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RESEARCH ARTICLE

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Genomic variants causing mitochondrial dysfunction are common in hereditary lower motor neuron disease

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Abstract

Hereditary lower motor neuron diseases (LMND) other than 5q-spinal muscular atrophy (5q-SMA) can be classified according to affected muscle groups. Proximal and distal forms of non-5q-SMA represent a clinically and genetically heterogeneous spectrum characterized by significant overlaps with axonal forms of Charcot–Marie–Tooth (CMT) disease. A consensus for the best approach to molecular diagnosis needs to be reached, especially in light of continuous novel gene discovery and falling costs of next-generation sequencing (NGS). We performed exome sequencing (ES) in 41 families presenting with non-5q-SMA or axonal CMT, 25 of which had undergone a previous negative neuromuscular disease (NMD) gene panel analysis. The total diagnostic yield of ES was 41%. Diagnostic success in the cohort with a previous NMD-panel analysis was significantly extended by ES, primarily due to novel gene associated-phenotypes and uncharacteristic phenotypic presentations. We recommend early ES for individuals with hereditary LMND

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presenting uncharacteristic or significantly overlapping features. As mitochondrial dysfunction was the underlying pathomechanism in 47% of the solved individuals, we highlight the sensitivity of the anterior horn cell and peripheral nerve to mitochondrial imbalance as well as the necessity to screen for mitochondrial disorders in individuals presenting predominant lower motor neuron symptoms.

KEYWORDS
axonal CMT, exome sequencing, hereditary neuropathy, lower motor neuron disease, mitochondrial dysfunction, non-5q-SMA

1 | INTRODUCTION

Hereditary lower motor neuron diseases are characterized by muscular atrophy, weakness, and hyporeflexia. They can be divided into two broad groups of genetic disorders: spinal muscular atrophies (SMA) and distal hereditary motor neuropathies (HMNs; Garg et al., 2017). SMA is a heterogeneous group of hereditary disorders characterized by anterior horn cell degeneration in the spinal cord, leading to progressive muscle weakness and atrophy. While around 96% of SMA cases are caused by autosomal recessive (AR) deletions or rarely point mutations in the *SMN1* gene on chromosome 5q13 (5q-SMA; Lefebvre et al., 1995; Wirth et al., 2020; Wirth, 2000), the remaining minority form a genetically heterogeneous and phenotypically diverse group of conditions summarized under the umbrella term 'non-5q-SMA'. In contrast to clinically well-characterized SMA cases, in the routine diagnostic labs only about 50% of individuals suspected to have SMA were found with biallelic *SMN1* mutations (Karakaya et al., 2018). These groups of heterogeneous hereditary disorders (non-5q-SMAs) share neither an inheritance pattern nor a common underlying pathomechanism and often present a more complex phenotype with variable onset and features beyond the classical 5q-SMA spectrum (Peeters et al., 2014; Rossor et al., 2012). A classification system typically deployed is by muscle involvement pattern, distinguishing between proximal predominant and distal predominant forms (Darras, 2011).

Both proximal and distal non-5q-SMAs show phenotypic and genetic overlap with other neuromuscular disorders (NMD) including amyotrophic lateral sclerosis (ALS), hereditary spastic paraplegias (HSP), and Charcot–Marie–Tooth (CMT) disease (Peeters et al., 2014; Rossor et al., 2012). Especially axonal forms of CMT significantly overlap with distal non-5q-SMA or HMN, as for both phenotypes clinical presentation and electrophysiological findings are characteristic of a length-dependent axonal motor neuropathy, the distinguishing feature being the additional involvement of the

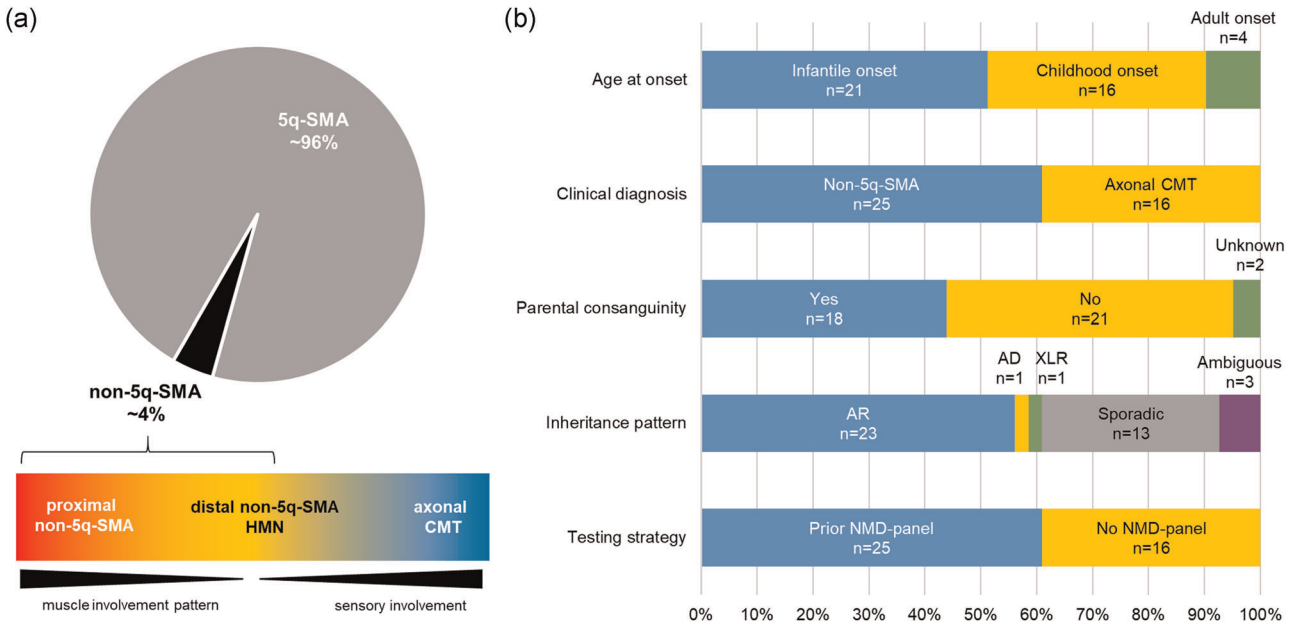


FIGURE 1 Study and cohort characterization. (a) Overview of the clinical spectrum in focus. Proximal and distal non-5q-SMAs show significant genetic and phenotypic overlap with axonal CMT. (b) Clinical, demographic data and prior testing strategy of affected individuals. AD, autosomal dominant; AR, autosomal recessive; CMT, Charcot–Marie–Tooth; 5q-SMA, 5q-spinal muscular atrophy; XLR, X-linked recessive

sensory nerves in axonal CMT (Irobi et al., 2006; Rossor et al., 2012). However, as HMN can present with minor sensory involvement, and axonal CMT can show motor-predominance, the disorders represent two ends of a continuous phenotypic spectrum rather than two distinct disease entities (Figure 1(a); Bansagi et al., 2017; Rossor et al., 2012), a fact further highlighted by the many shared causal genes such as *GARS1* (Antonellis et al., 2003), *TRPV4* (Auer-Grumbach et al., 2010), or *HSPB1* (Evgrafov et al., 2004). In addition, also proximal predominant non-5q-SMA and axonal CMT are allelic disorders, such as in the case of *TRPV4* (Auer-Grumbach et al., 2010) or *DYNC1H1* (Harms et al., 2012; Weedon et al., 2011).

Since the introduction of next-generation sequencing (NGS) into research and molecular diagnostics, the number of genes associated with axonal motor- and sensorimotor neuropathies has grown immensely and continuously (Benarroch et al., 2019; Garg et al., 2017). However, while the rising number of detected disease-genes enables a definite molecular diagnosis for an increasing number of affected individuals, it has also created novel challenges, a prominent one being the question of the best approach to molecular diagnosis (Volk & Kubisch, 2017). The need for cost- and analysis-efficiency often clashes with the necessity for fast-track diagnosis and high diagnostic yield, which is essential to prevent the infamous 'diagnostic odyssey'. As gene panels covering a large number of associated disease-genes allow a cost-efficient and phenotype-centric but still comprehensive approach, they have become the diagnostics method of choice for heterogeneous lower motor neuron disorders in the last years (Garg et al., 2017). However, the narrowing price-gap between targeted panel sequencing and exome sequencing (ES) (Mazzarotto et al., 2020) as well as evidence supporting the reduction of incremental costs and time-to-diagnosis by early ES analysis (Soden et al., 2014; Stark et al., 2017) increasingly support an early, maybe even first-tier, unbiased and comprehensive ES approach for disorders with clinical and genetic heterogeneity. Thus, continuous testing and critical comparison of the diagnostic methods are essential for providing time- and cost-effective high-yield molecular diagnostics in the fastly evolving field of NMD genetics.

In a previous study, we developed and tested a large neuromuscular disease gene panel comprising 479 genes (subsequently referred to as NMD-panel; Karakaya et al., 2018). Among the 479 NMD-associated genes, 127 genes were associated with motor neuron disorders and hereditary neuropathies, 79 genes for hereditary paraplegias and ataxias (excluding repeat expansion-associated genes), 190 genes for muscular dystrophies, myopathies and myasthenic syndromes, 83 genes for arthrogryposis syndromes, other neuromuscular diseases and candidate disease-associated genes. A detailed list of the genes in the NMD panel can be found in our previous study (Karakaya et al., 2018). Seventy-four individuals with a clinical presentation of either proximal or distal non-5q-SMA or axonal CMT were analyzed via the NMD-panel, with a total diagnostic yield of 45% (33% for non-5q-SMA, 65% for axonal CMT) (Karakaya et al., 2018). In the present study, we re-evaluated 25 cases with a previous negative NMD-panel analysis using ES. We additionally performed ES analysis on 16 cases without prior NMD-panel analysis. We compared ES diagnostic yield in the NMD-panel and no-NMD-panel cohort to establish the significance of extended panel diagnostics compared to the expanded unbiased ES approach in rare hereditary lower motor neuron phenotypes. By identifying disease-associated genes not included in the NMD-panel, we aimed to uncover common reasons for panel escaping to facilitate panel upgrading, in turn increasing diagnostic yield and further improving time- and cost-efficiency in NMD diagnostics. Studies in recent years provided important insights for hereditary neuropathies, in which the cellular defects and their underlying molecular pathomechanisms were unraveled (Beijer et al., 2019). We, therefore, investigated the cellular processes that were impaired by the detected mutations in our cohort and observed mitochondrial dysfunction as a frequent pathomechanism involved in rare disorders of the anterior horn cell and/or peripheral nerve.

2 | MATERIALS AND METHODS

2.1 | Editorial policies and ethical considerations

This study conformed to standards outlined in the Declaration of Helsinki and was approved by the Ethics Committee of the University of Cologne (Reference Number 13-022). Informed written consent for the collection of human material, for the participation in the study, and for publication purposes was obtained from the respective subjects or their legal guardians following the regulations of the Ethics Committee of the University of Cologne.

2.2 | Proband selection

Forty-one independent individuals presenting with LMND phenotype with or without sensory involvement, with a suspected clinical diagnosis of either non-5q-SMA ($n = 25$) or axonal CMT ($n = 16$) and 103 family members were selected for the study. The ethnicities of the recruited probands were Turkish ($n = 21$), Iranian ($n = 8$), German ($n = 7$), Moldavian ($n = 1$), Arabic ($n = 1$), Russian ($n = 1$), Russian/Belarussian ($n = 1$), and Turkmen ($n = 1$). Twenty-five (61%) were selected after inconclusive NMD-panel analysis comprising 479 genes implicated in neuromuscular disease (Karakaya et al., 2018), either on a research basis as part of the NeurOmics-LMND study ($n = 18$) (Karakaya et al., 2018) or on diagnostic basis as part of the diagnostic routine of the Institute of Human Genetics of the University Hospital of Cologne ($n = 7$). The remaining 16 individuals (39%) were recruited for ES without prior NMD-panel testing but with previous negative phenotype-specific genetic testing (Figure S2). At minimum, homozygous *SMN1* deletions were excluded before selection in all cases. Each affected individual was phenotypically characterized based on a questionnaire that was originally established for the NeurOmics research study (Karakaya et al., 2018) and later on adopted for the current study and re-approved by the Ethics Committee of the University of Cologne.

The questionnaire included information about the age at onset, family history, parental consanguinity, developmental data (for index probands <5 years), findings of physical and neurological examination, investigation findings (electromyography, nerve conduction studies, muscle biopsy/MRI, cranial/spinal MRI) and laboratory results.

2.3 | Exome sequencing

ES was conducted for 32 parent-child trios, two parent-child duos, and seven singletons. For target enrichment, we used the SureSelect All Exon v7 kit (Agilent) according to the *'Sure*SelectXT Low Input Automated Target Enrichment for Illumina Paired-End Multiplexed Sequencing' protocol (Version D0, July 2018) provided by Agilent Technologies. Sample processing steps were automated using the NGS Workstation Option B (Agilent), deploying the reagents specified in the protocol. Before target enrichment via hybridization-based capture, an individual molecular-barcoded and indexed library was prepared for each sample. Input samples contained 10–200 ng genomic DNA. After DNA fragmentation to a size of 150–200 base-pair (bp) and DNA end-modification including end-repair, dA-tailing, and ligation of the molecular-barcoded adaptor, adaptor-ligated libraries were amplified via PCR using index primers. Amplified DNA was subsequently purified using AMPure XP beads (Beckman Coulter). After quantity and quality assessment (Agilent tape station), the prepared and aliquoted gDNA library was hybridized to the target-specific SureSelect Capture Library via thermal cycler incubation. The gDNA-Capture Library hybrids were then captured with streptavidin-coated magnetic beads. Postcapture, SureSelect-enriched DNA samples were PCR-amplified and purified. After library validation and quantification (Agilent tape station), equimolar amounts of the library were pooled. Pools were quantified by using the Peqlab KAPA Library Quantification Kit and the Applied Biosystems 7900HT Sequence Detection System and sequenced by using a paired-end 100-nucleotide protocol on the Illumina NovaSeq6000 sequencer, with a resulting average 75× target coverage.

2.4 | Alignment and variant calling

Reads were aligned to human reference genome hg38/GRCh38 (Genome Reference Consortium Human GRCh38). For read improvement, PCR duplicates were removed using Picard tools, and local realignment and base quality score recalibration (BQSR) were performed using Genome Analysis Toolkit (GATK). Single nucleotide polymorphisms (SNPs) and short insertions/deletions (INDELs) were called by Platypus, Haplotype Caller, and Mpileup programs and subsequently filtered via variant quality score calibration (VQSR) using GATK. For CNV detection based on coverage analysis XHMM, Conifer, and ExomeDepth algorithms were applied. ALLEGRO program was used for ROH detection based on multipoint linkage analysis. Data combining and functional variant annotation were enabled by the COMBINE and FUNC modules developed by Cologne Center for Genomics (CCG).

2.5 | Variant interpretation and classification

ES data was analyzed using exome analysis pipeline Varbank 2.0 developed by CCG. We focused our analysis on single nucleotide nonsynonymous missense, loss-of-function, and splice site variants, as well as short INDELs. Parental consanguinity analysis was estimated by the total sum of runs of homozygosity (ROH) in the affected individual (>100 Mb) and the average allele sharing percentage between parents (>10%). After application of a first filtering step to select variants based on inheritance pattern via the 'allele read frequency' (frequency of the allele carrying reads with the detected variant compared to the reference carrying reads in %: 75%–100% for homozygous variants: 25%–75% for heterozygous variants), and allele frequency (<1% for recessive variants, <0.1% for dominant variants), variants were evaluated utilizing the genomic variant search engine 'VarSome' (Kopanos et al., 2019), Human Gene Mutation Database (HGMD) Professional 2019.4, Database of Single Nucleotide Polymorphisms and ClinVar archive (latest accessions in August 2020). For a comprehensive and integrated approach to computational in silico pathogenicity prediction, we applied the Varbank 2.0 'MedPred', a median rank score incorporating the prediction scores of up to 31 in silico mutation pathogenicity prediction algorithms (value between 0 and 1; 0 = benign; 1 = pathogenic; - Table S3). Following the ACMG 2015 guidelines (Richards et al., 2015), we classified the variants as pathogenic (class 5), likely pathogenic (class 4), or uncertain significance (class 3). For a final variant validation and confirmation of segregation, candidate variants were resequenced via the Sanger method on an ABI 3730XL sequencing machine (Sanger et al., 1977). For probands with an inconclusive SNV/INDEL analysis, we investigated copy number variations (CNVs) utilizing Integrative Genomics Viewer (IGV) software (Broad Institute) and Varbank's gene coverage tool in compliance with the results yielded from CNV detection based on coverage analysis.

3 | RESULTS

3.1 | Cohort of affected individuals

Forty-one independent individuals (28 males, 13 females) with a suspected diagnosis of LMND were selected for ES. We included 25 individuals (61%) with a suspected clinical diagnosis of proximal or distal non-5q-SMA and 16 individuals (39%) presenting with features consistent with axonal (or in two cases intermediate) CMT. Clinical presentation was not only of variable onset and severity but in some cases overlapping with other neuromuscular or neurologic phenotypes, as to be expected in a clinically heterogeneous spectrum such as non-5q-SMA. A majority of individuals (n = 37, 90%) had an onset in

pediatric or adolescent age (<18 years), while only four subjects (10%) presented with an adult-onset disorder (Figure 1(b)).

Of the 41 families, 23 had an inheritance pattern suggestive of autosomal-recessive (AR), one of autosomal-dominant (AD), one of X-linked (XLR) inheritance, and 13 were sporadic cases. The inheritance pattern in three families was ambiguous. Parental consanguinity was present in 44% of families (28% of families in the non-5q-SMA cohort, 69% of families in the CMT cohort; Figure 1b). There was no information about parental inbreeding for two individuals. However, a total ROH sum of 128 Mb and 7% of allele sharing between parents indicated a mild degree of inbreeding in one individual, while a low ROH summary of 44 Mb and an allele sharing percentage between parents of 2% favored nonconsanguinity in the second.

After negative NMD-panel analysis ($n = 25$; 61%) or only phenotype-specific genetic testing ($n = 16$; 39%), affected individuals were subjected to ES. A prior panel analysis was performed for 52% of non-5q-SMA and 75% of axonal CMT individuals. We performed trio ES for individuals where the DNA samples of both biological parents were available. Otherwise, affected individuals were selected for proband-parent duo or singleton ES. In total, ES was performed for 107 individuals: 32 trios, two duos, and seven singletons.

3.2 | Results and diagnostic yield of exome sequencing analysis

ES analysis yielded a molecular diagnosis in 17 of the 41 families (41%). Table 1 provides an overview of the affected individuals that received a definite genetic diagnosis. Pedigrees and detailed clinical information of the individuals with positive results are provided in the supplementary materials (Figure S1 and Table S1). According to ACMG 2015 guidelines (Richards et al., 2015), the identified variants included ten pathogenic and ten likely pathogenic variants (Table 1). One variant in *SEPSECS* was annotated as a variant of uncertain significance (VUS) following ACMG criteria but considered putatively disease-causing; it was detected in trans with a pathogenic variant and affecting the tRNA interaction site of the protein. Moreover, VUS were detected in five additional individuals (Table S2). A VUS of particular interest is a homozygous 6bp duplication in *EGR2* (NM_001136178.1: c.906_911dup) leading to the insertion of two additional alanine molecules into an alanine stretch spanning residues 300–309 (p. Ala308_Ala309dup), which we detected in an individual with a clinical presentation resembling *EGR2*-related AR congenital hypomyelinating neuropathy 1 (CHN1; MIM# 605253). *EGR2* has not previously been linked to the spectrum of polyalanine expansion disorders and ACMG guidelines annotated the variant only as a VUS. Additionally of note is the identification of compound-heterozygous VUS in *MACF1* in three unrelated affected individuals in our cohort (Table S2), which we consider worth remarking due to previous associations of *MACF1* with a to-date not securely established NMD phenotype (Jorgensen et al., 2014; Kang et al., 2019). Nineteen families remained unsolved, in four of which we detected putative variants in genes that we regard as novel disease-associated candidates. These have been added to the GeneMatcher database and will be followed in future studies. RefSeq identification numbers of each gene with pathogenic/likely pathogenic variants or VUS are given in the footnote of the corresponding table (Table 1 and Table S2).

Sixteen of the solved cases showed a recessive pattern (12 homozygous, four compound-heterozygous variants), and one de novo variant was found in a sporadic case. Of the overall 21 detected variants, 12 were known pathogenic variants, whereas nine were novel variants not previously published (Table 1).

Diagnostic yield in consanguineous families (50%) was higher than in remaining nonconsanguineous or ambiguous families (35%). Comparing non-5q-SMA with axonal CMT individuals, we were able to solve seven of the 25 individuals with a suspected clinical diagnosis of non-5q-SMA and 10 of the 16 axonal CMT individuals, adding up to diagnostic yields of 28 and 63%, respectively. The significantly higher diagnostic yield in the CMT cohort correlates with the higher number of consanguineous families in this group (69% consanguineous families in the CMT cohort vs. 28% in the non-5q-SMA cohort). Of the seven solved individuals with a suspected diagnosis of non-5q-SMA, two had a variant in known SMA-phenotype associated genes (*AGTPBP1*, *EXOSC3*). Of the 10 individuals with axonal CMT with a positive result, only three had variants in genes causing a characteristic pure axonal CMT phenotype (*PDXK*, *MPV17*, *SPG11*). Thus, 12 of the 17 individuals with a positive ES analysis received a molecular diagnosis, not in line with the initial clinical diagnosis (Table 1).

3.3 | Comparison of ES outcome post-NMD-panel versus without NMD-panel analysis

A definite diagnosis by ES was achieved for 10 of the 25 individuals with a previous negative NMD-panel analysis (three non-5q-SMA and seven axonal CMT cases) and for seven of the 16 individuals without prior NMD-panel testing (four non-5q-SMA and three axonal CMT cases), amounting to diagnostic yields of 40% and 44%, respectively.

Of the seven ES-detected variants in the cohort without prior NMD-panel testing, four were located in genes included in our NMD-panel (57%), which we therefore categorized as 'NMD-panel-detectable': *POLG* (AR mitochondrial ataxia syndrome; MIM# 607459), *SPG11* (AR spastic paraplegia 11; MIM# 604360), *C12orf65* (AR spastic paraplegia 55; MIM# 615035), and *EXOSC3* (AR pontocerebellar hypoplasia type 1B; MIM# 614678). Of the 10 diagnosed cases in the cohort with prior NMD-panel testing, two causal variants were located in NMD-panel genes (20%): *NDUFS6* (AR mitochondrial complex I deficiency nuclear type 9; MIM# 618232) and *TTN* (AR limb-girdle muscular dystrophy 10; MIM# 608807). The homozygous variant in *NDUFS6* (Table 1) had in fact been detected in the prior panel analysis, yet had been annotated as a VUS because of uncharacteristic clinical presentation of a known pathogenic variant, and ES was performed as a more comprehensive method, which did

TABLE 1 List of individuals with positive results

ID	Eth.	Age at onset (years)	Clinical presentation	Disease category	NMD-panel	Final diagnosis (MIM#)	Mutated gene (MIM#)	Zyg.	Novelty	cDNA[a]	Protein	Med Pred	CADD score	ACMG 2015 class (evidence of pathogenicity)
1	GER	2	Slowly progressive muscular weakness and atrophy	non-5q-SMA	yes	LGMDR10 (608807)	*TTN* (188840)	CHZ	Known; novel	c.[97816delC]; [90811_90864del]	p.[(Arg32606Alafs*4)]; [(Pro30271_Val30288del)]	N/A, N/A	62; N/A	Pathogenic (PVS1, PS4, PM2); likely pathogenic (PM2, PM3, PM4)
2	TUR	Birth	Infantile muscular atrophy and neurodegeneration	non-5q-SMA	yes	ECHS1D (616277)	*ECHS1* (602292)	hmz	Known	c.[476A>G]; [476A>G]	p.[(Gln159Arg)]; [(Gln159Arg)]	0.66	18.33	Likely pathogenic (PS4, PM2, PM3_sup, PP3)
3	GER	12	Motor neuron disease	non-5q-SMA	yes	SCAR4 (607317)	*VPS13D* (608877)	CHZ	Novel; novel	c.[12350C>G]; [12530G>A]	p.[(Ser4117*)]; [(Gly4177Asp)]	0.81; 0.85	58; 32	Pathogenic (PVS1, PM2, PP1); likely pathogenic (PM2, PM3, PP1, PP3)
4	GER	1	Infantile muscular atrophy and weakness	non-5q-SMA	no	Sensory ataxic neuropathy with mitochondrial DNA deletions (607459)	*POLG* (174763)	CHZ	Known; novel	c.[2419C>T]; [678G>C]	p.[(Arg807Cys)]; [(Gln226His)]	0.91; 0.52	35; 19.59	Pathogenic (PS4, PM1, PM2, PP2, PP3); likely pathogenic (PM2, PM3, PP2, PP3)
5	TUR	0.3	Severe motor delay and respiratory insufficiency	non-5q-SMA	no	CONDCA (618276)	*AGTPBP1* (606830)	hmz	Novel	c.[1120C>T]; [1120C>T]	p.[(Arg374*)]; [(Arg374*)]	0.67	40	Pathogenic (PVS1, PM2, PM3_sup.)
6	TUR	0.6	Infantile lower motor neuron disease and cerebellar atrophy	non-5q-SMA	no	CONDCA (618276)	*AGTPBP1* (606830)	hmz	Known[c]	c.[2276G>T]; [2276G>T]	p.[(Arg759Leu)]; [(Arg759Leu)]	0,78	29.1	Likely pathogenic (PM2, PM3_sup PP1, PP3)
7	TUR	0.3	Global muscular weakness and hypotonia, cerebellar atrophy	non-5q-SMA	no	PCH1B (614678)	*EXOSC3* (606489)	hmz	Known	c.[395A>C]; [395A>C]	p.[(Asp132Ala)]; [(Asp132Ala)]	0,76	30	Pathogenic (PS4, PM2, PM3_sup., PP1, PP3)
8	TUR	3	Axonal polyneuropathy	CMT	yes	CONDSIAS (618170)	*ADPRS* (610624)	hmz	Known[d]	c.[235A>C]; [235A>C]	p.[(Thr79Pro)]; [(Thr79Pro)]	0.76	25.8	Pathogenic (PS3, PM2, PM3_sup, PP1, PP3)
9	IRA	1	Infantile progressive muscular atrophy and spasticity	CMT	yes	MLD (250100)	*ARSA* (607574)	hmz	Known	c.[465+1G>A]; [465+1G>A]		0.66	23.6	Pathogenic (PVS1, PM2, PM3_sup.)

(Continues)

TABLE 1 (Continued)

ID	Eth.	Age at onset (years)	Clinical presentation	Disease category	NMD-panel	Final diagnosis (MIM#)	Mutated gene (MIM#)	Zyg.	Novelty	cDNA[a]	Protein	Med Pred	CADD score	ACMG 2015 class (evidence of pathogenicity)
10	IRA	0.5	Infantile onset progressive axonal polyneuropathy	CMT	yes	PCH2D (613811)	*SEPSECS* (613009)	CHZ	Known; novel	c.[715G>A]; [812G>T]	p.[(Ala239Thr)]; [(Arg271Leu)]	0.72; 0.81	32; 34	Likely pathogenic (PS3, PM2, PP3); VUS (PM2, PM3, PP3)
11	IRA	4	Neuropathy and optic atrophy	CMT	yes	Optic atrophy-ataxia-peripheral neuropathy-global developmental delay syndrome (ORPH-A:543470)[b]	*FDXR* (103270)	hmz	Known	c.[463C>T]; [463C>T]	p.[(Arg155Trp)]; [(Arg155Trp)]	0.69	27.4	Likely pathogenic (PM2, PM3, PP1, PP3)
12	IRA	10	Distal motor neuropathy and optic atrophy	CMT	yes	HMSN6C (618511)	*PDXK* (179020)	hmz	Novel	c.[225T>A]; [225T>A]	p.[(Asn75Lys)]; [(Asn75Lys)]	0.6	12.19	Likely pathogenic (PM2, PM3, PP1, PP4)
13	TUR	10	Unspecified polyneuropathy	CMT	yes	MC1DN9 (618232)	*NDUFS6* (603848)	hmz	Known	c.[309+5G>A]; [309+5G>A]		0.3	7.936	Likely pathogenic (PS4, PM2, PM3_sup.)
14	IRA	26	Proximal leg muscle atrophy and axonal polyneuropathy	CMT	yes	CMT2EE (618400)	*MPV17* (137960)	hmz	Known	c.[263_265delAGAinsTGT]; [263_265delAGAinsTGT]	p.[(Lys88_Met89delinsMetLeu)]; [(Lys88_Met89delinsMetLeu)]	N/A	21.8	Likely pathogenic (PM2, PM3_sup, PM5 PP3)
15	TKM	13	Progressive distal limb muscle atrophy	CMT	no	SPG11 (604360)	*SPG11* (610844)	hmz	Known[d]	c.[5986dup]; [5986dup]	p.[(Cys1996Leufs*4)]; [(Cys1996 Leufs*4)]	N/A	34	Pathogenic (PVS1, PM2, PM3_sup)

TABLE 1 (Continued)

ID	Eth.	Age at onset (years)	Clinical presentation	Disease category	NMD-panel	Final diagnosis (MIM#)	Mutated gene (MIM#)	Zyg.	Novelty	cDNA[a]	Protein	Med Pred	CADD score	ACMG 2015 class (evidence of pathogenicity)
16	TUR	0.3	Severe infantile muscular atrophy and axonal polyneuropathy	CMT	no	EMPF1 (614388)	*DNM1L* (603850)	*de novo*	Known[e]	c.[115A>G]	p.[(Ser39Gly)]	0.9	23.5	Pathogenic (PS2, PM1, PM2, PP2, PP3)
17	TUR	7	Progressive distal limb muscle atrophy	CMT	no	SPG55 (615035)	*C12orf65* (613541)	hmz	Novel	c.[127_146del]; [127_146del]	p.[(Met43Profs*8)]; [(Met43Profs*8)]	N/A	31	Pathogenic (PVS1, PM2, PM3_sup)

Note: *TTN*: NM_133378.4, *ECHS1*: NM_004092.3, *VPS13D*: NM_015378.3, *POLG*: NM_002693.2, *AGTPBP1*: NM_015239.2, *EXOSC3*: NM_016042.3, *ADPRS*: NM_017825.2, *ARSA*: NM_000487.5, *SEPSECS*: NM_016955.3, *FDXR*: NM_024417.4, *PDXK*: NM_003681.4, *NDUFS6*: NM_004553.4, *MPV17*: NM_002437.4, *SPG11*: NM_025137.3, *DNM1L*: NM_012062.4, *C12orf65*: NM_152269.4.

Abbreviations: ACMG, American College of Medical Geneticists; CADD, combined annotation dependent depletion; CHZ, compound heterozygous; ethn., ethnicity; het, heterozygous; hmz, homozygous; zyg, zygosity.

[a]GRCh38/hg38 genomic reference sequence was used.

[b]Condition ID was given according to Orphanet (https://www.orpha.net/) due to no available OMIM entry.

[c]This individual was reported in our previous study (Karakaya et al., 2019).

[d]These individuals were reported in our previous study (Paketci et al., 2020).

[e]This variant was reported recently as a case report (Keller et al., 2021).

not reveal any other causal variants and therefore consolidated the molecular diagnosis. The compound-heterozygous variants in *TTN* had remained undetected by panel analysis due to the missed call of a heterozygous 54 bp deletion by the calling algorithm of the NMD panel. This 54 bp deletion was later detected by ES. Altogether, of the 17 ES-detected disease-causing variants, six were 'NMD-panel-detectable' (gene included in NMD-panel) (35%) while 11 variants were not (65%; Figure S3).

We evaluated the reasons for prior noninclusion of detected genes in the NMD-panel to uncover the most frequent reasons for missed panel diagnosis. Our analysis showed that 50% (5/10) of genes were only recently identified as associated with their respective phenotypes, with first reports in the past three years: *AGTPBP1* (AR childhood-onset neurodegeneration with cerebellar atrophy; MIM# 618276; Shashi et al., 2018), *ADPRS* (AR stress-induced childhood-onset neurodegeneration with variable ataxia and seizures; MIM# 618170) (Ghosh et al., 2018), *FDXR* (AR optic atrophy-ataxia-peripheral neuropathy-global developmental delay syndrome; ORPHA:543470; Peng et al., 2017), *PDXK* (AR hereditary motor and sensory neuropathy type VIC with optic atrophy; MIM# 618511; Chelban et al., 2019), *VPS13D* (AR spinocerebellar ataxia 4; MIM# 607317; Gauthier et al., 2018; Seong et al., 2018). The other half was not included due to phenotypic discordance: *ARSA* (AR metachromatic leukodystrophy; MIM# 250100) was not included because of its characteristic cranial MRI abnormalities usually leading to single-gene testing (van Rappard et al., 2015), *DNM1L* (AD/AR encephalopathy due to defective mitochondrial and peroxisomal fission 1; MIM# 614388), *ECHS1* (AR mitochondrial short-chain enoyl-CoA hydratase-1 deficiency; MIM# 616277), and *SEPSECS* (AR pontocerebellar hypoplasia type 2D; MIM# 613811) were not included because of clinical presentation where pure NMD features are not typically most prominent. *MPV17* (AR axonal CMT type 2EE; MIM# 256810) was not included due to the gene's more frequent hepatocerebral phenotype.

3.4 | Underlying pathomechanisms in solved cases: mitochondrial dysfunction the most frequent pathomechanism

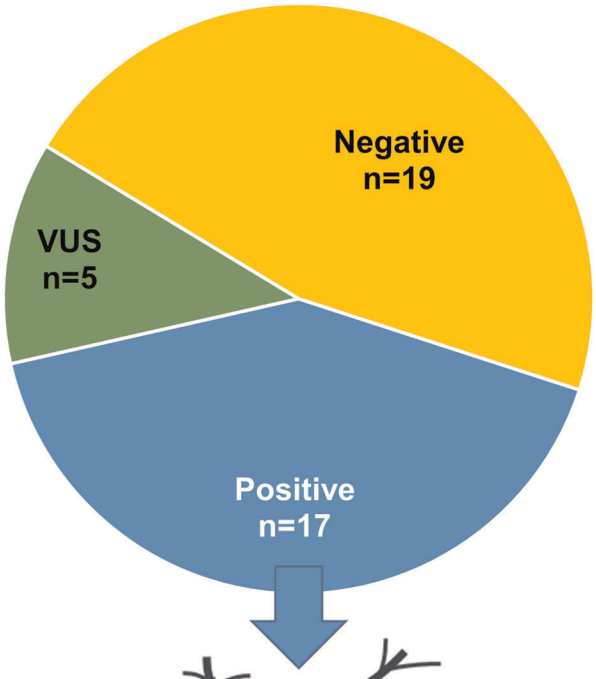
When assessing the physiological cellular functions and related pathomechanisms of the proteins affected by our detected mutations, we found that eight of the 17 solved probands (47%) harbored variants in genes encoding proteins with a function in mitochondrial processes, whereas the other mutated proteins had functions in various other physiological processes (Figure 2). Five of the eight individuals with mitochondrial dysfunction variants belonged to the axonal CMT cohort, while three were of the non-5q-SMA group. The mechanisms impaired were mitochondrial dynamics (*VPS13D*, *DNM1L*), mtDNA replication and maintenance (*POLG*, *MPV17*), mitochondrial translation machinery (*C12orf65*), and respiratory chain (*NDUFS6*, *FDXR*, *ECHS1*; Figure 2).

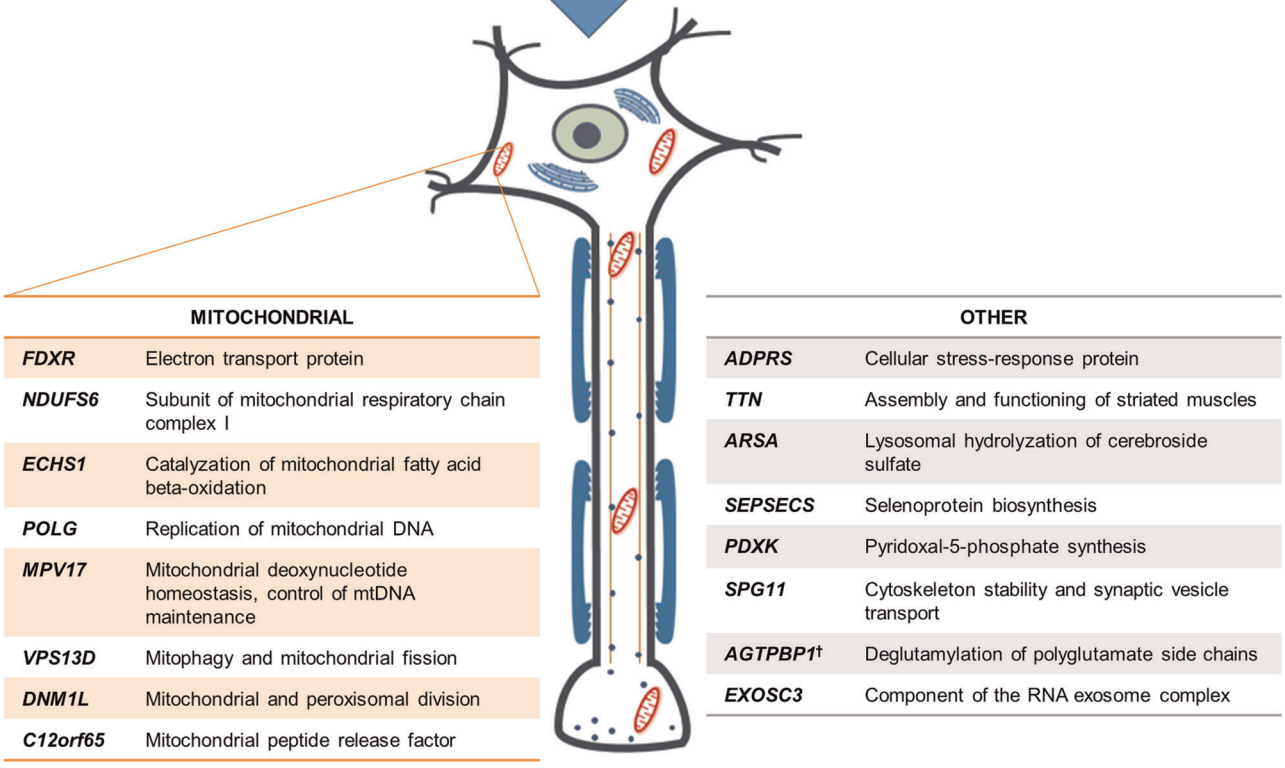
4 | DISCUSSION

In the scope of this study, we validated the diagnostic potential of ES in a complex and clinically heterogeneous cohort of previously undiagnosed non-5q-SMA and axonal CMT individuals. In fact, drawing clear and reliable conclusions from comparison of diagnostic yields in the cohorts with or without prior NMD-panel is not possible because of the obvious small cohort evaluation bias. Especially in clinically heterogeneous cohorts, diagnostic yield and thus NMD-panel success is highly dependent on the individual characteristics of the selected probands. Yet, more than half of the genes detected in the cohort without prior NMD-panel testing were NMD-panel-detectable, highlighting the diagnostic potential of the panel despite the almost similar diagnostic yields. Our study emphasizes the need for generous inclusion of overlapping phenotypes and constant panel updating when applying panel diagnostics to lower motor neuron disorder phenotypes, which is especially important in countries where only gene panels are routinely performed due to reimbursement policies.

The obvious advantages of ES in the diagnosis and research of Mendelian disorders have been outlined thoroughly (Gilissen et al., 2011; Rabbani et al., 2012; Rabbani et al., 2014; Warr et al., 2015). In this regard, during the course of our study, we were able to highlight the potential significance of time-efficient genetic diagnosis provided by early ES in a rare case of a treatment opportunity for peripheral neuropathy with optic atrophy (Keller et al., 2020).

When it comes to deciding whether to perform gene panel testing or direct ES, suspected clinical diagnosis and individual phenotype characteristics should be considered as the key decision factors. In a previous study, we compared the diagnostic yield of our NMD-panel for non-5q-SMA and axonal CMT and observed a significantly higher diagnostic yield of 65% for the latter compared to 33% for non-5q-SMA (Karakaya et al., 2018). Although also presenting significant clinical variability, CMT is mostly characterized by its genetic variety and typically presents with the characteristic clinical features of a length-dependent sensorimotor neuropathy and only limited overlaps, mainly with the dHMN (Bansagi et al., 2017; Morena et al., 2019). Consequently, this often enables a reliable clinical diagnosis, which then only needs to be confirmed by genetic analysis. Thus, for individuals presenting a typical CMT phenotype with signs of sensorimotor axonal neuropathy on NCS, a panel approach can be considered comprising all significantly overlapping phenotypes, especially including the dHMN spectrum. However, CMT might manifest itself as a predominant or early feature of more complex phenotypes, of which the causal genes might be outside the scope of the diagnostic gene panel. This is supported by the fact that among 10 individuals with axonal CMT who were solved by ES, only two of them could have been solved by our NMD panel. Therefore, when presenting atypical features, a direct ES approach should be considered. Accordingly, due to the extreme clinical heterogeneity, first-tier ES is highly recommended for probands who received a clinical SMA diagnosis after initial *SMN1* exclusion. In the clinical





MITOCHONDRIAL	
FDXR	Electron transport protein
NDUFS6	Subunit of mitochondrial respiratory chain complex I
ECHS1	Catalyzation of mitochondrial fatty acid beta-oxidation
POLG	Replication of mitochondrial DNA
MPV17	Mitochondrial deoxynucleotide homeostasis, control of mtDNA maintenance
VPS13D	Mitophagy and mitochondrial fission
DNM1L	Mitochondrial and peroxisomal division
C12orf65	Mitochondrial peptide release factor

OTHER	
ADPRS	Cellular stress-response protein
TTN	Assembly and functioning of striated muscles
ARSA	Lysosomal hydrolyzation of cerebroside sulfate
SEPSECS	Selenoprotein biosynthesis
PDXK	Pyridoxal-5-phosphate synthesis
SPG11	Cytoskeleton stability and synaptic vesicle transport
AGTPBP1†	Deglutamylation of polyglutamate side chains
EXOSC3	Component of the RNA exosome complex

FIGURE 2 Classification of the identified causal genes according to organelle pathology. Mitochondrial pathologies amount to 47% of the overall identified disease mechanisms. †This gene was detected in two unrelated probands

setting of our institution, we have observed that since the recent advent of therapeutic options for 5q-SMA (Nusinersen and gene replacement therapy; Finkel et al., 2016; Mendell et al., 2017), clinicians opt testing for 5q-SMA more frequently even if the clinical phenotype does not strongly suggest a classical SMA. Naturally, this practice would increase the number of probands classified as 'non-5q-SMA' cases as well as widen the clinical spectrum observed in these individuals. Therefore, the genetic diagnosis of the non-5q-SMA group has become more essential to provide a correct diagnosis for these probands, and only an unbiased approach such as ES takes into account the growing range of non-5q-SMA-classified phenotypes.

In both, the solved non-5q-SMA and axonal CMT cohorts, mitochondrial dysfunction was a frequent underlying pathomechanism. This observation is in line with previously conducted studies on hereditary motor-predominant neuropathies (Bansagi et al., 2017;

Liu et al., 2020). Naturally, the high metabolic requirements of neuronal cells result in a high dependency on mitochondrial ATP production through oxidative phosphorylation to uphold neuronal homeostasis and function. As a consequence, motor neurons are extremely sensitive to disbalances of mitochondrial physiology, and it is not surprising that mitochondrial dysfunction is a pathological process shared by ALS (Smith et al., 2019) and SMA (Acsadi et al., 2009; Miller et al., 2016; Thelen et al., 2020). Many neuromuscular disorders where mitochondrial dysfunction is not the primary underlying defect present with accompanying mitochondrial abnormalities (Katsetos et al., 2013), the most notable being *SMN1*-related SMA (Acsadi et al., 2009; Miller et al., 2016; Thelen et al., 2020).

In the scope of this study, we were able to highlight the many different mechanisms that play a role in mitochondrial pathogenesis of LMND. Mitochondrial dynamics are essential for balanced organelle distribution, especially among the long peripheral motor and sensory nerves. Disturbance of this dynamic process, caused mainly by variants in *MFN2* (Züchner et al., 2004) and *GDAP1* (Cuesta et al., 2002), has long been recognized as a frequent underlying pathomechanism of axonal CMT. Herein, we were able to emphasize that axonal neuropathy also presents as a predominant feature in the remaining complex neurologic and neuromuscular spectrum caused by mitochondrial dynamics defects, as represented in our cohort by variants in *VPS13D* and *DNM1L*. We additionally observed impairment of electron chain machinery or mitochondrial DNA synthesis as relevant disease mechanisms presenting with prominent lower motor neuron symptoms (Figure 2).

Taking into account the entire mitochondrial disease spectrum, peripheral neuropathy is a common (about one-third of affected individuals; Finsterer, 2005) but rarely predominant feature (Pareyson et al., 2013), often overshadowed by severe neurologic symptoms including encephalopathy, epilepsy, or cerebellar ataxia (Nardin & Johns, 2001). However, it is important to keep in mind that it can also present as the first and/or predominant sign of a multisystem mitochondrial disorder (Needham et al., 2007). Considering previous as well as our own findings, we recommend that for individuals presenting with prominent axonal peripheral neuropathy, especially when accompanied by atypical features such as ptosis, ophthalmoparesis, myopathy or optic atrophy, mitochondrial dysfunction should be considered and specifically tested as a potential disease cause, even when the initial clinical presentation does not primarily suggest a mitochondrial disorder.

5 | CONCLUSIONS

Thorough phenotype characterization and constant panel-updating are prerequisites for successful gene panel diagnostics in genetically heterogeneous disorders with a characteristic phenotypic presentation such as CMT. Early ES analysis should be considered in cases with nonspecific, uncharacteristic, or overlapping phenotypic presentation such as in non-5q-SMA. As mitochondrial dysfunction is a frequent pathomechanism in rare hereditary disorders of the anterior horn cell and/or peripheral nerve, and mitochondriopathies can present with peripheral neuropathy as an early or predominant feature, genes implied in mitochondrial physiology should be given special attention in molecular diagnosis.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

AUTHOR CONTRIBUTIONS

NK and MK recruited, analyzed the NGS data, and interpreted variants. BW and MK designed and coordinated the study. JA, SM, HT, and PN provided NGS platform, raw data, and the software to analyze NGS data. NF performed NMD-panel analysis and interpretation and assisted in interpreting variants. CP, GW, BS, OU, SY, RB, EGK, RM, and UY provided clinical data and biological material from probands and family members, NK wrote the manuscript, MK and BW provided the main revision of the manuscript, all coauthors read and commented the manuscript.

WEB RESOURCES

Neuromics Consortium https://rd-neuromics.eu/disease/spinal-muscular-atrophy-lower-motor-neuron-disease/).

Varbank analysis pipeline (version 2) https://varbank.ccg.uni-koeln.de/varbank2/.

Varsome https://varsome.com/.

The Human Gene Mutation Database Professional http://www.hgmd.cf.ac.uk/.

Database of Single Nucleotide Polymorphisms (dbSNP) https://www.ncbi.nlm.nih.gov/snp/.

ClinVar archive https://www.ncbi.nlm.nih.gov/clinvar/.

GeneMatcher https://genematcher.org/.

DATA AVAILABILITY STATEMENT

All identified pathogenic, likely pathogenic, and unclear variants and their respective phenotypes were submitted to ClinVar. The data is publicly accessible under the accession number SUB254892.

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