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RESEARCH ARTICLE

Genetic landscape and novel disease mechanisms from a large LGMD cohort of 4656 patients

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Abstract

Objective: Limb-girdle muscular dystrophies (LGMDs), one of the most heterogeneous neuromuscular disorders (NMDs), involves predominantly proximal-muscle weakness with >30 genes associated with different subtypes. The clinical-genetic overlap among subtypes and with other NMDs complicate disease-subtype identification lengthening diagnostic process, increases overall costs hindering treatment/clinical-trial recruitment. Currently seven LGMD clinical trials are active but still no gene-therapy-related treatment is available. Till-date no nation-wide large-scale LGMD sequencing program was performed. Our objectives were to understand LGMD genetic basis, different subtypes' relative prevalence across US and investigate underlying disease mechanisms. **Methods**: A total of 4656 patients with clinically suspected-LGMD across US were recruited to conduct next-generation sequencing (NGS)-based gene-panel testing during June-2015 to June-2017 in CLIA-CAP-certified Emory-Genetics-Laboratory. Thirty-five LGMD-subtypes-associated or LGMD-like other NMD-associated genes were investigated. Main outcomes were diagnostic yield, gene-variant spectrum, and LGMD subtypes' prevalence in a large US LGMD-suspected population. **Results**: Molecular diagnosis was established in 27% (1259 cases; 95% CI, 26–29%) of the patients with major contributing genes to LGMD phenotypes being: *CAPN3*(17%), *DYSF*(16%), *FKRP*(9%) and *ANO5*(7%). We observed an increased prevalence of genetically confirmed late-onset Pompe disease, *DNAJB6*-associated LGMD subtype1E and *CAPN3*-associated autosomal-dominant LGMDs. Interestingly, we identified a high prevalence of patients with pathogenic variants in more than one LGMD gene suggesting possible synergistic heterozygosity/digenic/multigenic contribution to disease presentation/progression that needs consideration as a part of diagnostic modality. **Interpretation**: Overall, this study has improved our understanding of the relative prevalence of different LGMD subtypes, their respective genetic etiology, and the changing paradigm of their inheritance modes and novel mechanisms that will allow for improved timely treatment, management, and enrolment of molecularly diagnosed individuals in clinical trials.

Introduction

Limb-girdle muscular dystrophies (LGMD) are a group of heterogeneous genetic disorders involving proximal muscle weakness with autosomal-recessive or-dominant inheritance. LGMD have more than 30 different subtypes linked to specific gene loci, which manifest in very overlapping and heterogeneous phenotypes.[1–3] This is likely the result of a tight link between associated muscle proteins within the sarcomere-sarcolemma-sarcoplasm-extracellular-matrix network (Fig. S1). The dominant forms (LGMD1A to LGMD1G) are less common than the recessive forms, representing fewer than 10% of LGMD cases.[1,4] Defects in a wide variety of muscle-related genes, including ones associated with severe congenital muscular dystrophy, are responsible for the 23 recessive forms (LGMD2A to LGMD2W).[1,4–6] Even though each of the individual LGMD subtypes is relatively rare, they are estimated to affect 1 in 14,500 to 1 in 123,000 individuals causing them to collectively affect many people (60,000 to 500,000 individuals) worldwide and is one of the most common muscular dystrophies.[3]

The substantial overlap and variability among each LGMD subtype in the age of onset, severity, and affected muscle groups, and the fact that cost of gene testing was previously unaffordable for patients, make definitive diagnosis highly elusive.[7] The lack of establishing a genotype prior to targeted therapeutic approaches obstructs recruitment of LGMD patients to available clinical trials.[8–11] Only five of the 12 LGMD trials are LGMD-subtype-specific.[8] Recently, the FDA approved Phases 1b and 2 of clinical trial NCT02579239 for (Resolaris™) an orphan drug developed by aTyR Pharma for treatment of LGMD2B based on targeting the immune component of the disease subtype. No global or other subtype-specific approved therapy is available yet. In this genomic era, establishment of such therapies is greatly facilitated by a comprehensive diagnostic approach including clinical-pathological evaluation, and cost-effective parallel testing of several genes using next-generation sequencing (NGS) so that faster diagnosis can be achieved to help patients to avail such personalized therapies, management, or to be recruited for specific clinical trials.

Several studies reported the use of exomes or gene panels in small LGMD cohorts elsewhere.[12–16] We previously showed that for NMDs such as LGMD, comprehensive gene-panel testing has a higher clinical yield (46%) than single-gene testing (15%) or has about 18% higher detection rate of causative pathogenic variants than exome sequencing.[17]

Previously, multiple targeted disease-specific panel NGS efforts including that of LGMD for molecular diagnostics were performed.[12,18–24] But to our knowledge, this study uniquely surpasses them by recruiting a very large number (4656) of patients clinically suspected of a specific disorder (LGMDs) in the USA irrespective of ethnicities. We aimed to provide complete molecular diagnosis to this large group of clinically characterized LGMD patients. We also aimed to understand the respective prevalence, the gene-variant spectrum of LGMD subtypes across the USA, and novel disease mechanisms leading to unusual clinical presentation and progression of LGMD that can broaden understanding of disease inheritance and apply to other heterogeneous inherited diseases.

Methods

Patient enrollment

The Muscular Dystrophy Association (MDA) and Jain Foundation jointly launched large-scale LGMD molecular diagnostic testing through Emory Genetics Laboratory (EGL). The study included 4656 individuals not reported previously who underwent molecular testing from June 2015 to June 2017. The study protocols have received prior approval by the Emory University Institutional Review Board, and that informed consent was obtained from each subject for genetic testing using the targeted LGMD gene-panel NGS and for collecting and using their clinical data in this study.

The inclusion criteria for this study were clinical suspicion of LGMD based on clinical data, questionnaire answers, and prior imaging studies on muscle biopsies by respective physicians and clinics, and no prior confirmed molecular testing of disease. Since most of the 4656 patients are adults, in most cases parental sample was not available for segregation analysis, and hence this study is mostly proband related, albeit some segregation studies on some families was performed. For the Jain Foundation program patients clinically suspected of having LGMD were enrolled in the study with informed consent through an institutional review board protocol (IRB00075815) approved by Emory University with the help of Jain Foundation. The Jain Foundation developed the "Automated limb-girdle muscular dystrophy diagnostic assistant" tool, popularly referred to as ALDA (https://jain-foundation.org/alda/content/login-tool) (http://www.jain-foundation.org/lgmd-subtyping-diagnosis-tool) where physicians fill up their respective patient clinical parameters which is helpful in clinical prediction of the LGMD subtype. ALDA includes prediction of all currently identified LGMD subtypes as well as other muscular dystrophies having similar clinical presentations.[25] The ALDA tool results report out two main parameters, probability and concordance. Based on the provided patient clinical information by the physician, the probability percentage indicates up to the top three most likely clinical diagnoses

for each patient. The concordance score represents how well the patient phenotype corresponds to the predicted LGMD subtypes, based on the available information in the published scientific literature. A subset of the patients with muscle phenotypes were initially screened using the online ALDA tool questionnaires and all positively screened LGMD patients by respective physicians were enrolled in the current large scale study. Additionally, patients, only in consultation with their respective clinics and physicians, could enter the study by answering the sequencing program website (http://lgmd-diagnosis.org/) (https://www.jain-foundation.org/patient-physician-resources/free-genetic-sequencing) questions that were stored as part of clinical data (See Questionnaires in Supporting Information Methods). For the MDA program, neurologists from different MDA clinics across US also referred LGMD-suspected patients for this large scale molecular testing program and also used the ALDA tool for providing us quantifiable clinical data.

During recruitment, ethnicity of patients was not one of the questions that we asked in our intake questionnaire. Although, in ALDA, we ask whether the patient was Finnish, Other Northern European, Japanese, other not listed, or unknown (see questionnaires in Supporting Information Methods). The reason for these specific choices is because there are specific types of LGMDs that are more common in these ethnicities so that is important to take these into account when predicting the possible type of LGMDs.

For details of the patient questionnaires for recruitment to the sequencing program, see (Supporting Information Methods).

Targeted gene panel testing

Clinical NGS of targeted gene-panel was performed at the Emory Genetics Laboratory (EGL), a facility certified by the Clinical Laboratory Improvement Amendments and College of American Pathologists (CLIA-CAP) as previously described.[17] Genomic DNA was isolated from peripheral blood samples or saliva samples for each patient referred for molecular testing. The list of potential LGMD genes to screen is too large for a single gene testing approach, but is very well suited for a targeted NGS panel. Genes selected for this panel were based on their evidence for disease causality, their relative contribution to disease as described in the literature[26] and relative cost analysis. A total of 35 genes related to specific LGMD subtypes or other muscular dystrophies with clinical overlap with LGMD features were included in this NGS panel (Table S2). Targeted NGS was performed as described by us previously[17] with 100% coverage for each gene in the panel using Agilent SureSelect target enrichment. Patient genomic DNA was sheared to a 200 bp average, followed by end-repair, A-tailing, adapter ligation, and library amplification. Samples were then hybridized with the capture library. The probes were biotin tagged to allow for capture by streptavidin beads following hybridization. Prepared libraries were then indexed and amplified. Direct sequencing of the amplified captured regions was performed using next generation short base pair read sequencing. Although this assay was not designed to detect intragenic deletions and duplications, breakpoint bait was included in NGS library for common adult-onset Pompe deletion of exon 18 and two intronic dysferlin pathogenic variants.

NGS data analysis and variant interpretation

We analyzed targeted NGS variant data, and identified variants classified according to board standards and guidelines[27,28] (http://www.egl-eurofins.com/emvclass/emvclass.php). Alignment to the human reference genome (hg19) and variant calling was performed using NextGENe®. For all 35 genes included in the panel (Table S2), the analyzed region includes the coding exons and 10 bp of flanking intronic region on both sides of each exon. Certain known deep intronic variants were also targeted. We analyzed targeted NGS variant data carefully considering other important parameters such as available clinical phenotype information, family history and sequence result (if available) and previous undiagnosed diagnostic genetic test results. Identified variants were classified according to standards and guidelines of the American College of Medical Genetics and Genomics and classification were made available[27,28] (http://www.egl-eurofins.com/emvclass/emvclass.php). Diagnostic yield was calculated based on definitive diagnosis of patients harboring pathogenic variants in autosome-recessive, -dominant, and X-linked genes and patients with one pathogenic and one variant of uncertain significance (VUS) in the same LGMD gene. Prevalence of each identified LGMD subtype identified by our clinical NGS panel diagnostic program was established and compared. Major genes contributing to LGMD genes were identified from the patients with definitive molecular diagnosis having pathogenic variants in autosome-recessive, -dominant, and X-linked genes. Homozygosity was compared for different subtypes of LGMD and relatively common variants were identified in different LGMD-associated genes.

Peripheral blood mononuclear cells (PBMC) assay for Dysferlin (DYSF) protein estimate

Cases suspected of dysferlinopathy, as in LGMD2B or Miyoshi myopathy with *DYSF* (OMIM# 603009) variant (s), were analyzed for DYSF protein expression using our established blood PBMC assay.[25,29,30]

Results

Definitive molecular diagnosis and demographics

Seventy percent of the patients enrolled in the study were over 18 years of age, and 30 percent were less than 18 years with patient mean age during participation was about 40 years equally distributed among the two genders (male: 50.6%, female: 49.4%). About half of the patients (2359/4656; 50.66%) were recruited through more than 150 MDA clinics across the US (https://www.mda.org/) and the remaining half (2297/4656; 49.33%) recruited through the Jain Foundation. Overall diagnostic yield was based on definitive diagnosis of 1003 patients harboring pathogenic variants in autosome-recessive, -dominant, and X-linked genes and 256 patients with one pathogenic and one VUS in the same recessive LGMD gene, totaling to 1259 diagnosed cases. Gene contribution percentages, that is, yield of each LGMD subtypes were based on the 1003 patients harboring pathogenic variants with definitive diagnosis. The diagnostic yield does not include the possible digenic/multigenic cases that harbor a single pathogenic variant in ≥2 genes. Critical analysis of this extensive molecular data confirmed molecular diagnosis for 27% (1259/4656 cases) of all the patients belonging to eleven different autosomal-recessive LGMD subtypes associated with *DYSF*(OMIM# 603009), *FKRP*(OMIM# 606596), *GAA*(OMIM# 606800), *SGCA*(OMIM# 600119), *SGCB*(OMIM# 600900), *SGCG*(OMIM# 608896), *TRIM32* (OMIM# 602290), *FKTN*(OMIM# 607440), *ANO5* (OMIM# 608662), *POMT2*(OMIM# 607439), and *ISPD* (OMIM# 614631). However, no cases with a definitive diagnosis were identified for the four autosomal-recessive LGMD subtypes associated with *POMT1*(OMIM# 607423), *SGCD*(OMIM# 601411), *DAG1*(OMIM# 128239), and *POMGNT1*(OMIM# 606822). Majority of the patients have pathogenic variants in one of the following genes: *CAPN3* (OMIM# 114240) 17% (175/1003 cases), *DYSF* (OMIM# 603009) 16% (167/1003 cases), *FKRP* (OMIM# 606596) 9% (87/1003 cases), and *ANO5* (OMIM# 608662) 7% (72/1003 cases); indicating that these genes are likely the major contributors to LGMD phenotypes in the US population (Fig. 1A and Fig. S2). Pathogenic variants were identified in dominant LGMD genes *DNAJB6*(OMIM# 611332), *MYOT*(OMIM# 604103), and *CAV3*(OMIM# 601253), but not in the *TNPO3*(OMIM# 610032) gene. Pathogenic variants also occurred in 10 genes with both autosomal-recessive and -dominant inheritance including *CAPN3*, *COL6A1*, *COL6A2*(OMIM# 120240), *COL6A3*(OMIM# 120240), *DES*(OMIM# 125660), *GNE*(OMIM# 603824), *LMNA*(OMIM# 150330), *TCAP* OMIM# 604488), and *TTN*(OMIM# 188840). In 24% (1125/4656 cases) of the patients, we found no reportable variants which are true negative group in our targeted LGMD gene-panel NGS. In the rest (49%; 2272/4656 cases) patients, we identified

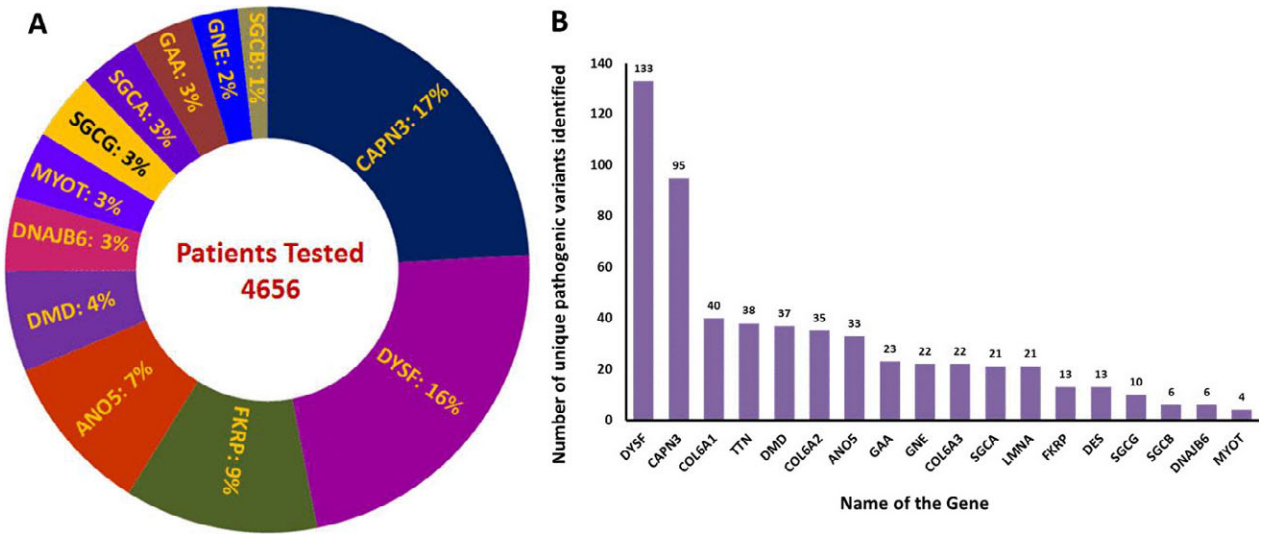

Figure 1. Major contributing LGMD genes. (A) Molecular diagnosis has been established in 27% of the patients. A majority of these patients had a pathogenic variant in one of the following genes *CAPN3* 17%(175/1003), *DYSF* 16%(167/1003), *FKRP* 9%(87/1003), and *ANO5* 7%(72/1003) indicating that these genes are likely the major contributors to LGMD phenotype. (B) Number of unique pathogenic variants identified. Numbers of identified pathogenic variants were compared among the major contributing LGMD genes to understand the allelic heterogeneity of these genes. *DYSF*, *CAPN3,* and *COL6A1* were identified with the most pathogenic variants including 133, 95 and 40, respectively, in each gene, indicating more allelic heterogeneity in these genes.

reportable variants but did not confirm molecular diagnosis such as the cases with one pathogenic variant in a recessive genes or cases with one or two VUSs in a gene, or cases with a single pathogenic variant or VUS in multiple genes. Seventy-two percent of identified reportable variants are classified as VUS, and 23% of the variants are classified as pathogenic variants (Fig. 2). Patients had at least one VUS either in *DYSF*(90 patients), *CAPN3*(44 patients), or in any of the three collagen genes (246 patients) (Table S7) without any reportable variant in other LGMD genes. Overall, 45% of patients were of "Northern European" origin except Finnish, 29% were "Other Not Listed", 20% were "Unknown", and the remaining 5% comprised of ethnic origins of Finnish, North Asia, South Asia, Hispanic, Native American, and African American. These data originated from self-reported responses by the patients on the testing form (see Supporting Information Methods).

Allelic heterogeneity of major LGMD contributing genes

We identified 133 unique pathogenic variants in the *DYSF* gene followed by 95 pathogenic variants in *CAPN3* and 40 pathogenic variants in *COL6A1* (OMIM# 120220) thus indicating a high allelic heterogeneity in these genes (Figs. 1B and 2). This large-scale study confirmed calpainopathy as the most common LGMD subtype (*CAPN3*-associated LGMD2A: 175/1003 cases; 17%) in the USA. Sixteen percent of patients (167/1003 cases) had a definitive diagnosis of LGMD2B by identifying two pathogenic variants in the *DYSF* gene indicating LGMD2B is the second major contributor among the LGMD subtypes (Fig. 1A). Splice site pathogenic variant c.2643 + 1G>A was identified in 20 LGMD2B patients making it as the most common *DYSF* pathogenic variant followed by c.5713C>T (p.R1905X) and c.5979dupA, as the second most common variants each found 12 patients.[31] Extensive homozygosity of variants was observed in the *DYSF* (10%), *FKRP* (7%), and *CAPN3* (6%) genes (Fig. 3).

Mode of inheritance in LGMD subtypes

In addition to being the most common LGMD subtype (*CAPN3*-associated LGMD2A: 17%), we report that 17 unrelated patients with a 21 bp in-frame deletion (c.643_663del21) in *CAPN3* gene who did not possess a second reportable variant (Table 2). This variant occurred in multiple different families with muscle disease, segregating with dominant inheritance.[32–34] A second 15 bp in-frame deletion (c.598_612del15) was also identified in different 16 unrelated patients. Most of these 16 patients had no identified second variant, indicating an increased prevalence of autosomal-dominant *CAPN3*-associated LGMD subtype (Table 2).

Prevalence of late-onset Pompe in large LGMD-suspected cohort

We identified 28 LGMD-suspected patients, all with two *GAA* pathogenic variants (Table 1). We also identified 10 patients with one pathogenic variant and one VUS in

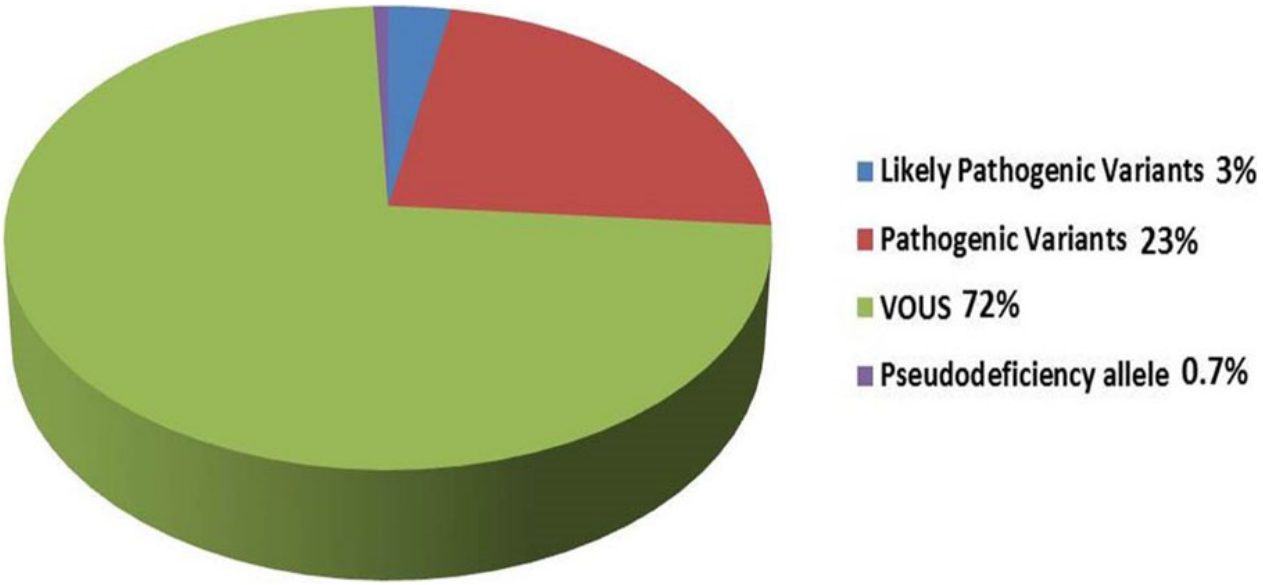

Figure 2. Types of variants identified in the tested LGMD patients. Variants were classified according to standards and guidelines of the American College of Medical Genetics and Genomics. Around 23% of the identified variants are pathogenic. Around 72% of the variants are interpreted as variants of uncertain significance (VUS) because majority of LGMD subtypes are poorly studied and currently limited knowledge available.

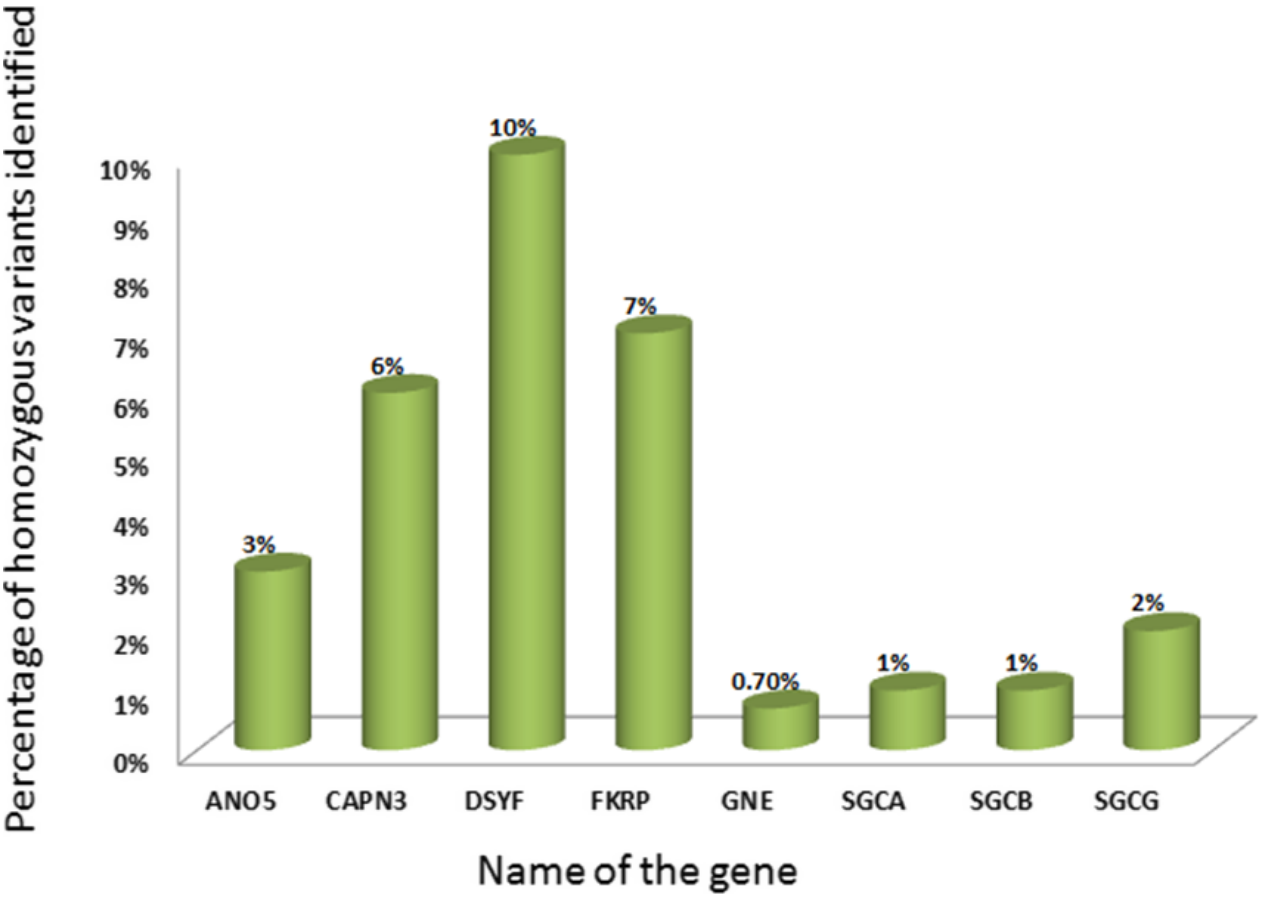

Figure 3. Homozygosity identified in major LGMD genes. Percentage of homozygous pathogenic variants identified in major LGMD genes.

GAA which is likely to be pathogenic given no other mutation in *GAA* or other genes and that the patients show clinical symptoms (Table S3), totaling to 38 identified late-onset Pompe cases (38/4656; 0.8% prevalence). It is interesting to note that none of the patients had homozygous variants in *GAA* (Table 1) with the c.-32-13T>G leaky splice variant identified in 31 of 38 LOPD patients in the compound-heterozygous state. The common exon-18 deletion (c.2481 + 102_2646 + 31del) was found in five patients along with the leaky-splice variant. The third common variant, c.525delT, was identified in five LOPD patients. Ninety-one patients had at least one pathogenic variant in *GAA* indicating further deletion, duplication, and enzymatic analysis are warranted in these individuals.

DNAJB6-associated LGMD subtype 1E molecular diagnosis and prevalence

DNAJB6 (OMIM# 611332) is an autosomal-dominant gene associated with limb-girdle muscular dystrophy 1E (LGMD1E). We identified 29 LGMD1E cases with pathogenic variants and 13 additional cases with novel missense variants in *DNAJB6* (OMIM# 611332) gene. *MYOT* (OMIM# 604103) was the next major contributor to the autosomal-dominant LGMD group with pathogenic variants identified in 30 patients.

Multigenic inheritance in muscular dystrophy

Instead of one locus, alterations in two genes are required for phenotypic expression in digenic inheritance as in retinitis pigmentosa (*ROM1* (OMIM# 180721) and *PRPH2* (OMIM# 179605)), as well as in different muscular dystrophies such as Emery-Dreifuss, limb-girdle, Ullrich congenital and facioscapulohumeral,[35–38] indicating more complex nature of disease presentation and progression in muscular disorders.[35–38] Our data identified pathogenic variants in more than one LGMD genes in at least 31 individuals suggesting potential digenic or multiple genes contribution to LGMD disease presentation and progression (Fig. 4 and Table S1). Majority of these patients showed atypical clinical presentation and in some

Table 1. Summary of *GAA* variants identified in late-onset Pompe patients. Identification of 28 patients with two *GAA* pathogenic variants indicates the increased prevalence of late-onset Pompe disease in this study. (AOP: Adult-onset Pompe).

Patient ID	Gender	Age	Gene	Variant 1	Variant 2
AOP1	Female	61	*GAA*	c.-32-13T>G	c.1124G>T (p.R375L)
AOP2	Female	79	*GAA*	c.-32-13T>G	c.2140delC
AOP3	Female	33	*GAA*	c.-32-13T>G	c.525delT
AOP4	Male	71	*GAA*	c.-32-13T>G	c.1912G>T (p.G638W)
AOP5	Unknown	54	*GAA*	c.-32-13T>G	c.2512C>T(p.Q838X)
AOP6	Male	66	*GAA*	c.-32-13T>G	c.2481 + 102_2646 + 31del (Exon 18 deletion)
AOP7	Male	70	*GAA*	c.-32-13T>G	c.2481 + 102_2646 + 31del (Exon 18 deletion)
AOP8	Female	44	*GAA*	c.-32-13T>G	c.2481 + 102_2646 + 31del (Exon 18 deletion)
AOP9	Male	18	*GAA*	c.-32-13T>G	c.2481 + 102_2646 + 31del (Exon 18 deletion)
AOP10	Male	40	*GAA*	c.-32-13T>G	c.2238G>A(p.W746X)
AOP11	Male	59	*GAA*	c.-32-13T>G	c.1655T>C(p.L552P)
AOP12	Male	70	*GAA*	c.736delC	c.546G>A(p.T183T)
AOP13	Female	53	*GAA*	c.-32-13T>G	c.1841C>A(p.T614K)
AOP14	Male	68	*GAA*	c.-32-13T>G	c.1143delC
AOP15	Female	40	*GAA*	c.853C>T (p.P285S)	c.2560C>T(p.R854X)
AOP16	Male	41	*GAA*	c.-32-13T>G	c.2560C>T(p.R854X)
AOP17	Male	44	*GAA*	c.-32-13T>G	c.655G>A(p.G219R)
AOP18	Male	70	*GAA*	c.-32-13T>G	c.1064T>C(p.L355P)
AOP19	Female	49	*GAA*	c.-32-13T>G	c.1655T>C(p.L552P)
AOP20	Female	56	*GAA*	c.-32-13T>G	c.525delT
AOP21	Female	36	*GAA*	c.-32-13T>G	c.1827delC
AOP22	Male	80	*GAA*	c.-32-13T>G	c.525delT
AOP23	Male	33	*GAA*	c.-32-13T>G	c.258dupC
AOP24	Female	46	*GAA*	c.-32-13T>G	c.766_785delTATATCACAGGCCTCGCCGAinsC
AOP25	Female	61	*GAA*	c.-32-13T>G	c.2481 + 102_2646 + 31del (Exon 18 deletion)
AOP26	Male	18	*GAA*	c.-32-13T>G	c.525delT
AOP27	Female	56	*GAA*	c.-32-13T>G	c.525delT
AOP28	Female	8	*GAA*	c.-32-13T>G	c.2242dupG

cases unusually rapid or very slow progression (Table S1). For example, a 32-year-old Caucasian male patient had initial symptoms in his late teens including onset of muscle weakness in arms and legs as well as elevated CK levels was diagnosed with LGMD, but unusual, rapid progression of proximal leg and girdle weakness occurred. Two homozygous variants in two muscle genes *ANO5* and *SGCA* (Fig. 4; Table S1) could be potentially contributing to the unusual presentation in this individual. A 16-year-old male clinically diagnosed with LGMD, but with an unexpected severe progression had heterozygous loss of *COL6A2* coupled with a homozygous loss of *ANO5* possibly contributing to the rapid disease progression and severe phenotype (Fig. 4; Table S1). In this family, the 40-year-old mother with heterozygous loss of both *ANO5* and *COL6A2* showed mild myopathy, whereas her 8-year-old daughter with the same genotype had no muscle weakness indicating variable presentation and the possibility of later-onset clinical features. There are multiple other patient cases identified with single pathogenic variant in one recessive gene and another single VUS in a different recessive gene with no other reportable variant.

Deep intronic variant in *DYSF* gene

Recently, we characterized a deep intronic mRNA splice-altering pathogenic variant c.4886 + 1249G>T in *DYSF* gene that inserts a novel 177 bp pseudoexon at the exon 44–45 junction. This splicing causes an in-frame insertion of 59 additional amino acids in the dysferlin protein.[39] This intronic variant segregated in a compound heterozygous state with the other pathogenic variant in two siblings (Fig. 5). Dysferlin protein expression levels in PBMC showed an evident pathogenic effect of the deep intronic splice variant in significantly reducing DYSF expression in the compound heterozygous states (Fig. 5).We targeted this deep intronic region in the current LGMD NGS panel and successfully identified it in one family. Overall we have been able to identify 45 different deep intronic variants including pathogenic or VUS across nine genes in our panel (*DMD*, *CAPN3*, *DNAJB6*,

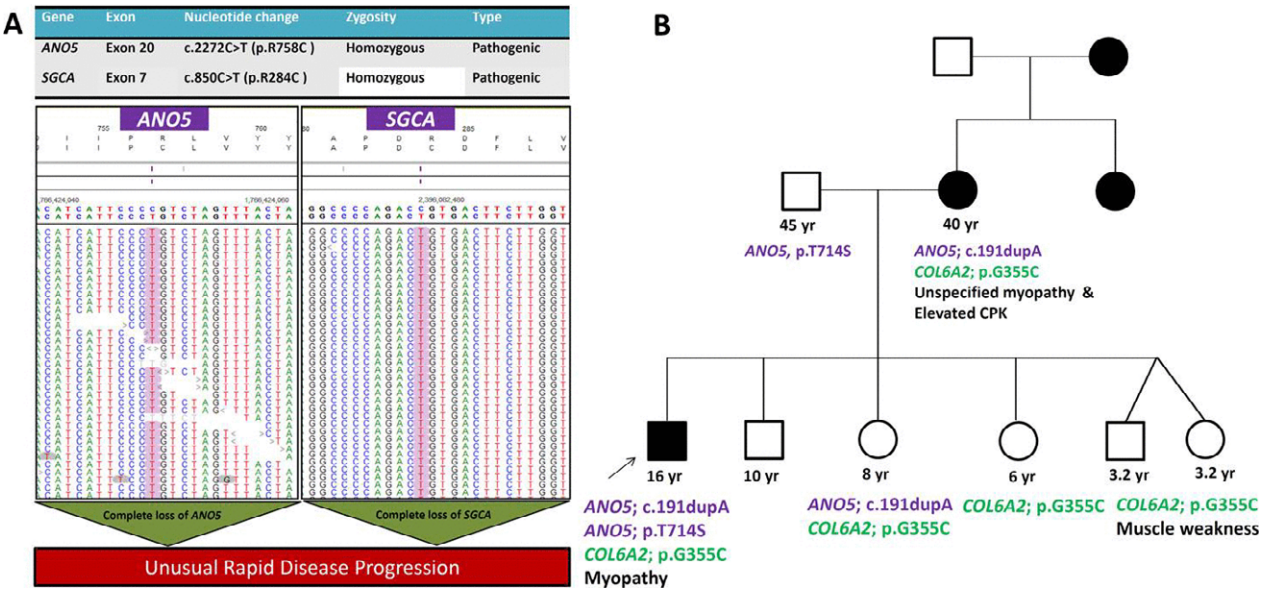

Figure 4. Multigenic inheritance in LGMD. Pathogenic variants identified in more than one LGMD genes in two patients with unusual disease presentation and progression indicating complex inheritance patterns of LGMD. (A) Patient with homozygous variants in both *ANO5* and *SGCA* genes. NGS reads indicated the identification of homozygous missense pathogenic variants c.2272C>T (p.R758C) and c.850C>T (R284C) in *ANO5* and *SGCA* genes, respectively. (B) Rapid disease progression was observed in a 16-year-old male (arrow) with two pathogenic variants in *ANO5* gene and one pathogenic variant in *COL6A2* gene indicating multiple gene contributions for an unusual presentation. His mother, a 40-year-old female with one pathogenic variant each in *ANO5* and *COL6A2* shows unspecified myopathy with elevated creatine phosphokinase (CPK).

GAA, *DYSF*, *FKTN*, *COL6A1*, *COL6A2*, *COL6A3*) in all different unrelated patients in our cohort (additionally see Tables S3–S7).

Collagen family genes

Collagen family genes *COL6A1/A2/A3* are associated with both autosomal-dominant and -recessive forms of muscular dystrophies. Variants located in the Gly-X-Y domain-encoding region of these genes are associated with dominant forms of myopathy. Here we report 55 patients with pathogenic variants in the Gly-X-Y domain of these three genes; *COL6A1* is the major contributor with 35 patients. Eleven patients harbored two pathogenic variants in one of these genes, with eight patients having two pathogenic variants in *COL6A2* indicating a role in recessive myopathy. Seventy-nine patients were identified with one pathogenic variant in any of these three genes (Table S5).

Discussion

The severity of the different LGMD subtypes varies with most of the dominant forms having later-onset ages and milder-clinical presentations than the recessive subtypes.[40] A large number of affected patients remain without a definitive molecular diagnosis regardless of >30 LGMD genes are known, because of the current challenges of interpreting variants such as deep-intronic or regulatory variants in known genes, or novel yet-to-be implicated genes. Most of the LGMD subtypes are genetically poorly studied, and limited knowledge is available regarding the mutation spectrum of different LGMD genes. This clinical-diagnostic program provided a valuable opportunity to gain a deeper understanding of rare LGMD subtypes by studying 4656 patients throughout the USA. Moreover, since majority of these patients had the adult-onset disease, our study adds to the knowledge base for use in newborn-genetic-screening programs that will enable earlier intervention if available. Major contributors to LGMD phenotypes were found to be *CAPN3*(17%), *DYSF*(16%), *FKRP*(9%), and *ANO5*(7%) (Fig. 1A). No reportable variants were identified in 24% patients indicating a possible role of deep-intronic/regulatory-variants/novel genes.

Our LGMD panel diagnostic yield (27%) is smaller than that of our previous NMD panel (46%) since previously yield was for all NMDs, not just for LGMD, and the cohort was much smaller (81 patients).[17] Identification of pathogenic variants in dominant LGMD genes *DNAJB6*, *MYOT*, and *CAV3*, but not in the *TNPO3* gene indicates a higher prevalence of dominant LGMD-subtypes-1E,1A,1C, respectively, and the rarity of LGMD-1F. The 27% diagnostic yield included the 256 patients with one pathogenic and one VUS in the same LGMD gene since it is most likely these VUSs are likely pathogenic as

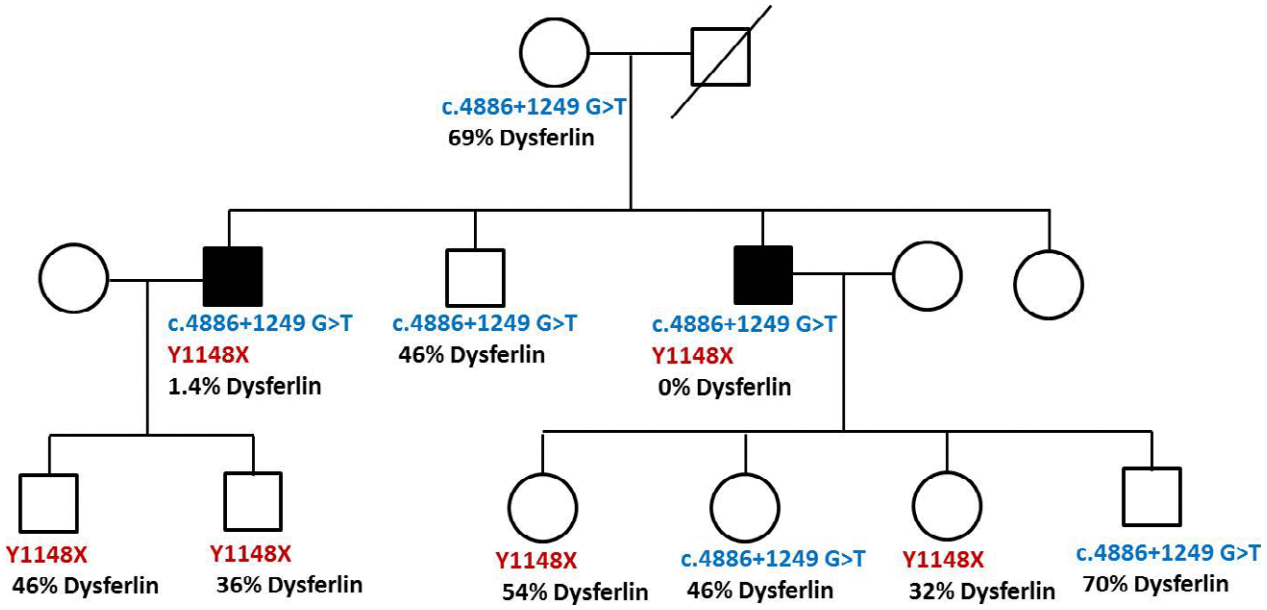

Figure 5. Segregation analysis of a deep intronic *DYSF* variant. Variant c.4886 + 1249G>T in *DYSF* gene was identified in a large family with LGMD2B. A deep intronic variant in *DYSF* gene alters mRNA splicing and ultimately results in inframe insertion of a new pseudoexon in dysferlin. Percentages indicate levels of expression of dysferlin protein in peripheral blood mononuclear cells (PBMCs) compared to the control.

the patients show clinical symptoms and no other variant was identified in the same or any other gene. The diagnostic yield of our study is slightly higher than some previous exome sequencing studies in neurological disorders, even with a substantial patient cohort recruited based on specific disease phenotypes.[41,42] This suggests the importance of using screening criteria of disease-specific clinical information carefully to judge the type of NGS-diagnostic testing for patient recruitment (fig. 3 in 43) and justifying subsequent advantage of targeted NGS panel over exome sequencing.

Due to overlapping phenotypes and complex presentations, it is difficult for clinicians to provide an accurate clinical diagnosis especially for Duchenne/Becker muscular dystrophy (DMD/BMD) patients with milder presentations, which often mimics limb-girdle phenotypes. Unlike some well-known LGMD gene panels from popular commercial diagnostic clinics, we included common X-linked muscular dystrophy genes *DMD*(OMIM# 300377), *EMD*(OMIM# 300384), *FHL1*(OMIM# 300163) in the NGS LGMD-panel, and 43 patients were identified with pathogenic variants in *DMD*. Also, the complete lack of coverage for consecutive exons in the *DMD* gene was also observed in 10 patients indicating possible pathogenic deletions. These pathogentic deletions stress the importance of comprehensive clinical evaluation for DMD/BMD patients with a milder-presentation before referring to NGS-panel testing for other muscular dystrophies.[43]

Dysferlinopathies (LGMD2B and Miyoshi myopathy) include autosomal-recessive muscle diseases caused by pathogenic variants in dysferlin gene (*DYSF*), characterized by proximal muscle weakness, difficulty in running, climbing, increased fatigue.[44,45] *DYSF*, *CAPN3*, and *COL6A1* have the highest number of pathogenic variants indicating more allelic heterogeneity in these genes compared to others in the panel (Fig. 1B). Comparing Figure 1A and B, it is seen that *FKRP*, the third most contributing gene (Fig. 1A), has low allelic heterogeneity (lesser number of unique pathogenic variants), whereas *TTN* harbors high number of unique pathogenic variants (higher allelic heterogeneity) (Fig. 1B) even though being one of the least-contributing genes. This indicates that even greater number of unique pathogenic variants such as deep-intronic/deletion/duplications could potentially be identified in *TTN* with confirmed LGMD2J diagnosis, if we use whole genome sequencing instead of panel testing. These indicate therefore, LGMD overall might still remain clinically under diagnosed. Moreover, extensive homozygosity of variants in *DYSF*, *FKRP*, and *CAPN3* genes highlights the immediate need for carrier testing for these LGMD subtypes (Fig. 3). The 79 patients identified with one pathogenic variant in any of the three collagen genes (Table S5) may not be of diagnostic value since *COL6A1/A2/A3* variants can be either recessive/dominant, depending on the individual's family history. Hence further functional studies using target muscle biopsy tissue, segregation analysis and nature history studies are needed

for those patients with one pathogenic variant in a collagen gene.

Late-onset Pompe disease (LOPD) is a rare, but potentially treatable lysosomal storage disorder characterized by progressive glycogen accumulation and muscle weakness, often with a limb-girdle pattern of weakness. It is essential that such patients should not be overlooked. Increased prevalence (2.4–3.63%) in LOPD was observed previously in LGMD-suspected or hyperCKemia patients using targeted sequencing approach.[46–48] But these studies are different with regard to much lower number of patient cohort that unlike our study also included non-LGMD other NMD patients such as other congenital myopathies, distal myopathies, and isolated hyperCKemia in European population. Our results of prevalence of LOPD through targeted LGMD gene-panel testing in a very large strictly clinically LGMD-characterized or -suspected patients across US with multiple ethnicities indicate that LOPD should not be overlooked when considering potential diagnoses for LGMD patients even if clinical subtyping can be performed, that in turn will assist in ERT or recruitment for other clinical trials.

This large-scale study confirmed that LGMD2A (calpainopathy), characterized by severe-to-mild, symmetric, progressive weakness of proximal limb-girdle muscles with 2–40 years onset-age, as the most common LGMD subtype with the higher prevalence of dominant LGMD forms. LGMD2A was considered a strictly autosomal-recessive-LGMD subtype for many years, but recently patients carrying a single pathogenic variant in the *CAPN3* have been reported. This variant, a 21 bp in-frame deletion c.643_663del21, cosegregates in 37 individuals from 10 families with muscle disease and dominant-transmission through several generations. Moreover, it is associated with severe loss of calpain3 expression shown by immunoblotting, suggesting a dominant-negative effect on calpain3 homodimer.[33] In our large-scale study, the identification of the same 21 bp in-frame deletion in 17 individuals (Table 2) without any reportable second variant further confirms the c.643_663del21 as a common, dominant mutation, but further deletion-duplication analysis did not occur. Even *CAPN3* inheritance is considered only autosomal recessive in OMIM (https://www.omim.org/entry/114240). Hence it is important to emphasize that through our very large sequencing program, we now know that dominant inheritance of *CAPN3* is far more prevalent than previously thought, and should be widely considered in molecular diagnostic pipeline and NGS data analysis and interpretation in the clinics. Our finding of a similar 15 bp in-frame deletion c.598_612del15 in 16 patients with ages ranging from 48 to 76 years (Table 2) without a second pathogenic variant indicate a possible autosomal-dominant form of calpainopathy. The same 15 bp in-frame deletion c.598_612del15 as a single *CAPN3* variant was previously described in one patient, but dominant inheritance was not suggested.[49,50] Further studies are required to confirm the dominant subtype associated with this *CAPN3* variant.

Another interesting dominant LGMD subtype identified is the poorly studied *DNAJB6*-associated-LGMD1E, which is a mostly adult-onset, slowly progressive proximal muscle-weakness. Nevertheless, some early onset cases were reported.[51,52] *DNAJB6* encodes a protein that belongs to DNAJ family, a class of molecular chaperones, involved in a wide range of cellular events.[53,54] The mutation spectrum for *DNAJB6* is limited to 11 missense variants and one

Table 2. *CAPN3* patients with in-frame deletions associated with autosomal-dominant subtype.

Patient ID	Gender	Age (years)	*CAPN3* variant 1	*CAPN3* variant 2
C100	Male	24	c.643_663del21	—
C200	Male	72	c.643_663del21	—
C300	Male	44	c.643_663del21	—
C400	Female	70	c.643_663del21	—
C500	Male	58	c.643_663del21	c.584A>C (p.N195T), VUS
C600	Female	73	c.643_663del21	—
C700	Male	57	c.643_663del21	—
C800	Male	60	c.643_663del21	—
C900	Female	61	c.643_663del21	—
C111	Male	69	c.643_663del21	—
C222	Female	17	c.643_663del21	—
C333	Male	57	c.643_663del21	c.640G>A (p.G214S), VUS
C444	Female	34	c.643_663del21	—
C555	Male	45	c.643_663del21	—
C666	Female	13	c.643_663del21	—
C777	Female	78	c.643_663del21	—
C888	Female	73	c.643_663del21	—
D001	Male	59	c.598_612del15	—
D002	Female	57	c.598_612del15	—
D003	Female	48	c.598_612del15	—
D004	Male	69	c.598_612del15	—
D005	Female	59	c.598_612del15	—
D006	Male	57	c.598_612del15	c.794C>T (p.S265F), VUS
D007	Female	58	c.598_612del15	c.1477C>T (p.R493W), VUS
D008	Female	54	c.598_612del15	—
D009	Female	57	c.598_612del15	—
D010	Male	76	c.598_612del15	—
D011	Female	53	c.598_612del15	c.1505T>C (p.I502T), VUS
D012	Female	54	c.598_612del15	—
D013	Male	39	c.598_612del15	—
D014	Female	59	c.598_612del15	—
D015	Female	69	c.598_612del15	—
D016	Male	66	c.598_612del15	—

VUS, Variant of uncertain significance.

splice variant (Table S6) with most of them located in the mutational-hotspot region of exon-5 in the G/F-rich linker domain.[54] Only one reported variant c.525C>G(p.F175L) is located in exon-7 and no nonsense/frameshift variants have been reported. We report 29 patients with pathogenic missense variants in the exon-5 hotspot; but also identified 13 patients with novel missense variants outside of exon-5 (exons 4, 6, 7, 8, 9, 10) of *DNAJB6* (Table S4) with no other reportable variants in an LGMD gene. These data indicate a broad mutation spectrum for *DNAJB6* and a need for natural-history studies to understand the penetrance and nature of disease association. These 13 missense variants are further classified as VUS due to lack of published studies on LGMD1E and signifies the need for functional validation for reclassification of the VUSs and natural history studies on these patients to determine nature of variant contribution to disease.

Our targeted NGS panel data identified at least 31 individuals with pathogenic variants in more than one LGMD gene suggesting potential digenic/multiple gene contributions to LGMD disease presentation/progression (Fig. 4; Table S1). The explicit possible association of multigenic pathogenicity to unusual disease presentation with rapid/very slow progression seen in all 31 cases (Table S1) in our cohort including examples given in above (Results), suggests a potential high prevalence of gene-gene epistatic interaction and synergistic heterozygosity[55] in LGMD. Albeit, further functional and segregation studies are needed to validate the complex inheritance patterns of LGMD subtypes.[55] Moreover, we expect whole genome sequencing is needed on these patients to confirm if there are any other reportable pathogenic variant that could definitively diagnose these patients, before performing functional and segregation studies on them to understand multigenic contribution.

The findings that 72% identified reportable variants are classified as VUSs, and 23% of the variants are classified as pathogenic variants indicate the importance of further functional studies including RNA sequencing, enzyme assays or mass spectrometry to prove pathogenicity of identified VUSs.[56,57] This will further aid in resolving the diagnosis of patients with at least one VUS either in *DYSF* or *CAPN3* or any of the three collagen genes (Table S7).

Overall, the current large-scale LGMD sequencing project has improved our knowledge of the gene-variant spectrum, mutational hotspots, genetic etiologies, and relative prevalence of different LGMD subtypes across the US using a large clinically characterized patient cohort, allowing timely management, participation of definitively diagnosed individuals in ongoing clinical trials through disease-specific registries. This large US-cohort not previously described for LGMD has also showed evidence for a new mechanism which has been previously described in handful of cases in the literature. We identified the higher prevalence of dominant forms of LGMD, *CAPN3,* and *DNAJB6* in particular and novel digenic/multigenic (more than 2) possible contribution to unusual LGMD clinical presentations/progressions. Adult-onset Pompe patients, also identified in our LGMD-suspected cohort, now have the opportunity of seeking enzyme replacement therapy. The slightly higher diagnostic yield of this study compared to our previous exome studies on NMD patients and exomes of neurological disorders in general as mentioned before, as well as the identification of 24% negative cases suggests yet-to-be discovered genes or genetic events associated with LGMD-like clinical symptoms. To resolve these cases, we predict the need for clinical genome sequencing (GS) to capture the full genetic variation spectrum as seen in recent trends in large clinical diagnostics studies as a first-tier approach.[58] We expect a shift from panel sequencing to clinical-GS with better, uniform coverage therefore leading fewer exon drop-out, to detect deep-intronic and copy-number variants, and ability to interrogate periodically without resequencing.

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Author Contributions

MH had full access to all of the data in the study and had responsibility for the integrity and accuracy of the data and its analysis. MH, SC, BRRN, LR, PM, MW, and MH designed the study concept. All authors contributed to the acquisition, analysis, or interpretation of data. BRRN, SC, and MH drafted the manuscript. All authors contributed to the critical revision of the manuscript for important scientific or intellectual content. BRRN and SC performed statistical analysis. MH obtained funding for this study. MH provided administrative support and supervised the overall study. The corresponding author MH had final responsibility for the decision to submit for publication.

Conflict of Interest

Nothing to report.

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Supporting Information

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

Methods. Questionnaires for patient recruitment.
Figure S1. Pictorial representation of different muscle-related proteins associated with various subtypes of LGMD is shown here pictorially in an interlinked chemo-mechanical network of sarcomere, sarcoplasm, sarcolemma, and extracellular matrix.

Figure S2. Contribution of different LGMD genes. Molecular diagnosis has been established in 27% of the patients with the majority having pathogenic variants identified in one of the following genes *CAPN3* (17%), *DYSF* (16%), *FKRP* (9%), and ANO5 (7%) indicating that these genes are likely the major contributors to LGMD like phenotype.

Table S1. Summarized list of some of the affected cases with pathogenic variants in multiple genes and unusual clinical presentations.

Table S2. List of genes in the targeted LGMD panel testing with their respective LGMD subtype or other associated neuromuscular disease with/without overlapping LGMD subtype.

Table S3. Summary of late onset patients with one pathogenic variant and one variant of uncertain significance in the *GAA* gene.

Table S4. Summary of patients with *DNAJB6* variants.

Table S5. List of patients with pathogenic variants in *COL6A1*, *COL6A2,* and *COL6A3* genes.

Table S6. Mutation spectrum of *DNAJB6* gene. List of variants reported in the Human Gene Mutation Database (HGMD).

Table S7. List of variants of uncertain significance (VUS) identified in major LGMD genes.

