UNIVERSITÀ DEGLI STUDI DI MILANO

Ph.D. Course in Clinical Research

Ph.D. Thesis

The role of next-generation sequencing in the discovery of novel genetic causes of dystonia

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Abstract of the thesis

Aim: The scope of this thesis is to identify through a Next-Generation Sequencing (NGS) approach novel genes and genetic variants associated with hereditary dystonia.

Background: Dystonia is a hyperkinetic movement disorder characterized by sustained or intermittent muscle contractions causing abnormal movements and postures. The recent advances in molecular genetics (i.e., NGS technologies) are leading to the discovery of an increasing number of genetic dystonia forms. There is great interest in the study of the etiology of dystonia since the discovery of underlying biological mechanisms may facilitate the development of specific targeted therapies and contribute to a better knowledge of the disease mechanisms of non-genetic forms of dystonia as well.

Results: Here we present: 1) a new genetic form of dystonia associated with a novel disease-causing gene (*VPS11*); 2) the expansion of the phenotypic spectrum associated with known causative genes (*VPS16*, *VPS13A*, and *KMT2B*); 3) a novel group of hereditary dystonias sharing common disease mechanisms (i.e., HOPS-associated Neurological Disorders - HOPSANDs); 4) novel pathogenic variants in already known isolated, combined and complex dystonia genes (*VPS16*, *VPS13A*, *VPS13C*, *NUS1*, *KMT2B*).

Conclusions: The NGS approach in the field of dystonia increases the chance of early diagnosis, supports neurogeneticists in performing well-informed genetic counselling, helps the neurologist in clinical management, improves the understanding of biological

disease mechanisms, and allows for the identification of specific candidate therapeutic targets for each genetic form.

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1. Introduction and scope of the thesis

1.1 The complex landscape of movement disorders

Movement disorders are a group of diseases of the central nervous system characterized by an excess or defect of movements, unrelated to weakness or spasticity (Jankovic et al. 2022). The group with reduced movements is defined as hypokinesias, while the group with excess movements is referred to as hyperkinesias (Jankovic et al. 2022). Movement disorders display high heterogeneity in terms of epidemiology, genetics, pathology, and clinical presentation. Most movement disorders are associated with basal ganglia and/or cerebellar dysfunction. These are cerebral structures primarily involved in movement control but having also cognitive and behavioural functions. The predominant role of basal ganglia and cerebellum is to coordinate and control complex movements in response to internal and external stimuli (Principles of neural science 2000; Tewari et al. 2017). Globally, the most common movement disorders are Parkinson's disease, essential tremor, restless legs syndrome, and dystonia (Wenning et al. 2005).

1.2 Definition of dystonia

In 1911 Hermann Oppenheim, one of the leading German neurologists of the time, coined the term dystonia describing the clinical picture of his patients as "*dystonia musculorum deformans*" (Klein and Fahn 2013). Remarkably, Oppenheim also observed that all his dystonic patients shared the same ethnic background (i.e., Ashkenazi Jews) suggesting an inherited disorder (Klein and Fahn 2013).

Dystonia is a hyperkinetic movement disorder characterized by sustained or intermittent muscle contractions causing abnormal movements and postures. It is often initiated or worsened by voluntary action and is associated with overflow muscle activation. Dystonic movements are typically patterned, twisting, and may be tremulous. Dystonia can present in isolation or combined with other movement disorders such as chorea, myoclonus, and parkinsonism (Albanese et al. 2013).

1.3 Epidemiology of dystonia

Several attempts were made to assess the epidemiology of dystonia leading to uncertain figures because of its wide clinical expressivity. Overall, dystonia is not so rare; however, several specific dystonic syndromes, both inherited and idiopathic, fall into the definition criteria for rare diseases.

Dystonia affects twice as often women than men (Albanese et al. 2019). Adult-onset focal dystonia syndromes are by far the most frequent presentations (Defazio et al. 2004). The majority of patients with focal dystonia present cervical dystonia (69%) or blepharospasm (17%), while other forms are much rarer: limb dystonia (3-7%), spasmodic dysphonia (1-3%), musician's dystonia (3%) and oromandibular dystonia (1%) (Williams et al. 2017; Wang et al. 2016).

Epidemiological studies estimated a prevalence of dystonia in the general population of 15–30 cases per 100,000 (Steeves et al. 2012; ESDE Collaborative Group 2000). However, in a study of a random sample of the population over 50 years of age, the prevalence of isolated dystonia was estimated to be 732 per 100,000, suggesting that dystonia is underdiagnosed and not so uncommon in the aging population (Müller et al.

2002b). The high variability amongst epidemiological studies likely indicates difficulty in diagnosing dystonia, probably due to the lack of validated criteria and the existence of a significant fraction of patients with mild phenomenology who never sought medical evaluation (Albanese et al. 2019).

Of note, even though the genetic causes of dystonia – especially for adult-onset focal forms – are still largely unknown, positive family history is reported in about 20% of dystonic patients (Williams et al. 2017; Schmidt et al. 2009; Groen et al. 2012).

1.4 Classification of dystonia

The first classification of dystonia was presented in 1976, and it was modified several times in subsequent years (Fahn and Eldridge 1976; Albanese et al. 2013). The last classification (2013) serves as a guide for clinical assessment and distinguishes two main axes: axis 1 and axis 2 (Albanese et al. 2013). Axis I describes clinical features and provides a synthetic picture of the patient's clinical condition at the time of examination, whereas Axis II contains information on etiology (Figure 1). The classification of dystonia in a single patient is dynamic and adaptable to subsequent changes in light of the progression of clinical signs and symptoms and new advances in dystonia research (Albanese et al. 2013).

Five clinical descriptors are listed in Axis I: age at onset, body distribution, temporal pattern, co-occurrence of other movement disorders, or neurological manifestations. Five groups are distinguished for age at onset: infancy (0-2 years), childhood (3–12 years), adolescence (13–20 years), early adulthood (21–40 years), and late adulthood (>40 years). Body distribution can be focal, segmental, multifocal, generalized (with or

without leg involvement), or unilateral (hemidystonia). The temporal pattern includes the disease course (static or progressive) and the variability of symptoms (persistent, fluctuating, action-specific, or paroxysmal). Associated features indicate whether dystonia is combined with another movement disorder (combined dystonias) or with other neurological or systemic manifestations (complex dystonias) (Albanese et al. 2013).

Axis II classifies dystonia by etiology and specifies whether dystonia is acquired (due to a known specific cause), inherited (due to pathogenic genetic variants), or idiopathic (possibly related to yet undiscovered genetic defects). Considering the recent advances in genetic discoveries, this is the most rapidly evolving area in the classification of dystonia. Evidence of degeneration, defined as a progressive structural abnormality (macroscopic, microscopic, or imaging level), provides a useful tool to identify degenerative dystonias. Conversely, static lesions are non-progressive neurodevelopmental anomalies or acquired lesions. In cases of isolated dystonia, usually, there is no evidence of either degeneration or structural lesion (Albanese et al. 2013).

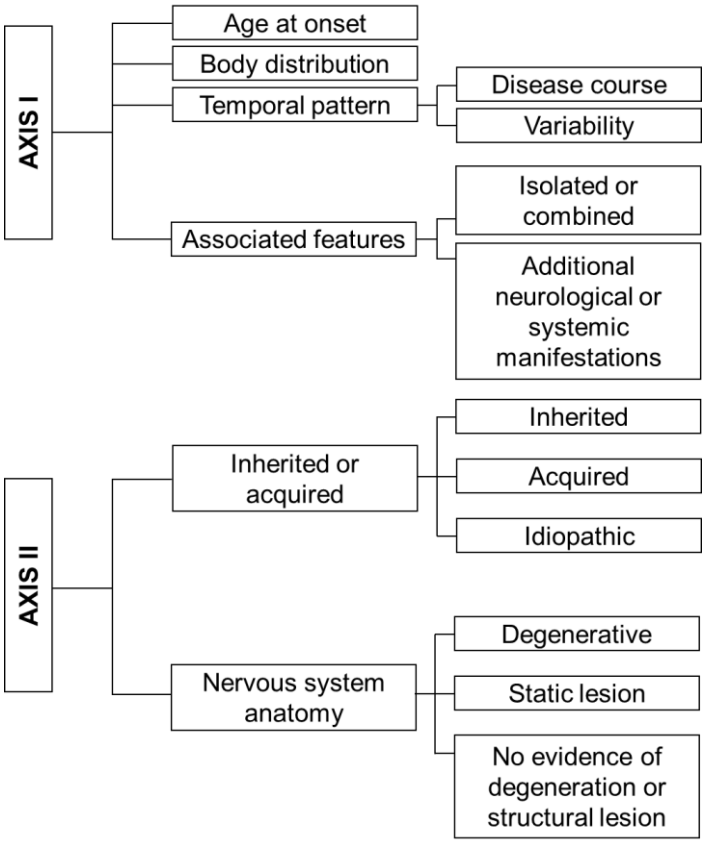

Figure 1: Organization of Axis I (clinical characteristics) and Axis II (etiopathogenesis) of the dystonia classification.

1.5 Nomenclature of monogenic dystonias

Genetic dystonia syndromes were initially classified as DYT followed by a number that represented the chronological order in which the description of the phenotype and/or genetic association (gene o locus) first appeared in the literature. However, considerable phenotypic overlap and genetic pleiotropy occur among monogenic dystonia forms, making classification based on phenotype problematic. Moreover, this classification based on the chronological order of description includes candidate dystonia genes or loci for which the association was never confirmed. Because of these classification inconsistencies, a naming system that combines the "DYT" term and the name of the associated dystonia gene was proposed (Marras et al. 2016). This new nomenclature

eliminated the problems of the chronological and phenotypic classification approaches and discarded previously listed loci that were unconfirmed (Table 1).

Form of Dystonia		Gene	Name	Distinguishing Features	MOI
Isolated		*TOR1A*	DYT-TOR1A	Childhood or adolescent-onset, generalized	AD
		THAP1	DYT-THAP1	Adolescent-onset, cranial or generalized	AD
		ANO3	DYT-ANO3	Adult-onset, focal or segmental	AD
		GNAL	DYT-GNAL	Mostly adult-onset, focal or segmental	AD
		KMT2B	DYT-KMT2B	Early-onset, generalized, mild syndromic features	AD
		TUBB4A	DYT-TUBB4A	Whispering dysphonia	AD
		EIF2AK2	DYT-EIF2AK2	Early-onset focal or generalized, progressive, mild developmental delay	AD, AR
		VPS16	DYT-VPS16	Early-onset dystonia, possible progressive generalization	AD, AR
Combined	Dystonia + parkinsonism	*GCH1*	DYT-GCH1	Dopa-responsive	AD, AR
		TH	DYT-TH	Dopa-responsive	AR
		SPR	DYT-SPR	Dopa-responsive, cognitive impairment	AR
		TAF1	DYT-TAF1	Neurodegeneration	XL
		PRKRA	DYT-PRKRA	Dystonia w/mild parkinsonism	AR
		ATP1A3	DYT-ATP1A3	Rapid-onset	AD
		DNAJC12	DYT-DNAJC12	Dystonia-parkinsonism Dopa-responsive	AR
		SLC18A2	DYT-SLC18A2	Dystonia-parkinsonism responsive to dopamine agonists	AR
	Dystonia + myoclonus	*SGCE*	DYT-SGCE	Psychiatric disease	AD
		KCTD17	DYT-KCTD17	Possible psychomotor delay	AD

Table 1: Nomenclature system for inherited monogenic forms of isolated and combined dystonia (MOI = Mode of Inheritance, AD = Autosomal Dominant, AR = Autosomal Recessive).

1.6 Inherited isolated dystonias

DYT-TOR1A (early-onset generalized dystonia) typically first manifests in childhood or early adolescence as twisting movements or torsion of an extremity. Symptoms more often start in lower parts of the body, then progress to involve other limbs and the trunk (Bressman et al. 2000). Only 35% of persons carrying a heterozygous *TOR1A* pathogenic variant are affected (reduced penetrance). Phenotypic expressivity varies in terms of age of onset, site of onset, and rate of progression. Individuals with later onset tend to be less severely affected. A founder *TOR1A* pathogenic variant (c.907_909delGAG), accounts for a large part of generalized dystonia patients, particularly in the Ashkenazi Jewish population (Ozelius et al. 1997).

DYT-THAP1 (adolescent-onset segmental/generalized dystonia) presents some phenotypic overlap with DYT-TOR1A. However, disease onset is later and cranial involvement is more pronounced, especially in the muscles of the tongue, and larynx. Dysphonia is a predominant feature. Penetrance is estimated to be at 50%. DYT-THAP1 was first identified in Mennonite families originated from a common ancestor (Fuchs et al. 2009; Bressman et al. 2009) but, currently, more than a hundred different pathogenic *THAP1* variants have been reported in literature/databases (Blanchard et al. 2011).

DYT-ANO3 (adult-onset focal or segmental dystonia) was initially described in individuals with predominantly cranio-cervical dystonia and variable age of onset (Charlesworth et al. 2012). Subsequently, heterozygous pathogenic *ANO3* variants were identified in ~1% of individuals affected by different dystonia types and were shown to cosegregate with the disease in small family pedigrees (Stamelou et al. 2014).

DYT-GNAL (adult-onset segmental dystonia) is characterized by cervical or cranial dystonia that often begins in adult life (Fuchs et al. 2013). About 30 *GNAL* heterozygous pathogenic variants were reported in individuals with dystonia and are spread over the entire gene. *GNAL* pathogenic variants are highly (but not fully) penetrant (Vemula et al. 2013).

KMT2B is a novel dystonia gene identified in patients with early-onset generalized dystonia (DYT-KMT2B, early-onset, generalized dystonia with mild syndromic features), and is the most common cause of early-onset generalized dystonia in non-Ashkenazi Jewish population (Zech et al. 2016; Meyer et al. 2017). Pathogenic *KMT2B* variants cause non-random DNA hypermethylation across the genome that can be used as an epigenetic signature specific for this disorder enabling accurate diagnosis and reclassification of ambiguous genetic findings (Ciolfi et al. 2021). Mild syndromic features such as intellectual disability, developmental delay, facial dysmorphism, and seizures have been described, therefore the inclusion of DYT-KMT2B in the isolated dystonia group may be modified over time.

DYT-TUBB4A also known as “whispering dysphonia”, is an autosomal dominant disease with high penetrance characterized by dystonia onset in the second to third decade, typically with laryngeal dysphonia followed by the involvement of the neck or limbs. Progressive generalization is frequent (Lohmann et al. 2013). Whispering dysphonia is allelic to another TUBB4A-related disorder: hypomyelination with atrophy of the basal ganglia and cerebellum (H-ABC) (Hamilton et al. 2014).

DYT-EIF2AK2 is a novel inherited dystonia form characterized by the onset of focal or generalized dystonia in the first decades of life with slow progression. Most *EIF2AK2*

pathogenic variants are heterozygous with incomplete penetrance, but a homozygous mutation with autosomal recessive inheritance has been reported (Kuipers et al. 2021). The clinical presentation can be complex with developmental delay, lower limb spasticity, and cognitive impairment. Therefore, the inclusion of DYT-EIF2AK2 in the isolated dystonia group may not be entirely appropriate.

DYT-VPS16 is an autosomal dominant early-onset dystonic disorder (Steel et al. 2020). A Chinese family with autosomal recessive VPS16-related dystonia was initially reported (Cai et al. 2016). Patients present at different ages with cranio-cervical or upper limb dystonia, often showing gradual progression to a generalized form. A minority of patients may also present cognitive impairment or psychiatric manifestations (Steel et al. 2020).

1.7 Inherited combined dystonias

● Dystonia with parkinsonism

DYT-GCH1 (Dopa-responsive dystonia) is a childhood-onset dystonia characterized by a remarkable and long-lasting response to low doses of levodopa. It typically presents with gait disturbance caused by foot dystonia and later development of parkinsonism. A slow progression to generalized dystonia can be observed. The pattern of inheritance is autosomal dominant with reduced penetrance, particularly in males (Ichinose et al. 1994).

DYT-TH is a dopa-responsive infancy-onset dystonia caused by tyrosine hydroxylase deficiency. The mode of inheritance in autosomal recessive. Initial symptoms are

typically lower-limb dystonia and gait problems. Diurnal fluctuation of symptoms with worsening of the symptoms in the evening may be present. Sleep can be beneficial. Other clinical features include bradykinesia and hypotonia, autonomic disturbances, ptosis, and oculogyric crises. Unfortunately, the clinical response to levodopa is incomplete (Lüdecke et al. 1995; Lüdecke et al. 1996).

DYT-SPR is an autosomal recessive (partially) dopa-responsive childhood-onset dystonia associated with axial hypotonia, developmental delay, weakness, and oculogyric crises; typically, symptoms present diurnal fluctuation with sleep benefit. Heterozygous carriers may be affected by abnormal postures (Friedman et al. 2006). Other clinical features include parkinsonism, hyperreflexia, intellectual disability, psychiatric abnormalities, autonomic dysfunction, and sleep disturbances (Bonafé et al. 2001).

DYT-TAF1, also known as X-linked dystonia-parkinsonism, is a neurodegenerative disorder affecting patients of Filipino ancestry (Lee et al. 2011). It is also called "Lubag", meaning “twisted” in the local dialect. DYT-TAF1 is characterized by a combination of dystonia and parkinsonism and the inheritance is X-linked recessive. Penetrance is complete in men. Almost all women heterozygous carriers are unaffected; however, few affected females with phenotypes of variable severity have been reported. A retrotransposon insertion in *TAF1*, which contains an unstable repeat expansion, is the likely causative variant of Lubag syndrome (Bragg et al. 2017).

DYT-PRKRA is an autosomal recessive dystonia with predominant oromandibular involvement, dysphagia, and cervical dystonia. Parkinsonism is often present and mild without a beneficial response to levodopa therapy (Camargos et al. 2008).

DYT-ATP1A3 (rapid-onset dystonia-parkinsonism) is characterized by rapid onset of dystonia, particularly affecting the bulbar region, in association with parkinsonism (primarily bradykinesia and postural instability). No beneficial response to levodopa therapy is reported (Brashear et al. 2007; Carvalho Aguiar et al. 2004). The age of onset has a wide range, from childhood to adulthood. Fever, physiologic stress, or alcoholic binges often trigger the onset of symptoms. After their initial appearance, findings commonly stabilize; however, occasionally, subsequent stressing episodes can cause sudden worsening. The pattern of inheritance is autosomal dominant with incomplete penetrance (Heinzen et al. 2014).

DYT-DNAJC12 is an autosomal recessive disorder presenting with psychomotor delay, hyperphenylalaninemia, and dystonia, with favourable response to BH4 and levodopa. Interestingly, Straniero et al. reported non-progressive dopa-responsive isolated parkinsonism as one of the possible DNAJC12-associated phenotypes (Straniero et al. 2017; Anikster et al. 2017).

DYT-SLC18A2 is an autosomal recessive form of infantile-onset dystonia-parkinsonism. Affected individuals may display mood disorder and autonomic nervous system dysfunction. A remarkable long-term response to dopamine agonists but not to levodopa was consistently observed (Rilstone et al. 2013; Zhai et al. 2021).

● Dystonia with Myoclonus

DYT-SGCE is characterized by a combination of myoclonus and dystonia (Zimprich et al. 2001) and is the most common form of myoclonus-dystonia syndrome. Many *SGCE* mutation carriers develop psychiatric features in addition to the movement disorder

(Weissbach et al. 2013). Maternal imprinting of the *SGCE* chromosomal region explains the fact that most affected individuals inherit the pathogenic variant from their fathers; conversely, subjects inheriting the pathogenic variant from their mothers will likely remain unaffected throughout their lives (Müller et al. 2002a).

DYT-KCTD17 is an autosomal dominant disease characterized by progressive generalized early-onset dystonia with prominent cranial involvement associated with varying degrees of myoclonus. Some motor and cognitive developmental delays may be present (Mencacci et al. 2015).

1.8 Inherited complex dystonias

In contrast to dystonia in adults, which is typically isolated or combined, dystonia in children is complex in about half of the cases (Marsden and Harrison 1974). In complex forms, dystonia co-occurs with systemic manifestations or other neurologic signs beyond movement disorders. Additional neurological manifestations include pyramidal signs, cerebellar ataxia, oculomotor abnormalities, cognitive problems, hearing loss, intellectual disability, developmental delay, and seizures (Herzog et al. 2021).

Some phenomenological dystonic features are more indicative of complex dystonia such as sustained dystonia at rest rather than action-associated occurrence and prominent tongue or perioral involvement leading to what has been called “risus sardonicus” (Herzog et al. 2021).

In terms of etiology, complex dystonias can be genetically determined or acquired. Complex dystonias are often grouped into those that are inherited (neurodegenerative or metabolic) and those that are acquired (e.g., brain lesions, drugs) (Herzog et al. 2021).

The list of genetic forms of complex dystonia is very long and constantly growing (Table 2).

Disorder	MOI	Genes
Neurodegenerative diseases		
Dentatorubral-pallidoluysian atrophy	AD	*ATN1*
Huntington disease	AD	*HTT*
Huntington disease-like 2	AD	*JPH3*
Rett or Rett-like syndrome	XL, AD	*MECP2, FOXG1, GNB1*
Early-onset Parkinson disease	AR	*PARK2, PINK1, VPS13C*
Chorea-acanthocytosis	AR	*VPS13A*
McLeod neuroacanthocytosis syndrome	XL	*XK*
Neuronal intranuclear inclusion disease	AD	*NOTCH2NLC*
Disorders leading to brain calcification		
Primary familial brain calcification	AD, AR	*PDGFB, PDGFRB, SLC20A2, XPR1, MYORG, JAM2*
Disorders of heavy metal metabolism		
Wilson disease	AR	*ATP7B*
Hypermanganesemia with dystonia, polycythaemia, and cirrhosis	AR	*SLC30A10, SLC39A14*
Neurodegeneration with brain iron accumulation (NBIA)		
Mitochondrial membrane protein-associated neurodegeneration	AR	*C19ORF12*
Aceruloplasminemia	AR	*CP*
Woodhouse-Sakati syndrome	AR	*DCAF17*
Fatty acid hydroxylase-associated neurodegeneration	AR	*FA2H*
Neuroferritinopathy	AD	*FTL*
Pantothenate kinase-associated neurodegeneration	AR	*PANK2*
PLA2G6-associated neurodegeneration	AR	*PLA2G6*
Beta-propeller protein-associated neurodegeneration	XL	*WDR45*
Lipid storage disorders		
Neuronal ceroid-lipofuscinoses	AR	*ATP13A2, CLN3, CLN5, CLN6, CLN8, CTSD, CTSF, DNAJC5, GRN, KCTD7, MFSD8, PPT1, TPP1*
Fucosidosis	AR	*FUCA1*
Niemann-Pick disease type C	AR	*NPC1, NPC2*
Sphingolipidosis		
Arylsulfatase A deficiency	AR	*ARSA*
Lysosomal storage diseases		
Krabbe disease	AR	*GALC*
GM1-gangliosidosis	AR	*GLB1*
GM2-gangliosidosis, AB variant	AR	*GM2A*
Leukodystrophies		
Creatine deficiency syndromes		*GAMT, GATM, SLC6A8*
Pelizaeus-Merzbacher disease	XL	*PLP1*
Disorders of purine metabolism		
Lesch-Nyhan syndrome	XL	*HPRT1*
Mitochondrial disorders		
Leigh syndrome	AR, mt	Pathogenic variants in the mtDNA and nuclear genes
Leber hereditary optic neuropathy	mt	Pathogenic variants in the mtDNA
MELAS	mt	Pathogenic variants in the mtDNA
MERRF	mt	Pathogenic variants in the mtDNA
POLG-related disorders	AR, AD	*POLG*
Mohr-Tranebjaerg syndrome	XL	*TIMM8A*
Other dystonia-deafness syndromes	AD, XL	*SERAC1, SUCLA2, DDP*
Organic acidurias		
D-2-hydroxyglutaric aciduria	AR	*D2HGDH*
Glutaric aciduria type 1	AR	*GCDH*
Methylmalonic acidemia	AR	*MCEE, MMAA, MMAB, MMADHC, MMUT*
Aminoacidurias		
Homocystinuria caused by cystathionine β-synthase deficiency	AR	*CBS*
Phenylketonuria	AR	*PAH*
Hartnup disorder	AR	*SLC6A19*
Disorders of biotin metabolism		
Biotinidase deficiency	AR	*BTD*
Disorders of thiamine metabolism		
Biotin-thiamine-responsive basal ganglia disease	AR	*SLC19A3*
Disorders of galactose metabolism		
Classic galactosemia and clinical variant galactosemia	AR	*GALT*
Miscellaneous		
Aicardi-Goutières syndrome	AD, AR	*ADAR, RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1, TREX1*
NUS1-related disorder	AD	*NUS1*
NEDAMSS	AD	*IRFBPL2*
SCAR29	AR	*VPS41*
YY1-Related Dystonia	AD	*YY1*
Torsion dystonia 2	AR	*HPCA*
GNAO1-related disorder	AD	*GNAO1*

Table 2: A tentative list of inherited complex dystonias (MOI = Mode of Inheritance, AD = Autosomal Dominant, AR = Autosomal Recessive, XL = X-linked, mt = mitochondrial).

Paroxysmal movement disorders (PxMDs) are a distinct group of movement disorders that are sometimes classified in the group of combined/complex dystonias. PxMDs are characterized by episodic involuntary movements (dystonia, dyskinesia, chorea, and/or ataxia). Historically, PxMDs were classified by their clinical presentation; however, the advent of NGS changed this nomenclature. Similarly to DYT classification, specific PxMDs forms are named with the suffix PxMD followed by the name of the mutated gene (Harvey et al. 2021) (Table 3).

Disorders	*Gene*	Name	MOI
Paroxysmal Movement Disorders	*PNKD*	PxMD-PNKD	AD
	PRRT2	PxMD-PRRT2	AD
	SLC2A1	PxMD-SLC2A1	AD
	KCNMA1	PxMD-KCNMA1	AD
	ADCY5	PxMD-ADCY5	AD

Table 3: List of the most common paroxysmal movement disorders (PxMDs)

1.9 Next Generation Sequencing (NGS)

The term NGS refers to many different technologies that are rapidly overcoming the traditional way of DNA sequencing (i.e., Sanger sequencing method). These novel approaches, also called massively parallel sequencing or second-generation sequencing, have a broad range of applications in medicine. In the field of human genetics, the main application of NGS is the sequencing of DNA molecules (from single fragments to entire genomes) for the discovery of deleterious variants by comparison to known reference genomic sequences (Mardis 2008).

The high throughput of NGS techniques is the main advantage in comparison to traditional sequencing technologies. However, this great amount of data needs dedicated hardware, software, and specialized scientists. Moreover, this novel technology creates many scientific and technical challenges, including big data handling and long-term storage of data for possible future re-analyses (Olgiati et al. 2016).

Concerning the target regions, one can distinguish targeted sequencing, whole-exome sequencing (WES), and whole-genome sequencing (WGS). WGS is the most comprehensive approach, and it is expected to be the method of choice in the future. However, the wide use of WGS is limited by the costs of sequencing and data storage and by the difficulty of interpreting the enormous amount of data. WES is currently a cheaper and more popular method to analyze the entire exome (about 2% of the human genome). Finally, targeted sequencing is currently the best strategy to screen a list of predetermined genes for diagnostic purposes (i.e., gene panels) (Sun et al. 2015).

A major problem to consider in choosing the best NGS approach is the possible identification of incidental findings, which are pathogenic variants unrelated to the disease for which the patient underwent NGS analysis, but which nevertheless may have clinical relevance. The use of a limited gene panel including only the genes associated with the disease under study may represent a good option in this sense, especially when the analysis is performed for diagnostic purposes only. In case of incidental findings, the currently predominant view in the field of medical genetics is to report the genetic results of a minimum gene list ("actionable genes") associated with severe and treatable conditions to the ordering clinician who can place them into the context of that patient's

medical and family history, physical examination and other laboratory tests (Green et al. 2013).

The majority of NGS technologies share a similar fundamental workflow, in which a high-molecular-weight DNA sample (e.g., genomic DNA) is fragmented by mechanical or enzymatic methods in a library of short DNA sequences (usually about 150 bp). Then, platform-specific adaptors are ligated to both ends of each fragment, enabling an easy PCR amplification of single-stranded DNA molecules with a single set of primers or allowing the fragments to bind to a surface using complimentary adaptors (hybridization). In WES and gene panels there is an important additional step (capturing) aimed at the library enrichment with the DNA fragments of interest (e.g., exome for WES and specific genes for gene panels). All the single-stranded DNA molecules are sequenced in parallel, using fluorescent or electrical signals. Then, an imaging or a different sensing system associated with a computer detects millions of sequencing signals cyclically. This large amount of data is then processed and interpreted using bioinformatic tools (van Dijk et al. 2014).

The first step of bioinformatic analysis is to align the raw reads against the reference genome (alignment), in order to obtain a list of genetic variants present only in the DNA under study (variant calling). Finally, the list of these variants is arranged and compiled with different descriptive information in a process called variant annotation (e.g., genetic position, gene affected, predicted consequence, the frequency of the variation in different populations, and many others). This technique allows researchers and geneticists to identify with confidence single nucleotide variants (SNVs) and short insertion or deletions (INDELs) (Pereira et al. 2020). Interestingly, repeat expansions

and copy number variations (CNVs) can be now identified through NGS techniques using advanced dedicated bioinformatic tools (Dolzhenko et al. 2019).

1.10 Identification of novel mendelian mutations through NGS

In the recent literature on medical genetics, relevant genetic variants are conveniently classified according to their allele frequency and effect size. Highly penetrant pathogenic variants causing mendelian diseases are usually very rare (minimum allele frequency - MAF< 0.1%) and easily identified by classic family-based approaches (linkage analysis). Conversely, genetic risk variants with a small effect size are common in the population (MAF>5%) and are identified by genome-wide association studies (GWAS). The remaining category of variants with intermediate prevalence and effect size, difficult to find using linkage analysis and GWAS, can now be more easily approached with the advent of NGS (Olgiati et al. 2016).

Linkage analysis is the traditional strategy to identify the genetic cause of disease in families with multiple affected members. The linkage approach tests the co-segregation of a chromosomal locus tagged by polymorphic genetic markers with a genetic disease by taking advantage of the phenomenon of genetic recombination (crossing-over during meiosis). Linkage studies are very powerful in detecting genetic loci harboring pathogenic variants with a large effect size (high penetrance). However, alleles with a large effect size are usually very rare in the population (Olgiati et al. 2016). Linkage studies have led to the identification of many dystonia genes. For example, linkage analyses greatly helped in the identification of several dystonia-causing genes, including DYT-TOR1A (Kramer et al. 1994), DYT-ATP1A3 (Kramer et al. 1999),

DYT-TAF1 (Németh et al. 1999), DYT-GCH1 (Nygaard et al. 1993), DYT-SGCE (Nygaard et al. 1999).

When multiple members of a family are affected, the genomic region of interest can be substantially narrowed by linkage analysis and the small number of genes located within the identified locus can be investigated by Sanger sequencing or by a quick analysis using an NGS approach. The NGS power here is not strictly necessary, but it can be of great help to speed up the analysis. Similarly, in the case of recessive disorders, particularly when consanguinity is present, NGS can be associated with homozygosity mapping to identify candidate homozygous regions (Olgiati et al. 2016).

In addition to the complementary role to traditional linkage methods, NGS technology allows innovative experimental designs that were impossible before:

1) The first approach is trio analysis, which is used to easily identify de novo mutations (i.e., mutations that are present in the proband but not in the healthy parents). The affected offspring and the unaffected parents undergo WES or WGS; the rare variants that are present only in the offspring are the candidate pathogenic mutations. De novo mutations are present in patients without a family history of the disease (sporadic) and therefore cannot be studied by the classic linkage analysis approach (Olgiati et al. 2016).
2) Another approach is to perform WES or WGS of two or more distant relatives (e.g., first or second cousins) affected by the same disease and search for rare, shared candidate variants. This approach streamlines the study of families compared to classic linkage analysis and it can be applied also when the number of available affected members is very low. However, the risk of including

phenocopies when the affected relatives are distant should be always taken into consideration (Olgiati et al. 2016).

3) A third approach is the so-called “double-hit strategy” in the field of recessive diseases. In this experimental design, the WGS o WES of the patient is screened for genes carrying two rare heterozygous variants or a homozygous variant. This approach is easier when the WGS or WES of both unaffected parents is available, to include only candidate variants associated in trans, an arrangement compatible with recessive inheritance (Olgiati et al. 2016).
4) Finally, NGS can be used to look for the recurrence of the same gene carrying deleterious variants in a series of unrelated subjects affected by the same disease. This approach is especially powerful when the phenotype of interest is particularly well-defined because in this context the probability that pathogenic variants in only one gene are responsible for the disease phenotype is higher (Olgiati et al. 2016).

1.11 Variant prioritization and assessment of pathogenicity

The interpretation of the vast amount of sequencing data is one of the biggest challenges in the NGS era. For example, the WES of a single subject unravels tens of thousands of genetic variants. The typical analysis approach, when looking for a highly penetrant disease-causing variant is to adopt one of the strategies previously described, excluding thousands of non-candidate variants. However, this approach alone is usually insufficient to identify a single candidate variant, leaving dozens of possible candidates to be assessed for pathogenicity.

Intronic variants and other variants localized in non-coding regions are usually removed from the list of candidates in the hypothesis that pathogenic mutations affect predominantly the coding region. However, although true in most cases, this approach carries the risk to exclude a possible non-coding pathogenic variant from the list.

Additional prioritization strategies include the use of appropriate filters. The most important filter is allele frequency (AF), which is used to exclude variants with high AF in the general population, relying on in-house NGS data and/or large public repositories based on thousands of individuals from different populations (e.g., 1000 Genomes, ExAC, and gnomAD). The upper limit AF cut-off should be chosen very carefully to include the disease-causing variant and to exclude a reasonable number of benign polymorphisms. For example, when the inheritance pattern is thought to be autosomal dominant due to a highly penetrant mutation and the disease is rare in the population, the AF cut-off should be set very low, while when the inheritance pattern seems to be autosomal recessive or autosomal dominant with low penetrance the threshold should be set higher, reducing the risk of filtering out the disease-causing variants.

Another approach is to look for the predicted effect of variants at the protein level. For example, manifestly deleterious variants, such as stop, splice-site, or frameshift, and lead to truncated or aberrant proteins are good pathogenic candidates if the suspected disease mechanism is “loss of function”. In addition, good candidate missense variants usually affect an evolutionarily conserved amino acid because the evolutionary stability of an amino acid indicates that a variation in that position is poorly tolerated by evolution because it impacts the fitness of the carrier. Another commonly used approach is the analysis of variants through in silico prediction tools that predict the functional

damage associated with the identified variants. However, the outputs of these in silico analyses should always be evaluated with caution, because many known pathogenic mutations are predicted benign, and many rare benign polymorphisms are predicted pathogenic. The error rate of in silico prediction tools is particularly high when the variant is pathogenic through a toxic gain-of-function mechanism, which is less predictable in comparison to a loss of function.

An additional criterion to consider is biological plausibility. For example, the candidate gene should be expressed in the tissue affected by the disease or involved in a pathway already known to be involved in the disease.

Despite all these strategies to classify the identified variants as benign, likely benign, likely pathogenic, and pathogenic (classification system of the American College of Medical Genetics – ACMG), there are some of them which remain difficult to interpret and categorize. These variants are called variants of unknown significance (VUS), meaning that the available evidence is not conclusive to claim or exclude their pathogenicity. However, it should be noted that clinical interpretation of many VUS is likely to change in the future as our knowledge increases over time (Richards et al. 2015).

1.12 NGS and dystonia

During the last twenty years, the advent of Next-Generation sequencing (NGS) techniques has provided an impressive step forward in the field of dystonia genetics. NGS methods provide a way to rapidly generate an unprecedented amount of DNA sequences at a relatively low cost. As a result of these novel approaches, the pace of

discovery of novel dystonia-causing genes is rapidly accelerating. Indeed, the causative genes for DYT-TUBB4A (Lohmann et al. 2013), DYT-ANO3 (Charlesworth et al. 2012), DYT-GNAL (Fuchs et al. 2013), DYT-KMT2B (Zech et al. 2016), DYT-KCTD17 (Mencacci et al. 2015), DYT-EIF2AK2 (Kuipers et al. 2021), and DYT-VPS16 (Cai et al. 2016; Steel et al. 2020) were all identified using NGS approaches in the last decade. Moreover, the phenotypic spectrum associated with previously known dystonia genes is expanding.

Genetic testing in dystonia

Genetic tests are now widely considered a standard part of the diagnostic workup in unexplained dystonia, particularly in early-onset and more severe cases. Nevertheless, a high proportion of dystonia cases remain genetically unsolved, especially late-onset focal forms. Whether this fact primarily reflects undiscovered monogenic disorders or a polygenic-multifactorial etiology remains under investigation (Zech et al. 2016).

Dystonia patients may undergo a wide range of genetic testing: single-variant testing, single-gene Sanger sequencing, NGS gene panels, clinical exome sequencing (CES), WES, or WGS. The best genetic approach for each patient should be decided on the basis of individual characteristics as it relies on the clinical presentation, family history, and ethnic background. In any case, in the last years, the NGS techniques (i.e., gene panels, CES, WES, and WGS) took over and are currently the preferred approach for genetic testing in dystonia (Pozojevic et al. 2021).

● Single-variant testing

Single-variant testing can be applied in highly selected dystonia forms in patients with specific ethnic backgrounds due to the presence of a common founder carrying the pathogenic variant. At least two dystonia forms are caused primarily by single founder variants: DYT-TOR1A (Ozelius et al. 1997) and X-linked dystonia-parkinsonism (DYT-TAF1) (Lee et al. 2011).

In Ashkenazi Jews, DYT-TOR1A is predominantly caused by the founder *TOR1A* in-frame deletion in exon 5 (c.907_909delGAG). This variant accounts for approximately 80–90% of early-onset dystonia in this specific population (Ozelius and Bressman 2011). Therefore, in Ashkenazi patients affected with early-onset dystonia direct sequencing of exon 5 of *TOR1A* is a rational approach (Bressman et al. 2000).

DYT-TAF1 has been reported exclusively in Filipino patients presenting adult-onset dystonia–parkinsonism carrying the founder retrotransposon insertion in the *TAF1* gene (Bragg et al. 2017; Aneichyk et al. 2018; Pozojevic et al. 2022). Thus, in an adult Filipino patient with dystonia-parkinsonism, the analysis of the presence of the founder *TAF1* retrotransposon insertion is a reasonable first-line genetic testing option (Weissbach et al. 2021).

● Single-gene testing

Sanger sequencing of a single gene means to sequence exons and intron-exon boundaries of a gene of interest. Nowadays, the rational use of the time-consuming and labour-intensive single-gene testing is restricted to patients with dystonia phenotypes clearly suggesting a specific etiology.

For example, a patient with paternally inherited myoclonus dystonia that occurs early in life predominantly affecting the neck and upper limbs may be tested specifically for *SGCE* pathogenic variants (DYT-SGCE) (Weissbach et al. 2021). Similarly, a childhood-onset lower limbs dystonia, spreading cranially to become generalized dystonia with concomitant parkinsonism, diurnal fluctuations of symptoms, and excellent response to levodopa, is highly indicative of dopa-responsive dystonia (DYT-GCH1) (Weissbach et al. 2021).

● NGS-based approaches

Dystonia NGS gene panels are designed for sequencing exons and exon-intron boundaries of genes known to be associated with dystonia, and they ensure high coverage of the analyzed regions. CES represents a "minimalist" variant of WES directed only to the coding regions of ~4000 genes already associated with human genetic diseases (Gorcenco et al. 2020; Trujillano et al. 2017). The most important drawback of these two otherwise cost-effective approaches is that, in patients with negative findings, the research of novel dystonia genes or reanalysis of data once novel dystonia genes are identified is impossible or significantly limited (Pozojevic et al. 2021).

WES and WGS provide information on the majority of exonic or genomic variants of the patient. WGS is more comprehensive than WES and may detect disease-causing variants in deep intronic or relevant regulatory regions of dystonia genes that would be overlooked by sequencing exons only (Kumar et al. 2019). The current practice includes WGS only in patients who remain without a diagnosis after other variant detection strategies have been attempted, but this is likely to change in the near future.

The largest WES study of dystonia so far sequenced 764 dystonic patients. Causative or likely causative variants were identified in ~19% of subjects, involving 78 distinct monogenic disorders. The genetic diagnosis was more likely to be found in subjects with complex dystonia (~45%) in comparison to those with combined (~19%) or isolated (~4%) forms. Moreover, diagnosed individuals were younger at dystonia onset and presented with more widespread dystonia. Interestingly, ~65% of the diagnostic variants involved genes associated with neurodevelopmental disorders (Zech et al. 2020).

A single study to date described the application of WGS in a cohort of dystonia patients (n=111), who presented isolated or combined dystonia (Kumar et al. 2019). A genetic diagnosis was obtained in ~12% of cases and was more probable in patients with younger age of onset (Kumar et al. 2019). The probability of receiving positive genetic testing results was significantly higher in the group with a combined dystonia phenotype, whereas it was lower in patients with focal/segmental isolated dystonia with adult onset. CNVs were detected in ~23% of the diagnosed individuals (Kumar et al. 2019). To my knowledge, CNVs have been already identified in four isolated/combined dystonia genes: *THAP1* (Baker et al. 2014), *KMT2B* (Zech et al. 2016), *GCH1* (Zirn et al. 2008), and *SGCE* (Grünewald et al. 2008). Therefore, these data, taken together, suggest that NGS-based genetic testing should also be examined for evidence of CNVs with appropriate bioinformatic tools (Roller et al. 2016).

In summary, a gene panel sequencing approach may be a cost-effective option in patients with childhood-onset isolated dystonia and positive family history. It is important to note that age at onset is one of the most important predictors of a positive

genetic result. Conversely, WES and WGS represent a more meaningful and effective strategy in probands affected by dystonia as a part of a more complex clinical phenotype, as these phenotypes are not likely to be caused by variants in isolated/combined dystonia genes (Pozojevic et al. 2021; Zech et al. 2020; Kumar et al. 2019).

1.13 Scope of the thesis

The scope of this thesis is to identify through a Next-Generation Sequencing (NGS) approach novel genes and genetic variants associated with hereditary dystonia and to shed a light on novel disease mechanisms and clinical phenotypes associated with inherited dystonias.

2. Methods

This thesis is composed of a reasoned collection of scientific investigations on the genetics of dystonia using NGS technology performed by the candidate (Dr. Edoardo Monfrini) during the Ph.D. program at the University of Milan and NYU Langone Health. The NGS “short-reads” approach was applied to resolve the genetic diagnosis of families and singletons affected by heterogeneous forms of isolated, combined, and complex dystonia. The specific methods used to perform each study are detailed in each chapter.

3. A novel *VPS11* variant causes autosomal recessive dystonia

3.1 Abstract

In this work we describe the novel association of a homozygous *VPS11* mutation with adult-onset generalized dystonia, providing a detailed clinical report and biological evidence of disease mechanism. Vps11 is a subunit of the HOPS complex, which promotes the fusion of late endosomes and autophagosomes with the lysosome. Functional studies on mutated fibroblasts showed marked lysosomal and autophagic abnormalities, which improved after overexpression of the wild-type Vps11 protein. In conclusion, a homozygous *VPS11* mutation, damaging the autophagic and lysosomal pathways, causes a novel form of generalized dystonia associated with signs of neurodegeneration.

3.2 Introduction

Dystonia is a hyperkinetic movement disorder characterized by sustained or intermittent muscle contractions causing abnormal movements and/or postures. If the trunk and at least two other sites are involved, dystonia is defined as generalized[1]. Inherited dystonias can be classified as isolated (dystonia is the only motor feature, except for tremor), combined (dystonia is associated with other movement disorders), or complex (dystonia co-occurs with other neurologic or systemic manifestations)[2].

Typically, isolated and combined dystonia have not characteristic features at brain MRI. On the other hand, complex dystonia often shows pathognomonic MRI changes. Indeed, complex dystonia is one of the most frequent clinical presentations of

neurodegeneration with brain iron accumulation (NBIA), which is a group of genetic disorders displaying progressive iron accumulation in basal ganglia[2].

Vps11 aggregates with other Vps proteins (i.e., Vps16, Vps18, Vps33, Vps41, and Vps39) to form the "homotypic fusion and protein sorting complex (HOPS)". The HOPS complex promotes the fusion of late endosomes and autophagosomes with lysosomes [3, 4].

Homozygous *VPS11* mutations were associated with hypomyelinating leukodystrophy 12 (HLD12), characterized by appendicular spasticity, truncal hypotonia, opisthotonic posturing, and seizures. Brain MRIs of affected subjects present a thin corpus callosum and diffused hypomyelination. Two homozygous mutations were described so far (c.2536T>G p.C846G and c.1158_1184del p.L387-G395del) (Figure 1A) [5–7].

Here we describe a novel homozygous *VPS11* mutation causing adult-onset generalized dystonia. We provide strong evidence of variant pathogenicity and demonstrate its highly deleterious impact on the autophagy-lysosomal pathway.

3.3 Materials and methods

● Clinical data

The subject underwent several neurological examinations, brain MRI and neurophysiological studies. Blood samples and a skin biopsy were collected. The Ethics Committee of the IRCCS Foundation Ca' Granda Ospedale Maggiore Policlinico (Milan, Italy) approved the study. Written informed consent was obtained from the patient.

● Genetic analysis

The genomic DNA of the proband was analyzed by whole-exome sequencing (WES) using the Nextera Rapid Capture Exome Library kit (Illumina) on the Illumina NextSeq500 platform. Reads alignment and variant calling/annotation were performed using standard procedures. The candidate *VPS11* variant was validated by Sanger sequencing in the affected subject and his relatives. Homozygosity mapping was performed starting from WES data using the HomozygosityMapper online tool (http://www.homozygositymapper.org/)

● Cell cultures and plasmid transfection

Skin fibroblasts of the patients and three controls were cultured in DMEM high glucose supplemented with 15% Fetal Bovine Serum (Euroclone), 1% penicillin/streptomycin (Sigma-Aldrich), and 1% of Amphotericin B (Sigma-Aldrich). Patient fibroblasts were transfected through lipofectamine with a wild-type *VPS11* untagged plasmid (HG21081-UT, Sinobiological).

● Protein blotting and biochemical studies

Western blot analyses were performed in triplicate on whole-protein lysates from cultured fibroblasts using the following primary antibodies: Vps11 (HPA039020, Sigma-Aldrich); Actin (A2066, Sigma-Aldrich), Lysosome-associated membrane glycoprotein 1 - LAMP1 (ab25630, Abcam), β-glucocerebrosidase - GBA (EPR5143(3), Abcam), α-glucosidase - GAA (02D05, Genzyme), Acid ceramidase - ASAH1 (sc-28486, Santa Cruz Biotechnology), p62 (MABN130, Sigma-Aldrich), LC3A/B (ab58610, Abcam), Beclin-1 (D40C5, Cell Signaling Technology). Enzyme activities of lysosomal and plasma membrane glycohydrolases were determined in total cell lysates using already published method[8]. A two-tailed Student's t-test was

performed to assess the statistical significance of the protein amount and lysosomal activities differences of in the patient compared to control cells.

● Electron microscopy

Approximately $5x10^6$ fibroblasts were incubated with the fixing solution (glutaraldehyde 3%). Samples were dehydrated through graded alcohols, infiltrated, and embedded in Spurr's resin. Ultrathin sections (60 nm) were cut with Ultrotome Nova (LKB). Observations were performed using a EM 109 transmission electron microscope (Zeiss).

3.4 Results

● Clinical features

The family of the proband lived in a very small and geographically isolated village of Southern Italy for many generations. Family members considered very plausible the distant consanguinity of the parents (I.1 and II.2). Two of proband's brothers (II.2 and II.6) died within the first year of life for unknown reasons. All the living siblings were healthy and had by far passed their brother's age at onset (Figure 1B).

The proband was born at term after an uneventful pregnancy and had normal psychomotor development. From the age of 30 years, the proband developed progressive dystonic postures, initially affecting the left upper limb. In the following 5 years, dystonia progressively became generalized, involving the trunk, limbs, neck, and larynx. Moreover, progressive dysarthria and dysphagia appeared.

The proband reached our clinic at 40 years of age presenting with severe generalized dystonia affecting the trunk, face, neck (torticollis), and limbs (left > right),

accompanied by moderate dysphagia and complete anarthria. Moderate lower limb hyperreflexia was also observed.

Brain MRI displayed mild brain atrophy with more conspicuous involvement of basal ganglia. Interestingly, substantia nigra, red nucleus, and globus pallidus presented symmetrical hypointensity in T2-weighted sequences. The T2*-weighted sequence showed symmetrical bipallidal hypointensity, possibly due to iron accumulation (Figure 1C). Neurophysiological studies were unremarkable.

Therapeutic challenges with levodopa and anticholinergics did not bring any benefit. Botulinum toxin injections brought a slight improvement of the dystonic posture of the neck and right limbs. At the age of 50, the patient underwent bilateral deep brain stimulation of the internal globus pallidus (GPi DBS). No significant improvement of the dystonic features was observed after DBS.

● Genetic analysis

The suspected consanguinity of the parents suggested a homozygous mutation as the cause of the disease. WES was performed on the proband. No pathogenic mutations were found in known disease genes for inherited dystonia. A filtering analysis for rare (allele frequency <0.1%) homozygous variants with protein impact was performed. Only the c.136C>T, p.P46S variant in *VPS11* gene was identified. Rare biallelic variants were identified either in obvious non-candidate genes or were carried also by healthy siblings. The *VPS11* variant lies on chromosome 11 in one of the two homozygous regions identified by homozygosity mapping (Figure 1E). Sanger sequencing confirmed the mutation to be homozygous in the proband (II.7) and heterozygous in subjects II.3, II.4, and II.8 (Figure 1B and 1E).

The p.P46S *VPS11* mutation is a novel variant absent from public databases (i.e., 1000G and gnomAD). The Proline at position 46 is highly conserved in mammalian orthologues (Figure 1F) and is localized in the Vps11 N-terminal β-propeller domain (Figure 1A), which is known to be essential for protein-protein interactions[9]. Moreover, the Pro > Ser substitution is non-conservative and the peculiar properties of Proline residues (e.g., their rigid conformation with low flexibility) are frequently important to stabilize protein structure. For these reasons, *VPS11* was selected as the candidate etiological gene.

● **Functional studies**

Immunoblot studies on total lysates from cultured fibroblasts showed a slight but statistically significant increase of Vps11 protein amount in mutated cells compared to controls. In addition, patient fibroblasts showed a striking increase in autophagic proteins p62, LC3A, and LC3B, with normal Beclin-1 levels, and augmented lysosomal proteins and hydrolases (LAMP1, β-glucocerebrosidase, acid α-glucosidase, and acid ceramidase), suggesting a significant proliferation of the late-endosomal and lysosomal compartments (Figure 2A).

Transient overexpression of wild-type Vps11 protein in patient fibroblasts significantly improved the pathological phenotype at the protein level, demonstrating the deleterious effect of the p.P46S variant (Figure 2A).

Lysosomal enzymatic activities in patient fibroblasts (i.e., β-glucocerebrosidase, β-galactosidase, and β-hexosaminidase) were three to four times higher than in controls. The plasma membrane enzymatic activities were also significantly increased, of about two to three times compared to controls (Figure 2B).

Electron microscopy of patient fibroblasts showed large clear vacuolar structures, which were completely absent in control fibroblasts (Figure 3). This result appears to be consistent with a morphological alteration of the autophagic and/or endo-lysosomal compartments, in line with the results of protein studies.

3.5 Discussion

The identification of a *VPS11* mutation in a patient with adult-onset generalized likely neurodegenerative dystonia confirms the important role of the lysosomal and autophagic pathways in the pathogenesis of neurodegenerative disorders [7].

In a previous study, knockdown of *VPS11* gene in HeLa cells caused an increase of autophagy markers (i.e., p62, LC3A, and LC3B). Moreover, the accumulation of immature autophagosomes and the reduction of autolysosomes were observed. These data suggest that *VPS11* knockdown severely impairs autophagy flux[6]. In the present study, patient fibroblasts presented an over-expression of autophagic proteins p62 and LC3B without alteration of Beclin-1 levels, suggesting an accumulation of autophagosomes without autophagy induction. Moreover, an increased amount and activity of lysosomal hydrolases suggested the accumulation of late endo-lysosomes. In addition, considering the increased plasma membrane lysosomal activities, these stored vesicles are probably partly exocytosed. The transfection of patient-derived fibroblasts with the wild-type *VPS11* plasmid and consequent Vps11 overexpression ameliorated the pathological phenotype at the protein level, supporting the pathogenic role of the identified *VPS11* mutation. These results are in line with previous *VPS11* knockdown models, indicating that the p.P46S mutation is probably a loss-of-function mutation (Figure 2C).

Hörtnagel et al. conducted a microscope analysis on tissue samples derived from subjects affected with HLD12 carrying a homozygous *VPS11* mutation (p.C846G). In these patients, electron microscopy on skin biopsies showed several intra-cellular clear vacuoles[7]. In our study, electron microscopy analysis on patient fibroblasts showed a strikingly consistent result, suggesting that the cytoplasmic accumulation of clear vacuoles, consistent with an alteration of the endo-lysosomal compartment, is the pathological hallmark of *VPS11*-related disease.

Very interestingly, biallelic mutations of *VPS16* and *VPS41* genes were recently found to cause complex dystonia, often associated with T2 hypointensity of basal ganglia[10–12]. Vps16 and Vps41 associate with Vps11 to form the HOPS complex. Therefore, our finding supports the hypothesis that the disruption of HOPS normal function is crucial in the pathogenesis of this group of complex dystonias.

Our findings show that *VPS11* mutations are associated with at least two different phenotypes: the infantile-onset hypomyelinating leukodystrophy 12 (HLD12) and the later onset *VPS11*-associated neurodegeneration. The identification of additional cases will help to disentangle the genotype-phenotype correlation in *VPS11*-related disorders. Phenotypic heterogeneity associated with mutations of a single gene is not a novelty in the field of inherited dystonias (and NBIA); for example, *PLA2G6* mutations can cause both Infantile Neuroaxonal Dystrophy (INAD) and the later onset *PLA2G6*-related dystonia-parkinsonism.

In conclusion, this work represents the first association of a recessive *VPS11* mutation with a novel form of generalized dystonia and brain iron accumulation and provides in

vitro evidence for a crucial role of the autophagy-lysosomal pathway in the pathogenesis of this neurodegenerative disorder.

3.6 References

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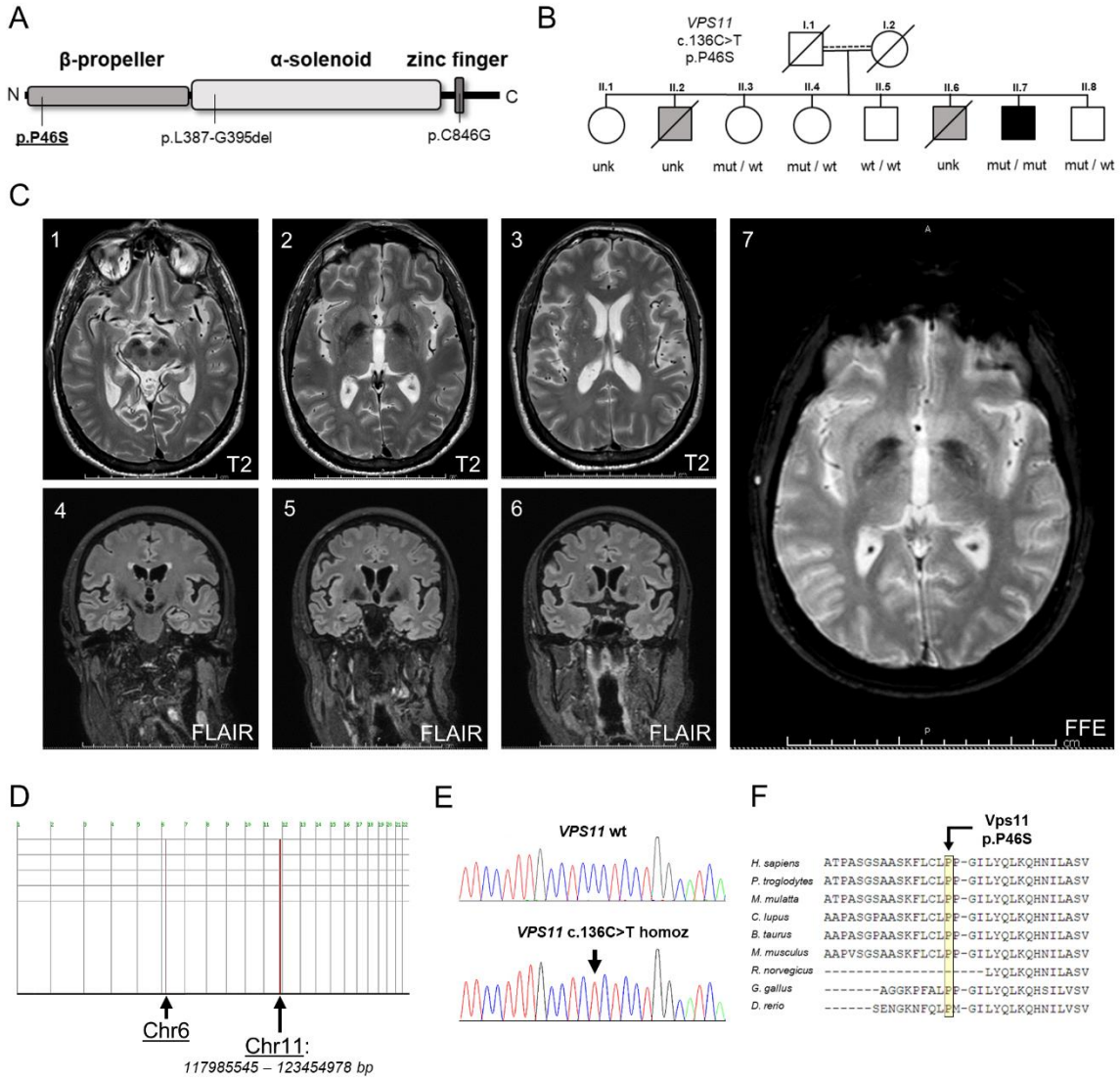

Figure 1: Genetic analysis, family pedigree, and brain MRI: A) Vps11 protein domains and the position of the pathogenic mutations identified so far; B) Pedigree of the family under study. Black symbol denotes the affected individual. Grey symbol indicates unknown status; C) Brain MRI of the subject II.7. Axial T2 (1-3) and coronal FLAIR (4-6) display bilateral T2 hypointensity of substantia nigra, red nucleus, and globus pallidus. Axial FFE (7) shows bilateral pallidal hypointensity; D) Homozygosity mapping plot displays two homozygosity peaks on chromosomes 6 and 11 in the proband. E) Electropherograms of the *VPS11* wild-type

(upper) and the c.136C>T homozygous mutation (lower) of the proband (II.7). F) Alignment of Vps11 protein homologs shows the conservation of the mutated amino acid (Proline46).

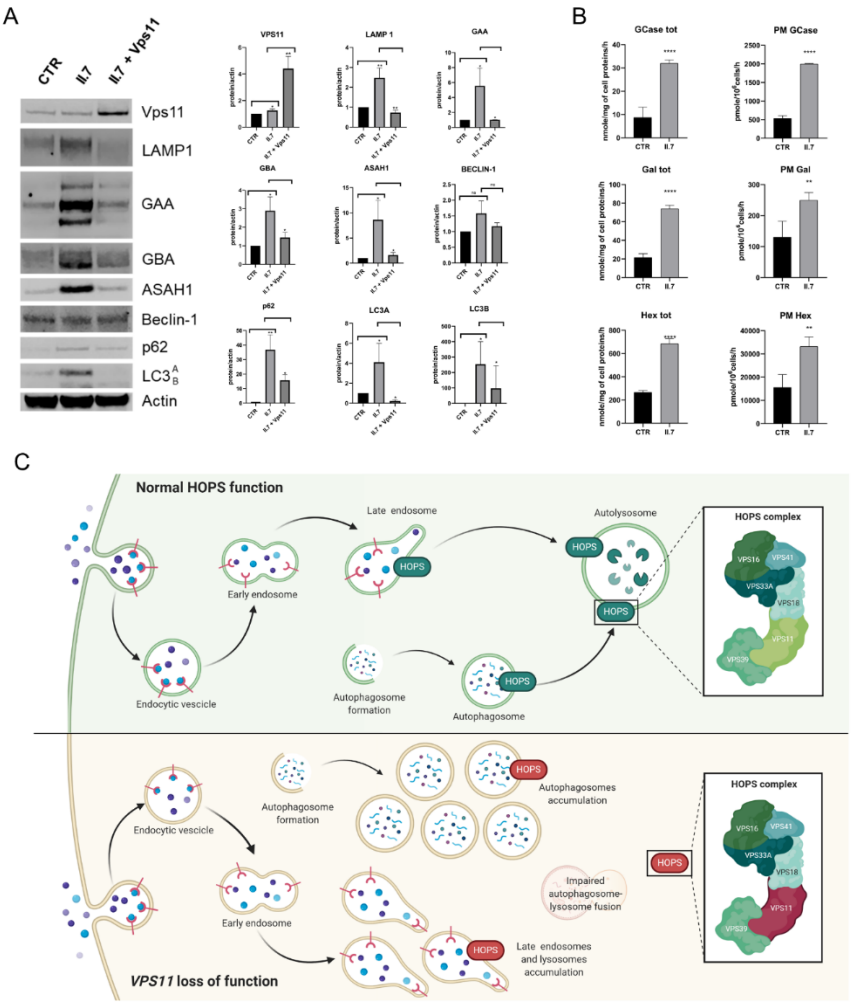

Figure 2: Immunoblot of autophagic and lysosomal proteins and lysosomal enzymatic activities: A) Immunoblots of fibroblast lysates display a statistically significant increase of autophagic and lysosomal proteins in proband cells (II.7) compared to controls (CTR). The overexpression of wild-type Vps11 protein in patient fibroblasts (II.7 + Vps11) ameliorates the abnormal phenotype. * = $p < 0.05$, ** = $p < 0.02$. B) Lysosomal and plasmatic membrane enzymatic activities in patient fibroblasts (II.7) are significantly higher than in controls (CTR). GCase = β-glucocerebrosidase, Gal = β-galactosidase, Hex = β-hexosaminidase, PM = plasma membrane, ** = $p < 0.01$, **** = $p < 0.0001$. C) Cartoon model of the pathological autophagic and lysosomal functions associated with *VPS11* loss-of-function mutation. Adapted from the

template “Mutation of HOPS Complex Subunits”, by BioRender.com (2020), and from Steel D et al. Ann Neurol 2020.

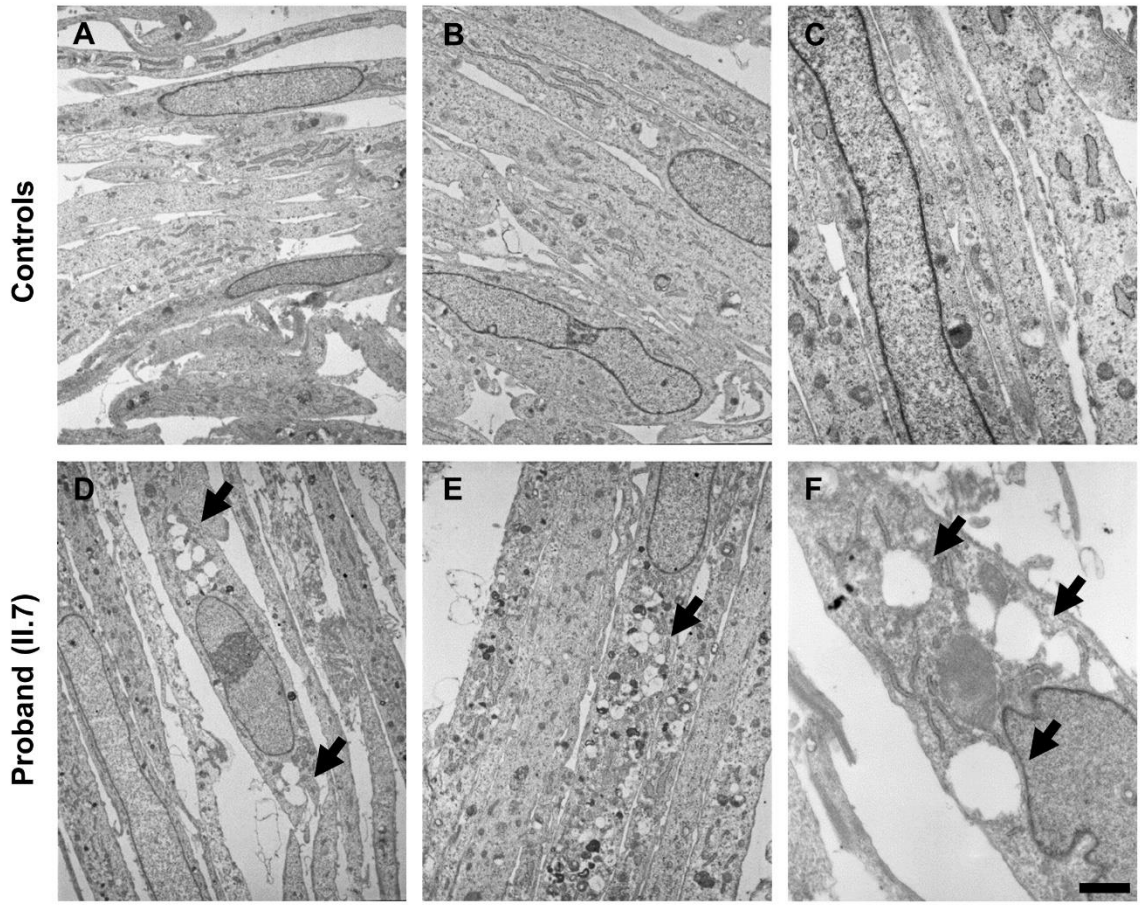

Figure 3: Electron microscopy analysis: Patient fibroblasts (D, E, F) show large clear vacuolar structures in the cytoplasm (black arrows), which are absent in control fibroblasts (A, B, C). This abnormality is consistent with an alteration of the autophagic and/or endo-lysosomal compartments.

4. A novel nonsense *VPS16* variant causes choreodystonia

4.1 Introduction

Dominant and recessive *VPS16* pathogenic variants have been recently associated with inherited dystonia (1,2). Initially, a homozygous *VPS16* mutation was found to co-segregate with juvenile-onset progressive generalized dystonia in a large consanguineous family from China (2). Subsequently, several heterozygous *VPS16* mutations were identified in dystonia families displaying a dominant pattern of inheritance with incomplete penetrance (1,3-6). In addition, two very recent reports linked biallelic *VPS16* mutations with a mucopolysaccharidosis-like disease, characterized by developmental delay, psychomotor regression, delayed myelination, brain atrophy, neutropenia, skeletal abnormalities, and dysmorphic features, indicating that the phenotypic consequences of *VPS16* mutations and the pattern of inheritance of VPS16-associated disease are still far from being completely understood (7,8).

From the clinical point of view, most patients harboring a heterozygous *VPS16* mutation display early-onset dystonia with prominent oromandibular, bulbar, cervical and upper limb involvement, followed by slowly progressive generalization and retained ability to walk in adulthood (1). Brain MRI of these patients can show bilateral and symmetrical hypointensity of the globus pallidus in T2-weighted sequences1. Interestingly, two subjects carrying pathogenic *VPS16* mutations were reported to display myoclonus (9,10).

4.2 Case Report

The proband, male and right-handed, came to our attention at the age of 76 years, seeking a diagnosis for a long-lasting, progressive, and disabling movement disorder. He was born at term and had normal psychomotor development. As a first symptom, he reported difficulty in writing, which started during the early years of primary school, interpreted as writer's cramp. In addition, his relatives always noticed minimal involuntary movements affecting the right upper limb, of which the patient was initially unaware. At the age of 52 the hyperkinesias worsened and the proband started noticing the involuntary movements. In the following years, speech became progressively dysarthric and severely disturbed by intrusive repetitive protrusions of the tongue. In the last years, the involuntary movements spread to the left upper limb.

Past medical history was unremarkable from the neurological point of view revealing cholecystectomy, hypercholesterolemia, and mild carotid artery disease for which he was prescribed antiplatelet therapy. Familial history was positive for movement disorders. The mother presented involuntary tongue protrusions, one younger sister was affected by choreodystonic movements, and one younger brother was affected by "slowness of movements", diagnosed as dystonia-parkinsonism.

The neurological examination showed the presence of involuntary choreic movements affecting the distal upper limbs (right>left), repetitive protrusions of the tongue, which further impaired an already dysarthric speech, writer's cramp, and increased blinking frequency. Muscle tone was ubiquitously reduced. Deambulation was autonomous but slightly wide-based. No pyramidal signs were observed. Romberg test was negative. Brain MRI was unremarkable except for the presence of a retrocerebellar arachnoid cyst, and non-specific cerebral biemisferic white matter lesions probably associated

with cardiovascular risk factors. EEG, EMG, and NCS were unremarkable. Polysomnography showed the presence of involuntary myoclonic movements of the four limbs predominantly of the right arm causing many frequent micro-awakenings during the phases of falling asleep and light sleep.

Genetic testing for several spinocerebellar ataxias (SCA1, SCA2, SCA3, SCA6 and DRPLA) and Huntington's disease resulted negative. Therefore, whole-exome sequencing (WES) was performed on proband's genomic DNA. A virtual gene panel including the genes associated with chorea and dystonia was applied to filter WES variants. A novel heterozygous nonsense *VPS16* variant was found (NM_022575: c.2181G>A, p.Trp727*). This variant is likely pathogenic since *VPS16* loss of function is the genetic mechanisms leading to VPS16-associated disease. No other rare variants in genes associated with chorea or dystonia were identified. Sanger sequencing confirmed the presence of the variant in the proband. The siblings of the proband denied consent for genetic analysis and segregation studies.

4.3 Discussion

VPS16 gene encodes a subunit of the homotypic fusion and vacuole protein sorting (HOPS) complex, which plays a key role in autophagosome-lysosome fusion. Very interestingly, mutations in genes encoding for other subunits of the HOPS complex have been associated with inherited neurological disorders presenting dystonia as a prominent clinical feature (i.e., *VPS41* and *VPS11*) (1). These disorders likely share a common disease mechanism and present similar clinical features (11).

The case reported here displayed atypical clinical features in comparison to the predominantly dystonic presentation of the previously reported VPS16-mutated patients. He did present some dystonia (writer's cramp); however, the prevalent movement disorder was chorea, as in the case of his mother and sister. Conversely, the brother presented a dystonic phenotype, indicating that the identified variant per se cannot entirely explain the unexpected choreic presentation in this family and that some other genetic or environmental factors modulate the phenotypic manifestations of *VPS16* pathogenic variants.

In conclusion, we described chorea as a new clinical presentation associated with a pathogenic *VPS16* variant - the novel nonsense c.2181G>A, p.Trp727* - expanding the genetic and phenotypic spectrum of VPS16-associated disease.

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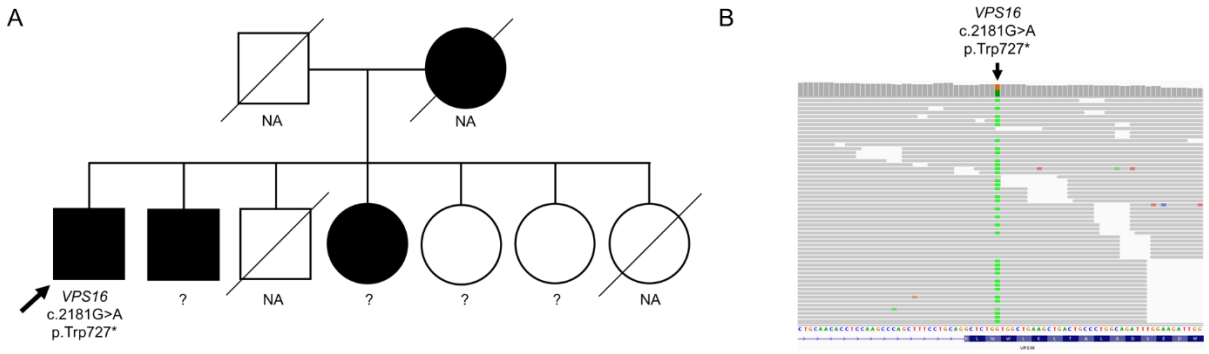

Figure: A) Pedigree of the family, black filling denotes affected individuals; B) IGV viewer visualization of the .bam file displaying the heterozygous c.2181G>A in the proband.

5. HOPS-associated Neurological Disorders (HOPSANDs): linking endolysosomal dysfunction to dystonia pathogenesis

5.1 Abstract

The “homotypic fusion and protein sorting” (HOPS) complex is the structural bridge necessary for the fusion of late endosomes and autophagosomes with lysosomes. Recent publications linked mutations in genes encoding HOPS complex proteins with the etiopathogenesis of inherited dystonias (i.e., *VPS16*, *VPS41*, and *VPS11*). Functional and microstructural studies conducted on patient-derived fibroblasts carrying mutations of HOPS complex subunits displayed clear abnormalities of the lysosomal and autophagic compartments. We propose to name HOPS-associated Neurological Disorders (HOPSANDs) this group of diseases, which are mainly characterized by dystonic presentations. The delineation of HOPSANDs further confirms the connection of lysosomal and autophagic dysfunction with the pathogenesis of dystonia, prompting researchers to find innovative therapies targeting this pathway.

5.2 Introduction

Dystonia is a movement disorder defined by the presence of sustained or intermittent muscle contractions causing abnormal movements and postures (1). Dystonia appears in the setting of non-degenerative syndromes affecting a neural network involving basal ganglia, cerebellum, and other brain structures, or as a manifestation of several neurodegenerative disorders (2). Temporal pattern can distinguish between progressive and static dystonias (1). Disease progression can be measured in terms of dystonia intensity and/or involvement of other muscles groups (1). Typically, neurodegenerative

dystonias are progressive, but also non-degenerative isolated dystonias may display a progressive course (2). Neuroimaging may support the diagnosis of the neurodegenerative group, by showing reduced volume or altered signal of the basal ganglia (3). The most significant example is represented by neurodegeneration with brain iron accumulation (NBIA), a group of genetic disorders displaying progressive iron accumulation in the basal ganglia, which present with dystonia as one of the most prominent clinical features often in combination with other neurological signs (e.g., parkinsonism, pyramidal signs, and chorea) (4). Nevertheless, not all the neurodegenerative dystonias have specific brain imaging hallmarks.

Several lines of evidence suggest that dysregulation of the endolysosomal and autophagic system is linked to the pathogenesis of dystonia (5). Dystonic features are part of the clinical presentation of many lysosomal storage disorders (e.g., Niemann-Pick type C, neuronal ceroid lipofuscinosis, gangliosidosis, fucosidosis et cetera) (5). In addition, genetic defects affecting proteins of the endolysosomal and autophagic pathways can cause neurological diseases mainly characterized by dystonia. Notable examples of this group are complex dystonia syndromes caused by mutations of *WDR45* (6), *ATP13A2* (7), *VAC14* (8,9), *IRF2BPL* (10), and *SQSTM1* (11) genes (5). Remarkably, the endolysosomal-autophagic pathway is already known to play a critical role in the pathogenesis of other neurodegenerative movement disorders. The most notable example is Parkinson's disease, which can be associated with mutations of the lysosomal genes *GBA*, *VPS35*, and *ATP13A2* (12).

Lysosomes are dynamic cytoplasmic organelles at the crossroad of endocytic, autophagic and phagocytic trafficking pathways. Fusion with these other organelles

results in the formation of hybrid structures, in which the degradation of macromolecules and wasted cellular components occurs and from which lysosomes are re-formed (13). Autophagy is a self-degradative cellular process critical for balancing energy supplies in response to nutrient deprivation. It also plays a housekeeping role in removing misfolded or aggregated proteins and clearing damaged organelles (14). The "homotypic fusion and protein sorting" (HOPS) complex is the structural bridge necessary for the fusion of late endosomes and autophagosomes with the lysosomes in the cytoplasm (15). HOPS complex is composed by the four "Vps-C core" proteins (i.e., Vps11, Vps16, Vps18, and Vps33a) and two additional subunits (i.e., Vps39 and Vps41) (15).

5.3 Genetic and clinical findings

Recent publications linked mutations in genes encoding for the HOPS complex with the etiopathogenesis of inherited dystonias (i.e., *VPS16*, *VPS41*, and *VPS11*) (16-19).

A single homozygous and several heterozygous *VPS16* mutations were identified in patients affected by dystonia (16, 18, 20, 21). *VPS16* pathogenic variants were found with different genetic strategies. The homozygous mutation was found to cosegregate with juvenile-onset progressive generalized dystonia in a large consanguineous family from China using a combined approach of whole-exome sequencing (WES) and homozygosity mapping (18). In contrast, the heterozygous *VPS16* mutations were initially identified in 19 dystonic patients from 14 families, starting from a weighted burden analysis of WES data derived from 138 patients with generalized dystonia. Several of these deleterious variants were then confirmed to cosegregate with dystonia in multigenerational families displaying a dominant pattern of inheritance with

incomplete penetrance (16). Two additional heterozygous carriers have since been identified through a screening of *VPS16* gene by two different groups (20, 21). Clinically, most subjects harbouring a *VPS16* mutation display early-onset dystonia with prominent oromandibular, bulbar, cervical, and upper limb involvement, followed by progressive generalization. The course of the diseases was slowly progressive in most patients, who retained the ability to walk in adulthood. Interestingly, some patients responded favourably to deep brain stimulation. Four of these patients showed on brain MRI bilateral and symmetrical hypointensity of the globus pallidus in T2*-weighted sequences, suggesting possible iron deposition (16).

Biallelic *VPS41* mutations were initially found independently by two different groups reporting three patients affected by dystonia in more complex phenotypes (16, 17). Firstly, compound heterozygous mutations were found through WES in two siblings displaying dystonia, ataxia, and retinal dystrophy (17). Another patient carrying a homozygous splicing disrupting *VPS41* mutation was identified through screening of genes encoding for a selected group of HOPS proteins (i.e., *VPS18*, *VPS39*, and *VPS41*) (16). He presented with global developmental delay, generalized dystonia, optic atrophy, and axonal neuropathy (16). Brain MRI of all subjects showed progressive cerebellar atrophy and thinning of the corpus callosum. Interestingly, bilateral T2-weighted hypointensity in the globus pallidus appeared in a subsequent brain MRI of one of the two siblings, possibly indicating neurodegeneration with brain iron accumulation (17). Very recently, nine affected individuals from five unrelated families were found to carry deleterious *VPS41* homozygous variants. All these patients

presented with a progressive neurodevelopmental disorder characterized by cognitive impairment, cerebellar atrophy, and motor dysfunction with dystonia and ataxia (22).

A novel homozygous *VPS11* variant was found in a single patient with adult-onset progressive generalized dystonia and prominent bulbar involvement from a consanguineous family through a combined approach of homozygosity mapping and WES analysis (19). Interestingly, brain MRI showed bilateral hypointensity in the globus pallidus in Fast Field Echo (FFE) sequence (19). Biallelic VPS11 mutations were already associated with a severe infantile neurogenetic disorder, called Hypomyelinating Leukodystrophy 12 (HLD12), indicating that at least two different phenotypes are associated with mutations of this gene (23).

Both VPS41- and VPS11-associated diseases seem to be very rare in large, unselected cohorts of whole-exome-sequenced individuals with dystonia (24), whereas VPS16-associated disease accounts for up to 4% of cases in some cohorts of genetically unresolved generalized dystonia (25). At least two disease-causing *VPS16* alleles (p.Arg187* and p.Arg635*) were found recurrently among European generalized dystonia patients, suggesting the existence of population-specific founder effects.

The fact that brain MRI of some patients carrying HOPS-associated genes mutations displays involvement of basal ganglia, possibly compatible with brain iron accumulation, is very intriguing. However, brain MRI imaging of additional patients with the same genetic lesions are needed to corroborate this observation. Moreover, neuropathological studies will be necessary to definitively establish the nature of the observed MRI abnormalities in this specific group of neurological disorders. A

summary of the genetic and clinical characteristics of these dystonic disorders is presented in Figure 2A and B.

Biallelic mutations of *VPS33A*, encoding for one of the remaining HOPS complex subunit, have been already associated with a human disease known as Mucopolysaccharidosis-Plus Syndrome (MPSPS) which presents with an early lethal phenotype characterized by severe neurological impairment, respiratory and cardiac issues, anaemia, dysostosis multiplex and renal involvement. *VPS18* and *VPS39* genes have not been associated with a human genetic disorder yet. Despite a candidate gene approach was used to search for rare deleterious variants in these two genes in available dystonia genetic databases, no pathogenic variants were found (16). Interestingly, Vps18 conditional knock-out mouse showed severe neurodegeneration and neuronal migration defects, with evidence of autophagy block and lysosomal abnormalities (26). Phenotypically, neural-specific Vps18-deficient mice displayed severe postnatal growth retardation and died prematurely. No dystonic features were reported (26). Similarly, neither *VPS16* nor *VPS41* mutant mice displayed dystonia, suggesting that the human dystonic phenotype may not be fully recapitulated by these models (17,18).

5.4 Disease mechanisms

Functional studies conducted on patient-derived fibroblasts carrying *VPS16*, *VPS41*, and *VPS11* mutations displayed clear overlapping abnormalities of the lysosomal and autophagic compartments (16,19). Electron microscopy of VPS16-, VPS41-, and VPS11-mutated fibroblasts showed large clustered vacuolar structures, with or without inclusions, suggestive of an alteration of these pathways (17,16,19). In addition, a marked increase of lysosomal enzymes quantity and activity was observed in VPS11-

mutated fibroblasts (19). Interestingly, the activity of the same lysosomal hydrolases was raised also at the plasma membrane level, suggesting a possible exocytosis of these accumulated enzymes (19). Moreover, in these same VPS11-mutated fibroblasts, an increased expression of autophagic proteins p62 and LC3B, without a proportional raise of Beclin-1 levels, indicated an accumulation of autophagosomes without autophagy induction, suggesting an impairment of the autophagy flux (19). All the evidence combined from genetic and functional studies supports the hypothesis that the identified mutations are loss-of-function, damaging the function of HOPS complex hence impairing the fusion of late endosomes and autophagosomes with the lysosomes.

Notably, also cultured fibroblasts of MPSPS patients (*VPS33A* mutation) displayed the typical vacuolations of HOPS-related disorders (27). Moreover, plasma lysosomal enzymatic activities in these patients were raised above the reference range, in line with the observed increase of lysosomal enzymatic activity in VPS11-mutated fibroblasts (19). In view of this, the possible use of lysosomal hydrolases activity in plasma as a possible diagnostic and prognostic biomarker in HOPS-related disorders should be investigated in future studies.

5.5 Final remarks

It remains to be elucidated whether HOPS-associated phenotypes are the result of neurodegeneration or whether they might also be related to disordered early neurodevelopmental processes. Future studies aimed at understanding the exact mechanism linking the lysosomal-autophagic dysfunction due to HOPS complex disruption and the dysfunction/degeneration of basal ganglia will shed light on the etiology and potential therapeutic interventions in these disorders. Possible therapeutic

approaches may include autophagy inducers, small-molecule chaperones, and/or substrate-reducing molecules, which are already under study for other lysosome-associated disorders (28, 29).

In conclusion, mutations in genes encoding for HOPS complex subunits are associated with a novel group of inherited dystonias, which we propose to name HOPS-associated Neurological Disorders (HOPSANDs). This group of inherited disorders confirms and deepens the connection between the pathogenesis of dystonias and the dysfunction of lysosomes and autophagy, prompting researchers to find innovative therapies targeting these pathways.

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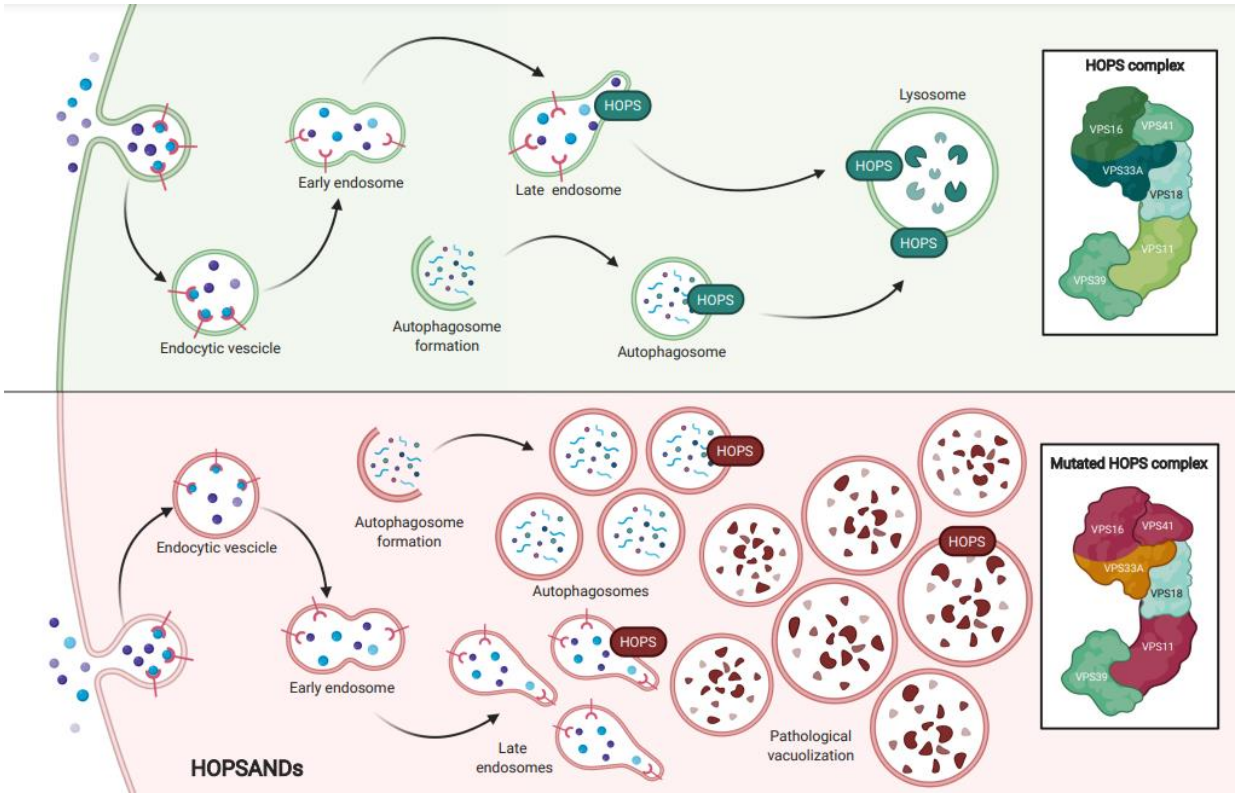

Figure 1: Cartoon model of disease mechanism. Mutations of *VPS16*, *VPS41* an *VPS11* cause a dysfunction of the HOPS complex leading to a defect of the fusion of lysosomes with autophagosomes and accumulation of abnormal lysosomal and autophagic vesicles. Adapted from the template "Mutation of HOPS Complex Subunits", by BioRender.com (2020).

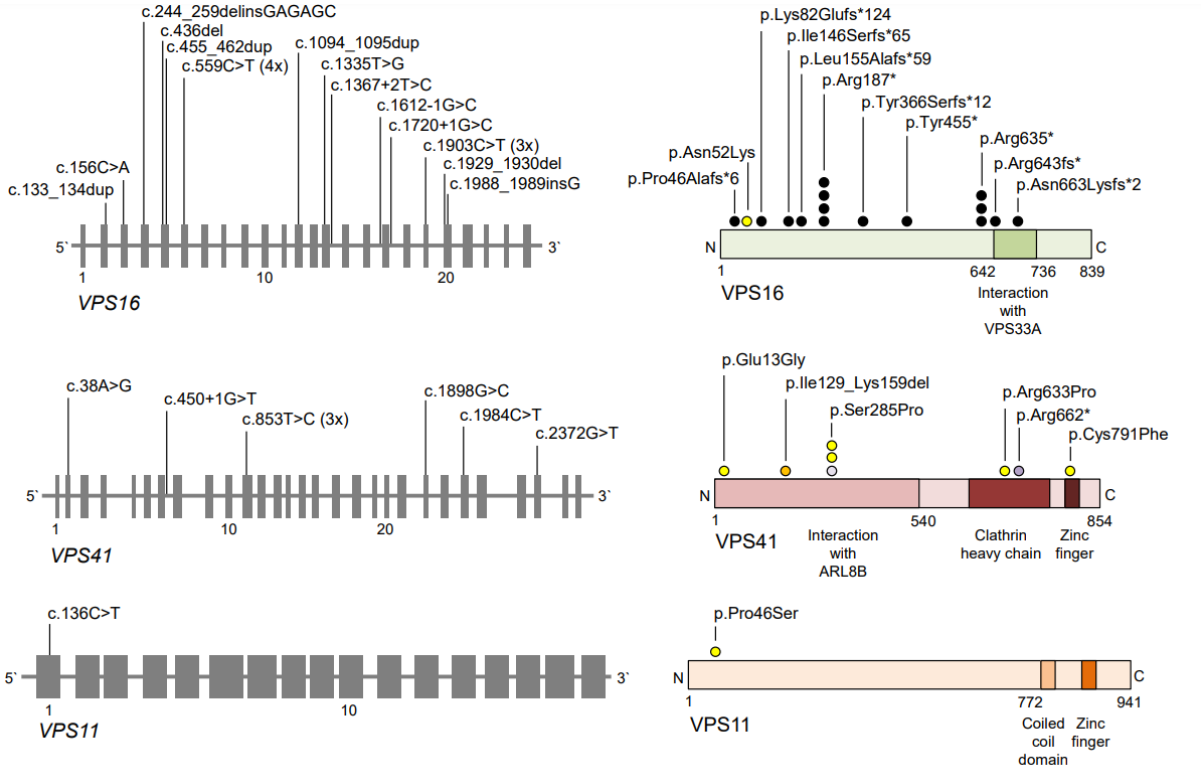

Figure 2: Schematic representations of *VPS16*, *VPS41*, and *VPS11* pathogenic variants identified in dystonia patients to date. (A) Graphical view of reported dystonia-causing variants in *VPS16* (including unpublished data), *VPS41*, and *VPS11*. Three heterozygous *VPS16* splice-site mutations, whose effect was not determined at the protein level, are only shown in the gene-structure graphic. A VPS16-involving microdeletion16 is not illustrated. The heterozygous *VPS16* p.Arg187* and p.Arg635* mutations were identified in four and three independent families, respectively (including unpublished data). The *VPS41* p.Ser285Pro mutation was found in three unrelated families (in two families in a homozygous state and in one family in compound heterozygosity with an additional pathogenic allele). The positions of functional protein domains annotated in the UniProt database are also shown.

6. VPS13C-associated parkinsonism-dystonia

6.1 Abstract

VPS13C is a protein-coding gene involved in the regulation of mitochondrial function through the endolysosomal pathway in neurons. Homozygous and compound heterozygous *VPS13C* mutations are etiologically associated with early-onset Parkinson's disease (PD) with additional features, such as dystonia and spasticity. Neuropathological studies on two mutated subjects showed diffuse Lewy body disease. In this article, we report the clinical and genetic findings of two subjects affected by early-onset parkinsonism and dystonia carrying three novel *VPS13C* mutations (i.e., one homozygous and one compound heterozygous), and review the previous literature on the genetic and clinical findings of VPS13C-mutated patients, contributing to the knowledge of this rare genetic alpha-synucleinopathy.

6.2 Introduction

Vacuolar Protein Sorting 13 Homolog C (*VPS13C*) is a protein-coding gene known to be involved in mitochondrial homeostasis through Pink1/Parkin-mediated mitophagy in response to mitochondrial depolarization. *VPS13C* protein is localized to the outer membrane of mitochondria and is important for the maintenance of mitochondrial morphology, transmembrane potential, and respiration (1).

In 2014, a Parkinson's disease (PD) GWAS study found several single-nucleotide polymorphism (SNPs) localized in the VPS13C gene region to be associated with PD (2). This result was confirmed by two subsequent GWAS studies (3,4). In 2016, biallelic mutations of *VPS13C* were etiologically linked with a distinct form of early-onset PD

(also called PARK23) characterized by rapid and severe disease progression, early cognitive decline, dystonia, pyramidal signs, and neuropathologic findings consistent with diffuse Lewy body disease (1). Conversely, it is still highly controversial whether rare deleterious heterozygous *VPS13C* variants confer an increased risk for PD (5,6). Interestingly, recent studies suggested that rare biallelic *VPS13C* variants can be also the genetic cause of dementia with Lewy bodies (DLB) (7,8).

The clinical presentation of only 16 patients harboring biallelic *VPS13C* mutations was described in the literature so far (1,7–11).

In this article, we aim to describe, clinically and genetically, two cases of early-onset parkinsonism with dystonia carrying novel *VPS13C* mutations and to review the existing literature on genetic and phenotypic features of VPS13C-associated neurodegeneration.

6.3 Materials and Methods

A neurologist expert in movement disorders evaluated the probands. A brain MRI and 123I-ioflupane SPECT were performed in both subjects. Venous blood samples were collected with standard procedures. The Ethics Committee of the IRCCS Foundation Ca' Granda Ospedale Maggiore Policlinico (Milan, Italy) approved the study. Written informed consent was obtained from the patients.

Genomic DNA was extracted from venous blood with standard salting-out method. DNA was analysed with a targeted customized gene panel for PD genes (Haloplex technology, Agilent). The genomic DNA of both probands underwent MLPA analysis

to exclude the presence of deletions or duplication in *SNCA*, *PRKN*, *PINK1*, and *PARK7* genes. The identified VPS13C variants were validated by Sanger sequencing.

All peer-reviewed articles reporting the clinical presentation of parkinsonian patients carrying biallelic *VPS13C* mutations were included. The data extracted from each article included demographic information (age at onset of symptoms, ethnicity, and sex), genetic mutations, and clinical-radiological features. Reports in which the biallelic state of the identified *VPS13C* variants was not made explicit were excluded.

6.4 Results

● Case report 1

The first case is a 55-year-old female, daughter of consanguineous parents (first cousins). The mother of the proband was affected by late-onset unspecified cognitive deterioration. Of her three siblings, the eldest brother was reported to be affected by rapidly worsening parkinsonism, which started when he was 44 and complicated by progressive cognitive decline and behavioural disturbances, mainly characterized by hallucinations, psychomotor agitation, and violence. The behavioral aspect became so predominant that his wife was forced to institutionalize him. Institutionalized and bedridden, he died of pneumonia when he was 52. No further clinical information could be obtained (Figure 1A).

The proband had normal psychomotor development. No history of epileptic seizures was reported. At the age of 42, she manifested hyposmia and slightly progressive bradykinesia of the left limbs. She was referred to a neurologist only several years later, for the worsening of parkinsonism, and in 2016 a dopamine agonist (pramipexole) was

initiated, after performing a brain MRI scan (reportedly normal) and a 123I-ioflupane SPECT, which showed severe symmetrical dopaminergic denervation (Figure 1B). The dopamine agonist was initially effective and well-tolerated, however, two years later, it was discontinued due to drug-induced visual hallucinations. Levodopa was started with good initial motor benefit but with rapid development of motor fluctuations and dyskinesias. In addition, she developed urinary urgency, symptomatic orthostatic hypotension (Schellong test), and frequent falls. A bilateral sensorineural hypoacusia became apparent at that age. On neurological examination she showed continuous vocalizations and echolalia. Hypomimia, limitation of the downward vertical gaze, and oculomotor apraxia were also appreciated, associated with plastic hypertonia of the neck and limbs. Cortical release reflexes, such as snout and palmo-mental, as well as masseter reflex were elicitable. Pull test was positive. The gait was unsteady, wide-based, and slow. Sub-continuous choreodystonic dyskinetic movements of the hands were observed, associated with lips self-mutilations. Postural and rest tremors, as well as pyramidal and cerebellar signs, were not present. Due to the development of atypical signs, the proband underwent an extensive assessment, including a new brain MRI scan, displaying only a moderate frontal cortical atrophy, and an FDG-PET which showed a normal pattern of glucose distribution. The search for acanthocytes on peripheral blood smear gave normal results. Neuropsychological evaluation disclosed an important ideomotor slowing with memory, attention, and executive deficits, associated with oculomotor and ideomotor apraxia. A lumbar puncture was also performed, revealing normal levels of Tau, Phospho-Tau, Aß1-42, and 14-3-3 proteins (i.e., 188 ng/mL, 33 ng/mL, 588 ng/mL and absent, respectively). The clinical syndrome progressed and at

last examination she showed a stuporous, progressive supranuclear palsy-like face, with a complete downward vertical gaze paralysis and worsening of oculomotor and limbs apraxia. Hypophonia and significant weight loss were also detected. Gait was only possible with unilateral support. At last observation MDS Unified Parkinson Disease Rating Scale (UPDRS) motor score moved from 73/108 OFF to 60/108 ON, showing a slight, persistent levodopa response. Her therapeutic regimen included levodopa/carbidopa, safinamide and opicapone, with a levodopa-equivalent-daily-dose of 715 mg (12).

Genetic analysis showed the presence of a novel homozygous frameshift *VPS13C* mutation c.860_866dupATATACC predicted to code a prematurely truncated protein (p.Pro290Tyrfs*45) (NM_020821) (Figure 1C). No other variants in PD-causing genes were found. MLPA did not detect gene deletions or duplications in PD-related genes. This variant may be reasonably considered a loss of function mutation with a strong pathogenic effect. Moreover, the recessive inheritance was suggested by the affected status of the brother, whose DNA was not available, and the homozygosity of the mutation was consistent with the consanguinity of the parents.

● **Case report 2**

The second case is a 43-years-old man without family history of movement disorders and/or dementia (Figure 1D). Past medical history showed hearing impairment from the age of 18 years. He was married, had one daughter, and worked as a bank clerk after 13 years of education. He presented with bradykinesia and painful dystonia in his right foot (i.e., dorsal flexion of the big toe) after moderate physical activity, such as walking and cycling. One year after onset he experienced bradykinesia affecting his right arm,

micrography, and mild depression. At the age of 45 years, he started taking levodopa, up to 400 mg a day, and pramipexole, up to 0.7 mg three times a day, with good control of motor symptoms, except for foot dystonia. At that time, on clinical examination mild hypophonia, hypomimia, bradykinesia, rigidity of right arm, and bending posture were observed. Gait and postural reflexes were normal. No resting or postural tremor was observed. UPDRS motor score, performed in ON therapy conditions, was 10/108. At the age of 48 years, he underwent the following investigations: 123I-ioflupane SPECT, which disclosed significant bilateral reduction in dopamine in the putamen, caudate, and striatum (left side more affected than the right side); brain MRI, which showed only mild cortical cerebellar atrophy and mild parietal cortical atrophy in the left cerebral hemisphere; levodopa test showed significant improvement after 200 mg of oral levodopa (UPDRS III scores in off and in on state were 23/108 and 11/108, respectively); Mini Mental State Examination (MMSE) was performed to evaluate cognition, which was within the normal range (28/30). At the age of 49 years, he reported progression of his symptoms, with akinesia at night, hypomimia, Pisa syndrome, mild wearing off. UPDRS motor score was 23/108 and Hoehn&Yahr (HY) stage was 2.5/5 in ON state. Moreover, he started to complain of forgetfulness. Signs of cognitive decline were confirmed by formal neuropsychological tests. Progressive worsening was prominent in terms of motor, and non-motor symptoms, not only in the cognitive area: blood pressure instability and hypertension at night (reverse dipper) were reported by means of 24-hour blood pressure monitoring at the age of 50; Rapid Eye Movement Sleep Behavior Disorder (RBD), snoring and daytime sleepiness appeared; urine and faecal urgency became manifest. At the age of 50 he decided to

retire. The next year, cognitive evaluation with formal NPS tests resulted pathological in multiple domains, including fluency, memory, and executive domains. At that time, the patient was no longer self-sufficient in most activities of daily living. One year later, cognitive evaluation worsened even further in most domains, and RBD became more evident. He was treated with rivastigmine (up to 9.5 mg) and memantine 10 mg with only temporary and subjective benefits. At 55, he was no longer able to stand and walk independently and he needed a wheelchair. HY stage was 4/5. The neuropsychological follow-up examination revealed severe worsening and most of the tests could not be carried out. At the age of 58, he was bedridden, unable to speak, and a percutaneous endoscopic gastrostomy (PEG) tube was placed due to severe dysphagia.

Genetic analysis identified three rare variants: c.532delA (p.Lys178=fs*12), c.4669G>C (p.Ala1557Pro), and c.7806C>G (p.Tyr2602*) (NM_020821) (Figure 1E). The c.7806C>G and c.532delA are novel, while the c. 4669G>C is a known extremely rare variant of unknown significance (rs201577653). The frameshift substitution (c.532delA) is expected to lead to a premature stop codon 12 amino acids downstream of mutation (p.Lys178=fs*12). Conversely, the c.7806C>G is predicted to trunk the protein at the amino acid 2602 of the VPS13C protein (p.Tyr2602*). No other variants in PD-causing genes were found. MLPA did not detect gene deletions or duplications in PD genes. Segregation analysis showed that the c.532delA (p.Lys178=fs*12) and c.4669G>C (p.Ala1557Pro) were associated in cis and derived from the father, while the c.7806C>G (p.Tyr2602*) originated from the mother.

● **Review of the literature**

To date, only 16 clinically described cases of VPS13C-related PD cases have been reported in the literature (1, 5, 7–11). The clinical features and the genetic mutations observed in these patients are summarized in the Table 1. All the *VPS13C* pathogenic mutations reported so far are reported graphically in Figure 1F. From the review of the literature and the two cases described here, it emerges clearly that VPS13C-related parkinsonism is characterized, with only few exceptions (7), by the classical motor (bradykinesia, rigidity, rest tremor, freezing, postural instability) and non-motor clinical features of PD (dysautonomia, cognitive decline, visual hallucinations, and hyposmia). The clinical response to dopaminergic therapy appears to be favourable in most cases. Motor fluctuations and levodopa-induced dyskinesias are common. A single VPS13C-mutated patient underwent STN DBS, with clinical benefit. The age at onset is earlier in comparison to the idiopathic form (mean age at onset: 37.5 ± 10.5 years). The clinical progression appears to be generally faster. In addition, several associated motor features can be present, such as dystonia and, less frequently, pyramidal signs. Progressive cognitive deterioration is present in most cases. Brain MRI can show symmetrical or asymmetrical lobar atrophic changes without a clear basal ganglia involvement. 123I-ioflupane SPECT, when performed, showed features compatible with dopaminergic denervation, often in an asymmetrical fashion. Interestingly, in a single subject demyelinating polyneuropathy was described; however, it is not clear whether this feature is a part of the VPS13C-associated PD clinical presentation or an unrelated incidental finding (10).

6.5 Discussion

VPS13C mutations are a novel definite genetic cause of monogenic PD and DLB. They are associated with an earlier onset in comparison to idiopathic forms; however, the degenerative parkinsonism appears to be qualitatively similar to the idiopathic form, albeit faster and more severe. The phenotype spectrum of VPS13C-associated PD appears to be quite broad, starting from very severe early-onset parkinsonism, spasticity, and dementia to clinical pictures poorly distinguishable from other forms of early-onset PD, at least at the beginning of the disease. The early and considerable presence of cognitive decline, dysautonomia, and pyramidal signs may help to distinguish it from the other classic early-onset PD forms, such as those associated with *PRKN* and *PINK1* mutations. Part of this phenotypic variability can be due to the severity of the mutations; indeed, the cases described by Lesage et al., which are clinically the most severe described so far, carry highly deleterious mutations (stop, frameshift, splice disruptive) (1). However, this does not appear to be a satisfactory definitive explanation because some of the patients carrying highly deleterious mutations did not present a particularly severe phenotype at the time of description (10, 11). Therefore, some other genetic and/or environmental factors may play a role in determining the phenotypic presentation.

The probands described here exhibited some peculiar phenotypic findings, such as hearing impairment (both subjects), oculomotor disturbances (subject 1), and self-mutilating behaviour (subject 1). The latter feature is particularly intriguing considering that this clinical manifestation is suggestive of chorea acanthocytosis (13), another rare genetic movement disorder caused by mutations in *VPS13A* gene, which is another member of the VPS13 family, involved in lipid metabolism and organelle trafficking

(14). Interestingly, also VPS13D gene, another member of the VPS13 family, and has recently been associated with childhood movement disorders (15).

Rare monogenic causes of PD are a fundamental model to understand the mechanisms of the more common idiopathic form, particularly those associated with typical neuropathology as in the case VPS13C-associated forms. Indeed, neuropathological studies on two *VPS13C* mutated patients showed a picture compatible with diffuse Lewy body pathology (1). Alpha-synuclein and ubiquitin positive-Lewy bodies were observed in the brainstem, limbic system, hippocampus, and all cortical associative areas. In addition, Tau-positive neurofibrillary tangles and neurites were seen in the brainstem, hippocampus, and primary motor cortex (1, 8). Therefore VPS13C-associated PD may represent a simplified view of the pathogenetic mechanisms of idiopathic PD in general.

In conclusion, we presented here two novel cases and reviewed the existing literature on the clinical and genetic features of VPS13C-associated PD, contributing to the knowledge of this rare monogenic alpha-synucleinopathy.

6.6 References

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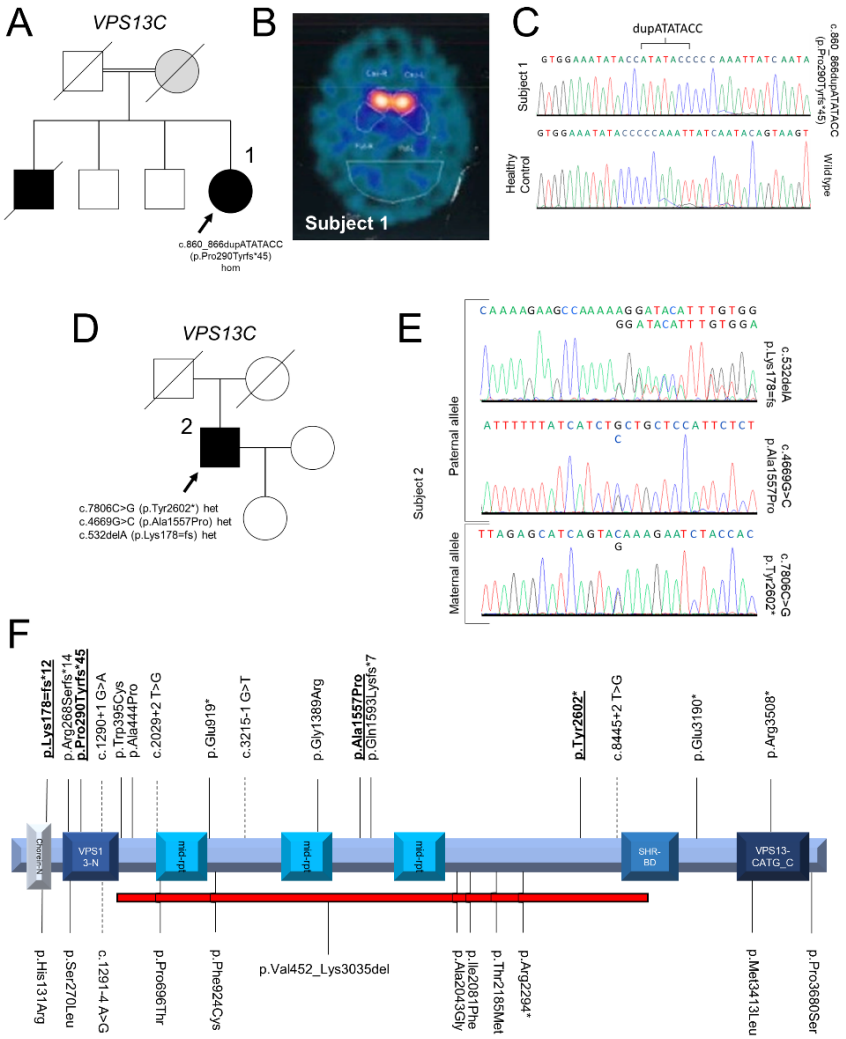

Figure Legend: (A) Pedigree of the proband 1 (black filling indicates affected individuals); (B) 123I-ioflupane SPECT imaging of proband 1 shows a symmetrical severe putaminal denervation and a symmetrical reduction of radiotracer uptake in the caudate nuclei; (C) Electropherogram of the identified *VPSC13C* homozygous c.860_866dupATATACC (p.Pro290Tyrfs*45) mutation in subject 1 compared to wild type sequence; (D) Pedigree of the proband 2; (E) Electropherograms of the three VPSC13C mutations found in subject 2: c.532delA (p.Lys178=fs*12), c.4669G>C (p.Ala1557Pro), and c.7806C>G (p.Tyr2602*); (F) Graphical representation of the Vps13c protein domains and the pathogenic mutations reported so far.

	Lesage S et al.			Schormair B et al.	Darvish H et al.	Gu X et al.								Kobayashi R et al.		Smolders S et al.			This report	
Subject n°	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		
Sex	F	M	F	F	F	M	F	F	M	F	F	F	F	F	NA	NA	F	M		
Ethnicity	Turkish	French	French	Caucasian	Iranian	Chinese	Chinese	Chinese	Chinese	Chinese	Chinese	Chinese	Japanese	Japanese	NA	NA	Italian	Italian		
Age at onset	<46	33	25	39	20	32	41	18	35	44	36	36	48	62	42	41	42	43		
Clinical features																				
Bradykinesia and Rigidity (17/18)	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+		
Tremor (8/18)	-	+	-	+	+	+	-	-	+	-	+	+	-	-	+	-	-	-		
Dystonia (5/11)	-	+	+	+	-	NA	NA	NA	NA	NA	NA	NA	-	-	-	-	+	+		
Motor fluctuations (6/15)	-	+	-	+	-	+	NA	+	-	-	-	-	-	-	NA	NA	+	+		
Dyskinesia (5/15)	-	+	-	+	-	+	NA	+	-	-	-	-	-	-	NA	NA	+	-		
Freezing (5/17)	+	+	-	-	-	+	NA	+	-	-	-	-	-	-	-	-	+	-		
Dysautonomia (9/17)	+	+	-	+	-	+	NA	+	+	+	-	-	-	-	-	-	+	+		
Cognitive decline (13/17)	+	+	+	+	-	-	NA	+	-	+	-	+	+	+	+	+	+	+		
Pyramidal signs (2/11)	-	+	+	-	-	NA	NA	NA	NA	NA	NA	NA	-	-	-	-	-	-		
Levodopa response (12/12)	+	+	+	+	+	+	NA	+	+	+	+	NA	NA	NA	NA	NA	+	+		
DBS response (1/1)	NA	NA	NA	+	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA		
Radiological features																				
Brain MRI	Atrophic changes	Normal	Normal	NA	Normal	NA	NA	NA	NA	NA	NA	NA	Atrophic changes	Atrophic changes	Atrophic changes	Atrophic changes	Atrophic changes	Atrophic changes	Atrophic changes	
123I-ioflupane SPECT	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	+	+	NA	NA	+	-		
VPS13C Mutations NM_020821 (NP_0658721)	c.8445+2T>G (homozygous)	c.806_807insCAGA (p.Arg269Serfs*14) c.9568G>T (p.Glu3190*)	c.4165G>C (p.Gly1389Arg) c.4777del (p.Gln1593Lysfs*7)	c.2029+2T>G c.3215-1G>T	c.1353+3558_9106–7010del (p.Val452_Lys3035del) (homozygous)	c.6241A>T (p.Ile2081Phe) c.2086C>A (p.Pro696Thr)	c.6880C>T (p.Arg2294*) c.1290+1G>A	c.6554C>T (p.Thr2185Met) c.2771T>G (p.Phe924Cys)	c.10237A>C p.Met3413Leu c.1291-4A>G	c.1291-4A>G c.809C>T (p.Ser270Leu)	c.392A>G (p.His131Arg) c.11038C>T (p.Pro3680Ser)	c.1291-4A>G c.6128C>G (p.Ala2043Gly)	c.2755G > T (p.Glu919*) c.10522C > T (p.Arg3508*)	c.2755G > T (p.Glu919*) c.10522C > T (p.Arg3508*)	c.1185G>C (p.Trp395Cys) c.1330G>C (p.Ala444Pro)	c.1185G>C (p.Trp395Cys) c.1330G>C (p.Ala444Pro)	c.860_866dupATATACC (p.Pro290Tyrfs*45) (homozygous)	c.532delA (p.Lys178=fs) + c.4669G>C (p.Ala1557Pro) c.7806C>G (p.Tyr2602*)	

Table 1: Clinical features of all the VPS13C-mutated patients reported so far (F = Female, M = Male, NA = data not available, DBS = response to Deep Brain Stimulation) associated with all the respective pathogenic VPS13C mutations.

7. Adult-onset KMT2B-related dystonia

7.1 Abstract

KMT2B-related dystonia (DYT-KMT2B, also known as DYT28) is an autosomal dominant neurological disorder characterized by varying combinations of generalized dystonia, psychomotor developmental delay, mild-to-moderate intellectual disability, and short stature. Disease onset occurs typically before ten years of age.

We report the clinical and genetic findings of a series of subjects affected by adult-onset dystonia, hearing loss, or intellectual disability carrying rare heterozygous *KMT2B* variants.

Twelve cases from five unrelated families carrying four rare *KMT2B* missense variants predicted to impact protein function are described. Seven affected subjects presented with adult-onset focal or segmental dystonia, three developed isolated progressive hearing loss, and one displayed intellectual disability and short stature. Genome-wide DNA methylation profiling allowed to discriminate these adult-onset dystonia cases from controls and early-onset DYT-KMT2B patients.

These findings document the relevance of *KMT2B* variants as a genetic determinant of adult-onset dystonia and prompt to further characterize *KMT2B* carriers investigating non-dystonic features.

7.2 Introduction

KMT2B-related dystonia (DYT-KMT2B, also known as dystonia 28, DYT28) is an autosomal dominant infantile-onset neurological disorder[1,2]. Disease onset typically

occurs before age 10; however, later onset has been seldom reported[3,4]. DYT-KMT2B generally presents with a progressive course evolving from lower-limb into generalized dystonia with prominent cranial, cervical, and bulbar involvement[5,6]. Dystonia severity is variable and ranges from minor gait disturbances to wheelchair dependence[5]. Psychomotor developmental delay, mild-to-moderate intellectual disability, and relative short stature can precede dystonia onset or be present in isolation as the only phenotypic signature in mutation carriers[5]. Sensorineural hearing loss has also been rarely reported[5]. Most of the DYT-KMT2B patients described to date harbour a *de novo* *KMT2B* variant, that can show incomplete penetrance within the same family[5].

In this clinical and genetic report, we present twelve cases from five unrelated families (Figure 1) carrying four rare functionally relevant *KMT2B* variants who presented with adult-onset dystonia or non-dystonic phenotypes including progressive hearing loss, intellectual disability, and short stature.

7.3 Materials and Methods

Genomic DNA was extracted from peripheral venous blood by standard salting-out procedures. Probands' DNA was analysed by WES using the Nextera Rapid Capture Exome Library kit (Illumina, San Diego, CA, USA) and the Illumina NextSeq500 platform (Illumina), according to the manufacturer instructions. Reads were aligned against the human reference genome (hg38) using BWA and variant calling was performed with GATK4. A virtual gene panel targeted for genetic dystonias (Supplementary material) was used to filter WES data. The candidate *KMT2B* variants were validated by Sanger sequencing and tested in available relatives.

The Ethics Committee of the IRCCS Foundation Ca' Granda Ospedale Maggiore Policlinico (Milan, Italy) approved the study. Written informed consent for publication of clinical details, clinical images, and video recording were obtained from all involved subjects.

Genome-wide DNA methylation (DNAm) analysis was performed on peripheral blood DNA using Infinium Methylation EPIC BeadChip array (Illumina), according to the manufacturer's protocol. Data pre-processing and normalization starting from IDAT files were performed as previously described[7-9]. The DNAm profiles of eight affected cases were compared to those from an in-house database including ~300 samples from healthy individuals and patients with different rare neurodevelopmental disorders, using the established DNAm signature for early-onset DYT-KMT2B by means of multidimensional scaling (MDS), considering the pair-wise Euclidean distances between samples. The training of the SVM classifier was performed with a linear kernel using the e1071 R package (V.1.7) using nu-classification option. To determine the best hyperparameter and to measure the accuracy of the model, the whole dataset was split in a training set (75% of samples) and a test set (25% of samples), and a 5-fold cross-validation was performed during the training process. Scores from SVM classifier below 0.25 were considered as control samples, from 0.25 to 0.5 were considered inconclusive findings, whereas > 0.5 indicated predicted pathogenic variants. The selected cases were then analyzed together with 27 age- and sex-matched controls to identify the most informative differentially methylated probes[7-9]. The selected subset of differentially methylated probes was validated using the entire in-house dataset by MDS analysis.

7.4 Results

● ***Clinical features***

Family A

Family A originated from a Sardinian village. No consanguinity was reported. All family members had normal birth and psychomotor development. At age 69, A.II.4 developed severe eyelid apraxia and pretarsal blepharospasm that led to functional blindness. Local injections of botulinum toxin produced some benefit, but the duration of relief was less than two months. Subject A.II.5 developed at the age of 62 a dystonic head tremor and action tremor in both hands, which remained stable over the years. At age 56, subject A.II.8 developed severe blepharospasm; two years later, he developed cervical dystonia (right torticollis and laterocollis) and neck pain. Local injections of botulinum toxin were effective. Brain MRI was unremarkable in all three siblings. Subjects A.II.1 and A.II.7 developed isolated hearing loss in infancy. Their father (A.I.1) was reported to have developed a tremor, diagnosed as Parkinson's disease, at the age of 50, and died at age 93. Two children of the affected subject A.II.4 were tested (A.III.10 and A.III.11) since A.III.10 developed hearing loss in childhood, and A.III.11 displayed abnormal hand posture when writing since childhood (writer's cramp).

Family B

Subject *B.II.3* is a 36-year-old female born prematurely at week 36 of gestation. Psychomotor development and school performance were normal. At the age of 19 years, she complained of a subacute motor impairment affecting the left upper limb with the development of a fixed dystonic posture within a few months. In the subsequent year,

dystonia spread to the left lower limb and speech became progressively rhinolalic. The motor phenotype thereafter stabilized, whereas a progressive disturbance of ocular movements developed. By age 23, horizontal saccades were slow and reduced in amplitude, and in two years she developed progressive oculomotor apraxia on the horizontal gaze. Brain MRI at age of 23 was normal. No family history of dystonia was reported; however, her mother (subject B.I.2) presented mild intellectual disability (Total IQ 58 - WEIS-IV) and short stature (126 cm).

Family C

Subject C.II.1 was born at term after a difficult delivery. Since childhood, he presented writing difficulties, and his handwriting was poorly understandable. At the age of 34, he developed dystonic posture and tremor of the upper right limb. Neurological examination at the age of 49 showed dystonic movements and tremor of the right upper limb. These were induced by action and modulated by position. Brain MRI showed mild enlargement of the left lateral ventricle. I-123-Ioflupane SPECT was normal. Trihexyphenidyl, levodopa, and tetrabenazine were not beneficial. He achieved some improvement with botulinum toxin.

Subject D.II.2

Subject D.II.2 was born at term and had normal psychomotor development. No family history of neurological diseases was present. At the age of 23, the patient progressively developed spasmodic dysphonia with dysarthric speech, a strained and strangled voice, and voice breaks during speaking. From the age of 29, a bilateral hand dystonic tremor developed, prevalent on the right side, especially during writing; dystonia then spread

to the oromandibular region and cervical region; laryngeal impairment worsened leading to a significant decrease in speech intelligibility. Anticholinergics did not bring any benefit. Botulinum toxin injections led to moderate improvement of dystonia. Brain MRI performed at the age of 44 showed mild brain atrophy and mild symmetrical pallidal hypointensity on T2*-weighted sequences.

Subject E.II.2

Subject E.II.2 was born at term after an uneventful pregnancy. No familial history of neurological disorders was present. At the age of 43, she progressively developed upper right arm focal dystonia. Cervical spine and brain MRI were unremarkable. Several drugs (anticholinergics, benzodiazepines, baclofen, tetrabenazine, and botulinum toxin injections) were ineffective. Neurological examination at age of 59 disclosed dystonic posture of the right upper limb with abduction of the arm and flexion-pronation of the forearm that worsened on action. The remainder neurological examination was normal.

● ***Genetic analysis***

A virtual gene panel for dystonia was applied on WES data of probands A.II.4, B.II.3, C.II.1, D.II.2, and E.II.2. Four rare heterozygous *KMT2B* variants were identified: c.3232C>T in subject A.II.4, c.7016G>A in subjects B.II.3 and C.II.1, c.2909G>A in subject D.II.2, and c.1918T>A in subject E.II.2. No pathogenic or rare variants were found in other dystonia genes. *KMT2B* variants validation and segregation analyses in the families of the probands were performed by Sanger sequencing.

The c.3232C>T (p.R1078C) is absent from genetic population databases but has been already reported by Ciolfi A. et al.[8]. It is predicted pathogenic by all *in silico* prediction

tools. Arginine1078 is conserved among orthologues. Previously performed functional analyses indicated that the genome-wide peripheral blood DNAm profile associated with this variant was different from that of *KMT2B* mutations causing childhood-onset DYT-KMT2B, suggesting a different functional impact of this missense change[8]. The same variant was found in all the affected members of the family (A.II.5, A.II.7, A.II.8, A.III.10, and A.III.11), indicating co-segregation of the c.3232C>T with the disease (dystonia or hearing loss). The affected subject A.II.1 was an obligate carrier of the variant. One asymptomatic 50-years-old female carrier was also found (A.III.2).

The c.7016G>A (p.R2339Q) is a very rare known variant (dbSNP: rs751409145). Its allele frequency in gnomAD is 0.0001442. This variant is predicted benign by most in silico prediction tools. Genetic analysis of family B showed that this variant was carried also by the affected parent (i.e., B.I.2). Therefore, segregation analysis supported its pathogenic role in family B. Subject C.I.2 was carrier of the variant and reportedly asymptomatic.

The c.2909G>A (p.R970Q) is a known very rare variant (dbSNP: rs780053167) with an allele frequency of 0.000004024 in gnomAD. The amino acid Arginine970 is highly conserved among orthologues. Most in silico tools predict this variant to be pathogenic. The unaffected mother and the sister of the proband did not carry this variant. The DNA of the neurologically unaffected father was not available as he was already deceased from other causes.

The c.1918T>A (p.S640T) is a very rare missense variant (dbSNP: rs771667749) never reported in dystonic patients so far. The gnomAD allele frequency is 0.00005. This

variant is predicted benign by most in silico prediction tools. Segregation analysis demonstrated that the healthy sisters of the proband did not carry this variant.

● ***Genome-wide DNAm array analyses***

To investigate the functional relevance of the identified *KMT2B* variants associated with late-onset dystonia, hearing loss, and short stature-intellectual disability, a genome-wide DNAm analysis by means of EPIC array was performed as previously described[8]. As a first step, we analyzed the DNAm profiles of eight affected individuals (six affected by adult-onset dystonia, one by hearing loss, and one by intellectual disability and short stature) carrying the four identified missense changes in the context of the episignature characterizing early-onset DYT-KMT2B by MDS analysis (Figure 2A)[8]. The clustering of the 8 tested cases, localizing far from the early-onset DYT-KMT2B group and within the controls, did not support the functional equivalence of the presently identified *KMT2B* missense variants with those causing early-onset DYT-KMT2B. This finding was confirmed by the low scores obtained by the SVM classifier (Supplementary Table 1). To explore the occurrence of a distinctive DNAm pattern shared by the patients reported here, these cases were compared with 27 age- and sex-matched unaffected subjects by linear modeling[8,9], allowing to identify 175 independent differentially methylated probes. This probe-set was validated considering DNAm data referred to 270 unaffected controls and patients with rare neurodevelopmental disorders by MDS analysis, which confirmed the occurrence of a separate cluster including all subjects with late-onset dystonia, hearing loss, and short stature-intellectual disability (Figure 2). These specific probes did not overlap with early-onset DYT-KMT2B

episignature, suggesting a different molecular mechanism underlying this late-onset phenotype.

7.5 Discussion

In this report, we present twelve subjects from five unrelated families carrying four rare functionally relevant *KMT2B* missense variants, identified by a virtual dystonia gene panel derived from WES data.

The possible pathogenic role of the identified *KMT2B* variants is supported by the association with a consistent disease phenotype and their very low frequency in genetic databases. Moreover, the p.R1078C and p.R2339Q co-segregated with the phenotypes in families A and B, respectively. In particular, p.R1078C segregation in a large adult-onset dystonia family strongly supports its claim of pathogenicity. *In silico* tools predicted a likely pathogenic effect for only two of these variants (i.e., p.R970Q and p.R1078C). One of these variants has been already reported (i.e., p.R1078C) and its genome-wide DNAm episignature profiling indicated a different impact compared to the highly deleterious *KMT2B* variants causing childhood-onset DYT-KMT2B. The present DNAm analyses confirm the differential behaviour of the identified missense *KMT2B* variants associated with late-onset dystonia, though they provide evidence of a distinctive DNAm pattern, which suggests their functional and clinical relevance.

These clinical, genetic, and functional findings support the hypothesis that *KMT2B* missense variants with mild pathogenic effect on methylation profile, and not predicted deleterious in silico, could be associated with later disease onset and lower penetrance, considering that two asymptomatic carriers were also found. In line with this reasoning,

previous literature supported the hypothesis of an inverse correlation between mutation severity and age at onset[10]. Nevertheless, the clinical interpretation of these variants should be managed carefully; in particular, at the current time, the p.S640T and p.R2339Q lack proof of disease segregation and need additional evidence to support their pathogenicity. A further word of caution is necessary concerning the association of *KMT2B* pathogenic variants with hearing loss as it has only rarely been reported and has been identified only in one of the families shown here, leaving open the possibility of a coincidental association with the *KMT2B* variant. Conversely, non-dystonic phenotypes such as mild intellectual disability and short stature are already consolidated clinical features of *KMT2B*-related disease, and were also observed in Family B. These observations should prompt future detailed family studies to characterize underrecognized non-dystonic features associated with *KMT2B* mutations.[11]

Seven of the patients reported here presented with adult-onset dystonia. All of them had an involvement of the upper part of the body; in particular, a cranio-cervical involvement (cervical dystonia, blepharospasm, oromandibular and laryngeal dystonia) was observed in 5/8 patients. The oromandibular and/or laryngeal regions are classically involved in childhood-onset KMT2B-related dystonia, sometimes leading to anarthria. No patient presented with predominant lower limb dystonia, which is instead classically observed in paediatric patients. In 3/8 patients, phasic upper limb dystonia and/or dystonic tremor developed during the disease course. One patient (B.II.3) exhibited overt oculomotor apraxia, which has been seldom reported and may represent another clue to suspect *KMT2B* variants in the context of segmental/generalized dystonia[2]. None of the dystonic patients displayed a favourable response to oral anti-dystonic therapies

(e.g., anticholinergics, benzodiazepines, tetrabenazine), while several of them received botulinum toxin injections with clinical benefit.

In conclusion, this report highlights the possible relevance of *KMT2B* missense variants as a genetic determinant of adult-onset dystonia and emphasizes the possibility of non-dystonic presentations of KMT2B-related disease. *KMT2B* mutations should be considered in patients with adult-onset progressive dystonia involving the upper body part, in particular the larynx and oromandibular region and/or the upper limb. The identification of additional family members presenting with mild or even non-neurological phenotypes may represent an important clue to suggest this specific genetic etiology.

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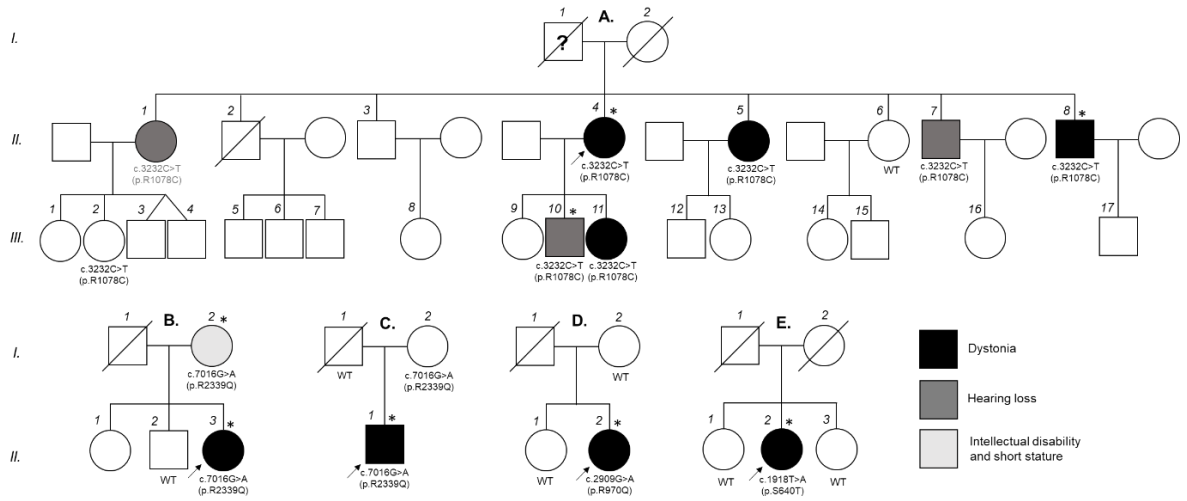

Figure 1: Family pedigrees of the probands A.II.4, B.II.3, C.II.1, D.II.2, and E.II.1. Black, dark grey, and light grey fillings indicate affected status by dystonia, hearing loss, and short stature/intellectual disability, respectively.

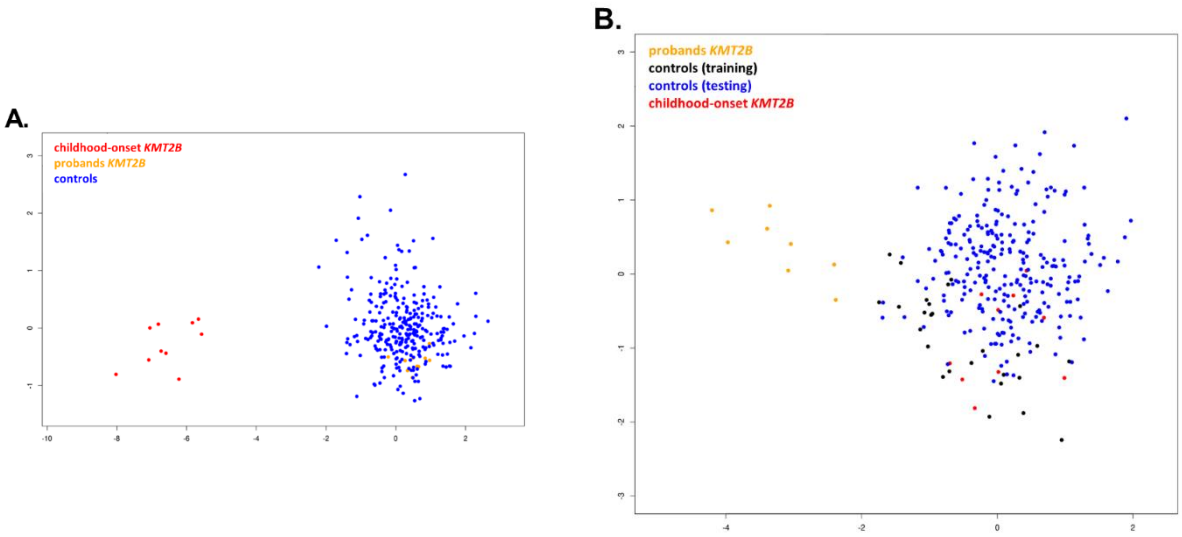

Figure 2. DNAm array analyses. (A) DNAm profiles in patients with *KMT2B* variants associated with late-onset dystonia differ from those characterizing childhood-onset DYT-KMT2B (DYT28). MDS plot is used to classify the presently identified *KMT2B* missense variants (orange) with respect to *KMT2B* variants causing childhood-onset DYT-KMT2B (red) and an in-house DNAm data-set including ~300 healthy individuals and subjects with rare neurodevelopmental disorders (blue). (B) Genome-wide DNAm analysis was able to cluster cases with late-onset dystonia (n=6), hearing loss (n=1), and intellectual disability-short stature (n=1) carrying heterozygous *KMT2B* missense variants (orange) from childhood-onset DYT-KMT2B (red) and control samples (blue), by MDS analysis.

8. Progressive myoclonus-dystonia-ataxia due to a *NUS1* frameshift insertion

8.1 Abstract

NUS1 pathogenic variants have been previously identified in severe epileptic encephalopathies often in association with movement disorders (ataxia, dystonia, myoclonus). We found through whole exome sequencing a novel pathogenic *NUS1* frameshift insertion in a patient with progressive myoclonic dystonia syndrome and mild cerebellar features without epilepsy. The C-terminal localization of the identified variant may be responsible for the milder phenotype described here.

8.2 Case report

Within neurogenetic disorders, myoclonus usually occurs as part of a more complex phenotype, such as epileptic encephalopathy, myoclonus-dystonia or cerebellar ataxia (1). Heterozygous pathogenic variants of the *NUS1* gene have been linked to infantile-onset epilepsy, intellectual disability, cerebellar ataxia, neuropsychiatric features, and movement disorders, including dystonia, tremor, and myoclonus (2–6). The *NUS1* gene encodes a transmembrane receptor for the neural and cardiovascular regulator Nogo-B (NUS1 or NgBR) (7). In addition, *NUS1* is essential for dolichol synthesis and protein glycosylation in the endoplasmic reticulum (ER) (7). Here we present a non-epileptic *NUS1* patient presenting with a progressive myoclonus-dystonia syndrome and mild cerebellar signs.

The proband was a 14-years-old right-handed male, the only child of non-consanguineous parents (Figure 1A), without family history of neurological disorders.

He was born at term after an uncomplicated pregnancy. His mother reported infantile-onset motor clumsiness but no additional psychomotor development delay. At the age of 11, he started to develop involuntary twitching movements of his face, shortly followed by bilateral jerky distal movements of the arms. His handwriting deteriorated, although it was already poor. Social interaction and academic performance were impacted by a suspected mild intellectual disability.

He was initially evaluated at the age of 11. Brain MRI and laboratory analyses, including thyroid function, metabolic screening, and copper profile, were reportedly unrevealing. The jerks progressively increased in frequency and amplitude, significantly impacting his quality of life. At the age of 13, clonazepam was begun, initially at a dose of 2 mg twice daily that was soon reduced to 1 mg twice daily because of marked daytime sleepiness and limited benefit.

He was first assessed at our center at the age of 14. Neurological examination showed almost continuous, multifocal myoclonic jerks affecting his face, tongue, and upper limbs (distal more than proximal), both at rest and with action. The hands presented a dystonic posture when in motion and during positions sustained over time. There was mild dysdiadochokinesis. Gait was narrow-based, but standing on one leg and tandem walking were impaired. The remainder of the neurologic and general examination were unremarkable. Brain MRI at that time showed mild atrophy of the rostral part of the cerebellar vermis (Figure 1B). EEG revealed diffuse excessive fast activity (likely due to clonazepam) without epileptiform discharges.

Whole-exome sequencing (WES) of genomic DNA of the proband was performed as part of a research protocol approved by the Institutional Review Board of NYU Langone

Health. Written informed consent and assent for study participation and video publication were obtained from the patient and his mother. Variant prioritization looking for rare (AF<0.001) nonsynonymous variants in genes associated with neurological disorders revealed a frameshift insertion in exon 4 of *NUS1*, causing a premature stop codon (NM_138459.5: c.754_755insGTTTTCTTCCCTGGCACATCAG, p.Thr261Serfs*9). The pathogenicity of the variant was supported by the following ACMG criteria: PVS1, PM2, and PP3 (8). PCR amplification and subsequent Sanger sequencing of *NUS1* exon 4 confirmed the presence of the frameshift insertion in the proband, while indicating wild type status of the mother (Figure 1C, 1D, and 1E). The father of the patient was not available for testing, since he was no longer in contact with the family but was reportedly healthy.

The causative role of the identified nonsense mutation is consistent with the mechanism of haploinsufficiency previously described for this gene. Although it was not possible to test the proband's father, we suspect that the identified mutation occurred de novo since he was reportedly healthy, while *NUS1* pathogenic variants are considered fully penetrant at young age (4).

Previously reported pathogenic variants of *NUS1* are mostly severe deleterious mutations (frameshift, stop, splice-disruptive, exon deletions, and chromosomal deletions) with an expected complete protein loss (4). A missense variant (p.Gly102Asp) was identified in a patient presenting with milder phenotype (dystonia, myoclonic jerks, mild intellectual disability, and epilepsy) (9). The frameshift variant we found affects the C-terminus of the protein; therefore, only partial loss of function

of the mutated protein is hypothesized (hypomorphic mutation), possibly explaining the mild clinical presentation.

Interestingly, a recent report described a young epileptic patient with early-onset upper body myoclonus with a strikingly similar pattern to our case, affecting predominantly the face and the upper limbs with a proximal-distal gradient. The patient was found to be a carrier of a splice-site *NUS1* variant. We suggest that the described presentation, and particularly the involvement of the face, may be a diagnostic clue to suspect mutations of this gene in subjects with early-onset, predominantly myoclonic syndromes (5).

This report represents a novel case of a myoclonus-dystonia syndrome without epilepsy associated with *NUS1* mutation and supports the inclusion of this gene among the genetic causes of non-epileptic myoclonus. Currently, the *NUS1* gene is present in epilepsy gene panels while it is not included in the majority of movement disorders panels. Therefore, a targeted gene panel approach based on phenotype would not have identified the pathogenetic variant in this specific patient.

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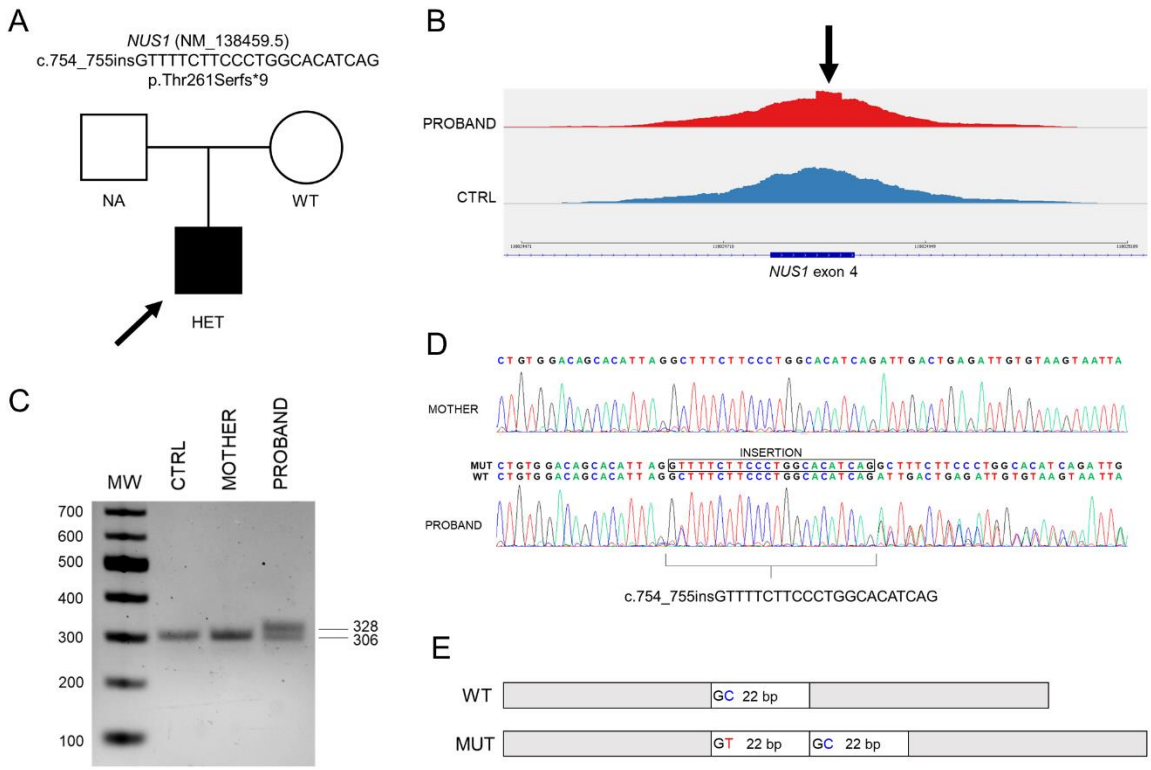

Figure 1: A) Family pedigree (black-filled symbol indicates affected status); B) Sagittal T1-weighted sequence brain MRI of the proband displayed mild atrophy of the rostral part of the cerebellar vermis; C) PCR electrophoretic analysis confirmed the presence of a 22 bp insertion in the proband, which was absent in the mother and in a healthy control; D) Sashimi plot showed an increased WES coverage (black arrow) on exon 4 of NUS1 gene (image exported from IGV viewer). BWA bioinformatic tool aligned the insertion onto the normal reference sequence because its nucleotide sequence is almost identical to the genomic reference in that region, except for a C>T in the second nucleotide position. E) Sanger sequencing validation of the identified variant in the proband and his mother (wild-type). (Abbreviations: NA=Not Available, HET=Heterozygous, WT=Wild-type, CTRL=Healthy Control, MW=Molecular Weight)

9. Dystonia-parkinsonism caused by a homozygous *VPS13A* truncating variant

9.1 Abstract

Chorea-acanthocytosis is a rare neurogenetic disorder caused by biallelic *VPS13A* pathogenic variants phenotypically characterized by progressive chorea, neuropsychiatric features, seizures, and subclinical myopathy. We report a remarkable case of Chorea-acanthocytosis who did not display the phenomenology of chorea but presented early-onset dystonia-parkinsonism and epilepsy. In addition, the identified *VPS13A* variant is novel and expands the mutational spectrum of Chorea-acanthocytosis. Our work suggests that *VPS13A* mutation should be suspected also in patients without chorea presenting dystonia and parkinsonism when in combination with other typical neurological signs of Chorea-acanthocytosis.

9.2 Introduction

Chorea-acanthocytosis (ChAc) is a rare genetic neurodegenerative disorder caused by biallelic VPS13A pathogenic variants which is phenotypically characterized by progressive chorea, neuropsychiatric features, seizures, and subclinical myopathy associated with hyperCKemia (1–3). The term “acanthocytosis” is due to the observation of abnormal erythrocytes with spiked cell membrane (acanthocytes) in the blood smear of affected individuals (4). ChAc is part of the Neuro-acanthocytosis syndromes, which is a group of rare progressive disorders displaying acanthocytes and neurological abnormalities also including McLeod syndrome and Pantothenate kinase-associated neurodegeneration (5).

9.3 Case report

The proband is a 50-year-old Italian male. He worked as a policeman. His psychomotor development was normal. No family history of neurological disorders was present except for one maternal cousin with epilepsy. His father died of lung carcinoma at age 63. One sister died of lung carcinoma at age 48. His parents were first cousins.

The disease onset was at age 34 with several generalized epileptic seizures. In the following years, the patient developed cognitive disturbances (frontal/behavioral type), gait impairment with freezing of gait, and slowness of movements. In a few years, he became completely dependent on others in all activities of daily living. Brain MRI showed symmetrical T2-weighted hyperintensity of the putamen, caudate atrophy and moderate atrophy of the cerebellar vermis (Figure 1A, 1B and 1C). Seizures were controlled with a pharmacological polytherapy composed of oxcarbazepine 1800 mg daily, levetiracetam 100 mg daily, and clonazepam 1 mg daily. Levodopa trial? Neurological examination showed a dystonic gait combined with severe freezing and festination, action-induced dystonia of lower limbs, mild appendicular parkinsonism, motor perseveration, pyramidal signs, dysarthria, and stimulus-induced myoclonus. No choreic movements were observed. Lab investigations including full blood count, CK, ceruloplasmin, copper studies, protein electrophoresis, and alpha-fetoprotein were normal except for mild hyperCKemia (258 U/L). Acanthocytes? Skeletal muscle biopsy showed a moderate reduction in size of some fibers. A liver ultrasound showed hepatomegaly and steatosis. Echocardiography, nerve conduction studies, and electromyography were normal.

Whole-exome sequencing (WES) of the proband was performed as part of a research protocol approved by the Ethics Committee of the IRCCS Foundation Ca' Granda Ospedale Maggiore Policlinico (Milan, Italy). Written informed consent for genetic analyses and video publication was obtained from the patient and his legal guardian. Variant prioritization looking for rare (AF≤0.001) nonsynonymous variants in genes associated with movement disorders revealed a novel homozygous frameshift truncating variant affecting the VPS13A gene (NM_033305.3): c.4351delT, p.Phe1451Serfs*3, which was confirmed by Sanger sequencing (Figure 1D and 1E). This variant is predicted to lead to nonsense-mediated decay and Vps13a loss-of-function is the disease mechanism of ChAc.

9.4 Discussion

The case of ChAc presented here is remarkable because the patient did not display the phenomenology of chorea, which is the most common movement disorder associated with this neurogenetic disorder. Conversely, the patient displayed early-onset parkinsonism associated with epilepsy, cognitive impairment, action-induced dystonia in the lower limbs, and mild myopathic changes. He also presented hepatomegaly which might be encountered in ChAc (6).

Dystonia-parkinsonism without choreic features has been already reported in at least three patients affected by Neuro-acanthocytosis who were reported several decades ago before the identification of VPS13A as the disease-causing gene of ChAc (7,8).

In addition, the identified VPS13A variant is novel and expands the mutational spectrum of ChAc. The description of other cases of Chorea-acanthocytosis carrying this specific variant may help in the understanding of genotype-phenotype correlations.

In conclusion, VPS13A mutation should be suspected also in patients without chorea presenting parkinsonism in combination with other typical neurological signs of ChAc.

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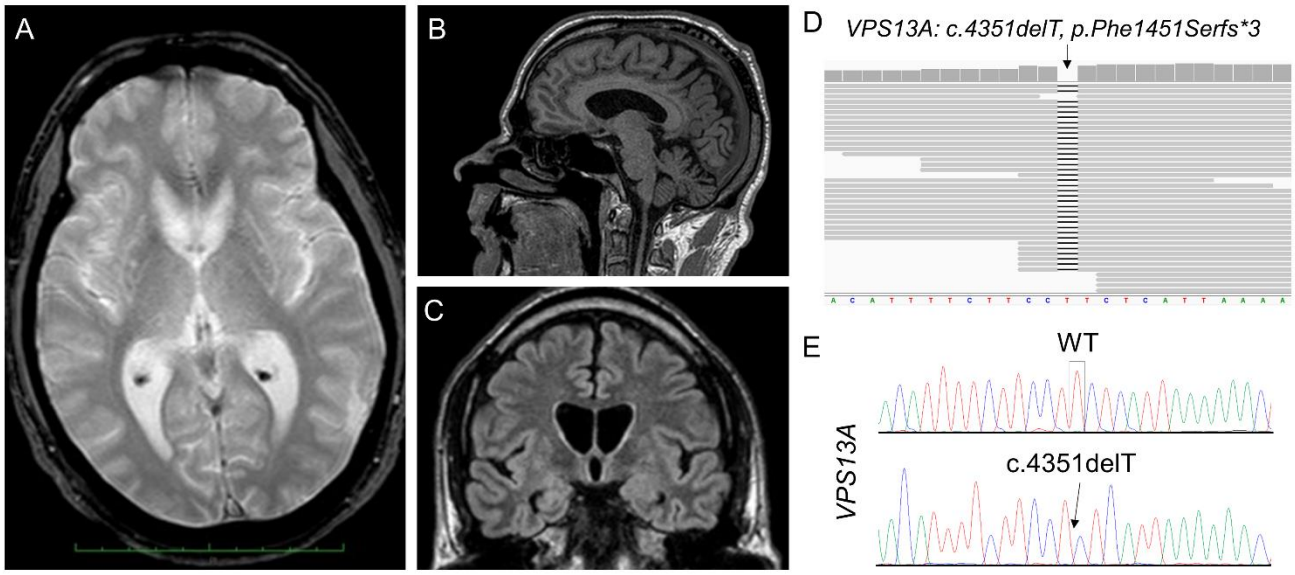

Figure 1: A-C) Brain MRI of the patient: axial T2-weighted sequence showing symmetrical hyperintensity of the putamen (A), sagittal T1-weighted sequence showing moderate atrophy of the cerebellar vermis (B), coronal T1-weighted sequence revealing symmetrical atrophy of the caudate nucleus (C); D) IGV viewer snapshot of genomic .bam file demonstrating the homozygous deletion of a single thymidine (T) in position c.4351 of the *VPS13A* gene; E) Sanger validation of the identified homozygous *VPS13A* single base deletion: wild-type (WT) in the upper panel and mutated in the lower panel.

10. Discussion

In the previous decades, the traditional family-based linkage analysis approach was successfully applied to identify the genetic causes of many human diseases, including inherited dystonias. However, in the last years, the field of human genetics is facing a technological revolution, fuelled by NGS technologies, holding great promises also for the field of hereditary dystonias, in which the identification of the causative mutation coincides with the definitive diagnosis. The scope of this thesis is to dissect the heterogeneous genetic etiologies of dystonia and describe original phenotypes associated with known dystonia genes. To achieve this goal, an NGS approach was utilized.

10.1 The contributions of this thesis

This thesis aimed at increasing our understanding of inherited dystonias. I presented a novel disease-causing gene (*VPS11*), expanded the phenotypic spectrum associated with known causative genes (*VPS16* and *KMT2B*), defined a novel group of hereditary dystonias sharing common disease mechanisms (i.e., HOPSANDs), and reported novel pathogenic variants in already known dystonia genes (*VPS13C* and *NUS1*).

● *VPS11* is a novel dystonia-causing gene

In chapter 3 the original association of a homozygous *VPS11* pathogenic variant with adult-onset generalized dystonia was described, providing a detailed clinical report and biological evidence of disease mechanism. Functional studies on patient-derived fibroblasts showed marked abnormalities of the lysosomal and autophagic compartments, which improved after overexpression of the wild type Vps11 protein.

This work represented the first association of *VPS11* with a distinct form of complex dystonia and supported the hypothesis that the disruption of HOPS complex is crucial in complex dystonia pathogenesis (Monfrini et al. 2021a).

● Dominant *VPS16* mutation causes chorea

VPS16 pathogenic variants have been recently associated with inherited dystonias. Myoclonic jerks were also reported as additional movement disorders in these patients. In chapter 4, familial chorea was reported as a new clinical presentation, expanding the genetic and phenotypic spectrum of VPS16-associated disease.

● HOPSANDs are a novel disease group of inherited dystonias

In chapter 5, HOPS-associated Neurological Disorders (HOPSANDs) are presented as a novel group of inherited neurological disorders phenotypically characterized by the presence of prominent dystonia. They share common disease mechanisms and display partially overlapping clinical presentations. The delineation of this new nosological entity supports the connection of lysosomal and autophagic dysfunction with the pathogenesis of dystonia and can prompt researchers to find innovative therapeutic strategies to target this pathway (Monfrini et al. 2021b).

● *KMT2B* variants are also associated with adult-onset dystonia

In chapter 7, twelve cases from five unrelated families affected by adult-onset dystonia, hearing loss, or intellectual disability carrying four rare *KMT2B* missense variants are reported. DNA methylation array analysis allowed the identification of a common pattern in these patients differentiating them from controls and early-onset KMT2B-related dystonic patients. These findings widen the clinical spectrum associated with

KMT2B missense variants, linking altered KMT2B function to adult-onset dystonia and atypical clinical presentations (i.e., intellectual disability, short stature, and hearing loss) (Monfrini et al. Brain Comm. 2022, accepted).

● **Pathogenic *VPS13A* variant causes dystonia-parkinsonism without chorea**

In chapter 9, the patient affected by chorea-acanthocytosis surprisingly did not display the phenomenology of chorea, which is the typical movement disorder associated with this neurogenetic disorder. Conversely, the patient displayed early-onset parkinsonism associated with epilepsy, cognitive impairment, action-induced dystonia in the lower limbs, and mild myopathic changes. Therefore, *VPS13A* mutation should be suspected also in patients without chorea presenting parkinsonism in combination with other typical neurological signs of chorea-acanthocytosis (Monfrini et al. MDS Clinical Practice 2022, submitted).

● **Novel pathogenic variants affecting *VPS13C* and *NUS1***

In chapters 6 and 9, four novel pathogenic variants of *VPS13C* (n=3) and *NUS1* (n=1) have been reported, thus expanding the mutational spectrum of these genes. In this thesis, two patients with parkinsonism-dystonia carrying *VPS13C* pathogenic variants and one patient with myoclonus dystonia e mild cerebellar signs harboring a *NUS1* mutation are reported (Monfrini et al. 2022b; Monfrini et al. 2022a). *VPS13C* mutations typically cause early-onset Parkinson's disease often associated with additional neurological symptoms, including dystonia. *NUS1* variants are associated with a phenotypic spectrum ranging from intellectual disability-epilepsy-ataxia to combined dystonia.

10.2 The importance of a genetic diagnosis

Known inherited dystonia forms go often undiagnosed for long periods mainly because of reduced access to genetic testing. However, the knowledge of the genetic etiology of dystonia can help the clinician choose effective clinical approaches and treatments, also providing a more precise prognosis for their dystonic patients. In some cases, significant symptomatic improvements can be achieved by gene-specific dietary treatment, medication, or surgical procedures (e.g., Deep Brain Stimulation – DBS) (Pozojevic et al. 2021).

For example, heterozygous variants affecting the *SLC2A1* (*GLUT1*) gene cause a complex movement disorder which includes paroxysmal dystonia. A ketogenic diet improves symptoms and early treated patients have a more satisfactory prognosis (Alter et al. 2015).

Dopa-responsive dystonia (DYT-GCH1) is caused by pathogenic variants of the *GCH1* gene, which encodes an enzyme necessary for dopamine synthesis (GTP-cyclohydrolase 1). The deficiency of endogenous dopamine can be effectively corrected by dopaminergic medication resulting in alleviation of motor symptoms. In some instances, in case of positive prenatal genetic testing, prenatal replacement therapy with levodopa may be indicated (Brüggemann et al. 2012).

Dystonic patients affected by DYT-TOR1A and DYT-KMT2B respond well and predictably to DBS (Brüggemann et al. 2015; Cif et al. 2020). When considering combined dystonia forms, favorable outcomes can be expected in DYT-TAF1 (Brüggemann et al. 2019) and DYT-SGCE patients (Kosutzka et al. 2019), but not for ATP1A3-related dystonia (DYT-ATP1A3) (Albanese et al. 2017; Brücke et al. 2015).

In addition, for patients, receiving a conclusive genetic result may be a relief from uncertainty and a reduction of psychological distress. Moreover, it may provide an organic cause to a previously presumed functional movement disorder and prevent the infliction of iatrogenic injury caused by ineffective and futile treatments, including surgeries. Knowledge of the genetic cause of dystonia in a patient enables informed life decisions for the proband and relatives (Pozojevic et al. 2021).

Despite the great progress of the last years, a lot more is still to be discovered, in particular, many disease genes are still to be found. The identification of novel causative genes for dystonia will increase the diagnostic yield of genetic testing.

10.3 The role of the neurologist expert in the neurogenetics of movement disorders

General neurologists should have adequate knowledge of the genetic background of dystonias and should perform an initial selection of who must be tested (Di Fonzo et al. 2018). However, the advent of NGS posed many new challenges, including the increased relevance and complexity of this field, the delicate issue of genetic testing in unaffected family members, the easy availability of "commercial" home testing kits, and the increasing number of variants of unknown significance (VUS) (Di Fonzo et al. 2018).

The interpretation of NGS results requires great expertise in both clinical neurology and neurogenetics. Given the exceptional complexity of the matter and the high degree of specialization needed because of the rapid innovations in the field, when "first-line" genetic approaches (e.g., single gene testing or gene panels) are not conclusive, the general neurologist should refer the dystonic patient to the neurologist expert in the genetics of movement disorders, in order to perform "second-line" genetic analyses

(e.g., WES, WGS, array-CGH, third generation sequencing approaches). In this view, a new generation of movement disorder specialists trained in neurogenetics is needed to face the current era of complexity, advanced technology, and hyper-specialization.

10.4 Conclusions and perspectives

The genetic discoveries of the last thirty years improved our understanding of the etiopathogenic mechanism of inherited dystonias, but still more work remains ahead. Several genes and molecular mechanisms associated with dystonia are identified every year, and most likely many genetic factors are still to be found. In the next years, novel technological advances will appear, new genetic determinants will be discovered, and previously identified genetic forms will be better characterized.

Moreover, due to the increasing knowledge of the role of non-coding regions, it is likely that new mutations will be identified within this context. A better understanding of the functional consequences of mutations localized in non-coding regions, and the development of new technologies to identify structural variants (e.g., “long-read” NGS), will allow the discovery of “non-conventional mutations” associated with inherited dystonias.

Another issue in the field of dystonia genetics that needs to be addressed is the extremely low diagnostic yield of genetic screening in late-onset dystonic patients, even in case of positive family history (Zech et al. 2020). An explanation of this phenomenon can be that milder dystonic forms with later onset are determined by more complex genetic mechanisms (e.g., mendelian variants with low penetrance, risk variants, oligogenic or polygenic inheritance) and environmental influences (e.g., specific professions, use of drugs). This hypothesis would categorize late-onset dystonia as a

multifactorial disease, in which genetic-environment interactions play a pivotal role in disease pathogenesis.

More importantly, the expanding knowledge about the genetic architecture, and consequently, of the pathogenic mechanisms of dystonia, will increase the chance of identifying specific therapeutic targets for each genetic form, in line with the promising perspective of precision medicine. Although currently unavailable for the large majority of inherited dystonia forms, genetic therapeutic approaches relying on precise molecular diagnoses may be critical for patients for whom only symptomatic treatment is currently feasible. Genome editing is emerging as a powerful therapeutic approach, albeit very expensive due to its prerequisite to be individually tailored. This strategy may represent a major game-changer as it holds the promise to replace symptomatic treatments with etiological approaches in the future (Doudna 2020).

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