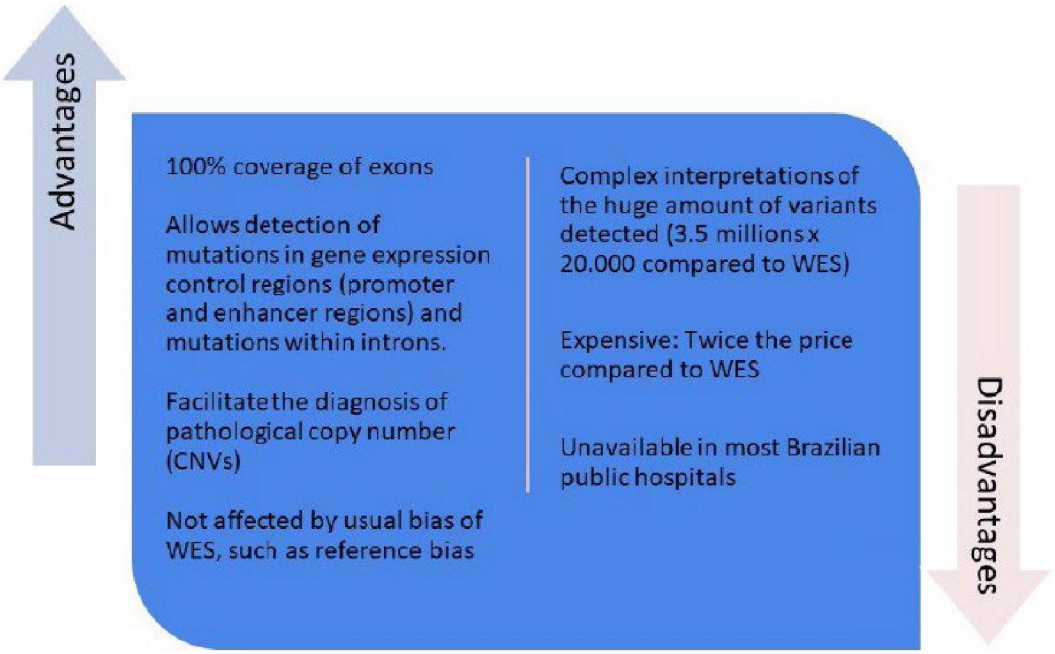


Figure 3. Whole genome sequencing [34,35,36,39,42,45,47,50,51].



4.3- Manuscript 2

Title:

Unraveling the Genetic Landscape of Undiagnosed Cerebellar Ataxia in Brazilian Patients

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Keywords: Hereditary cerebellar ataxia; whole exome sequencing; expansion hunter; next generation sequencing

Abstract

Introduction: *Hereditary ataxias (HAs) encompass a diverse and genetically intricate group of rare neurodegenerative disorders, presenting diagnostic challenges. Whole-*

exome sequencing (WES) has significantly improved diagnostic success. This study aimed to elucidate genetic causes of cerebellar ataxia within a diverse Brazilian cohort. ***Methods:*** *Biological samples were collected from individuals with sporadic or familial cerebellar ataxia, spanning various ages and phenotypes, excluding common SCAs and Friedreich ataxia. RFC1 biallelic AAGGG repeat expansion was screened in all patients. For AAGGG-negative cases, WES targeting 441 ataxia-related genes was performed, followed by ExpansionHunter analysis for repeat expansions. Variant classification adhered to ClinGen guidelines, yielding definitive or probable diagnoses.* ***Results****: The study involved 76 diverse Brazilian families. 16% received definitive diagnoses, and another 16% received probable ones. RFC1-related ataxia was predominant, with two definitive cases, followed by KIF1A (one definitive and one probable) and SYNE-1 (two probable). Early-onset cases exhibited higher diagnostic rates. ExpansionHunter improved diagnosis by 4%.* ***Conclusion****: This study highlights diagnostic complexities in cerebellar ataxia, even with advanced genetic methods. RFC1, KIF1A, and SYNE1 emerged as prevalent mutations. Early-onset cases showed higher diagnostic success. WES coupled with ExpansionHunter holds promise as a primary diagnostic tool, emphasizing the need for broader NGS accessibility in Brazil.*

4.3.1. INTRODUCTION

Hereditary ataxias (HAs) comprise a clinically and genetically heterogeneous group of rare neurodegenerative diseases that manifest with a wide spectrum of ataxia-dominated phenotypes. The HAs can be inherited in an autosomal dominant (AD), autosomal recessive (AR), X-linked (XL) manner, or through a mitochondrial genetic syndrome (Mit) (JAYADEV; BIRD, 2013). However, sporadic cerebellar ataxia, in which family history is absent, can be due to genetic causes in approximately 6-13% (BOGDAN; WIRTH; IOSIF; SCHALK *et al.*, 2022; GIORDANO; HARMUTH; JACOBI; PAAP *et al.*, 2017; WIRTH; CLÉMENT; DELVALLÉE; BONNET *et al.*, 2023).

The average global prevalence of hereditary ataxia is $2.7/10^5$ for AD inheritance and $3.3/10^5$ for AR, while X-linked and mitochondrial cerebellar ataxias are extremally rare, and studies on their prevalence are scarce (NASCIMENTO; RODRIGUES; PELLOSO; CAMARGO *et al.*, 2019). Epidemiological studies in Brazil show that

spinocerebellar ataxia type 3 (SCA3) is the most prevalent genetic cause of HA, followed by spinocerebellar ataxia type 2 (SCA2) and spinocerebellar ataxia type 10 (SCA10). The high prevalence of SCA3 is due to the Portuguese-Azorean effect, while SCA2 and SCA10 originate from native American population (DE CASTILHOS; FURTADO; GHENO; SCHAEFFER *et al.*, 2014; RODRÍGUEZ-LABRADA; MARTINS; MAGAÑA; VAZQUEZ-MOJENA *et al.*, 2020).

Recently, next generation sequencing (NGS) tools, such as whole-exome sequencing (WES), have been established as the method of choice to rapidly and efficiently diagnose neurological disorders (REXACH; LEE; MARTINEZ-AGOSTO; NÉMETH *et al.*, 2019), with an accuracy ranging from 30-50% for the molecular diagnosis of patients with a wide range of ataxia-related phenotypes (DA GRAÇA; PELUZZO; BONADIA; MARTINEZ *et al.*, 2022; SUN; JOHNSON; NELAKUDITI; GUIDUGLI *et al.*, 2019).

Despite recent advances in diagnosis with NGS, approximately 2/3 of patients with HA remain without a molecular diagnosis (REXACH; LEE; MARTINEZ-AGOSTO; NÉMETH *et al.*, 2019), a yield that is even higher for sporadic cerebellar ataxia (BOGDAN; WIRTH; IOSIF; SCHALK *et al.*, 2022; GIORDANO; HARMUTH; JACOBI; PAAP *et al.*, 2017). This study aims to identify the underlying genetic cause of a large and heterogeneous cohort of Brazilian patients from different regions of the country with unsolved cerebellar ataxia, using WES and screening for the additional repeat expansions disorders: *RFC1*-related ataxia (CORTESE; SIMONE; SULLIVAN; VANDROVCOVA *et al.*, 2019), Huntington's Disease (*HTT),* SCA12 (*PPP2R2B)*, SCA17 (*TBP),* SCA36 (*NOP56*), Spinal and Bulbar muscular atrophy of Kennedy (*AR*) and Dentatorubral-pallidoluysian atrophy (*ATN1*).

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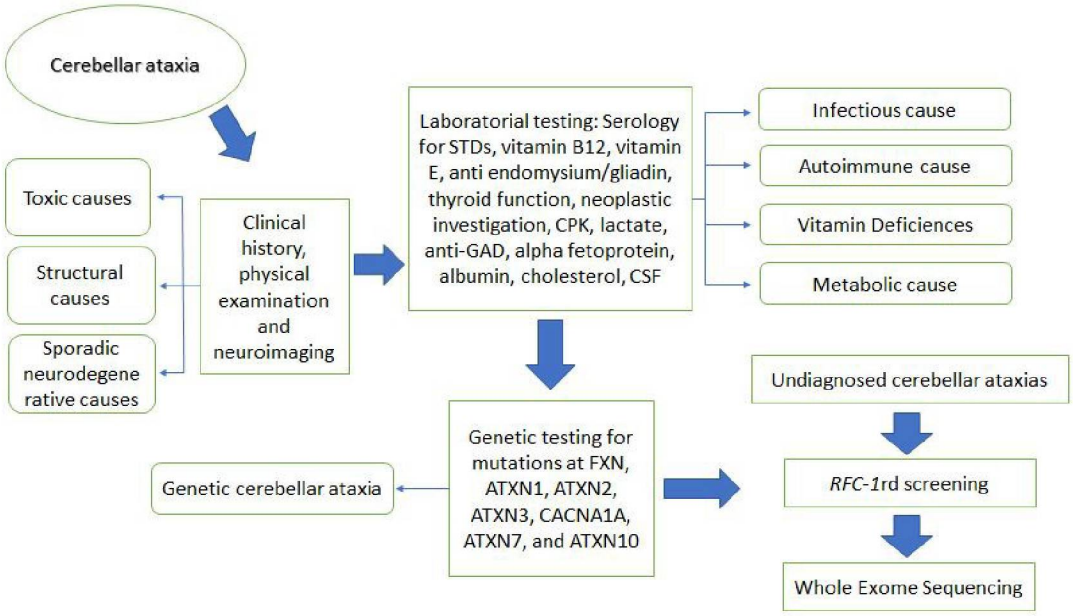
4.3.2. METHODS

Patient recruitment

Biological samples were collected from 87 patients with sporadic or familial cerebellar ataxia between June 2019 and June 2022. All samples were collected at the university centers involved in this study (Hospital das Clinicas da Universidade Federal do Paraná, Hospital Universitário Pedro Ernesto-RJ and Amazonas State Hospital). P

Patients with progressive cerebellar ataxia without a diagnosis after an extensive investigation for secondary/acquired causes, negative for SCA1, 2, 3, 6, 7, and 10, and Friedreich's ataxia were included (Figure 1). Patients were excluded if laboratory or imaging exams suggested a secondary cause or if they had incomplete clinical and genetic investigation as detailed above. Patients of all age groups, regardless of family history or the availability of relatives for segregation analysis, were included. In-depth phenotypic data were collected from patients' medical records or, when possible, patient reassessment.

Figure 1: Investigation work-flow for common genetic and secondary causes of cerebellar ataxia



STDs – sexually transmitted diseases ; CPK creatine-phosphokinase; anti-GAD: anti-glutamic acid decarboxylase; CSF: cerebrospinal fluid; *RFC-1*rd – Replication Factor C subunit-1 related disorders; *ATXN*- Ataxin; *CACNA1A*: calcium voltage-gated channel subunit alpha1 A.

This study was approved by the Ethics Committee of HC-UFPR (154,277). Furthermore, all patients were thoroughly informed about the study's objectives and implications, and they provided their explicit consent by signing the Informed Consent Form (ICF).

DNA Extraction and Preliminary Genetic Analysis

Genomic DNA was obtained from peripheral blood lymphocytes using the Quick-DNA Miniprep Plus Kit (Zymo Research) performed at a collaborating

laboratory located in Curitiba-PR (GENETIKA). Patients with an absent family history or a recessive inheritance pattern were also tested for the intronic GAA expansion in frataxin (*FXN*) using a long-range PCR technique. Samples with negative results for all applied tests were referred to The National Hospital for Neurology and Neurosurgery, Queen Square, London, for testing for biallelic expansions in the *RFC1* gene in recessive or sporadic cases, followed by WES of all samples with negative investigation for *RFC1* expansions.

NGS raw data collection

WES was performed at Macrogen Inc (Amsterdam, Netherlands). 1 ug of DNA was used for sequencing on an Illumina's NovaSeq 6000 platform using Agilent SureSelect Human All Exon V6 for library preparation, achieving a mean depth of coverage of 50X.

Alignment and variant calling

After sequencing, BCL file conversion and demultiplexing was performed inside Illumina instrument using the sample sheet file. Demultiplexed data was processed using GATK 4.1.4.0 best practices from FASTQ to VCF (variant call format) guideline (DEPRISTO; BANKS; POPLIN; GARIMELLA *et al.*, 2011). Briefly, raw sequence reads were aligned against GRCh38-alt-masked reference genome using Burrows-Wheeler Aligner BWA-MEM to give the BAM files followed by INDEL realignment and duplicate removal. Later, SNVs and INDEL variants were called and stored in VCF files (ROBINSON; HAWKINS; SANTANA-CRUZ; ADKINS *et al.*, 2017).

Variant annotation and filtering

Variants were annotated with ANNOVAR (v. 2019Oct24) and with some selected databases such as: ClinVar, UniProt, and HGMD public for known clinical significance of variants; ABraOM SABE-WES-609 (hg19) and gnomAD v2.1.1 for population frequency; Human Phenotype Ontology (HPO), OMIM and UNIPROT diseases for disease information and gene association; and REVEL for pathogenicity score prediction (WANG; LI; HAKONARSON, 2010).

We selected a list of 441 genes for analysis, based on Sun *et al* (2019), which provided a molecular diagnosis rate of 52% in that paper.

Variant filtering started according to following arguments: Alle Frequency (AF) > 0.3; it was a rare (<=1 %) PVS1 type variant (defined as "null variant" - nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multi-exon deletion - in a gene where loss-of-function (LoF) is a known mechanism of disease); it was a rare (<=0.1%) SNVs or indel variant. All variants without OMIM, UniProt or HPO disease association or located in genes that did not match the phenotype were discarded from classification analysis. If a recessive variant was found in this list, all filters were removed in the aim to find the second hit. Other online tools such as DECIPHER and STRING were used to help manual prioritization of variants (FIRTH; RICHARDS; BEVAN; CLAYTON *et al.*, 2009; SZKLARCZYK; GABLE; LYON; JUNGE *et al.*, 2019).

ExpansionHunter analysis

We used ExpansionHunter (v5.0.0) to screen for repeat expansions at exonic repeat loci (DOLZHENKO; DESHPANDE; SCHLESINGER; KRUSCHE *et al.*, 2019). Briefly, we used patients' exome BAM files along with the variant catalog of genes of interest. Output VCF files, containing lengths of repeat units, were used to filter for variants that pass the normal or pathogenic thresholds of repeat units (Supplementary Table 1). REViewer v0.2.7 was applied to visualize aligned reads in tandem repeat regions (DOLZHENKO; WEISBURD; IBAÑEZ; RAJAN-BABU *et al.*, 2022). We did not add *ATXN8* in our analysis since it was not covered in our exome sequencing.

Variant classification

Suspected variants were classified using ClinGen Variant Curation SOP Version 2.0 recommendations with some rule's adjustments following the examples listed in the guide (CLINGEN, 2022). Furthermore, the rules PS3 following OddsPath and PP4 for functional evidence from patient-derived material was used following its specific guidelines (BRNICH; ABOU TAYOUN; COUCH; CUTTING *et al.*, 2019).

We adopted a structured approach to variant classification and diagnosis. Definitive diagnoses exclusively comprise variants with a pathogenic classification. Conversely, variants categorized as likely pathogenic were designated as candidates or probable diagnoses. For patients harboring compound heterozygous variants, in which

at least one variant carries an uncertain classification (referred to as a Variant of Uncertain Significance or VUS), their diagnostic status remained uncertain.

In the context of repeat expansion disorders, definitive diagnosis was met only when the repeat expansion size reaches full penetrance. Conversely, repeat expansions falling within the spectrum of incomplete penetrance were considered as possible diagnoses.

Statistical Analysis

All analyses were conducted using the statistical package IBM SPSS Statistics, version 28.0. Data are expressed as absolute frequency and percentage for categorical variables. Quantitative variables are described as mean and standard deviation or median and range depending on their distribution. Shapiro-Wilk test was used to assess the normality of continuous variables. For group comparisons of quantitative variables, the nonparametric Kruskal-Wallis and post-hoc Dunn test were used. Fisher's exact test and Pearson's chi-square test were used to evaluate categorical variables. Statistical significance was defined as a p-value < 0.05. Bonferroni adjustment was used for correction due to multiple comparisons.

4.3.3. RESULTS

A cohort of 21 patients from Rio de Janeiro, 8 patients from Amazonas, and 58 patients from Paraná were included in this study. Overall, 87 patients from 76 families were recruited. Forty-six patients were female (52.8%) and forty-one patients were male (47.2%). The age of patients at the time of referral ranged from 16 to 85 years. Fifty-five patients (65%) presented symptom onset after the age of 18, with a median age of onset of 24 years (range, 0-67 years). Thirty-eight families had a history of cerebellar ataxia, with 20 showing autosomal recessive inheritance (26%) and 18 showing autosomal dominant inheritance (24%). Thirty-eight patients (50%) had no family history of ataxia. No case with suspected X-linked or mitochondrial inheritance was identified. However, three patients had unclear family histories. Cases #8, #15, and #17 had no contact with any known biological relatives and, as a result, were classified as sporadic cases. Six families had a history of consanguinity.

Regarding clinical characteristics, 23% of patients had pure cerebellar ataxia on examination. In the remaining cases, pyramidal syndrome was the syndrome most frequently associated with ataxia (34%), followed by peripheral neuropathy (23%), and cognitive impairment (17%). However, most of the cohort presented a complex phenotype (51%), in which at least two other syndromes (pyramidal, neuromuscular, movement disorders, cognitive impairment, psychiatric, epilepsy, neurodevelopment disorder, deafness or dysautonomia) were associated with cerebellar ataxia. Cerebellar atrophy on brain MRI or CT was found in 66% (57 out of 87 patients).

Of the 87 recruited patients, 3 were found to carry biallelic AAGGG repeat expansions in the *RFC1* gene. Two of these were siblings and clinically presented with cerebellar ataxia, neuropathy, and vestibular areflexia syndrome (CANVAS) with onset at 50 years of age, while the other patient presented sporadically with gait instability, onset at age 65, with a positive video-head impulse test, chronic cough, and Romberg's sign, but without other complaints or sensory signs upon examination. Electrophysiological studies were not available at the time of examination. These findings represent a prevalence of 2.5% (2/76 families) for this disease in our cohort. The remaining 74 families who tested negative for the *RFC1* expansions underwent WES with ExpansionHunter.

We solved 13.5% (10/74) of cases submitted to WES and found a candidate gene for definitive diagnosis in at least 16% of cases (12/74). One case was solved through the application of ExpansionHunter, revealing a $(CAG)_{54}$ repeat expansion within the *HTT* gene. Meanwhile, in two other familial instances, repeat expansions falling within the incomplete penetrance size range were identified within the *TBP* and *PPR2R2B* genes, characterized by $(CAG)_{43}$ and $(CAG)_{39}$ repeats, respectively. A standard PCR testing was performed to confirm the results. It is important to note that the latter cases had a compatible phenotype with the variant detected, which strengthens the possibility of the diagnosis. Table 1 presents the clinical and genetic characteristics of genetically definitive and probable cases from this cohort.

The most common pathogenic variant found in this cohort was *RFC1* repeat expansions. Other pathogenic variants found in singles families in this cohort included: *SPG7*, *FA2H*, *PMM2*, *TTPA*, *HEXA*, *PNKP*, *APTX*, *KIF1A*, and *HTT*.

Regarding variants classified as probable diagnosis, the most common gene was *SYNE1*, found in two families. Candidate variants in the following genes were found only in singles families: *KIF1A*, *KIF5A*, *NPC1*, *CACNA1G*, *STUB1*, *SPG11*, *PLP1*, *PRDX3*, *TBP*, and *PPR2R2B*.

Thirty patients did not have a candidate variant on exome (40.5% classified as "negative"), while twenty-two (30%) had at least one variant classified as VUS (for clinical and genetic features of uncertain cases, see supplementary table 2). We found a significant difference in the age of onset (AOO) between solved cases compared with the negative and uncertain cases. Patients with disease onset before 18 years of age had higher likelihood of receiving a definitive diagnosis with WES. Diagnostic yield was higher in autosomal recessive cases compared to other inheritance patterns (Table 2). When comparing specific phenotype features associated with ataxia, we did not observe any significant differences between groups, as shown in Supplementary Table 3.

4.3.4. DISCUSSION

This study presented an extensive genetic investigation on a heterogeneous cohort of Brazilian patients with unsolved cerebellar ataxia. A definitive diagnosis was found in only 16% of the cohort, while a probable diagnosis was considered for 16%.

RFC1-related ataxia was the most frequent genetic cause in this study (2 families), aligning with prior studies that have consistently identified it as the second most frequent autosomal recessive cerebellar ataxia (TRASCHÜTZ; ADARMES-GOMEZ; ANHEIM; BAETS *et al.*, 2023). Although the overall prevalence in this cohort is lower than that reported in previous studies (ABOUD SYRIANI; WONG; ANDANI; DE GUSMAO *et al.*, 2020; BARGHIGIANI; DE MICHELE; TESSA; FICO *et al.*, 2022), we posit that this discrepancy is attributable to the substantial heterogeneity evident in our cohort, both in terms of age of onset and clinical presentation. Notably, a significant proportion of our patients exhibit spasticity, a clinical manifestation not seen in *RFC1*-related disorder. Conversely, only 10% of patients manifested a phenotype characterized by ataxia with peripheral neuropathy. It is well established that the prevalence of *RFC1*-related disorder increases in populations where ataxia is concomitantly accompanied by peripheral neuropathy and/or vestibulopathy (TRASCHÜTZ; CORTESE; REICH; DOMINIK *et al.*, 2021).

We have identified variants in five genes among the confirmed or probable cases which are primarily linked to hereditary spastic paraplegias but may also exhibit cerebellar ataxia. These genes include *SPG7*, *SPG11*, *KIF1A*, *KIF5A*, and *FA2H*. This finding underscores the interconnection among these syndromes, further substantiating the notion of a continuous ataxia-spasticity spectrum. This concept is supported not only by the presence of overlapping phenotypes and underlying genes but also by the involvement of shared cellular pathways and disease mechanisms (SYNOFZIK; SCHÜLE, 2017).

Patients who developed symptoms before 18 years of age had higher diagnostic rates compared to patients with later disease onset, which is in keeping with previous studies (COUTELIER; HAMMER; STEVANIN; MONIN *et al.*, 2018). Pure cerebellar ataxia was more frequent in negative cases, while autosomal recessive cases had higher rates of definitive or possible molecular diagnosis, although it did not achieve statistical significance, probably due to the low number of patients.

In a comparable Brazilian study conducted in a distinct geographical region, da Graça *et al* (2022) reported a diagnostic rate of 35%, considering both definitive and probable pathogenic variants.This outcome closely aligns with our own findings, where 22 out of 74 families (30%) received a definitive or probable diagnosis through WES. Additionally, it's worth noting that ExpansionHunter was not performed in the da Graça study, while in our cohort, it contributed to one definitive and two probable diagnoses.

In this study, 7 out of 24 families with definitive or probable diagnoses exhibited rare or novel characteristics compared to the classical phenotype described in the literature for the implicated gene (Table 1). Extremely rare diseases may lead to delayed diagnoses in the absence of Next-Generation Sequencing (NGS) (FOGEL; LEE; DEIGNAN; STROM *et al.*, 2014). In the cases of Case #6 (Congenital Disorder of Glycosylation type Ia or PMM2-CDG) and Case #38 (Brain-Lung-Thyroid Syndrome and dental agenesis due to microdeletions in the NKX2-1 and PAX-9 genes), WES facilitated their diagnoses after 20 and 8 years from the initial symptom detection, respectively. NGS technologies are instrumental in expediting the diagnosis of rare diseases, especially those characterized by intricate or less-understood phenotypes, as they enable hypothesis-free diagnostic approaches (REXACH; LEE; MARTINEZ-AGOSTO; NÉMETH *et al.*, 2019). We also identified eight new variants (one classified

as pathogenic and seven as likely pathogenic) within the cohort of definitive and probable cases. These findings underscore the pivotal role of WES in the diagnosis of cerebellar ataxias, given their inherent heterogeneity (SYNOFZIK; SCHÜLE, 2017). Moreover, WES emerges as a valuable tool for unveiling novel variants and expanding the phenotypic spectrum of the diseases under scrutiny (COUTELIER; HAMMER; STEVANIN; MONIN *et al.*, 2018; REXACH; LEE; MARTINEZ-AGOSTO; NÉMETH *et al.*, 2019; SUN; JOHNSON; NELAKUDITI; GUIDUGLI *et al.*, 2019).

The integration of ExpansionHunter into our diagnostic approach led to a notable 4% increase in the diagnostic yield (1 definitive and 2 probable diagnosis out of 79 cases). This robust tool effectively addresses a crucial challenge associated with massive parallel sequencing: the detection of repeat expansions. Consequently, it emerges as a compelling candidate for first-tier diagnosis within the realm of hereditary cerebellar ataxia (MÉREAUX; DAVOINE; COUTELIER; GUILLOT-NOËL *et al.*, 2023). Nevertheless, a noteworthy limitation in Brazil lies in the accessibility and cost of NGS tools, factors that may become more favorable in the coming years.

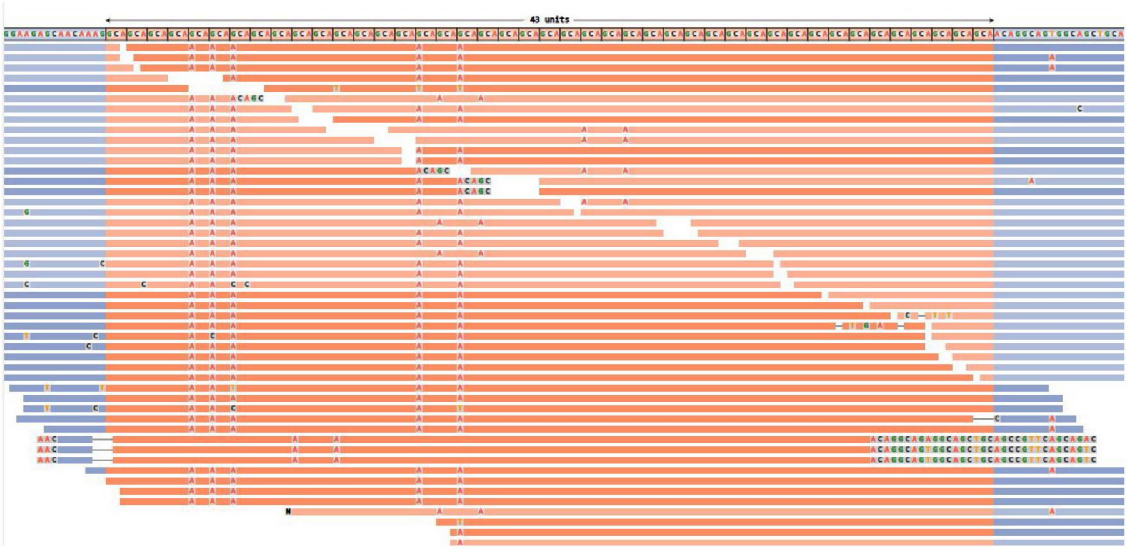
After applying ExpansionHunter, we identified a single family (individuals #73.A and #73.B) exhibiting one allele harboring 43 CAG repeats within the TATA-binding protein (*TBP*) gene, as depicted in Figure 2. It is noteworthy that patients presenting with the Spinocerebellar Ataxia Type 17 (SCA17) phenotype, harboring CAG repeat lengths falling within the range of 40-49 repeats, have been previously reported (DEPIENNE; MANDEL, 2021). In this family, we did not identify variants in the *STUB1* (STIP homology and U-box containing protein 1) gene, which is associated with Spinocerebellar Ataxia Type 48 in the heterozygous state and Spinocerebellar Cerebellar Ataxia Autosomal Recessive type 16 in the homozygous or compound heterozygous state (GENIS; ORTEGA-CUBERO; SAN NICOLÁS; CORRAL *et al.*, 2018; SHI; WANG; LI; REN *et al.*, 2013). Recently, Barbier *et al* (2023) demonstrated that intermediate expansions within the *TBP* gene could potentially exacerbate the severity of the phenotype in patients with *STUB1* variants, thus dispelling the previously suggested digenic mechanism (MAGRI; NANETTI; GELLERA; SARTO *et al.*, 2022).

Conversely, we detected a Variant of Uncertain Significance (VUS) in heterozygosity within the *Prodynorphin* gene (*PDYN* ; NM_024411.5:c.724G>A:p.Tyr242Lys), linked to Spinocerebellar Ataxia Type 23

(SCA23), which segregated among family members (BAKALKIN; WATANABE; JEZIERSKA; DEPOORTER *et al.*, 2010). However, uncertainties persist regarding the pathogenicity of the *PDYN* gene in SCA23 (PEDROSO; VALE; FREUA; BARSOTTINI *et al.*, 2016; SMEETS; VERBEEK, 2016). Furthermore, the literature lacks clear associations between *PDYN* and *TBP* genes, making it currently untenable to establish a relationship between these findings.

While the pathogenicity of intermediate expansions within the *TBP* gene warrants a nuanced discussion, we posit that the observed clinical phenotype aligns with that typically associated with SCA17 (ADAM; MIRZAA; PAGON; WALLACE *et al.*, 1993; GAO; MATSUURA; COOLBAUGH; ZÜHLKE *et al.*, 2008; ODA; MARUYAMA; KOMURE; MORINO *et al.*, 2004). Consequently, we surmise that this finding likely underlies the etiology of the disease within this family.

Figure 2. Visualization of aligned reads to the TBP repeat region.



Two related patients are heterozygous for the TBP repeat expansion. One allele has 36 repeat units and the other has 43. Repeat regions are in red and flanking regions are in blue.

In another family, intermediate repeat expansions in the *PPP2R2B* gene were observed. The patient displays a phenotype consistent with SCA12; however, uncertainties persist regarding the disease's penetrance within this range of repeat lengths, despite documented cases in the literature (MUSOVA; SEDLACEK; MAZANEC; KLEMPIR *et al.*, 2013; SRIVASTAVA; TAKKAR; GARG; FARUQ, 2017). Family member testing was not feasible during this study but would certainly clarify its pathogenicity.

In a parallel study conducted by our research group (2023) in collaboration with Brazilian colleagues, we selected 22 patients from the negative group of this cohort. These patients exhibited adult-onset ataxia (> 25 years of age), with either an autosomal dominant or sporadic pattern, and were screened for the *FGF14* GAA repeat expansion causing spinocerebellar ataxia type 27B (SCA27B) (PELLERIN; DANZI; WILKE; RENAUD *et al.*, 2023; RAFEHI; READ; SZMULEWICZ; DAVIES *et al.*, 2023). Among the 22 individuals tested, we identified pathogenic expansions in three families, representing at least 4% of the entire cohort of this present study. Although not all patients were subjected to testing, SCA27B seems to be a common cause of adult-onset cerebellar ataxia in Brazilian population. A comprehensive discussion of these cases can be found elsewhere (NOVIS; FREZATTI; PELLERIN; TOMASELLI *et al.*, 2023).

Finally, our study presents strengths and limitations. To date, this is the first study where *RFC1* AAGGG biallelic repeat expansion was screened and ExpansionHunter used in a large Brazilian cohort. Furthermore, in conjunction with the study conducted by da Graça *et al* (2022), we can propose prioritizing genes for analysis in centers without access to WES, particularly for patients with undiagnosed cerebellar ataxia. Prominent among these candidate genes are *RFC1*, *KIF1A*, and *SYNE1*. Although less frequent in this study (1 definitive case), *SPG7* should also be considered as a potential etiological cause in these cases, as it is commonly observed in similar studies (DA GRAÇA; PELUZZO; BONADIA; MARTINEZ *et al.*, 2022). Conversely, one of the main limitations of this study is the difficult access of family member for segregation analysis. Many relatives of recruited patients resided in remote areas from different regions and faced challenges in accessing hospitals for genetic material collection, thereby complicating their recruitment. Only 13 families among the confirmed/probable cases underwent segregation analysis.

Despite the presence of some patients from the northern region of the country, their number remains small, with the majority of this cohort originating from the southern and southeastern regions. The northern, northeastern, and central-western regions of Brazil have seen limited genetic studies on cerebellar ataxia, and the prevalence of specific mutations in these areas remains obscure. Broader population studies involving patients from these regions could elucidate distinct mutation prevalence across Brazilian territory and contribute to our understanding of Brazilian genetic landscape on HA.

4.3.5. CONCLUSION

In this comprehensive investigation of a cohort comprising Brazilian patients with undiagnosed cerebellar ataxia, WES coupled with ExpansionHunter emerged as a useful diagnostic tool, establishing a definitive or probable diagnosis in 30% of cases. Remarkably, the application of ExpansionHunter contributed to a 4% increase in the diagnostic yield. While we anticipate that WES with ExpansionHunter may evolve into a first-tier diagnostic modality in the near future, it is imperative to acknowledge the prevailing limitations in the availability of NGS technologies across most Brazilian medical centers. Thus, our study underscores the continued relevance of investigating other probable prevalent mutations within our populace, such as *RFC1*, *SPG7*, and

SYNE1, consistent with earlier documented findings (DA GRAÇA; PELUZZO; BONADIA; MARTINEZ *et al.*, 2022; TRASCHÜTZ; ADARMES-GOMEZ; ANHEIM; BAETS *et al.*, 2023). Furthermore, recent data underscores the significance of SCA27B, which appears to manifest as a recurrent etiology of adult-onset cerebellar ataxia amongst Brazilian patients (NOVIS; FREZATTI; PELLERIN; TOMASELLI *et al.*, 2023). Hence, we advocate for its inclusion in the screening protocols for individuals presenting with undiagnosed adult-onset cerebellar ataxia.

4.3.6. Author contributions

Design or conceptualization of the study: L.E.N.F., S.R., H.H. H.A.T.

Acquisition of data: L.E.N.F., S.A., D.P., M.V.D.C., S.R., M.S., A.C, H.H., H.A.T.

Analysis or interpretation of the data: L.E.N.F., S.A., D.P., A.C.

Drafting or revising the manuscript for intellectual content:

L.E.N.F., D.P., S.A., M.V.D.C., S.R., M.S., A.C, H.H., H.A.T.

4.3.7. Declaration of Interests

L.E.N. reports no disclosures

A.S. reports no disclosures.

D.P. reports no disclosures.

M.V.D.C. reports no disclosures.

S.R. reports no disclosures.

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A.C. reports no disclosures.

H.H. reports no disclosures.

H.A.T. reports no disclosures.

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4.3.10. REFERENCES

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Table 1 – Clinical and molecular features of patients with definitive and probable diagnosis after screening for RFC-1-AAGGG repeat expansion and WES with Expansion Hunter

Point Mutations											
ID	**Gene**	**Transcript (NM)**	**Variant**	**ACMG classification**	**Genotype**	**Acompanying features**	**Neuroimaging features**	**Age of Onset**	**Inheritance Pattern**	**Classical Phenotype (#MIM)**	**Previous Reports**
6	PMM2	NM_000303.3	c.367C>T:p.Arg123*	Pathogenic	Compound Heterozygous	Cognitive impairment, neurodevelopmental delay, strabismus, uterus agenesis, ovarian hypoplasia, flat feet	Cerebellar atrophy	birth	AR	Yes (212065)	Yes
		NM_000303.3	c.484C>T:p.Arg162Trp	Pathogenic							Yes
13	HEXA	NM_000520.6	c.1277_1278insTATC:p.Tyr427Ilefs*5	Pathogenic	Compound Heterozygous	sensory-motor peripheral neuropathy, tetraparesis	Cerebelar atrophy	1yo	Sporadic	No (272800)	Yes
		NM_000520.6	c.805G>A:p.Gly269Ser	Pathogenic							Yes
14	FA2H	NM_024306.5	c.102C>G:p.Tyr34*	Pathogenic	Homozygous	pyramidalism	Cerebellar and brainstem atrophy, periventricular white matter hyperintensities	10yo	AR	Yes (612319)	No
19.A	PNKP	NM_007254.4	c.1129G>A:p.Gly377Arg	Pathogenic	Compound Heterozygous	ocular apraxia, sensory-motor peripheral neuropathy, cognitive impairment	Cerebellar atrophy	2yo	AR	Yes (616267)	Yes
19.B		NM_007254.4	c.1221_1223del:p.Thr408del	Pathogenic		postural tremor, sensory-motor peripheral neuropathy, ocular apraxia	Cerebellar atrophy	5yo	AR	Yes (616267)	Yes
22	TTPA	NM_000370.3	c.227_229delinsATT:p.Trp76_Arg77delinsTyrTer	Pathogenic	Homozygous	generalized dystonia, head tremor, tongue atrophy, sensory-motor peripheral neuropathy	normal	5yo	AR	No (277460)	Yes
27	KIF1A	NM_001244008.2	c.821C>T:p.Ser274Leu	Pathogenic	Heterozygous	pyramidalism, sensory peripheral neuropathy, early cataract, hypogonadism, neurossensorial bilateral deafness	Cerebellar and brainstem atrophy	15yo	AR	No (610357)	Yes

28	SPG7	NM_001363850.1	c.1529C>T:p.Ala510Val	Pathogenic	Homozygous	pyramidalism, urinary incontinence	Cerebellar atrophy	32yo	Sporadic	Yes (607259)	Yes
33	APTX	NM_001195248.2	c.617C>T:p.Pro206Leu	Pathogenic	Coumpound Heterozygous	Cognitive impairment, vertical ophtalmoplegia, sensory peripheral neuropathy	Cerebellar atrophy	12yo	AR	Yes (208920)	Yes
		NM_001195248.2	c.837G>A:p.Trp279*	Pathogenic							Yes
4	PRDX3	NM_006793.5	c.604G>A:p.Asp202Asn	Likely Pathogenic	Homozygous	micropenis	Cerebellar and brainstem atrophy	18yo	Sporadic	Yes (619862)	No
8	PLP1	NM_000533.3	c.449del, p.Asp150Alafs*Ter10	Likely Pathogenic	Heterozygous	paraparesis, pyramidalism, epilepsy, urinary incontinence	Diffuse white matter hyperintensities involving the cerebral hemispheres, brainstem, and cerebellum	14yo	Sporadic	Yes (619862)	No
10	SPG11	NM_001160227.2	c.6493_6494del:p.Ser2165Leufs*61	Likely Pathogenic	Homozygous	Pyramidalism, cognitive impairment	Thin corpus callosum, global brain atrophy.	23yo	AR	Yes (604360)	Yes
21	STUB1	NM_001293197.2	c.512C>T:p.Pro171Leu	Likely Pathogenic	Heterozygous	anxiety	Superior vermis atrophy	59yo	AD	Yes (618093)	Yes
31.A	CACNA1G	NM_018896.5	c.5536G>A:p.Val1846Met	Likely Pathogenic	Heterozygous	cognitive impairment, oculomotor apraxia, bradikinesia, major depression disorder, facial tics, chorea	Cerebellar atrophy	4yo	AD	No (616795)	No
31.B				Likely Pathogenic		cognitive impairment, ocular apraxia	Cerebellar atrophy	2yo	AD	No (616795)	No
32	NPC1	NM_000271.5	c.3104C>T:p.Ala1035Val	Likely Pathogenic	Compound Heterozygous	anxiety, behavioral disorder, mild cognitive impairment	Cerebellar atrophy	21yo	Sporadic	Yes (257220)	Yes
		NM_000271.5	c.2987T>G:p.Met996Arg	Likely Pathogenic							Yes
35	KIF1A	NM_001379639.1	c.3719dupA:p.Asn1240Lysfs*2	Likely Pathogenic	Heterozygous	sensory peripheral neuropathy, dystonia, Pyramidalism	Cerebellar atrophy	52yo	Sporadic	No (610357)	No
37	SYNE1	NM_182961.2	c.4534C>T:p.Gln1512Ter	Likely Pathogenic	Compound Heterozygous	cognitive impairment, sensory-motor peripheral neuropathy	Cerebellar atrophy	12yo	Sporadic	Yes (610743)	Yes

		NM_182961.2	c.7030-2A>C	Likely Pathogenic							Yes
40	KIF5A	NM_001354705.2	c.329A>T:p.Asn110Ile	Likely Pathogenic	Heterozygous	pyramidalism	Cerebellar atrophy	7yo	Sporadic	No (604187) - Ataxia is rare	No
41.A	SYNE1	NM_033071.5	c.17101C>T:p.Gln5701*	Likely Pathogenic	Homozygous	pure cerebellar ataxia	Cerebellar atrophy	40yo	AR	Yes (610743)	No
41.B				Likely Pathogenic		motor peripheral neuropathy	Cerebellar atrophy	28yo	AR	Yes (610743)	No
Copy Number Variation											
ID	**Gene**	**Chromossomic location**	**Consequence**	**Classification**	**Genotype**	**Acompanying features**		**Age of Onset**	**Inheritance pattern**	**Classical Phenotype (#MIM)**	
38	NKX2-1/ PAX9	Chr14:36,473.858 – 41.904.175	5,4Mb deletion	Pathogenic	Heterozygous	Chorea, myoclonus, dental agenesis, hypothyroidism	normal	9yo	Sporadic	Yes (610978) (604625)	
Repeat Expansions											
ID	**Gene**	**Genomic coordinates (GRCh38)**	**Motifs/Number of repeats**	**Classification**	**Genotype**	**Acompanying features**		**Age of Onset**	**Inheritance pattern**	**Classical Phenotype (#MIM)**	
42	PPP2R2B	chr5:146878727	$(CAG)_{39}$	Likely Pathogenic	Heterozygous	parkinsonism, cervical dystonia, head tremor	Cortical and cerebellar atrophy	55yo	AD	Yes (604326)	
43.A	TBP	chr6:170561906	$(CAG)_{43}$	Likely Pathogenic	Heterozygous	chorea, cognitive impairment, anxiety	Cortical and cerebellar atrophy	45yo	AD	Yes (607136)	
43.B	TBP	chr6:170561906	$(CAG)_{43}$	Likely Pathogenic	Heterozygous	cognitive impairment	Cortical and cerebellar atrophy	44yo	AD	Yes (607136)	
44	HTT	chr4:3074939	$(CAG)_{53}$	Pathogenic	Heterozygous	ophtalmoplegia, bulging eyes, pyramidalism, cognitive impairment, parkinsonism, fingers dyspraxia, REM behavioral disorder	Bilateral putamen atrophy	20yo	AD	Yes (143100)	
75.A	RFC-1	chr4:39287456	$(AAGGG)_{1500}$	Pathogenic	Homozygous	Sensory peripheral neuropathy, vestibular arreflexia, chronic cough	Mild cerebellar atrophy	41yo	AR	Yes (614575)	

75.B	RFC-1	chr4:39287456	$(AAGGG)_{1500}$	Pathogenic	Homozygous	Sensory peripheral neuropathy, vestibular arreflexia, chronic cough	Mild cerebellar atrophy	49yo	AR	Yes (614575)	
76	RFC-1	chr4:39287456	$(AAGGG)_{1500}$	Pathogenic	Homozygous	Chronic cough, vestibular arreflexia	Mild global atrophy	65yo	Sporadic	Yes (614575)	

AD – autosomal dominant; AR autosomal recessive

Table 2: Clinical features and molecular results from patients submitted to WES

Clinical Features	ACMG classification				All	p*
	Likely pathogenic	Negative	Pathogenic	Uncertain		
% (n/N)	**16.2% (12/74)**	**40.5% (30/74)**	**13.5% (10/74)**	**29.7% (22/74)**	**74**	
AOO (mean ± sd)	29.2 ± 19.8	35.4 ± 20.2	10.6 ± 9.9	25.8 ± 13.6	28.2 ± 18.8	
AOO (median; min-max)	22 (4 - 59)	40.5 (0 - 67)	9.5 (0.3 - 32)	24.5 (2 - 63)	24.5 (0 - 67)	0.003
Male	5 (41.7%)	15 (50%)	4 (40%)	11 (50%)	35 (47.3%)	
Female	7 (58.3%)	15 (50%)	6 (60%)	11 (50%)	39 (52.7%)	0.915
AOO ≥18yo	8 (66.7%)	23 (76.7%)	2 (20%)	16 (72.7%)	49 (66.2%)	
AOO <18yo	4 (33.3%)	7 (23.3%)	8 (80%)	6 (27.3%)	25 (33.8%)	0.010
AD	4 (33.3%)	11 (36.7%)	1 (10%)	2 (9.1%)	18 (24.3%)	
AR	2 (16.7%)	5 (16.7%)	6 (60%)	6 (27.3%)	19 (25.7%)	
Sporadic	6 (50%)	14 (46.7%)	3 (30%)	14 (63.6%)	37 (50%)	0.046[1]
Consanguinity	2 families	3 families	1 family	-	6 families	-
Pure cerebellar ataxia	3 (25%)	9 (30%)	0 (0%)	5 (22.7%)	17 (23%)	0.363
Time from onset and last visit (y) (median; min-max)	13.4 (3.6 - 33.3)	15.3 (4.7 - 53.2)	21.8 (8.6 - 44)	15.7 (2.1 - 48.7)	15.7 (2.1 - 53.2)	0.372

Sd: standard deviation; AOO: Age of Onset; AD: autosomal dominant; AR: autosomal recessive; y: years

*Non-parametric Kruskal-Wallis test (quantitative variables) or Chi-square test (categorical variables)

[1] Non-significant after Bonferroni adjustment

SUPPLEMENTARY MATERIAL

Supplementary Table 1: Screened sequence repeats and their normal and pathogenic thresholds

Gene	Sequence repeat	Normal threshold	Pathogenic threshold
TBP	CAG	42	49
HTT	CAG	36	40
AR	CAG	36	38
ATN1	CAG	35	48
NOP56	GGCCTG	14	650
PPP2R2B	CAG	32	51

Supplementary Table 2 – Clinical and molecular features of patients with uncertain diagnosis

ID	Gene	Transcript (NM)	Variant	ACMG classification	Genotype	Acompanying features	Age of Onset	Inheritance Pattern	Classic Phenotype (#MIM)	Previous Reports
1	SCN2A	NM_001040142.2	c.5903C>G:p.Ser1968Cys	Uncertain	Heterozygous	Dystonia, peripheral neuropathy, epilepsy, cognitive impairment	40yo	Sporadic	No (618924)	No
2	CAPN1	NM_001198868.2	c.759+1G>T	Pathogenic	Compound Heterozygous	pyramidalism	30yo	Sporadic	Yes (616907)	No
		NM_001198868.2	c.1043T>C:p.Leu348Pro	Uncertain						No
3	SPTBN2	NM_006946.4	c.532G>A:p.Ala178Thr	Uncertain	Heterozygous	vertigo	18yo	Sporadic	Yes (600224)	No
5	PRDX3	NM_001302272.2	c.23delT:p.Leu8Cysfs*10	Likely Pathogenic	Compound Heterozygous	Pure cerebellar ataxia	35yo	Sporadic	No (619862)	Yes
		NM_001302272.2	c.538G>A:p.Gly180Ser	Uncertain						Yes
7	ATP13A2	NM_001141973.3	c.1098delG:p.His367Thrfs*24	Likely Pathogenic	Compound Heterozygous	Pyramidalism, peripheral neuropathy	63yo	Sporadic	No (606693)	Yes
		NM_001141973.3	c.1175G>A:p.Arg392His	Uncertain						No
9.A	VPS41	NM_014396.4	c.1984C>T:p.arg662*	Pathogenic	Heterozygous	Pyramidalism, ophthalmoplegia, optic atrophy	2yo	AR	Yes (619389)	Yes
9.B					(no 2nd variant)	Cognitive impairment	4yo	AR	Yes (619389)	
11	SPG7	NM_001363850.1	c.524T>C:p.Leu175Pro	Uncertain	Heterozygous	Pyramidalism	17yo	Sporadic	Yes (607259)	No
12	VPS13D	NM_018156.4	c.10382T>A:p.Leu3461*	Likely Pathogenic	Compound Heterozygous	peripheral neuropathy	41yo	Sporadic	Yes (607317)	No
		NM_018156.4	c.10907C>T:p.Ser3636Phe	Uncertain						No
15	STUB1	NM_005861.4	c.196C>T:p.Arg66Trp	Uncertain	Heterozygous	Psychiatric features (apathy and depression)	30yo	Sporadic[1]	Yes (618093)	No
16	TTBK2	NM_173500.4	c.83G>C:p.Gly28Ala	Uncertain	Heterozygous	Vertigo	19yo	Sporadic	No (604432)	No
17	GRM1	NM_001278066.1	c.2701T>C:p.Ser901Pro	Uncertain	Heterozygous	Epilepsy	22yo	Sporadic[2]	Yes (617691)	No
18	KANK1	NM_001354335.2	c.1225A>C:p.Asn409His	Uncertain	Homozygous	Pyramidalism, bilateral sensorineural deafness, peripheral neuropathy	15yo	Sporadic	No (612900)	No
20.A	KCNJ10	NM_002241.5	c.719T>C:p.Val240Ala	Uncertain	Homozygous	Pyramidalism, Dystonia, moderate sensorineural hearing loss, ichthyosis	15yo	AR	No (612780)	No
20.B	FLG	NM_002016.2	c.9740C>A:p.Ser3247*	Pathogenic	Heterozygous	Ichthyosis, pyramidalism, dystonia, head tremor	16yo	AR	No (612780)	
23	CAPN1	NM_005186.4	c.1442G>A:p.Arg481Gln	Uncertain	Homozygous	Pyramidalism, cavus feet	30yo	AR	Yes (616907)	Yes

24	SOX10	NM_006941.4	c.196G>C:p.Asp66His	Uncertain	Heterozygous	ophthalmoplegia, myoclonus, dystonia	15yo	AR	No (611584)	No
25	HSD17B4	NM_000414.4	c.487A>G:p.Asn163Asp	Uncertain	Compound Heterozygous	bilateral sensorineural deafness	23yo	Sporadic	Yes (233400)	No
		NM_000414.4	c.1940A>G:p.Asn647Ser	Uncertain						No
26.A	TRPC3	NM_003305.2	c.1997T>C:p.Leu666Pro	Uncertain	Heterozygous	dystonia, head tremor	20yo	AD	No (616410)	No
26.B					Heterozygous	pure cerebellar ataxia	45yo	AD	No (616410)	
26.C					Heterozygous	postural and orthostatic tremor	18yo	AD	No (616410)	
29	MRPL12	NM_002949.4	c.325_326insCTG:p.Ala113dup	Uncertain	Homozygous	external ophthalmoplegia, muscle weakness, low ear implantation, epicanthal folds, folliculitis decalvans, retinitis pigmentosa, micrognathia, parkinsonism, global developmental delay	2yo	Sporadic	Yes (618951)	No
30	CAPN1	NM_001198868.2	c.1442G>A:p.Arg481Gln	Uncertain	Compound Heterozygous	pyramidalism	26yo	AR	Yes (616907)	Yes
		NM_001198868.2	c.1341+7A>T	Uncertain						No
34	TMEM240	NM_001114748.2	c.511C>T:p.Arg171Trp	Uncertain	Heterozygous	pyramidalism, cognitive impairment	28yo	AD	Yes (607454)	Yes
36	ITPR1	NM_01168272.1	c.5644G>A, p.Asp1882Asn	Uncertain	Heterozygous	pure cerebellar ataxia	36yo	Sporadic	Yes (606658)	No
39	SLC7A9	NM_001126335.2	c.325G>A:p.Ala109Thr	Uncertain	Heterozygous	pure cerebellar ataxia	39yo	Sporadic	No (220100)	Yes

Legend: [1] Parents died at young age; [2] adopted; AD – autosomal dominant; AR- autosomal recessive

Supplementary table 3: Prevalence of clinical features in sub-groups, considering molecular results from WES + Expansion Hunter analysis (Patients with *RFC-1* related ataxia diagnosis were not included)

Clinical Feature	ACMG classification				All	P*
	Likely pathogenic	Negative	Pathogenic	Uncertain		
% (n/N)	**16.7% (14/84)**	**38.1% (32/84)**	**14.3% (12/84)**	**31% (26/84)**	**84**	
Peripheral Neuropathy	3 (21.4%)	7 (21.9%)	6 (50%)	4 (15.4%)	20 (23.8%)	0.129
Muscle Atrophy	2 (14.3%)	1 (3.1%)	4 (33.3%)	2 (7.7%)	9 (10.7%)	0.032
Pyramidalism	4 (28.6%)	12 (37.5%)	4 (33.3%)	10 (38.5%)	30 (35.7%)	0.925
Psychiatric features	3 (21.4%)	3 (9.4%)	1 (8.3%)	1 (3.8%)	8 (9.5%)	0.348
Cognitive Impairment	6 (42.9%)	5 (15.6%)	6 (50%)	3 (11.5%)	20 (23.8%)	0.014
Epilepsy	1 (7.1%)	4 (12.5%)	0 (0%)	2 (7.7%)	7 (8.3%)	0.602
Myoclonus	0 (0%)	1 (3.1%)	1 (8.3%)	1 (3.8%)	3 (3.6%)	-
Dystonia	2 (14.3%)	5 (15.6%)	1 (8.3%)	5 (19.2%)	13 (15.5%)	0.858
Parkinsonism	2 (14.3%)	2 (6.3%)	1 (8.3%)	1 (3.8%)	6 (7.1%)	-
Chorea/athetosis	2 (14.3%)	1 (3.1%)	1 (8.3%)	0 (0%)	4 (4.8%)	-
Tremor	1 (7.1%)	3 (9.4%)	3 (25%)	3 (11.5%)	10 (11.9%)	0.482
NDD	0 (0%)	1 (3.1%)	1 (8.3%)	1 (3.8%)	3 (3.6%)	-
Dysarthria	7 (50%)	15 (46.9%)	6 (50%)	13 (50%)	41 (48.8%)	0.994
Vertigo	1 (7.1%)	2 (6.3%)	0 (0%)	3 (11.5%)	6 (7.1%)	-
Paresis/Plegia	3 (21.4%)	2 (6.3%)	3 (25%)	3 (11.5%)	11 (13.1%)	0.293
Horizontal Nystagmus	3 (21.4%)	11 (34.4%)	3 (25%)	8 (30.8%)	25 (29.8%)	0.817
Vertical Nystagmus	1 (7.1%)	6 (18.8%)	3 (25%)	2 (7.7%)	12 (14.3%)	0.369
Oculomotor apraxia	2 (14.3%)	0 (0%)	2 (16.7%)	0 (0%)	4 (4.8%)	-
Ophthalmoplegia	2 (14.3%)	4 (12.5%)	2 (16.7%)	3 (11.5%)	11 (13.1%)	0.975
Dysautonomia	2 (14.3%)	2 (6.3%)	3 (25%)	1 (3.8%)	8 (9.5%)	0.166
Wheelchair Bound	0 (0%)	4 (12.5%)	4 (33.3%)	1 (3.8%)	9 (10.7%)	0.0231
Cane assistance	0 (0%)	1 (3.1%)	1 (8.3%)	2 (7.7%)	4 (4.8%)	
Dysphagia	0 (0%)	4 (12.5%)	1 (8.3%)	3 (11.5%)	8 (9.5%)	0.584
Deafness	0 (0%)	2 (6.3%)	1 (8.3%)	3 (11.5%)	6 (7.1%)	0.594
Ataxia + pyramidalism	1 (7.1%)	2 (6.3%)	0 (0%)	5 (19.2%)	8 (9.5%)	0.204
Ataxia + cognitive impairment	0 (0%)	1 (3.1%)	1 (8.3%)	1 (3.8%)	3 (3.6%)	-
Ataxia + peripheral neuropathy	1 (7.1%)	2 (6.3%)	1 (8.3%)	2 (7.7%)	6 (7.1%)	-
Ataxia + at least 2 symptoms[1]	9 (64.3%)	15 (46.9%)	10 (83.3%)	9 (34.6%)	43 (51.2%)	0.029

*Chi-square test, $p<0.05$

[1] Pyramidalism, peripheral neuropathy, cognitive impairment, movement disorders (Parkinsonism, dystonia, tremor, chorea/athetosis, myoclonus) psychiatric features, epilepsy, deafness, NDD and dysautonomia

4.4 - Manuscript 3

Title:

Frequency of GAA-*FGF14* ataxia in a large cohort of Brazilian patients with unsolved adult-onset cerebellar ataxia

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Abstract:

Objectives:

Dominantly inherited GAA repeat expansions in intron 1 of the fibroblast growth factor 14 (*FGF14*) gene have recently been found to be a common cause of hereditary adult-onset cerebellar ataxia (GAA-*FGF14* ataxia; spinocerebellar ataxia 27B). The global epidemiology and regional prevalence of this newly reported disorder remain to be established. We here investigated the frequency of GAA-*FGF14* ataxia in a large cohort of Brazilian patients with unsolved adult-onset cerebellar ataxia.

Methods

We recruited 93 index patients with genetically unsolved adult-onset cerebellar ataxia despite extensive genetic investigation and genotyped the *FGF14* repeat locus. Patients were recruited across 4 different regions of Brazil.

Results

Of the 93 index patients, 8 (9%) carried an *FGF14* $(GAA)_{\geq 250}$ expansion. The expansion was also identified in one affected relative. Seven patients were of European descent, one was of admixed American ancestry, and one was of African descent. One patient carrying a $(GAA)_{376}$ expansion developed ataxia at age 28 years, confirming that GAA-*FGF14* ataxia can occur before the age of 30 years. One patient displayed episodic

symptoms, while none had downbeat nystagmus. Cerebellar atrophy was observed on brain MRI in 7 of 8 patients (87%).

Discussion

Our results suggest that GAA-*FGF14* ataxia may be a common genetic cause of adult-onset cerebellar ataxia in the Brazilian population, although larger studies are needed to fully define its epidemiology.

Keywords: cerebellar ataxia, ataxia, *FGF14*, spinocerebellar ataxia 27B, repeat expansion

4.4.1. INTRODUCTION

Hereditary cerebellar ataxias are a genetically and phenotypically heterogeneous group of neurodegenerative diseases (TEIVE; MUNHOZ; ARRUDA; LOPES-CENDES *et al.*, 2012). Despite recent advances in genomic technologies, approximately two-thirds of patients with adult-onset ataxia remain without a genetic diagnosis, a yield that is even lower in the subgroup of patients with sporadic ataxia (BOGDAN; WIRTH; IOSIF; SCHALK *et al.*, 2022; GIORDANO; HARMUTH; JACOBI; PAAP *et al.*, 2017; REXACH; LEE; MARTINEZ-AGOSTO; NÉMETH *et al.*, 2019).

Recently, dominantly inherited GAA repeat expansions in intron 1 of the fibroblast growth factor 14 (*FGF14*) gene have been shown to be a common cause of late-onset cerebellar ataxia (GAA-*FGF14* ataxia; spinocerebellar ataxia 27B [MIM: 620174]) in European and South Asian populations. Patients with GAA-*FGF14* ataxia displayed slowly progressive cerebellar ataxia with frequent episodic symptoms and downbeat nystagmus. They also occasionally displayed vestibular hypofunction, pyramidal signs, and autonomic dysfunction (PELLERIN; DANZI; WILKE; RENAUD *et al.*, 2023; RAFEHI; READ; SZMULEWICZ; DAVIES *et al.*, 2023).

In Brazil, spinocerebellar ataxia 3 (SCA3) is the most prevalent form of hereditary ataxia, followed by spinocerebellar ataxia 2 (SCA2), and spinocerebellar ataxia 10 (SCA10). The high prevalence of SCA3 is largely due to the Portuguese-Azorean founder effect, while SCA2 and SCA10 originate from the Native American populations (DE CASTILHOS; FURTADO; GHENO; SCHAEFFER *et al.*, 2014; NASCIMENTO; RODRIGUES; PELLOSO; CAMARGO *et al.*, 2019; RODRÍGUEZ-LABRADA; MARTINS; MAGAÑA; VAZQUEZ-MOJENA *et al.*, 2020). The ethnic diversity of the Brazilian population provides a unique opportunity to study the distribution and prevalence of the recently described GAA-*FGF14* ataxia.

Here, we investigated the frequency and phenotypic profile of GAA-*FGF14* ataxia in a large cohort of Brazilian patients with unsolved adult-onset cerebellar ataxia.

4.4.2. METHODS

Ninety-three index patients were recruited in Curitiba (Hospital de Clínicas da Universidade Federal do Paraná), Rio de Janeiro (Hospital Universitário Pedro Ernesto), Amazonas (Universidade do Estado do Amazonas), and São Paulo (Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto) in Brazil. Patients presenting with unsolved neurodegenerative ataxia with onset after the age of 20 years were enrolled. Patients had previously undergone comprehensive investigation to exclude acquired and genetic causes, which included whole-exome sequencing (42/93; the list of analyzed genes was based on Sun *et al* (2019)) and screening for SCA1, SCA2, SCA3, SCA6, SCA7, SCA10, *RFC1*-related ataxia, and Friedreich ataxia. We obtained written informed consent from all participants in the study, and the institutional review board of the Hospital de Clínicas da Universidade Federal do Paraná and Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto approved this study.

Genetic Screening for FGF14 Repeat Expansions

The *FGF14* repeat locus was genotyped by long-range PCR. Repeat sizes were measured by capillary electrophoresis of fluorescent long-range PCR amplification products, as described previously (BONNET; PELLERIN; ROTH; CLÉMENT *et al.*, 2023). Patients who had large amplification products by PCR next underwent bidirectional repeat-primed PCRs targeting the 5'-end and the 3'-end of the repeat locus to confirm the repeat motif of the expansion. Select patients underwent Sanger sequencing to confirm the repeat motif. Expansions of at least 250 GAA repeat units were considered pathogenic (PELLERIN; DANZI; WILKE; RENAUD *et al.*, 2023; RAFEHI; READ; SZMULEWICZ; DAVIES *et al.*, 2023).

Data Availability

Individual anonymized data may be shared at the request of any qualified investigator upon reasonable request

4.4.3. RESULTS

We identified eight index patients (8 of 93; 9%) who carried an intronic *FGF14* $(GAA)_{\geq 250}$ expansion (Figure 1). The expansion was also identified in the affected son (Patient I.2) of an affected mother (Patient I.1). In this family, the transmission of an expanded $(GAA)_{253}$ allele resulted in expansion in the female germline (expansion from 253 to 268 triplets), consistent with previous reports showing further expansion of the locus upon maternal transmission (BONNET; PELLERIN; ROTH; CLÉMENT *et al.*, 2023; PELLERIN; DANZI; WILKE; RENAUD *et al.*, 2023; PELLERIN; GOBBO; COUSE; DOLZHENKO *et al.*, 2023). Compared to Patient I.1, patient I.2 had a slightly earlier age at onset by 5 years and displayed more prominent cerebellar signs. We also identified a patient carrying biallelic GAA repeat expansions (363 and 448 repeat units) and a patient carrying an expanded $(GAA)_{424}$ allele and an expanded $[(GAA)_4(GCA)_1]_{63}$ allele. Ancestry analysis of short-read sequencing data showed that one patient was of admixed American ancestry and two patients were of European ancestry (PEDERSEN; QUINLAN, 2017). One patient self-reported being of African descent, while the remaining patients self-reported being of European descent. We thus identified the first patients of admixed American and African descent with GAA-*FGF14* ataxia.

Table 1 presents the main phenotypic features and results of ancillary testing of the 9 patients with GAA-*FGF14* ataxia. The median age of onset was 59 years (range, 28 to 67 years). We identified one patient who had an age at disease onset of 28 years, confirming that GAA-*FGF14* ataxia can begin before the age of 30 years (BRAIS; PELLERIN; DANZI, 2023). All patients displayed a slowly progressive cerebellar syndrome, although none had dysarthria at the time of last examination. A single patient experienced episodic symptoms (11%), which were triggered by alcohol intake and stress, while 56% of patients (5/9) reported diplopia and/or oscillopsia and 44% (4/9) reported vertigo and/or dizziness. Horizontal gaze-evoked nystagmus was observed in 78% of cases (7/9), consistent with previous reports showing that cerebellar oculomotor signs are a common feature of GAA-*FGF14* ataxia (PELLERIN; DANZI; WILKE; RENAUD *et al.*, 2023; WILKE; PELLERIN; MENGEL; TRASCHÜTZ *et al.*, 2023). However, none of the 9 patients exhibited downbeat nystagmus. Postural tremor was observed in one patient on examination. Postural tremor has been reported with variable frequency in patients with GAA-*FGF14* ataxia, and is a frequent feature in

SCA27A caused by point and frameshift variants in *FGF14* (GROTH; BERMAN, 2018; PELLERIN; DANZI; WILKE; RENAUD et al., 2023; WILKE; PELLERIN; MENGEL; TRASCHÜTZ et al., 2023). The patient carrying biallelic GAA repeat expansions had a relatively rapid disease progression (Spinocerebellar Degeneration Functional Score disability score of 3 after three-year disease duration, indicating that patient has moderate gait impairment, is unable to run, and has a limited walking distance without help) (ANHEIM; MONGA; FLEURY; CHARLES *et al.*, 2009). In addition, extra-pyramidal features were observed in two patients.

Overall, the median disease duration at last follow up was 12 years (range, 3 to 39 years). Two patients required unilateral walking aid after 7 and 12 years of disease duration, respectively. One patient became wheelchair-bound after longstanding disease duration (15 years), in keeping with previous reports showing that wheelchair dependence is uncommon in GAA-*FGF14* ataxia (WILKE; PELLERIN; MENGEL; TRASCHÜTZ *et al.*, 2023). All other patients were still ambulating independently at last follow-up, including two patients who had a disease duration exceeding twenty years.

In keeping with some reports showing weak to no significant correlation between size of *FGF14* expansion and age at onset or disease severity, we identified three patients who presented marked differences in age of onset and disease progression despite carrying an expansion of similar size (patient II, 376 repeat units; patient III, 449 repeat units; patient IV, 424 repeat units) (Table 1) (BONNET; PELLERIN; ROTH; CLÉMENT *et al.*, 2023; PELLERIN; DANZI; WILKE; RENAUD *et al.*, 2023; WILKE; PELLERIN; MENGEL; TRASCHÜTZ *et al.*, 2023). Notably, patient III developed disease at age 28 years and had a more gradual disease progression (SDFS score of 1 after 23 years of disease duration) compared to patient IV who developed disease at age 59 years and had a SDFS score of 3 after 9 years of disease duration.

One patient had evidence of mild peripheral axonal sensory polyneuropathy on electrophysiological studies, suggesting that polyneuropathy is not a core feature of *GAA-FGF14* ataxia, in keeping with previous reports (PELLERIN; DANZI; WILKE; RENAUD *et al.*, 2023; RAFEHI; READ; SZMULEWICZ; DAVIES *et al.*, 2023). All but one patient underwent magnetic resonance imaging (MRI) of the head. Isolated

vermis atrophy was observed in 4 patients (4/8; 50%), while diffuse cerebellar atrophy was observed in 3 patients (3/8; 37%). Mild midbrain and pons atrophy was observed along with diffuse cortical atrophy in one of the patients. This patient had a comparatively more severe phenotype, with dystonia of the upper limbs, vestibular areflexia, and was wheelchair dependent. Although it is unknown if these findings are pathologically related to GAA-*FGF14* ataxia, as it has not been previously described in this condition, an extensive workup did not reveal an alternative diagnosis.

Of the GAA-*FGF14*-negative patients, we identified four patients who carried a non-GAA expansion (2 patients with $(GAAGGA)_n$ expansion, one patient with $(GAAGAAA)_n$ expansion, and one patient with $[(GAA)_4(GCA)_1]_n$ expansion). Non-GAA expansions in *FGF14* are likely not pathogenic as previously shown in segregation studies (PELLERIN; DANZI; WILKE; RENAUD *et al.*, 2023). Similarly, non-GAA expansions in *FXN* are not known to be pathogenic (OHSHIMA; SAKAMOTO; LABUDA; POIRIER *et al.*, 1999).

4.4.4. DISCUSSION

In this large and diverse Brazilian cohort, *FGF14* $(GAA)_{\geq 250}$ expansions were present in 9% (8 of 93) of patients with unsolved adult-onset ataxia, thus confirming previous reports showing that GAA-*FGF14* ataxia is a common cause of adult-onset ataxia. We identified the first cases of GAA-*FGF14* ataxia in patients of admixed American ancestry and African descent. This observation supports the wide distribution of this novel GAA repeat expansion in populations of diverse ethnic backgrounds. This is likely to be partly explained by the significant instability of the GAA repeat *FGF14* repeat locus upon meiotic transmission (PELLERIN; GOBBO; COUSE; DOLZHENKO *et al.*, 2023).

Our report expands the phenotypic spectrum of GAA-*FGF14* ataxia, with a patient of our cohort presenting before the age of 30 years. This result highlights the need to screen for *FGF14* expansions in patients with early-onset ataxia to fully define the age at onset spectrum of GAA-*FGF14* ataxia. The expansion size of our patient presenting at age 28 years was smaller compared to other patients in this cohort who developed disease later in life, which is in keeping with some previous reports showing weak or lack of significant correlation between age of onset and repeat expansion size

(PELLERIN; GOBBO; COUSE; DOLZHENKO *et al.*, 2023; WILKE; PELLERIN; MENGEL; TRASCHÜTZ *et al.*, 2023). This observation suggests that additional yet unknown factors may influence and modify age of onset in GAA-*FGF14* ataxia.

Episodic symptoms and downbeat nystagmus have previously been shown to be common phenotypic features of GAA-*FGF14* ataxia in some, but not all, patient cohorts. In our cohort, only one patient had episodic symptoms while none had downbeat nystagmus. These results suggest that episodic features and downbeat nystagmus may not be recurrent in all patients, illustrating that the phenotypic spectrum of this disease is yet to be fully defined. Nonetheless, the core phenotype of our patients, namely a late-onset slowly progressive cerebellar syndrome with frequent visual disturbances, cerebellar oculomotor signs, and vertigo and/or dizziness, is consistent with previous reports (BONNET; PELLERIN; ROTH; CLÉMENT *et al.*, 2023; PELLERIN; DANZI; WILKE; RENAUD *et al.*, 2023; RAFEHI; READ; SZMULEWICZ; DAVIES *et al.*, 2023; WILKE; PELLERIN; MENGEL; TRASCHÜTZ *et al.*, 2023). We also confirmed that isolated cerebellar atrophy predominant in the vermis is frequent in this patient population. Mild midbrain and pons atrophy was identified in one patient, although it is unknown if this finding is pathologically related to GAA-*FGF14* ataxia as it has not been previously described in this condition.

We also identified a patient carrying a biallelic GAA repeat expansion, which has been previously reported in a few cases (BRAIS; PELLERIN; DANZI, 2023; PELLERIN; DANZI; WILKE; RENAUD *et al.*, 2023; WILKE; PELLERIN; MENGEL; TRASCHÜTZ *et al.*, 2023). Our patient displayed a relatively rapid disease progression, similar to what has been previously observed in these patients. Additional longitudinal follow-up is needed to fully chart disease progression and phenotypic spectrum in these patients. Furthermore, extrapyramidal signs were found in two patients (22%), raising the possibility that this feature may be part of the phenotypic profile of GAA-*FGF14* ataxia.

In conclusion, our study suggests that GAA-*FGF14* ataxia is likely to be a common genetic cause of unsolved adult-onset cerebellar ataxia in the Brazilian population. Our findings also confirm the wide distribution of this novel GAA repeat expansion in populations of diverse ethnic backgrounds.

4.4.5. Author contributions

Design or conceptualization of the study: L.E.N.F., R.S.S.F, D.P., W.M.J, H.A.T.

Acquisition of data: L.E.N.F., R.S.S.F, D.P., P.J.T, S.A., M.V.D.C., M.S., M.-J.D., P.I, J.L.P, O.G.P.B, A.C, M.C.D, M.C.F.J, B.B., S.Z., H.H., W.M.J, H.A.T.

Analysis or interpretation of the data: L.E.N.F., R.S.S.F, D.P., W.M.J, H.A.T

Drafting or revising the manuscript for intellectual content:

L.E.N.F., R.S.S.F, D.P., P.J.T, S.A., M.V.D.C., M.S., M.-J.D., P.I, J.L.P, O.G.P.B, A.C, M.C.D, M.C.F.J, B.B., S.Z., H.H., W.M.J, H.A.T.

4.4.6. Declaration of Interests

L.E.N. reports no disclosures

R.S.S.F. reports no disclosures.

D.P. reports no disclosures.

P.J.T. reports no disclosures.

A.S. reports no disclosures.

M.V.D.C. reports no disclosures.

M.S. reports no disclosures.

M.-J.D. reports no disclosures.

P.I. reports no disclosures.

J.L.P reports no disclosures

O.G.P.B reports no disclosures

A.C. reports no disclosures.

M.C.D. reports no disclosures.

M.C.F.J took part as a PI in a clinical trial for Friedreich's Ataxia, sponsored by PTC and received research funding from Friedreich's ataxia research alliance. None of these related to the current study.

B.B. reports no disclosures.

S.Z. is a consultant on drug targets for Aeglea BioTherapeutics and consultant on clinical trial design for Applied Therapeutics, all of them unrelated to the work in the present manuscript.

H.H. reports no disclosures.

S.R. reports no disclosures.

W.M.J. reports no disclosures.

H.A.T. reports no disclosures.

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4.4.9. REFERENCES

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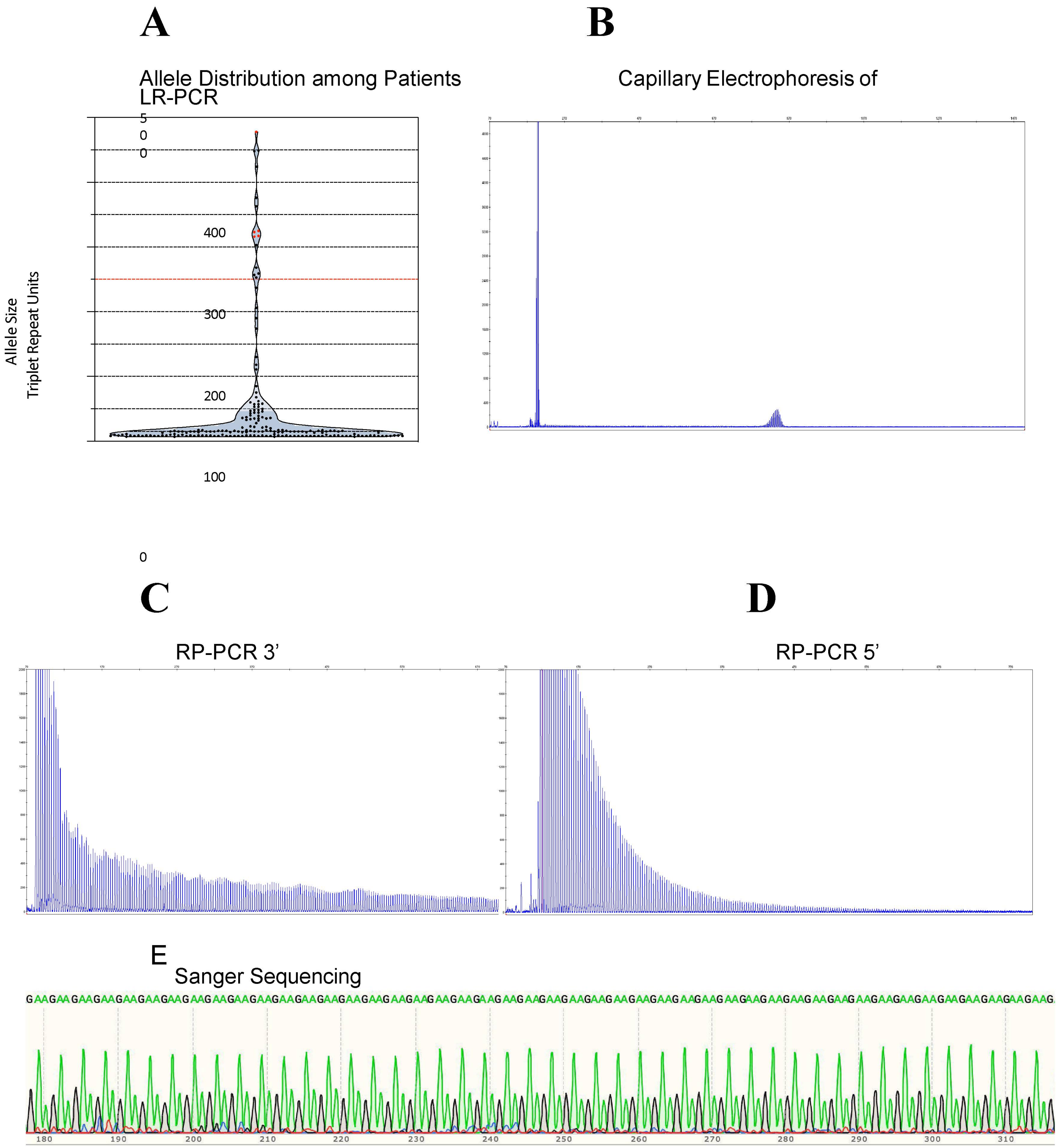
Figure 1: Allele Distribution and Genetic Analysis of the *FGF14* GAA Repeat Locus.



A) Allele distribution of the *FGF14* repeat locus in 94 Brazilian patients with unsolved adult-onset cerebellar ataxia. The repeat length was measured by capillary electrophoresis of fluorescent long-range PCR amplification products. The allele sizes are expressed in numbers of triplet repeat units. Expanded alleles consisting of non-GAA repeats are represented by red triangles. The dashed red line indicate the pathogenic threshold of at least 250 GAA repeat units. The bold dashed black line within the violin plot shows the median allele size. B) Fragment length analysis results of Patient I.1 carrying an expanded allele of 253 GAA repeat units. C and D) Results of repeat-primed PCR targeting the 3'-end (C) and 5'-end (D) of the *FGF14* repeat locus in Patient I.1. E) Excerpt of Sanger sequencing chromatogram showing the GAA motif of the expanded allele of Patient I.1.

Table 1: Clinical Features of Brazilian patients with GAA-*FGF14* related ataxia.

Clinical Features	Patient I.1	Patient I.2*	Patient II	Patient III	Patient IV	Patient V	Patient VI	Patient VII	Patient VIII
Inheritance	AD	AD	Sporadic	AD	AD	Sporadic	Sporadic	Sporadic	AD
Sex	F	M	F	F	M	M	M	M	F
Age of Onset	45y	40y	67y	28y	60y	59y	63y	57y	60y
Age at last examination (disease duration)	84y (39y)	65y (15y)	73y (6y)	51y (23y)	63y (3y)	68y (9y)	75y (12y)	64y (7y)	78y (18y)
Episodic Symptoms	no	no	no	yes	no	no	no	no	no
Downbeat Nystagmus	no	no	no	no	no	no	no	no	no
Horizontal Gaze-Evoked Nystagmus	yes	yes	no	no	yes	yes	yes	yes	yes
Diplopia/blurry vision/oscillopsia	no	yes	no	yes	yes	yes	no	yes	no
Cerebellar dysarthria	no	no	no	no	no	no	no	no	no
Gait Ataxia	yes	yes	yes	yes	yes	yes	yes	yes	yes
Apendicular Ataxia	yes	yes	yes	yes	yes	yes	yes	yes	yes
Vertigo / Dizziness	no	no	no	yes	yes	yes	no	yes	no
Postural tremor	yes	no	no	no	no	no	no	no	no
SDFS	2	3	3	1	3	3	4	4	6
Cerebellar atrophy on MRI	N/A	yes (cerebellar vermis)	Yes (global)	Yes (global)	no	yes (cerebellar vermis)	yes (cerebellar vermis)	Yes (global)	yes (cerebellar vermis)
Additional Features	tinnitus, deafness, insomnia, chin tremor	insomnia, tinnitus	dysphagia	none	none	sensory neuropathy	none	Facial and upper limb dystonia, lymphocitic pleocytosis	Parkinsonism, lower limb dystonia, MCI
GAA repeat units	253	268	376	449	363/448	424	303	259	257
Ancestry	EUR	EUR	AMR	EUR	AFR	EUR	EUR	EUR	EUR

AD: Autosomal Dominant; AMR: Admixed American; AFR: African; EUR: European; F: Female ; M: male; MRI: magnetic resonance imaging; MCI: mild cognitive impairment; N/A: not available; SDFS: Spinocerebellar Degeneration Functional Score. *Patient I.2 is the offspring of patient I.1

4.5 – Summary of findings

Given the defined objectives of this thesis, we believe that we have achieved success and obtained satisfactory answers. In the first manuscript, the escalating role of NGS in the diagnosis of hereditary cerebellar ataxias, particularly in cases of autosomal recessive cerebellar ataxia, early-onset cerebellar ataxia (<18 years of age) and a history of consanguinity, becomes evident. Furthermore, limitations associated with NGS have been mitigated by rapid advances in bioinformatics technologies, such as ExpansionHunter, capable of identifying trinucleotide repeat expansions, which constitute the primary and most prevalent causes of hereditary ataxias (RUANO; MELO; SILVA; COUTINHO, 2014; TEIVE; MUNHOZ; ASHIZAWA, 2012). Recent technologies like long-read sequencing may further enhance precision and utility in diagnosing these disorders, potentially unveiling new causes of hereditary ataxia (CHEN; TUCCI; CIPRIANI; GUSTAVSSON *et al.*, 2023). Finally, recent studies indicate that NGS is already considered a first-tier diagnostic approach for cerebellar ataxias in some centers worldwide due to its ability to provide a hypothesis-free, expeditious, and cost-effective diagnosis (REXACH; LEE; MARTINEZ-AGOSTO; NÉMETH *et al.*, 2019).

In the second manuscript, we demonstrated the practical utility of WES in diagnosing Brazilian patients with undiagnosed cerebellar ataxia. The combined use of WES with ExpansionHunter led to definitive or probable diagnoses in 30% of our cohort. ExpansionHunter also proved valuable, aiding in the diagnosis of 3 families with more rare causes of cerebellar ataxia (4%). WES further facilitated the diagnosis of extremely rare cases of cerebellar ataxia and cases with atypical presentations where clinical diagnosis is challenging. Simultaneously, we demonstrated that screening for biallelic noncanonical intronic expansion (AAGGG) in the *RFC1* gene was crucial in this specific cohort, resulting in definitive diagnoses in two families. This study thus confirmed the hypothesis that WES, especially when coupled with ExpansionHunter, is a valuable tool in this scenario and should be considered as a first-tier diagnostic tool for hereditary ataxias. Finally, we suggest that genes such as *RFC1*, *SPG7*, and *SYNE-1* are frequently seen in this population and should, therefore, be considered in the investigation of patients with undiagnosed cerebellar ataxia in cases where WES is unavailable.

In the third manuscript, we assessed the prevalence of SCA27B (GAA-*FGF14* ataxia) in a large cohort of Brazilian patients with undiagnosed cerebellar ataxia, including 42 patients from the second manuscript who remained undiagnosed after initial investigation. Among the 93 patients included in the study, 8 exhibited GAA repeat expansions >250 in the *FGF14* gene (9%). Focusing solely on the patients from the second manuscript, 3 families were identified with the mutation, equivalent to 4% of the cohort. These results indicate that SCA27B appears to be a frequent cause of ataxia in patients with undiagnosed cerebellar ataxia. Therefore, we recommend that this investigation be incorporated into the genetic screening of patients with hereditary cerebellar ataxia.

5 – CONCLUSIONS

In this doctoral thesis, we have successfully discerned that mutations within the *FGF14*, *RFC1*, *SYNE1*, and *KIF1A* genes stand out as the predominant etiological factors contributing to undiagnosed cases of cerebellar ataxia among Brazilian patients. Although less frequent in this study, *SPG7* should also be considered as a potential etiological cause in these cases, as it is commonly observed in similar studies. The findings derived from this thesis provide conclusive insights, particularly in relation to the predefined specific objectives:

1. NGS technologies prove valuable in the diagnosis of hereditary ataxias within the Brazilian population, particularly when employed in conjunction with ExpansionHunter.
2. In a diverse cohort of undiagnosed cerebellar ataxia patients in Brazil, the combined utilization of WES and ExpansionHunter yielded a diagnostic rate of 30%.
3. ExpansionHunter emerges as a valuable adjunct to WES, effectively enhancing the diagnostic yield for hereditary ataxias.
4. WES played a pivotal role in the diagnosis of exceedingly rare diseases and in cases characterized by atypical phenotypes. The latter underscores the significance of NGS in expanding our comprehension of disease phenotypes and enhancing our understanding of these conditions.
5. Within our cohort of patients with undiagnosed cerebellar ataxia, AAGGG-*RFC1* and GAA-*FGF14* ataxia emerged as the most prevalent diagnoses (2 and 3 families, respectively). This finding underscores the significance of these

disorders in our clinical context, emphasizing the necessity of including screenings for these conditions in the diagnostic assessment of patients with adult-onset hereditary ataxia.

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APPENDIX 1: CHC/UFPR institutional review board approval

CIW/I lC/UH'R

UFPR - HOSPITAL DE CLÍNICAS DA UNIVERSIDADE FEDERAL DO PARANÁ -

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: AVALIAÇÃO FENOTÍPICA E GENOTÍPICA DOS PACIENTES COM ATAXIAS CEREBELARES HEREDITÁRIAS

Pesquisador: Helio Afonso Ghizoni Teive

Área Temática:

Versão: 8

CAAE: 06128812.0.0000.0096

Instituição Proponente: Hospital de Clínicas da Universidade Federal do Paraná

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.320.610

Apresentação do Projeto:

pesquisador envia emenda adicionando pesquisadores:

Geslaine Janaina Bueno dos Santos, fisioterapeuta;

Maria Izabel Rodrigues Severiano, educadora física;

Bianca Simone Zelgeboim, fonoaudióloga_

Andressa Aline Vieira, aluna de pós-graduação

Objetivo da Pesquisa:

não se aplica

Avaliação dos Riscos e Benefícios:

não se aplica

Comentários e Considerações sobre a Pesquisa:

não se aplica

Considerações sobre os Termos de apresentação obrigatória:

não se aplica

Conclusões ou Pendências e Lista de Inadequações:

aprovado

Considerações Finais a critério do CEP:

Endereço: Rua Gal. Carneiro, 181
Bairro: Alto da Glória **CEP:** 80.060-900
UF: PR **Município:** CURITIBA
Telefone: (41)3360-1041 **Fax:** (41)3360-1041 **E-mail:** cep@hc.ufpr.br

CE"PII IC/UH'R

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Continuação do Parecer: 3.320.610

Diante do exposto, o Comitê de Ética em Pesquisa em Seres Humanos do HC-UFPR, de acordo com as atribuições definidas na Resolução CNS 466/2012 e na Norma Operacional Nº 001/2013 do CNS, manifesta -se pela aprovação da Emenda.

Solicitamos que sejam apresentados a este CEP, relatórios semestrais sobre o andamento da pesquisa, bem como informações relativas às modificações do protocolo, cancelamento, encerramento e destino dos conhecimentos obtidos. Manter os documentos da pesquisa arquivados.

É dever do CEP acompanhar o desenvolvimento dos projetos, por meio de relatórios semestrais dos pesquisadores e de outras estratégias de monitoramento, de acordo com o risco inerente à pesquisa.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇOES_BASICAS_128365 O E5 pdf	25/03/2019 14:42:39		Aceito
Outros	Carta_CEP_emenda_E5_pendencia pdf	25/03/2019 14:14:05	Vanessa Radünz	Aceito
Declaração de Instituição e Intraestrutura	Declaracao_ciencia_instituicao_copartici pantejpg	25/03/2019 13 06:10	Vanessa Radünz	Aceito
Declaração de Pesauisadores	Termo_responsabilidade_emenda_E5_p endencia odf	25/03/2019 12 54:20	Vanessa Radünz	Aceito
Declaração de Pesauisadores	Termo_confidencialidade_emenda_E5_ oendencia.ioa	25/03/2019 12 54:07	Vanessa Radünz	Aceito
Declaração de Pesouisadores	Justificativa_Documentos_Andressa.pdf	25/03/2019 12 53:35	Vanessa Radünz	Aceito
Declaração de Pesouisadores	Declaracao_uso_dados_emenda_E5_pe ndencia_.nn	25/03/2019 12 53:12	Vanessa Radünz	Aceito
Declaração de Pesauisadores	Declaracao_orientador_emenda_E5_pe ndencia.odf	25/03/2019 12:52:38	Vanessa Radünz	Aceito
Declaração de Pesauisadores	Declaracao_compromisso_pesquisadore s_emenda_E5_oendencia.ioa	25/03/2019 12:52:10	Vanessa Radünz	Aceito
Outros	Carta_CEP_emenda_E5_assinada.docx	13/02/2019 14:20:13	Helio Afonso Ghizoni Teive	Aceito
Outros	Carta_ao_CEP _emenda_E4.jpg	21/06/2017 10 00:14	Helio Afonso Ghizoni Teive	Aceito
Outros	Carta_ao_CEP _emenda_E3.jpg	03/05/2017 15 57:25	Helio Afonso Ghizoni Teive	Aceito

Endereço: Rua Gal. Carneiro, 181
Bairro: Alto da Glória **CEP:** 80.060-900
UF: PR **Município:** CURITIBA
Telefone: (41)3360-1041 **Fax:** (41)3360-1041 **E-mail:** cep@hc.ufpr.br

Outros	justificalivaemenda.pdf	20/03/2017 10 53:48	Helio Afonso Ghizoni Teive	Aceito
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Projeto Detalhado/ Brochura Invesliaador	Projelo_de_pesquisa_2.doc	07/09/2016 2140:15	Adriana Moro	Aceito
Outros	Inclusao_Pesquisador_Patricia_e_Giulia .odf	07/09/2016 21:38:33	Adriana Moro	Aceito
Outros	Inclusao_de_Pesquisador_Bruno.pdf	16/08/2016 20 57:50	Adriana Moro	Aceito
Outros	TCLE_Versão 3.doc	15/01/2013 11 22:12		Aceito
Outros	Inclusao de pesquisador.pdf	15/01/2013 11:21:49		Aceito
Outros	Orçamento Financeiro Detalhado - versão 2odf	09/11/2012 15 24:14		Aceito
Outros	Declaração de Riscos e Benefícios.pdf	09/11/2012 15 24:05		Aceito
Outros	Carla referente às pendencias pdf	09/11/2012 15 23:55		Aceito
Brochura Pesquisa	Projeto de pesquisa_2 doe	06/10/2012 17:37:19		Aceito
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TCLE / Termos de Assentimento/ Justificativa de Ausência	TCLE_2.doc	06/10/2012 17:34:52		Aceito
Declaração de Instituição e Infraestrutura	UCL_carta_Porlugues.pdf	22/08/2012 13 06:59		Aceito
Declaração de Instituição e Infraestrutura	UF_Carta_Portugues.pdf	22/08/2012 13:06:04		Aceito
Outros	Termo de Confidencialidade.pdf	01/08/2012 20:39:54		Aceito
Outros	Termo de compromisso para utilização de dados.odf	01/08/2012 20:39:25		Aceito
Outros	Instrumento de Coleta.doe	01/08/2012 20 38:33		Aceito
Outros	Identificação I1.pdf	01/08/2012 20 38:06		Aceito
Outros	Identificação I.pdf	01/08/2012 20 37:34		Aceito
Outros	Declaração Orienlador.pdf	01/08/2012 20 37:05		Aceito

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Continuação do Parecer: 3.320.610

Outros	Declaração de uso específico de materiais odf	01/08/2012 20 36:26		Aceito
Outros	Declaração de tornar público os resultados odf	01/08/2012 20 35:51		Aceito
Outros	Concordância do serviço envolvido.pdf	01/08/2012 20 35:15		Aceito
Outros	Carta ao comitê_Adriana Moro.pdf	01/08/2012 20:34:35		Aceito
Folha de Rosto	Folha de rosto.pdf	01/08/2012 20:31:43		Aceito
Outros	Análise de Mérito I1.pdf	01/08/2012 20:29:34		Aceito
Outros	Análise de Mérito I.pdf	01/08/2012 20 29:02		Aceito
Declaração de Instituição e Infraestrutura	Carta_Genetika.docx	01/08/2012 20 26:13		Aceito
Brochura Pesquisa	Projeto de pesquisa.doe	01/08/2012 20 17:30		Aceito
TCLE / Termos de Assentimento/ Justificativa de Ausência	TCLE.doc	01/08/2012 20 15:44		Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

CURITIBA, 12 de Maio de 2019

Assinado por:
maria cristina sartor
(Coordenador(a))

Endereço: Rua Gal. Carneiro, 181
Bairro: Alto da Glória **CEP:** 80.060-900
UF: PR **Município:** CURITIBA
Telefone: (41)3360-1041 **Fax:** (41)3360-1041 **E-mail:** cep@hc.ufpr.br

APPENDIX 2 – INFORMED CONSENT FORM

O senhor (a) está sendo convidado a participar de um estudo intitulado "Avaliação Fenotípica e Genotípica dos pacientes com Ataxias Cerebelares Hereditárias". É através das pesquisas clínicas que ocorrem os avanços importantes em todas as áreas, por isso a sua participação neste estudo é fundamental.

a) O objetivo desta pesquisa é verificar o número de repetições de um determinado segmento de DNA a fim de estudar qual é a sua relação com os sintomas, comparando com as repetições de uma população normal. Ao mesmo tempo pretende organizar uma biblioteca de DNA (banco de DNA), que poderá ser utilizado no futuro, caso não seja encontrada no momento uma alteração no seu exame; isso possibilita novos testes à medida que a ciência for se desenvolvendo. Possivelmente no futuro deverá existir um tratamento para cada tipo de ataxia e é necessário conhecermos exatamente o tipo que você é portador.

b) Caso o senhor (a) participe da pesquisa, será necessário apenas estar no local das atividades, nas dependências do Hospital das Clínicas, em data e horário pré-determinados. Para que o senhor (a) seja elegível para o estudo deverá ser portador de uma ataxia cerebelar progressiva comprovada por história clínica e exame neurológico, ter investigação neurológica e por neuroimagem (tomografia computadorizada ou ressonância magnética) negativa (normal) para outras causas de ataxia, devendo ser excluídas as doenças tóxico-infecciosas que determinem sintomas semelhantes.

a) Para tanto, o senhor (a) deverá estar presente no ambulatório de distúrbios do movimento, no 6° andar do Hospital das Clínicas da Universidade Federal do Paraná (Rua General Carneiro, 181, CEP: 80.060-900, Curitiba-PR) para realização

das avaliações, durante 1 dia da semana, sendo a duração dos procedimentos de aproximadamente 60 minutos. Após concordar em participar voluntariamente do estudo e ter assinado este termo de consentimento livre e esclarecido, será submetido aos seguintes procedimentos:

1. Inicialmente será realizada uma avaliação clínica com exame neurológico por um dos pesquisadores envolvidos, com registro de seus dados.
2. Investigação laboratorial para afastar outras doenças que podem simular ataxia cerebelar degenerativa, como exames de sangue rotineiros, neuroimagem, líquido cefalorraquidiano ou testes eletrofisiológicos, dependendo dos dados colhidos na avaliação clínica.
3. Em seguida serão colhidos 20ml (centímetros cúbicos) de sangue, que será propriamente identificado para controle de laboratório.
4. Esse sangue será enviado ao Laboratório Genetika, de onde será extraído o seu DNA.
5. O DNA será armazenado em freezer com a devida identificação.
6. O DNA será enviado para Instituições parceiras (UCL Institute of Neurology);
7. Serão feitas amplificações e classificação de segmentos do DNA que interessam ao estudo, através de métodos de laboratório específicos para as diversas ataxias cerebelares degenerativas.
8. O restante do DNA será armazenado para futuras análises e pesquisas, pois eventualmente é possível que nenhuma das ataxias testadas no momento seja positiva e o senhor (a) possua uma forma diferente ainda não descrita. Acompanhando a literatura, conforme forem sendo descritas novas formas de ataxia, será feito o teste em seu material.

c) É possível que o senhor (a) experimente algum desconforto da picada da agulha para retirada de sangue, porém, este será realizado com técnicas padrões e com material estéril.

d) Não existem riscos físicos relacionados à participação neste estudo. Se durante as avaliações houver necessidade de algum cuidado específico à sua saúde, relacionado ao estudo ele será encaminhado ao Hospital das Clínicas, no

município de Curitiba, onde qualquer problema decorrente deste estudo será tratado e os custos referentes ao tratamento serão de responsabilidade dos pesquisadores envolvidos neste estudo.

e) Os benefícios esperados são: saber com segurança o tipo de ataxia da qual o senhor (a) é portador, conhecer a incidência desta doença na população Brasileira, bem como eventualmente ajudar na elucidação do mecanismo de produção dos sintomas e talvez no desenvolvimento de um tratamento no futuro. Cumpre salientar que nem sempre você será diretamente beneficiado com o resultado deste teste, mesmo que seja encontrada uma alteração, pois essas doenças genéticas ainda não possuem tratamento. Porém, você estará contribuindo para o avanço científico.

b) Você poderá entrar em contato com os pesquisadores envolvidos, Luiz Eduardo Novis ou Hélio Afonso Ghizoni Teive, no Hospital de Clínicas da Universidade Federal do Paraná, Rua General Carneiro, 181, CEP: 80060-900, Curitiba, Paraná, ou pelo telefone 3360-1800, Ramal 6261 ou 6154, no horário das 8:00-12:00h ou 13:00-17:00h. Assim você poderá esclarecer eventuais dúvidas a respeito desta pesquisa.

Se você tiver dúvidas sobre seus direitos como um paciente de pesquisa, você pode contatar o Comitê de Ética em Pesquisa em Seres Humanos (CEP) do Hospital das Clínicas da Universidade Federal do Paraná, pelo telefone 3360-1896 ou 3360-1865 ou e-mail: cometica.saude@ufpr.br.

f) Todos os procedimentos de avaliação serão realizados pelo envolvidos na autoria da pesquisa, Luiz Eduardo Novis de Farias, e supervisionados pelo pesquisador, Prof. Dra. Mariana Spitz e Prof Dr Helio Afonso G Teive.

g) Estão garantidas todas as informações e esclarecimentos que o senhor (a) queira, sobre a metodologia, antes, durante e depois do estudo.

h) A sua participação neste estudo é voluntária. Contudo, se houver desistência da pesquisa, o senhor (a) poderá solicitar de volta o termo de consentimento livre esclarecido assinado.

i) As informações relacionadas ao estudo poderão ser acessadas pelos pesquisadores e pelas autoridades legais. No entanto, será garantida a confidencialidade dos sujeitos frente às autoridades. A inspeção pelas autoridades legais ocorrerá apenas nos dados já codificados (não aparecerá o nome do participante, e sim um código), para que os participantes da pesquisa não sejam identificados e não haja alguma forma de constrangimento. Ainda, se qualquer informação for divulgada em relatório ou publicação, isto será feito sob forma codificada (não aparecerá o nome do participante, e sim um código), para que a **confidencialidade** e o sigilo sejam mantidos.

j) O senhor (a) está livre para cancelar a sua participação na pesquisa a qualquer momento, sem aviso prévio.

k) Todas as despesas necessárias para a realização da pesquisa não são da sua responsabilidade.

l) Pela sua participação no estudo você não receberá qualquer valor em dinheiro. O senhor (a) terá a garantia de que qualquer problema decorrente do estudo será tratado no Hospital das Clínicas da Universidade Federal do Paraná, no município de Curitiba, e os custos referentes ao tratamento serão de responsabilidade do pesquisador autor, Dr Luiz Eduardo Novis de Farias e pesquisadores envolvidos.

m) Quando os resultados forem publicados, não aparecerá o seu nome, e sim um código.

Eu,_____ li o texto acima e compreendi a natureza e objetivo do estudo do qual participarei. A explicação que recebi menciona os riscos e benefícios do estudo. Eu entendi que sou livre para interromper a minha participação a qualquer momento sem justificar esta decisão e sem que a mesma me afete de qualquer maneira. Eu entendi o que não se pode fazer durante o estudo e sei que qualquer problema relacionado a este estudo será tratado sem custos para mim.

Eu, _____ fui informado sobre todos os procedimentos que serão realizados e concordo voluntariamente em participar deste estudo.

_____

(Assinatura do sujeito de pesquisa)

Curitiba, _____/_____/_____.

_____

Prof. Dr Helio Afonso G. Teive

(Pesquisador Orientador)

_____

Dr. Luiz Eduardo Novis de Farias

Pesquisador responsável

_____

Prof Dra. Mariana Spitz

Pesquisadora envolvida

APPENDIX 3: CLINICAL DATA FORM

PROTOCOLO DE ATAXIA ESPINOCEREBELAR

Nome			Registro-
Data	Data de Nascimento	Sexo	Raça
Profissão	Endereço		
			Telefone
Idade	Idade de início dos sintomas		

HISTÓRIA FAMILIAR

Pais consangüíneos: □ - sim □ - não

Etnia: avós maternos _____/_____avós paternos _____/_____

Antecedentes familiares:

□sim □não □não sabe

Heredograma

HERANÇA: □ autossômica dominante □- autossômica recessiva □ -outra_____

Comorbidades _____

Uso de medicamentos? _____

EXAMES COMPLEMENTARES

Tomografia de Crânio : realizada em _____

□- normal □ - alterada _____ □ - não realizada

Ressonância Nuclear Magnética Encefálica : realizada em _____

□ -normal □ - alterada _____ □ - não realizada

Eletromiografia/ECN realizada em _____

□ -normal □ - não realizada

□ - alterada _____

ECG realizado em _____

□ -normal □ - não realizada

□ - alterada _____

Ecocardiograma realizado em _____

□ -normal □ - não realizada

□ - alterada _____

Estudo Urodinâmico realizado em _____

□ -normal □ - não realizada

□ - alterada _____

	MANIFESTAÇÕES NEUROLÓGICAS	INÍCIO
□	**Ataxia**	

☐ de marcha
☐ Axial
☐ Apendicular
☐ **Tremor cerebelar**
☐ Disartria
☐ Disfunção cognitiva
☐ Distúrbios da motricidade ocular
☐ Oftalmoparesia/plegia horizontal
☐ Oftalmoparesia/plegia vertical
☐ Movimentos sacádicos lentos
☐ Nistagmo
☐ Horizontal
☐ Vertical
☐ Outro
☐ Outros distúrbios da motricidade ocular
☐ **Comprometimento de pares cranianos**
☐ “bulging eyes”
☐ Sinais piramidades ☐ MMSS ☐ MMII
☐ Hiperreflexia
☐ Espasticidade
☐ Sinal de Babinski
☐ **Distúrbios do movimento**
☐ Movimentos coreicos
☐ Distonia
☐ Mioclonia
☐ Tremor
☐ Balismo
☐ Atetose
☐ Estereotipia
☐ Acatisia

☐	Parkinsonismo
☐	Tremor de repouso
☐	Rigidez
☐	Bradicinesia
☐	Instabilidade postural
☐	Outro distúrbio do movimento
☐	**Comprometimento da sensibilidade**
☐	Superficial tipo neuropático
☐	Profunda- Ataxia sensitiva
☐	**Sinais de NMI**
☐	Amiotrofia periférica
☐	Arreflexia profunda
☐	Fasciculações
☐	Face
☐	MMII
☐	MMSS
☐	Tronco
☐	**Pés cavus**
☐	**Escoliose**
☐	**Distúrbios autonômicos**
☐	**Bexiga Neurogênica**

Ceruloplasmia Sérica realizada em _____

☐ -normal ☐ - alterada _____ ☐ - não realizada

VDRL

☐ -normal ☐ - alterada _____ ☐ - não realizada

Vitamina B12 realizada em _____

☐ -normal ☐ - alterada _____ ☐ - não realizada

Vitamina E realizada em _____

☐ -normal ☐ - alterada _____ ☐ - não realizada

CPK realizada em _____

□ -normal □ - alterada _____ □ - não realizada

Proteína total e frações realizada em _____

□ -normal □ - alterada _____ □ - não realizada

Alfa-feto-proteína realizada em _____

□ -normal □ - alterada _____ □ - não realizada

IgA realizada em _____

□ -normal □ - alterada _____ □ - não realizada

Lipidograma realizado em _____

□ -normal □ - alterada _____ □ - não realizada

Anticorpos antigliadina (IgG, IgA) realizados em _____

□ -normal □ - alterada _____ □ - não realizada

Anticorpo anti-GAD realizado em _____

□ -normal □ - alterada _____ □ - não realizada

Anticorpos anti-Hu (ANNA-1) realizado em _____

□ -normal □ - alterada _____ □ - não realizada

Líquor realizado em _____

□ -normal □ - alterada _____ □ - não realizada

HTLV realizado em _____ ( ) LCR ( ) Soro

□ -normal □ - alterada _____ □ - não realizada

DIAGNÓSTICO CLÍNICO

□ SCA tipo ___

□ ADPRL

□ ILOCA

□ FA

□ outras _____

ESTUDO GENÉTICO:

COLETADO SANGUE EM :

RESULTADO:

TRATAMENTO INSTITUÍDO

Evolução:

APPENDIX 4 – OTHER PUBLICATIONS AND AWARDS

1. Other Publications:

I. Novis LE, Maranhão Filho PA, Pires MEP, Spitz M, Teive HAG. Professor Faustino Esposel: Neurology, football and spiritualism. Arq Neuropsiquiatr. 2021 Sep;79(9):848-850. doi: 10.1590/0004-282X-ANP-2021-0013. PMID: 34133502.

II. Efthymiou S, Novis LE, Koutsis G, Koniari C, Maroofian R, Turchetti V, Velonakis G, Vasconcellos LF, Raskin S, Srinivasan VM, Pagnamenta AT, Arun YB, Kinhal UV, Gowda VK, Teive HAG, Houlden H. Pure cerebellar ataxia due to bi-allelic PRDX3 variants including recurring p.Asp202Asn. Ann Clin Transl Neurol. 2023 Oct;10(10):1910-1916. doi: 10.1002/acn3.51874. Epub 2023 Aug 8. PMID: 37553803; PMCID: PMC10578881.

III. Novis LE, Maranhão Filho PA, Pires MEP, Spitz M, Teive HAG. Reply to letter: 'Another one that extends the toe: the Austregésilo-Esposel sign'. Arq Neuropsiquiatr. 2021 Nov;79(11):1064. doi: 10.1590/0004-282X-ANP-2021-0310r. PMID: 34586186.

IV. Novis LE, Spitz M, Teive HAG. The history behind ALS type 8: from the first phenotype description to the discovery of VAPB mutation. Arq Neuropsiquiatr. 2021 Aug;79(8):743-747. doi: 10.1590/0004-282X-ANP-2020-0548. PMID: 34133501.

V. Ronco R, Perini C, Currò R, Dominik N, Facchini S, Gennari A, Simone R, Stuart S, Nagy S, Vegezzi E, Quartesan I, El-Saddig A, Lavin T, Tucci A, Szymura A, Novis De Farias LE, Gary A, Delfeld M, Kandikatla P, Niu N, Tawde S, Shaw J, Polke J, Reilly MM, Wood NW, Crespan E, Gomez C, Chen JYH, Schmahmann JD, Gosal D, Houlden H, Das S, Cortese A. Truncating Variants in *RFC1* in Cerebellar Ataxia, Neuropathy, and Vestibular Areflexia Syndrome. Neurology. 2023 Jan 31;100(5):e543-e554. doi: 10.1212/WNL.0000000000201486. Epub 2022 Oct 26. PMID: 36289003; PMCID: PMC9931080.

2. Awards

I. Best Oral Presentation – 2nd Place – III Congresso Brasileiro de Neurogenética – São Paulo, 2023;

II. Newson Davis Fellowship Grant 2020