

Sequential targeted exome sequencing of 1001 patients affected by unexplained limb-girdle weakness

A full list of authors and their affiliations appears at the end of the paper.

Purpose: Several hundred genetic muscle diseases have been described, all of which are rare. Their clinical and genetic heterogeneity means that a genetic diagnosis is challenging. We established an international consortium, MYO-SEQ, to aid the work-ups of muscle disease patients and to better understand disease etiology.

Methods: Exome sequencing was applied to 1001 undiagnosed patients recruited from more than 40 neuromuscular disease referral centers; standardized phenotypic information was collected for each patient. Exomes were examined for variants in 429 genes associated with muscle conditions.

Results: We identified suspected pathogenic variants in 52% of patients across 87 genes. We detected 401 novel variants, 116 of which were recurrent. Variants in *CAPN3*, *DYSF*, *ANO5*, *DMD*, *RYR1*, *TTN*, *COL6A2*, and *SGCA* collectively accounted for over

half of the solved cases; while variants in newer disease genes, such as *BVES* and *POGLUT1*, were also found. The remaining well-characterized unsolved patients (48%) need further investigation.

Conclusion: Using our unique infrastructure, we developed a pathway to expedite muscle disease diagnoses. Our data suggest that exome sequencing should be used for pathogenic variant detection in patients with suspected genetic muscle diseases, focusing first on the most common disease genes described here, and subsequently in rarer and newly characterized disease genes.

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INTRODUCTION

Genetic muscle diseases with limb-girdle weakness (LGW) are highly heterogeneous, rare neuromuscular disorders (NMD). In addition to the characteristic progressive pelvic and shoulder girdle muscle weakness, other manifestations may include respiratory insufficiency, cardiomyopathy, contractures, and gastrointestinal complications.¹ Consequently, these disorders diminish an individual's quality of life and can reduce life expectancy.² There is a critical need to fully characterize disease etiology to achieve accurate diagnoses and offer appropriate genetic counseling and disease management.

Despite 535 genes known to be associated with 955 distinct NMDs,³ many muscle disease patients still remain genetically undiagnosed. This is partly because gene-by-gene and small panel testing strategies are usually dictated by phenotype, leaving little power for the expansion of disease associations and novel disease characterizations.⁴ An unbiased methodology has thus been sought to enhance standard clinical practices. Consistent with an increased affordability over the past decade, next-generation sequencing (NGS) technologies have been integral in the diagnosis of disorders such as mitochondrial disease among others.⁵ Large-scale NGS panels were applied to 504 Italian undiagnosed muscular dystrophy and myopathy patients,⁶ and more recently to 4655 limb-girdle muscular dystrophy (LGMD) patients in the United States.⁷ By investigating 93 and 35 NMD

genes, respectively, they achieved a diagnostic rate of 43% and 27%. However, to date no study has been carried out where exome sequencing (ES) has been applied to similar limb-girdle cohorts; this allowed us not only to determine the impact that the panel size has on the detection rate, but also to interrogate the exomes beyond the known NMD genes.

We describe here the application of sequential targeted exome sequencing (TES) to a large cohort of undiagnosed patients presenting with proximal muscle weakness and/or elevated serum creatine kinase (CK) levels: the MYO-SEQ project. We established a partnership between academia, industry, and patient organizations, and developed an international network that connected over 40 specialist neuromuscular referral sites. A total of 1001 exomes were first analyzed for rare pathogenic variants in 169 common NMD genes, and then in a further 260 genes associated with rarer muscle conditions. We identify the most common muscle diseases in this population and suggest that an increased number of patients can be more rapidly tested and diagnosed through an appropriate TES approach.

MATERIALS AND METHODS

Patient recruitment

We utilized the infrastructure established by TREAT-NMD (<https://treat-nmd.org/>) to recruit 43 NMD referral centers

Correspondence: Volker Straub (volker.straub@ncl.ac.uk)

These authors contributed equally: Ana Töpf, Katherine Johnson

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from throughout Europe and the Middle East (Figure S1). Singleton patients matching the inclusion criteria of unexplained limb-girdle weakness and/or elevated CK were included in the project. Informed consent was obtained for all patients. Anonymized clinical information was entered on the PhenoTips platform (<https://phenotips.org/>) using NMD-specific forms. Clinical features, histological findings, electromyography results, CK levels, mode of inheritance, and disease onset and progression were recorded using Human Phenotype Ontology terms. DNA samples were submitted to the Newcastle Medical Research Council (MRC) Centre Biobank for Neuromuscular Diseases for which ethical approval was granted by the National Research Ethics Service (NRES) Committee North East–Newcastle & North Tyneside 1 (reference 08/H0906/28).

Exome sequencing, data analysis, and interpretation

ES was performed at the Broad Institute of MIT and Harvard's Genomics Platform (Cambridge, MA, USA) using a 38-Mb targeted Illumina exome capture, as described previously.⁸ Data were first filtered for variants in 169 NMD-causing genes (Table S1), with a minor allele frequency in the ExAC control population⁹ of $\leq 1\%$ and a moderate to high Ensembl Variant Effect Predictor. The resulting shortlists of single-nucleotide variants (SNVs) and small insertions/deletions (indels) were examined for pathogenicity by two independent data analysts (M.B./J.D./K.J./M.M./A.T. at the John Walton Muscular Dystrophy Research Centre [JWMDRC] and L.X./E.M.E./E.V./T.M. at the Broad Institute). We deemed SNVs and indels pathogenic if they had been previously and consistently reported as pathogenic in ClinVar (two gold stars review status; <https://www.ncbi.nlm.nih.gov/clinvar/>) and/or published literature. In line with American College of Medical Genetics and Genomics (ACMG) guidelines,¹⁰ pathogenicity of novel variants was evaluated based on (1) computational data, i.e., predicted to be deleterious by all, or the majority, of the *in silico* tools used; (2) population data, i.e., have a frequency in the control population compatible with the variant being rare disease-causing, according to its inheritance mode; and (3) allelic data, i.e., be *in trans* with a pathogenic variant; however, as phase could rarely be established for putative compound heterozygous cases we refer to these as double heterozygous. Variants of uncertain significance are not discussed here. To be considered solved, a patient was required to carry two known or suspected pathogenic variants in a recessive gene, or (at least) one known or suspected pathogenic variant in a dominant gene. By suspected pathogenic variants we refer to rare and damaging variants identified in a gene associated with a disease that matches the patients' clinical presentation, disease onset and progression, magnetic resonance image (MRI) findings, and/or muscle histopathology. The findings were disclosed to the submitting centers in tailored reports, signposting the patients to relevant disease registries. Where necessary and possible, the suspected pathogenic variants were confirmed and segregated through Sanger sequencing,

muscle biopsies further investigated, and clinical histories reassessed by the referring clinicians (Figure S2).

The remaining unsolved cases were subsequently analyzed for likely pathogenic variants in a further 260 genes (Table S2) including those associated with neuropathies, cardiomyopathies, and other phenotypes presenting with muscle weakness to identify rarer neuromuscular conditions. This extended gene list also included genes newly associated with muscle disease. ES data were also analyzed for copy-number variants (CNVs)¹¹ and *SMN1* exon 7 deletions¹² (Supplementary Materials and Methods). For those cases carrying a single heterozygous pathogenic variant in the most common (exclusively recessive) genes found in our cohort (i.e., *CAPN3*, *DYSF*, *ANO5*, and *SGCA*) we further interrogated the noncoding regions of the gene captured in the ES data. In addition, for the *DYSF* carriers we Sanger sequenced two previously reported pathogenic intronic variants.¹³ Finally, one patient was also subjected to genome sequence (GS) and RNA sequencing (RNA-Seq), as described elsewhere.¹⁴

RESULTS

Demographics of 1001 patients with suspected genetic muscle disease

One thousand and one patients were recruited into the MYO-SEQ project, with the majority (93%) inferred to be of European or Middle Eastern ancestry. The study cohort comprised 545 (54%) males and 456 (46%) females and originated from 972 families. Disease onset for 42% of the patients was in adulthood (Figure S3A) and the mean age at the time of recruitment was 39 years (Figure S3D). Proximal muscle weakness, either in isolation or with distal muscle weakness, affected most participants (77%; Figure S3B), while 68% of the cohort had increased serum CK levels (Figure S3C). Based on family history, 46% of patients were sporadic cases, while 14%, 7%, and 1% showed recessive, dominant, and X-linked inheritance, respectively; for the remaining 32%, no indication of inheritance pattern was stated by the referring clinician.

Initial screening detected suspected pathogenic variants in 47% of participants

Using the World Muscle Society gene table (<http://www.musclegenetable.fr/>) we generated a list of 169 genes known to be associated with limb-girdle muscle disease (Table S1) and initially restricted our search for likely causal variants to only these genes. As a result, 468 patients (47%) had rare known or suspected pathogenic variants across 72 genes that we considered were causal; these cases were deemed solved (Table 1, Fig. 1).

Frequency of NMD genes varied greatly in our cohort

The most common disease in our cohort was LGMD R1 calpain3-related, caused by autosomal recessive pathogenic variants in *CAPN3*. Variants in *CAPN3* and seven other genes—*DYSF*, *ANO5*, *DMD*, *RYR1*, *TTN*, *COL6A2*, and *SGCA*—accounted for over half of the solved cases ($n = 260$). The

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Table 1 Breakdown of genes in which suspected causal variants were identified in the MYO-SEQ cohort.

Gene	Number of patients	Gene	Number of patients	Gene	Number of patients	Gene	Number of patients
<i>CAPN3</i>	78	<i>POGLUT1</i> ^a	5	<i>PYROXD1</i> ^a	2	<i>HSPB1</i> ^a	1
<i>RYR1</i>	26	<i>SCN4A</i>	5	<i>SGCB</i>	2	<i>HSPB8</i> ^a	1
<i>DYSF</i>	45	<i>CLCN1</i>	3	<i>ACADVL</i>	1	<i>INPP5K</i> ^a	1
<i>ANO5</i>	33	<i>DNM2</i>	4	<i>BAG3</i>	1	<i>LPIN1</i>	1
<i>DMD</i>	31	<i>MYOT</i>	4	<i>BVES</i> ^a	1	<i>MATR3</i>	1
<i>TTN</i>	25	<i>RAPSN</i>	4	<i>CACNA1S</i> ^a	1	<i>MGME1</i> ^a	1
<i>COL6A2</i>	17	<i>DNAJB6</i>	3	<i>CHRND</i>	1	<i>MME</i> ^a	1
<i>SGCA</i>	16	<i>GMPPB</i>	3	<i>COL12A1</i>	1	<i>MYF6</i>	1
<i>COL6A3</i>	12	<i>MTM1</i>	3	<i>CPT2</i>	1	<i>MYH14</i>	1
<i>FLNC</i>	12	<i>SEPN1</i>	3	<i>CRYAB</i>	1	<i>NEB</i>	1
<i>DES</i>	10	<i>TCAP</i>	3	<i>DOK7</i>	1	<i>PFKM</i>	1
<i>GAA</i>	10	<i>TRAPPC11</i>	3	<i>DPM3</i>	1	<i>PGK1</i>	1
<i>LAMA2</i>	10	<i>ACTA1</i>	2	<i>DYNC1H1</i> ^a	1	<i>POLG</i>	1
<i>POMT2</i>	10	<i>AGL</i>	2	<i>EGR2</i> ^a	1	<i>PTPLA</i>	1
<i>COL6A1</i>	10	<i>ATP2A1</i>	2	<i>ETFDH</i>	1	<i>SYNE2</i>	1
<i>LMNA</i>	9	<i>COLQ</i>	2	<i>FKTN</i>	1	<i>TMEM8C</i> ^a	1
<i>MYH7</i>	9	<i>FHL1</i>	2	<i>GBE1</i>	1	<i>TNNT3</i>	1
<i>FKRP</i>	8	<i>PHKA1</i>	2	<i>GFPT1</i>	1	<i>TPM2</i>	1
<i>SGCG</i>	8	<i>POMK</i>	2	<i>GNE</i>	1	<i>TTR</i> ^a	1
<i>SMCHD1</i>	8	<i>POMT1</i>	2	<i>GYG1</i>	1	<i>VCP</i>	1
<i>TRIM32</i>	7	<i>PRPS1</i> ^a	2	<i>HNRNPA1</i> ^a	1	<i>VMA21</i>	1
<i>CAV3</i>	5	<i>PYGM</i>	2	<i>HNRNPDL</i>	1		

Suspected causative variants were identified in 87 genes in 489 patients: 72 genes during the first round of analysis using a gene list of 169 neuromuscular disorder (NMD) genes, and 15 additional genes when using a gene list extended by a further 260 NMD genes.

^aFifteen additional genes identified.

other 208 solved cases had suspected pathogenic variants across 64 additional genes. Importantly, we identified a stop-gain *TTN* founder pathogenic variant (p.[Gln35879Ter]) in 14 patients from a Serbian subpopulation.⁸ Without this variant, *TTN* would be outnumbered by *COL6A2* and *COL6A3* (collagen VI-related myopathies), *SGCA* (LGMD R3 α -sarcoglycan-related), and *FLNC* (myofibrillar myopathy 5 and distal myopathy 4). Some diseases were notably absent from our cohort; for example, no *POMGnT1* (LGMD R15 POMGnT1-related) or *ISPD* (LGMD R20 ISPD-related) patients were identified, highlighting the extreme rarity of these diseases. When the data were stratified by sex, there appeared to be a skewed ratio of males ($n = 22$; 69%) to females ($n = 10$; 31%) harboring suspected pathogenic variants in *ANO5* ($p < 0.0691$), confirming previously reported findings.¹⁵

Homozygous variants associated with established autosomal dominant inheritance

A proband from a consanguineous pedigree harbored a previously reported dominant pathogenic variant (c.476G>A p.[Arg159His]) in *VCP* in homozygosity, while another proband carried a novel homozygous variant (c.1325C>G p.[Pro442Arg]) in *FLNC*; both genes have established autosomal dominant inheritance. In both cases, the clinical presentation was in keeping with what was expected, but at the more severe

end of the spectrum with an earlier disease onset. Both sets of parents were heterozygous for the respective variants. The mother of the homozygous *VCP* patient remained asymptomatic at the time of examination, while the father presented with proximal weakness and atrophy of the upper and lower limbs, and rimmed vacuoles on histopathology,¹⁶ as would be expected for this previously reported dominant variant.¹⁷ On the other hand, the parents of the homozygous *FLNC* patient were both clinically unaffected on examination. This would suggest a different pathological mechanism for this novel *FLNC* variant and functional work is underway (unpublished data by University Duisburg–Essen).

Solved rate differed with geographical location of the participating center

When the data were stratified by geographic location, there was a clear distinction between regions with and without the infrastructure to sufficiently prescreen patients prior to submission. The detection rate for referring centers in Eastern Europe and the Middle East was as high as 95%, including, for example, 57 LGMD R1 calpain3-related and 32 LGMD R2 dysferlin-related cases. Conversely, the detection rate for Western Europe was as low as 35%, with only 23 LGMD R1 calpain3-related ($p < 0.00001$) and 15 LGMD R2 dysferlin-related cases ($p < 0.00001$), and a larger proportion of rarer genes and phenotypes (Fig. 2).

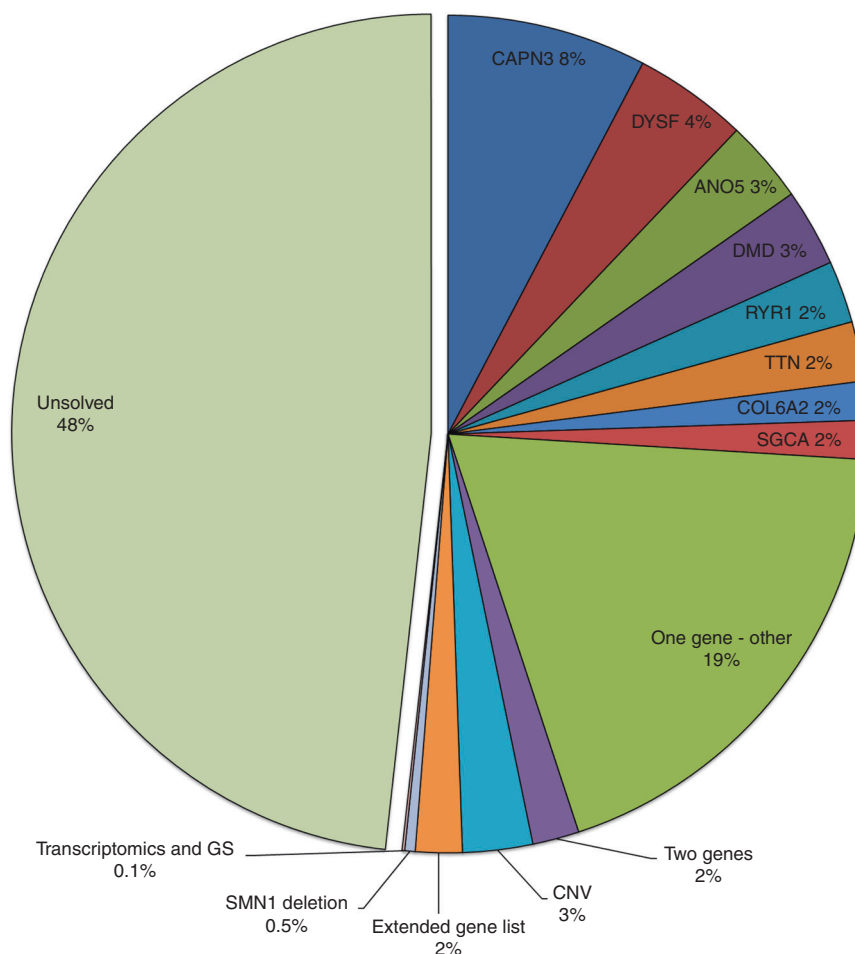


Fig. 1 Summary of the solved rate and sequential analysis in the MYO-SEQ cohort. Suspected pathogenic variants were detected in a total of 520 patients (52%). Initially, 468 (47%) patients were solved through a screening of the MYO-SEQ gene list of 169 neuromuscular disorder (NMD) genes; of these, 450 (45%) had pathogenic variants in one of 72 genes and 18 (2%) had pathogenic variants in two genes. Pathogenic variants in eight of these genes alone accounted for half of the solved patients (26%); the most common disease detected in our cohort was limb-girdle muscular dystrophy (LGMD) R1 calpain3-related (*CAPN3*). Variants in 64 genes accounted for the remaining patients with one causal gene. The analysis strategy was extended by a further 260 genes, solving an additional 2% of the cohort. Copy-number variations (CNVs) were detected in 26 (3%) patients, *SMN1* deletions in five (0.5%) patients and transcriptional perturbations in one patient. $n = 1001$. *GS* genome sequencing.

Analysis of an extended gene list increased the diagnostic yield to 49%

For the remaining unsolved cases, we extended the analysis to a total of 429 disease-causing genes (Table S2). This resulted in a 2% increase in detection rate to an overall 49% ($n = 488$), across 87 muscle disease-associated genes; 15 genes more than the initial analysis. These genes were associated with neuropathies (*EGR2*, *DYNC1H1*, *HSPB1*, *HSPB8*, *PRPS1*, and *MME*), multisystemic disorders presenting with muscle weakness (*HNRNPA1*, *INPP5K*, *TMEM8C*, and *TTR*), channelopathies (*CACNA1S*), and mitochondrial disease (*MGME1*), all detected in patients who had been initially clinically diagnosed as LGW ($n = 12$). In addition, three genes only recently associated with muscular dystrophy (*BVES* and *POGLUT1*)^{18,19} and myofibrillar myopathy (*PYROXD1*)²⁰ were found in eight patients. Thus, after the eight most common disease genes, accounting for 25% of the full cohort, the next ten genes (*COL6A3*, *FLNC*, *DES*, *GAA*, *LAMA2*,

POMT2, *COL6A1*, *LMNA*, *MYH7*, and *FKRP*) account for an additional 10%, followed by a very small but nonetheless meaningful increase in solved rate as very rare and/or unexpected disease genes, as well as those recently described, are detected (Table 1).

Expanding exome sequencing beyond single base pair changes and small indels increased the detection yield by a further 3%

Further to point variants and small indels, we detected suspected pathogenic CNVs in 26 (3%) additional patients. Of these, 17 carried copy-number zero deletions (at exon or multiexon level) in autosomal recessive genes (*DYSF*, *SGCA*, *SGCB*, *SGCD*, *SGCG*, and *LPIN1*; $n = 10$) and in X-linked genes (*DMD*, *EMD*, and *FHL1*; $n = 7$, all males). Eight cases carried a heterozygous CNV in combination with a suspected pathogenic SNV in the same gene and one case, presenting with a classical dominant myofibrillar myopathy phenotype,

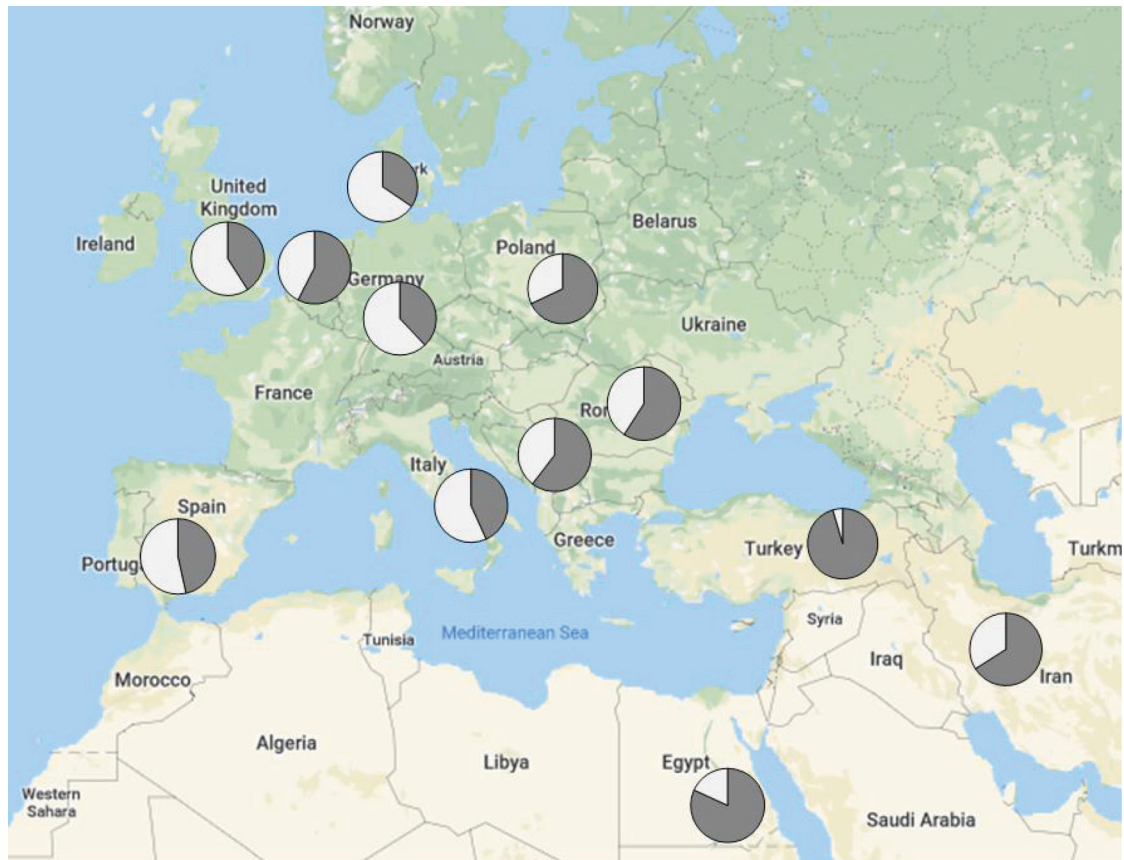


Fig. 2 Solved rate by country of origin. The MYO-SEQ solved rate (in dark gray) was higher in countries such as Egypt and Turkey where the infrastructure for genetic testing for prescreening is not as widely available, and lower in Western European countries where genetic prescreening of common limb-girdle muscular dystrophy (LGMD) genes is routinely performed. Calculated for referring centers submitting more than 20 samples.

carried a heterozygous deletion in *DES*. Some of these CNVs were recurring; we identified a 10.4-kb deletion in *CAPN3* in five unrelated patients, and a 0.7-kb and 0.12-kb deletion in *SGCD* and *SGCG*, respectively, in two unrelated patients each (Table S3). In addition, we looked for homozygous deletions of exon 7 in *SMN1*, causative of spinal muscular atrophy. Such deletions were detected in five patients (0.5%), all of which were confirmed by multiplex ligation-dependent probe amplification (MLPA). Finally, through additional RNA-Seq and GS, we were able to identify an intronic change resulting in a 73-bp intron inclusion in *DMD* leading to a premature stop codon in one patient with a clear Becker-like phenotype and supporting family history, but with no evident causal exonic *DMD* variant,¹⁴ highlighting that strong phenotypic clues should always be followed up.

Over 400 novel suspected pathogenic variants were identified

In total, we identified 865 SNV and indel changes in 429 genes that were most likely to contribute to the 496 participants' diseases. A breakdown of the genotypes and types of variants is shown in Fig. 3a, b. The 865 variants were accounted for by 520 distinct variants (Table S4); of these, 119 had been submitted to ClinVar previously, while the

remaining 401 were novel and had not been yet reported at the time of the analysis (Fig. 3c). Of the novel variants, 285 were seen only once and, notably, 116 were seen multiple times in our patient population (Fig. 3d). These novel variants will be submitted to ClinVar to aid future genetic diagnoses.

Single heterozygous pathogenic variants in the most common recessive genes account for 2.5% of the unsolved cases

To unravel the remaining unsolved patients, we looked at those who carried single reported pathogenic variants in the four most common exclusively recessive genes found in our cohort. We found 25 (2.5%) heterozygous carriers: 13 *CAPN3*, 9 *ANO5*, 2 *SGCA*, and 1 *DYSF*. These cases were accounted for by 16 pathogenic variants: 10 *CAPN3*, 4 *ANO5*, 1 *DYSF*, and 1 *SGCA*. These variants were all more frequent in our cohort of patients than in the control population. For example, a rare *SGCA* variant (c.229C>T; p.[Arg77Cys]) occurred four times more frequently in the disease population ($p = 0.02$), the common Eastern European *CAPN3* variant (c.550delA; p.[Thr184ArgfsTer36]) over eight times more ($p = 0.00028$), and the European founder *ANO5* variant (c.191dupA; p.[Asn63fs]) almost five times more ($p = 0.00016$). This suggests that these variants are likely to play

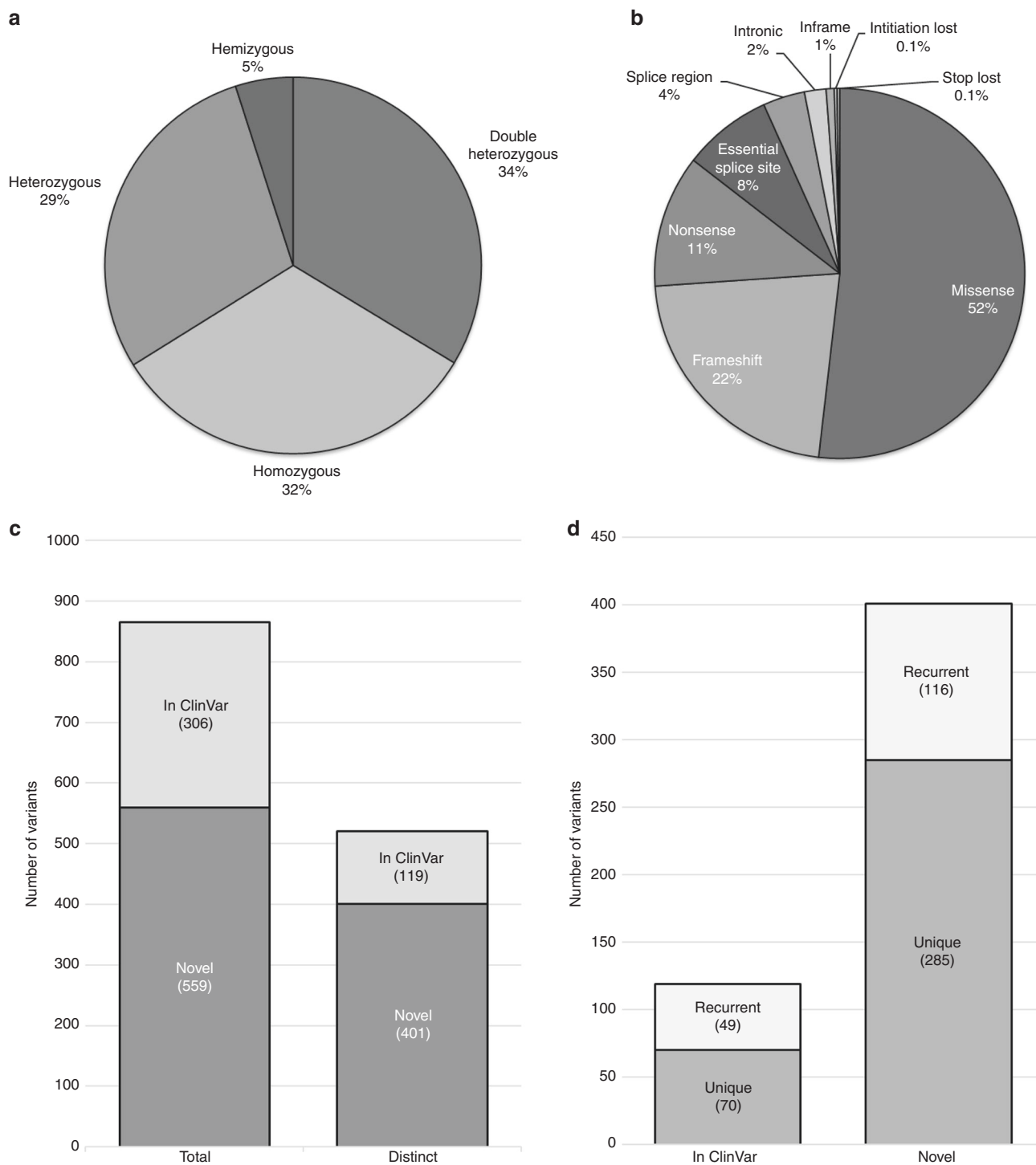


Fig. 3 Breakdown of the suspected pathogenic variants and genotypes in the MYO-SEQ cohort. (a) Zygosity of the solved patients' variants ($n = 506$; 489 patients, 20 with an additional gene to report). (b) Type of variants suspected to be pathogenic. Initiation and stop loss occurred twice each ($n = 865$). (c) The 865 occurrences were accounted for by 520 distinct variants, of which 119 were reported as pathogenic in ClinVar and 401 were novel in their association to disease at the time of the analysis. (d) Of the 119 distinct variants reported in ClinVar, 70 were detected in only one individual (unique) while 49 were detected in multiple patients. Of the 401 distinct variants that were novel in their association to disease, 285 were detected in only individual cases, while 116 were detected in multiple families.

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a role in disease manifestation and that a second cryptic disease-causing variant might not have been detected by our analysis; we therefore interrogated these carriers further by looking into the noncoding regions present in their exome data. We thus identified one novel 3' UTR and two rare *CAPN3* intronic variants, one of which (c.1746–20C>G) occurred in three cases. Histopathological analysis showed markedly reduced calpain3 immunostaining, suggesting that this noncoding variant may affect splicing or protein stability. In addition, we identified an intronic deletion (c.585–31_585–24delTCTGCTGA) in one of the *SGCA* carriers, which results in partial expression of the α -sarcoglycan protein.²¹ Conversely, none of the reported pathogenic intronic variants in *DYSF*¹³ were found in the *DYSF* carrier.

Many patients harbored suspected pathogenic variants in genes associated with treatable or manageable conditions

Our study identified 64 patients (6.5%) that might benefit from treatment or specific management options related to their diagnosis. For example, variants in *COLQ*, *DOK7*, *GFPT1*, and *RAPSN*—all associated with congenital myasthenic syndrome (CMS)—were identified in eight patients (<1%). We also identified ten patients (1%) with compound heterozygous variants in *GAA*, associated with Pompe disease.²² Nine patients (1%) harbored variants in either *SCN4A* or *CLCN1* and thus were suspected to have ion channel disorders that could benefit from selective drug interventions. We identified 13 female patients (1%) who were likely to be manifesting carriers of Duchenne muscular dystrophy, an X-linked disorder that often escapes clinical diagnosis in females. Finally, we identified 24 patients (2%) with heterozygous suspected pathogenic variants in *RYR1*, which can confer susceptibility to malignant hyperthermia.

DISCUSSION

We sought to improve the diagnostic pathway of patients with limb-girdle muscle diseases by implementing TES as a first-pass diagnostic strategy. By focusing on 429 NMD genes within the exome data, we solved 49% of our cohort, comparable with equivalent NGS panel studies.^{6,7} A small number of common NMD genes, namely *CAPN3*, *DYSF*, *ANO5*, *DMD*, *RYR1*, *TTN*, *COL6A2*, and *SGCA* accounted for more than half of the solved cases. Our finding that LGMD R1 calpain3-related was the most common LGMD in our cohort is in agreement with other European studies.⁶ Standard calpain3 immunoblot testing can only achieve a low sensitivity,²³ which could explain why a tenth of the MYO-SEQ cohort was not diagnosed with this LGMD prior to enrollment. Moreover, many of the calpain3-related patients were referred from centers without the technical infrastructure to prescreen their patients.

Given the considerable phenotypic overlap and the rarity of some of these muscle conditions, it is almost impossible for clinicians, however expert, to individually diagnose every disease. Some of the LGMDs, such as R15 POMGnT1-related

and R20 ISPD-related, were absent from our cohort. No SNVs and only one CNV were identified in the *SGCD* gene, in keeping with other sequencing projects from China ($n = 756$),²⁴ Italy ($n = 504$),⁶ and the United States ($n = 4656$)⁷ where no *SGCD* variants were found. Interestingly, LGMD R6 δ -sarcoglycan-related patients accounted for >1% of the diagnosed cases in a South American cohort ($n = 2103$),²⁵ highlighting that the geography-specific prevalence of some diseases should be considered during clinical work-ups. Per sex bias, we corroborated previous findings that *ANO5* diagnosis is more frequent in males.¹⁵ This is likely due to the milder, sometimes asymptomatic, presentation of LGMD R12 anoctamin5-related in females; because of this, females can present to clinic much later in life, or not at all, skewing the diagnostic rate in favor of males.

The relatively small benefit observed when extending the gene list from 169 to 429 genes is in line with a panel study of 700 NMD genes where exome sequencing of the remaining undiagnosed cases did not improve the pick-up rate for known NMD genes.²⁶ However, having exome data available, such as in our study, allows for both gene discovery and data analysis reiteration when new disease genes are characterized, neither of which are possible even with a large NGS panel approach. This was the case for the *BVES*, *POGLUT1*, and *PYROXD1* patients who were missed in the first round of analysis, as the genes had not yet been described as disease-causing. By interrogating the whole exome of the unsolved patients, we identified and have functionally characterized two novel genes, one associated with a rare secondary dystroglycanopathy and the other, *POPDC3*, found in three unrelated individuals, associated with a typical LGMD phenotype.²⁷ Two additional novel candidate genes are under investigation and we expect that this will only increase as the exome data of unsolved patients are more intricately examined.

It is possible that further causative variants have not been assigned pathogenicity due to the limitations of proband-only analysis or that they have not been detected due to nonuniform coverage and/or poorly designed capture baits that might not target relevant tissue-specific exons. For example, repeat regions in *NEB* or *TTN* are known to be difficult to map to the genome and therefore variants in these regions are poorly called.²⁸ Somatic mosaicism,²⁹ only detected by deep coverage sequencing, would also have been missed. A further proportion of the remaining unsolved cases might be caused by genetic changes in intergenic, intronic, or regulatory regions not covered by ES. The overrepresentation in our disease cohort of carriers of known pathogenic variants in recessive genes likely implies that a second cryptic variant is yet to be detected. In fact, when interrogating these cases further we identified recurrent suspected pathogenic intronic variants and CNVs. Otherwise, these pathogenic heterozygous variants might act as disease modifiers, contributing to the disease severity or progression.³⁰

Using modified pipelines, we have identified several disease-causing CNVs, accounting for 3% of the diagnoses. However,

more complex structural variants such as translocations and inversions, would not have been detected.³¹ In a similar manner, tri- and tetranucleotide expansion repeats, such as the cause of some adult onset neuromuscular conditions (e.g., myotonic dystrophy), are typically missed in standard short-read NGS data. Interestingly, four unsolved cases were reported to present with myotonia, percussion myotonia, or myotonic discharges. Other novel late-onset myopathies might be caused by similar mechanisms and long-read NGS would be needed to identify them. Digenic or non-Mendelian inheritance, such as genomic imprinting, may also account for a proportion of the undiagnosed patients^{32,33} and will need a different methodological and analytical strategy.

It is also possible that some of the unsolved patients may have acquired forms of muscle disease, such as acquired immune, inflammatory, noninflammatory, or even statin-induced myopathies. In fact, during the project four patients (<1%) obtained a confirmed diagnosis of inclusion body myositis. Regular screening for anti-NT5c1A and anti-HMGCoR should provide a more realistic indication of the prevalence of these diseases in the affected population.

Over 6% of our patients received a genetic outcome that resulted in specific monitoring and tailored treatments. For example, for the CMS patients, genetic diagnoses were vital to provide adequate treatment, as this depends on the disease subtype and causative gene. Indeed, the referring clinicians reported that these patients all showed marked improvement after appropriate treatments were commenced. The pathogenicity of the *RYR1* variants could not always be fully confirmed, yet it was prudent to return these potential diagnoses to physicians to manage possible risk of malignant hyperthermia. For most of the solved cases, however, who might not benefit from specific treatment, their genetic diagnosis will nevertheless allow appropriate family planning, counseling, and monitoring of their diseases.

MYO-SEQ was a worldwide partnership between academia, industry, and patient organizations. Frequent communication with the referring physicians was essential for the success of the project; to reach a meaningful diagnosis, interpretation of the genetic data was always in the context of clinical, histopathological, and MRI data. The participating centers provided consent for data sharing facilitated by the European Genome-Phenome Archive (EGA) and RD-Connect (<https://platform.rd-connect.eu/>), and we advocate that adopting such an approach will enable future matchmaking between extremely rare cases, such as *BVES*-related myopathy³⁴ or LGMD R21 *POGLUT1*-related.³⁵ In addition, thanks to the large cohort size and standardized deep phenotypic data, we were able to expand the clinical and mutational spectrum of known causative genes, such as *TRIM32*,³⁶ *POMK*,³⁷ *DPM3*,³⁸ *POMT2*,³⁹ and other dystroglycanopathies.⁴⁰

Based on our findings from this large-scale international collaboration, we suggest a new diagnostic approach in the

clinic and/or private health providers. Rather than implementing small NGS panel approaches, led by phenotypic clues that can often be misleading due to the high clinical heterogeneity of muscle diseases, patients should be directly referred for exome sequencing. The exome sequencing data of NMD patients of European and Middle Eastern origin should first be promptly analyzed for pathogenic variants in the eight genes most commonly associated with muscle disease in our cohort—*CAPN3*, *DYSF*, *ANO5*, *DMD*, *RYR1*, *TTN*, *COL6A2*, and *SGCA*. This is expected to diagnose over a quarter of individuals. The analysis pipeline should then be extended to include additional NMD genes, enabling a diagnosis in a further quarter of patients. For those patients who remain undiagnosed, their ES data can be further interrogated. Importantly, exome data can be retrospectively and repeatedly analyzed for pathogenic variants in novel disease genes as new discoveries are made, making it a much more cost-effective approach.

SUPPLEMENTARY INFORMATION

The online version of this article (<https://doi.org/10.1038/s41436-020-0840-3>) contains supplementary material, which is available to authorized users.

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DISCLOSURE

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Ana Töpf, PhD¹, Katherine Johnson, PhD^{1,67}, Adam Bates, MSc¹, Lauren Phillips, BSc¹, Katherine R. Chao, BSc^{2,3}, Eleina M. England, MSc^{2,3}, Kristen M. Laricchia, MSc^{2,3}, Thomas Mullen, PhD^{2,3}, Elise Valkanas, BA^{2,3}, Liwen Xu, MD, MPhil^{2,3}, Marta Bertoli, MD^{1,68}, Alison Blain, PhD¹, Ana B. Casasús, MSc¹, Jennifer Duff, PhD¹, Magdalena Mroczek, MD¹, Sabine Specht, MD¹, Monkol Lek, PhD^{2,3,69}, Monica Ensini, PhD^{1,70}, Daniel G. MacArthur, PhD^{2,3,71,72}, the MYO-SEQ consortium and Volker Straub, MD¹

¹John Walton Muscular Dystrophy Research Centre, Translational and Clinical Research Institute, Newcastle University and Newcastle Hospitals NHS Foundation Trust, Newcastle upon Tyne, UK; ²Analytic and Translational Genetics Unit, Massachusetts General Hospital, Boston, MA, USA; ³Program in Medical and Population Genetics, Broad Institute of MIT and Harvard, Cambridge, MA, USA; ⁶⁷Present address: Translational and Clinical Research Institute, Newcastle University, Newcastle upon Tyne, UK; ⁶⁸Present address: Northern Genetics Service, Newcastle upon Tyne NHS Foundation Trust, Newcastle upon Tyne, UK; ⁶⁹Present address: Department of Genetics, Yale University School of Medicine, New Haven, CT, USA; ⁷⁰Present address: Directorate-General for Research and Innovation, Directorate E, Unit E2 Combatting Diseases, Brussels, Belgium; ⁷¹Present address: Centre for Population Genomics, Garvan Institute of Medical Research, Sydney, Australia; ⁷²Present address: Centre for Population Genomics, Murdoch Children's Research Institute, Melbourne, Australia

The MYO-SEQ consortium

Ela Akay^{4,73}, Jorge Alonso-Pérez⁵, Jonathan Baets^{6,7}, Nina Barisic⁸, Alexandra Bastian^{9,10}, Sabine Borell¹¹, Teodora Chamova¹², Kristl Claeys¹³, Jaume Colomer¹⁴, Sandra Coppens^{15,16}, Nicolas Deconinck^{17,18}, Willem de Ridder^{6,7}, Jordi Díaz-Manera^{5,19}, Cristina Domínguez-González^{19,20}, Alexis Duncan²¹, Hacer Durmus²², Nagia A. Fahmy²³, Maria Elena Farrugia²⁴, Roberto Fernández-Torrón^{25,26,27}, Lidia Gonzalez-Quereda^{19,28}, Jana Haberlova²⁹, Maja von der Hagen³⁰, Andreas Hahn³¹, Antonia Jakovčević³², Ivonne Jerico Pascual³³, Solange Kapetanovic³⁴, Viktorija Kenina³⁵, Janbernd Kirschner¹¹, Andrea Klein^{36,37}, Heike Kölbel³⁸, Anna Kostera-Pruszczyk³⁹, Richa Kulshrestha⁴⁰, Jaana Lähdetie⁴¹, Mahsa Layegh⁴², Cheryl Longman²¹, Adolfo López de Munain^{25,26,27,43}, Wolfgang Loscher⁴⁴, Anna Lusakowska³⁹, Paul Maddison⁴, Armelle Magot⁴⁵, Anirban Majumdar⁴⁶, Pilar Marti^{19,47,48}, Amaia Martínez Arroyo⁴⁹, Radim Mazanec²⁹, Sandra Mercier⁴⁵, Tiziana Mongini⁵⁰, Nuria Muelas^{19,47,48}, Andrés Nascimento¹⁴, Shahriar Nafissi⁴², Shirin Omid⁴², Carlos Ortez¹⁴, Stéphanie Paquay⁵¹, Yann Pereon⁴⁵, Stojan Perić⁵², Valentina Ponzalino⁵⁰, Vidosava Rakočević Stojanović⁵², Gauthier Remiche⁵³, Aida Rodríguez Sainz⁴⁹, Sabine Rudnik⁵⁴, Iciar Sanchez Albisua⁵⁵, Manuela Santos⁵⁶, Ulrike Schara³⁸, Andriy Shatillo⁵⁷, Jadranka Sertić⁵⁸, Ulrich Stephani⁵⁹, Sonja Strang-Karlsson^{60,61}, Yves Sznajer⁶², Ani Tanev¹², Ivailo Tournev^{12,63}, Peter Van den Bergh⁵¹, Vinciane Van Parijs⁵¹, Juan Vilchez^{19,47,48}, Katharina Vill⁶⁴, John Vissing⁶⁵, Carina Wallgren-Pettersson^{60,66}, Julia Wanschitz⁴⁴, Tracey Willis⁴⁰, Nanna Witting⁶⁵ and Miren Zulaica^{25,26}

⁴Queen's Medical Centre, Nottingham University Hospitals NHS Trust, Nottingham, UK; ⁵Neuromuscular Disorders Unit, Neurology Department, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain; ⁶Neuromuscular Reference Centre, Department of Neurology, Antwerp University Hospital, Antwerpen, Belgium; ⁷Laboratory of Neuromuscular Pathology, Institute Born-Bunge, University of Antwerp, Antwerpen, Belgium; ⁸Clinical Medical Centre Zagreb, University of Zagreb, Zagreb Medical School, Zagreb, Republic of Croatia; ⁹Colentina University Hospital-CDPC-CCC, Bucharest, Romania; ¹⁰University of Medicine and Pharmacy Carol Davila Bucharest, Faculty of Dental Medicine, Bucharest, Romania; ¹¹Department of Neuropediatrics, Faculty of Medicine, University of Freiburg, Freiburg, Germany; ¹²Department of Neurology, UMHAT, Alexandrovska Medical University, Sofia, Bulgaria; ¹³Department of Neurology, University Hospitals Leuven, and Laboratory for Muscle Diseases and Neuropathies, KU Leuven, Leuven, Belgium; ¹⁴Unitat de Patologia Neuromuscular, Servei de Neurologia, Hospital Sant Joan de Deu, ISCIII, CIBERER, Barcelona, Spain; ¹⁵Department of Pediatric Neurology, Neuromuscular Reference Center, Hôpital Erasme, Université Libre de Bruxelles, Brussels, Belgium; ¹⁶Department of Pediatric Neurology, Neuromuscular Reference Center, Hôpital Universitaire des Enfants Reine Fabiola, Université Libre de Bruxelles, Brussels, Belgium; ¹⁷Neuromuscular Reference Center, Universitair Ziekenhuis Gent (UZ Gent), Ghent, Belgium; ¹⁸Neuromuscular Reference Center, Hôpital Universitaire des Enfants Reine Fabiola (HUDERF), ULB, Brussels, Belgium; ¹⁹Centro de Investigación Biomédica en Red en Enfermedades Raras (CIBERER), Madrid, Spain; ²⁰Instituto de Investigación Hospital 12 de Octubre Centro de Actividades Ambulatorias, Madrid, Spain; ²¹West of Scotland Centre for Genomic Medicine,

ARTICLE

Queen Elizabeth University Hospital, Glasgow, UK; ²²Department of Neurology, Istanbul University, Istanbul Faculty of Medicine, Istanbul, Turkey; ²³Neuromuscular Unit, Faculty of Medicine, Ain Shams University, Cairo, Egypt; ²⁴Institute of Neurological Sciences, Queen Elizabeth University Hospital, Glasgow, UK; ²⁵Neuroscience Area, Biodonostia Research Institute, San Sebastián, Gipuzkoa, Spain; ²⁶Department of Neurology, Hospital Universitario Donostia, OSAKIDETZA, San Sebastián, Gipuzkoa, Spain; ²⁷CIBERNED, Instituto de Salud Carlos III, Ministry of Science, Innovation and Universities, Madrid, Spain; ²⁸Genetics Department, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain; ²⁹Department of Neurology, 2nd Faculty of Medicine, Charles University, Prague, Czech Republic; ³⁰Department of Neuropediatrics, Medical Faculty Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany; ³¹Department of Child Neurology, Justus-Liebig-University Giessen, Gießen, Germany; ³²Department of Pathology and Cytology, University Hospital Center Zagreb, School of Medicine Zagreb, Zagreb, Republic of Croatia; ³³Department of Neurology, Complejo Hospitalario de Navarra, IdisNa, Pamplona, Spain; ³⁴Neuromuscular Consult Unit, Hospital Universitario Basurto, Bilbao, Spain; ³⁵Riga East University Hospital, Rare Disease Unit, Riga, Latvia; ³⁶Department of Neuropediatrics, University Childrens Hospital Basel, Basel, Switzerland; ³⁷Department of Neuropediatrics Inselspital Bern, Bern, Switzerland; ³⁸Department of Neuropediatrics, Centre for Neuromuscular Disorders in Children and Adolescents, Children's Hospital University Clinic Essen, University of Duisburg, Essen, Germany; ³⁹Department of Neurology, Medical University of Warsaw, Warsaw, Poland; ⁴⁰The Muscle Team, RJA Orthopaedic Hospital NHS Foundation Trust, Oswestry, UK; ⁴¹Department Pediatric Neurology, University of Turku and Turku University Central Hospital, Turku, Finland; ⁴²Department of Neurology, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran; ⁴³Department of Neurosciences, University of the Basque Country UPV-EHU, San Sebastián, Spain; ⁴⁴Department of Neurology, Medical University of Innsbruck, Innsbruck, Austria; ⁴⁵Centre de Référence Maladies Neuromusculaires AOC, Hôtel-Dieu, University Hospital, Nantes, France; ⁴⁶Department of Paediatric Neurology, Bristol Children's Hospital, Bristol, UK; ⁴⁷Neuromuscular Reference Centre, Neurology Department, Hospital Universitari i Politècnic La Fe, Valencia, Spain; ⁴⁸Neuromuscular Disorders Unit, Instituto de Investigación Sanitaria La Fe, Valencia, Spain; ⁴⁹Departamento de Neurología, Hospital Galdakao, Galdakao, Spain; ⁵⁰Neuromuscular Unit, Department of Neurosciences, University of Turin, Turin, Italy; ⁵¹Cliniques Universitaires St-Luc, Centre de Référence Neuromusculaire, Université de Louvain, Brussels, Belgium; ⁵²Faculty of Medicine, University of Belgrade, Neurology Clinic, Clinical Center of Serbia, Belgrade, Serbia; ⁵³Centre de Référence Neuromusculaire Erasme-HUDERF, Department of Neurology, CUB Hôpital Erasme, Université Libre de Bruxelles, Brussels, Belgium; ⁵⁴Institute of Human Genetics, Medical University Innsbruck, Innsbruck, Austria; ⁵⁵Department of Child Neurology, Children's Hospital, University of Tübingen, Tübingen, Germany; ⁵⁶Centro Hospitalar Universitário Porto, Porto, Portugal; ⁵⁷Institute of Neurology, Psychiatry and Narcology of NAMS of Ukraine, Kharkiv, Ukraine; ⁵⁸Department of Laboratory Diagnostics, Clinical Medical Centre Zagreb, University of Zagreb, Zagreb Medical School, Zagreb, Republic of Croatia; ⁵⁹Klinik für Neuropädiatrie, University Medical Center Schleswig-Holstein, Kiel, Germany; ⁶⁰Folkhaelsan Department of Medical Genetics, Helsinki, Finland; ⁶¹Department of Clinical Genetics, HUSLAB, University of Helsinki and Helsinki University Hospital, Helsinki, Finland; ⁶²Cliniques Universitaires St-Luc, Centre de Génétique Humaine, Université de Louvain, Brussels, Belgium; ⁶³Department of Cognitive Science and Psychology, New Bulgarian University, Sofia, Bulgaria; ⁶⁴Dr. v. Hauner Children's Hospital, Department of Pediatric Neurology and Developmental Medicine, LMU—University of Munich, Munich, Germany; ⁶⁵Copenhagen Neuromuscular Center Rigshospitalet, University of Copenhagen, Copenhagen, Denmark; ⁶⁶Department of Medical Genetics, University of Helsinki, Helsinki, Finland; ⁷³Present address: Department of Neurology, Royal Victoria Infirmary, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne, UK.