Targeted Next Generation Sequencing Approach Towards Improving Genetic Diagnosis of Limb Girdle Muscular Dystrophy

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Scholarly Report submitted in partial fulfillment of the MD Degree at Harvard Medical School

Date: 26 February 2018

Student Name: Liwen Xu, MPhil

Scholarly Report Title: Targeted Next Generation Sequencing Approach Towards Improving Genetic Diagnosis of Limb Girdle Muscular Dystrophy

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Abstract
Limb-girdle muscular dystrophies (LGMDs) are a heterogeneous group of rare muscle disorders in which progressive skeletal muscle weakness and wasting affect primarily the shoulders, hips and proximal limbs. More than 30 genes are causally linked to LGMDs; many more are implicated in metabolic, congenital, and other myopathies that present with LGMD-like patterns of muscle weakness. For patients affected by myopathies characterized by limb-girdle weakness, receiving a genetic diagnosis can impact their prognosis, therapeutic options and reproductive choices. We hypothesize that using whole exome sequencing as a first-pass diagnostic approach effectively prioritizes causal variants of LGMDs and clinically similar myopathies.

Targeted whole exome sequencing was applied to 1,001 undiagnosed patients of mixed ancestry with suspected genetic muscle disease. We analyzed exome data for pathogenic variants in 169 muscle disease-associated genes. Likely causal variants were identified in 468 families (47%), involving 72 genes. LGMD2A, due to mutations in CAPN3, was the most common disease in our cohort. Variants in CAPN3, DYSF, ANO5, DMD, RYR1, TTN, COL6A2 and SGCA collectively accounted for more than half of the solved cases. Over 150 patients were diagnosed with conditions for which effective therapies or tailored management were available, all of which are currently being applied.

We assembled one of the largest genetically characterized muscle disease cohort. Our results demonstrate that WES represents a powerful approach for diagnosis in a clinically and genetically heterogeneous disease, and in a subset of patients can identify diagnoses that alter clinical outcomes.
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**Glossary of abbreviations**

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<td>CK</td>
<td>Creatine Kinase</td>
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<td>ExAC</td>
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<td>Indel</td>
<td>Insertion / Deletion</td>
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<td>Limb Girdle Muscular Dystrophies</td>
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<td>SNV</td>
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I. Introduction
Limb girdle muscular dystrophies (LGMDs) are a heterogeneous group of rare muscle diseases with many subtypes categorized by disease gene and inheritance pattern. To date, more than 34 LGMD gene loci have been reported [1]. These genes encode proteins active in pathways that stabilize the dystrophin-glycoprotein complex, glycosylate α-dystroglycan, repair damaged muscle fibers, and maintain sarcolemma integrity [2]. Both autosomal dominant (LGMD1) and autosomal recessive (LGMD2) subtypes are known. Exceedingly rare, all forms of LGMD collectively affect only around 2 to 3 in 100,000 people; regional variation is notable, owing in some cases to founder effects [3].

Patients with LGMD are primarily afflicted with progressive weakness and wasting of the proximal muscles around the shoulders and hips. Clinical presentations are otherwise diverse in terms of age of onset, rate of disease progression, and severity of ultimate disability. Equally variable are the presence or absence of associated morbidities. These include, but are not limited to, contractures, gastrointestinal complications, and life-threatening cardiomyopathy and respiratory insufficiency [4]. Comprehensive guidelines exists to assist clinicians in managing and diagnosing LGMDs [5]. However, obtaining a precise diagnosis without genetic testing is difficult. Findings on clinical examination and muscle biopsy immunohistochemistry do not predictably correlate with the genetic subtypes of LGMD. Moreover, there are overlapping phenotypes in LGMDs and metabolic, congenital and other myopathies [6–8]. Therefore, many patients remain undiagnosed.

For patients with clinical limb-girdle weakness, receiving a genetic diagnosis can impact their understanding of prognosis, disease management options, and reproductive choices. The conventional strategy for diagnosing rare disorders is typically a gene-by-gene approach. A list of candidate genes, usually dictated by clinical phenotype, is tested serially or as panels for presence of mutations. However, in clinically and genetically heterogeneous disease such as LGMDs, gene-by-gene testing proves both time-consuming and costly [2,9]. This approach also limits the diagnostic search to known genotype-phenotype associations [10], leaving little power for novel disease characterizations.

By contrast, next generation sequencing (NGS) technology provides a relatively unbiased approach to search for potentially causative mutations. The capacity for targeted whole exome sequencing (WES) to complement and enhance standard clinical practice has been
demonstrated in the diagnosis of primary immunodeficiencies, cerebellar ataxias, and many other Mendelian diseases [11–13]. Using WES to facilitate LGMD diagnosis is attractive for several reasons. A large number of genes are associated with the phenotype; many known LGMD genes are large in size (e.g. TTN); and a high expected number of rare gene mutations are harbored by a single family or a small population, given overall low disease prevalence. NGS aided in achieving a diagnostic rate of 43% when recently applied to a cohort of 504 patients with undiagnosed muscular dystrophies and myopathic disease [14]. In a separate cohort of 60 patients for whom previous protein-based analysis and targeted Sanger sequencing had failed to identify the genetic cause of their disorder, WES revealed causative variants in a known LGMD gene in up to 45% of cases [15].

Our study – known as the MYO-SEQ project – applied WES to the largest ever cohort of patients with undiagnosed muscle disease, sequencing 1,001 index cases with a LGMD phenotype. The cohort was assembled from 43 neuromuscular disease referral centers from throughout Europe and the Middle East. Per our inclusion criteria, all patients presented with unexplained limb-girdle weakness and/or elevated serum creatine kinase (CK). Many patients have undergone genetic testing previously, although a substantial minority has had little or no prescreening.

With an aim to demonstrate the utility of WES as a first-pass diagnostic strategy, we initially analyzed rare candidate pathogenic variants in 169 genes known to be associated with muscle disease. We were able to diagnose 47% of our cohort with this “virtual panel” approach, revealing the most common muscle diseases in this population. We also provide evidence that early application of WES contributes to rapid diagnosis of most patients affected by limb-girdle weakness.

II. Methods
Forty-three referral centers (Supplemental table 1) submitted a variable number of index cases depending on patient eligibility. Our inclusion criteria is as stated above, patients must be age 10 or older with unexplained limb-girdle weakness and/or elevated serum creatine kinase (CK) with no prior genetic diagnosis. Informed consent was given by all participants, who were anonymized by collaborating centers using unique identification codes. Phenotypic information was uploaded onto the PhenoTips online software platform [16]. DNA was extracted from lysed
whole blood cells. DNA samples were submitted to the Newcastle MRC Centre Biobank. WES was performed at the Broad Institute of Harvard and MIT’s Genomics Platform. The reads were mapped using the Burrows-Wheeler Aligner [17]. Using Genome Analysis ToolKit [18,19], single nucleotide variants (SNVs), small insertions/deletions (indels) were identified by joint calling across all samples, including 60,706 reference samples from the Exome Aggregation Consortium (ExAC) [20]. Variants were annotated using Ensembl Variant Effect Predictor (VEP) [21]. The final call-set was uploaded onto the Broad Institute of Harvard and MIT’s open source software platform, seqr (seqr.broadinstitute.org) for analysis.

For each patient, we identified rare (minor allele frequency <1%) coding variants in 169 genes that are known to be associated with LGMD (Supplementary table 2) [1]. The resulting shortlists were examined for potential pathogenicity with consideration for (i) predicted effect on protein structure determined by VEP; (ii) frequency of the respective genotypes in the ExAC control population for expected inheritance mode; (iii) ClinVar reports of pathogenicity and any published reports of the variants; (iv) in silico predicted deleteriousness of missense variants by PolyPhen-2 [22], SIFT [23], MutationTaster2 [24], and FATHMM [25]; and (v) the patient’s phenotype and inheritance mode relative to that already associated with mutations in the gene of interest. Detected variants that were most likely to be disease-causing were reported back to referring clinician. Where necessary and possible, the suspected pathogenic variants were confirmed in the patient and segregated in parental DNA through Sanger sequencing.

III. Results

Patient demography

Of the 1,001 patients whose exomes were sequenced, 456 (46%) were female and 545 (54%) were male, originating from 927 families. Average age at time of enrollment was 39 years (range 2 - 88 years, median 38 years). Disease onset for 47% of the patients was before age 16; 42% reported first symptoms in adulthood; and 4% report the onset to have occurred after 60 years of age (7% not reported). A majority of patients, 77% of participants, reported proximal muscle weakness either in isolation or in combination with distal muscle weakness (3% reported distal muscle weakness only, 20% not reported). Sixty-eight percent of the cohort had increased serum CK levels (15% had normal CK, 16% not reported).
**Diagnostic yield**

Suspected causal variants were detected in 468 of 1,001 patients (47%). Nineteen of these patients (2%) harbored rare pathogenic variants in two genes, and it is unclear whether either or both genes were causal in their disease. In total, we identified 846 allelic changes. These were accounted for by 512 unique variants, 115 of which were previously reported as pathogenic in Clinvar (68 were detected in only one index case, 47 were detected in multiple families) while the remaining 397 were had not been previously described. Of these 397 distinct variants that were completely novel in their association to disease, 281 were detected in only one individual or family, while 116 were detected in multiple families.

In terms of genotypes, patients were mainly compound heterozygous (34%), homozygous (32%) and single heterozygous (29%); 25 patients (5%) were hemizygotes for X chromosomal variants. The most common type of variant was a missense change (52%), followed by frameshift (22%) and nonsense (12%) variants. Collectively, essential splice site, splice region, intronic, inframe, initiation lost and stop lost changes accounted for 14% of the variants.

**Reported variants**

The most common disease gene in our cohort was CAPN3, mutations in which cause LGMD2A. Variants in 8 genes (CAPN3, DYSF, ANO5, DMD, RYR1, TTN, COL6A2, and SGCA) collectively accounted for over half of the solved cases. Of note, a stop-gain TTN founder mutation (p.Gln35879Ter) was identified in 14 unrelated patients from a Serbian sub-population (Appendix A) [26]. Variants in 64 additional genes\(^1\) accounted for the remaining patients with one causal gene. Novel pathogenic variants associated with much these rarer conditions were detected in genes such as DPM3 (Appendix B) [27], POMT2 (Appendix D) [28], and POMK (Appendix E) [29]. Copy number variations were detected in 27 (3%) patients, SMN1 deletions in four (<1%) patients and transcriptional perturbations in one (<1%) patient.

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\(^1\) ACADVL, ACTA1, AGL, ATP2A1, BAG3, CAV3, CHRND, CLCN1, COL12A1, COL6A1, COL6A3, COLQ, CPT2, CRYAB, DES, DNAJB6, DNM2, DOK7, DPM3, ETFDH, FHL1, FKRPI, FKTN, FLNC, GAA, GBE1, GFPT1, GMPPB, GNE, GYG1, HNRNPD1, LAMA2, LMNA, LPIN1, MATR3, MTM1, MYF6, MYH14, MYH7, MYOT, NEB, PFKM, PGK1, PHKA1, POLG, POMK, POMT1, POMT2, PTPLA, PYGM, RAPSN, SCN4A, SEPN1, SGCB, SGCG, SMCHD1, SYNE2, TCAP, TNNT3, TPM2, TRAPPCL11, TRIM32, VCP, and VMA21
Clinical outcomes in select patients

Over 150 patients (15%) were identified with mutations in genes associated with conditions for which treatment or tailored management options were available. Variants in COLQ, DOK7, GFPT1 and RAPSN, all associated with congenital myasthenic syndromes, were identified in eight patients. The choice of treatment for patients with congenital myasthenic syndromes depends on the disease subtype and the causative gene, and so an accurate genetic diagnosis is vital to avoid exacerbating the disease [30,31]. In addition, twelve patients (eight unrelated index cases and four siblings) harbored compound heterozygous variants in GAA associated with Pompe disease (Appendix C) [32] and were thus potential candidates for disease-modifying enzyme replacement therapy [33,34]. Nine patients who harbored variants in either SCN4A or CLCN1 were suspected to have inherited skeletal muscle channelopathies and would additionally benefit from close follow-up for cardiomyopathy and arrhythmia [35,36]. Cardiomyopathy is a serious health condition that often presents in patients with a genetic muscle disease. Thirteen female patients in our cohort who were manifesting carriers of Duchenne muscular dystrophy, an X-linked disorder that often escapes clinical diagnosis in females, would also require close monitoring of cardiac function [37]. Finally, we identified 24 patients with variants in RYR1. It was prudent to highlight these cases to clinicians because mutations in RYR1 are not only associated with central core disease and minicore myopathy, but can also confer susceptibility to the dreaded anesthetic complication malignant hyperthermia [38,39].

IV. Student role

606 patient samples finished sequencing and joint calling between May 2015 and March 2016. I analyzed the sequencing data to identify potentially pathogenic variants in 169 genes associated with LGMDs and related muscle diseases. A diagnostic rate of 49% was estimated at the time. The following publications and manuscript resulted from this body of work.

● Contributed to analysis of WES data, classification of disease-causing variants in TTN, identification of the core founder haplotype, and revision of manuscript

● Contributed to identification of the founder stop-gain TTN variant (c.107635C>T, p.Gln35879Ter) as well as two additional frameshift, four stop-gain, one missense and one splice donor variants in 14 out of 19 patients of Serbian ancestry (data presented in Table 1)

● Contributed to construction and revision of Figure 2

Cited in PubMed; PMID: 28803818. Pub Status: Published. See Appendix B.

● Contributed to analysis of WES data and classification of disease-causing variants in DMP3

● Contributed to identification of the published homozygous DMP3 variant (c.131T > C (p.Leu44Pro))

Cited in PubMed; PMID: 29149851. Pub Status: Published. See Appendix C.

● Contributed to analysis of WES data and classification of disease-causing variants in GAA

● Contributed to identification of the published GAA mutations implicated in Pompei disease (data presented in Table 1)

Cited in PubMed; PMID: 29175898. Pub Status: Published. See Appendix D.
Contributed to analysis of WES data, classification of disease-causing variants in 
POMT2, and revision of manuscript
Contributed to identification of the published POMT2 mutations implicated in LGMD2N (data presented in Table 2)

Contributed to analysis of WES data and classification of disease-causing variants in 
POMK
Contributed to identification of the two published POMK variant (c.965C>T (p.Pro322Leu) and c.136C>T (p.Arg46Ter))

V. Discussion
The MYO-SEQ project was established in 2014 as a partnership between academia, industry and patient organizations. We set out to improve the diagnostic pathway of patients affected by rare genetic diseases by using WES as a first-pass strategy. In the process we aimed to accelerate the integration of NGS technologies into the clinical realm.

We applied WES to one of the largest genetically characterized muscle disease cohorts. One clear benefit of the WES approach was the achievement of high diagnostic rates. By focusing on a panel of 169 known neuromuscular disease genes, we identified candidate causal variants in 47% of 1,001 patients with undiagnosed LGMDs. This detection rate is comparable to those reported by other recent studies with a focus on NGS-based diagnosis of LGMDs and overlapping neuromuscular diseases [14,15]. In contrast to prior studies, patients in our cohort had variable access to previous genetic testing. Our solve rate ranged as high as 95% among patients referred from the Middle East and as low as 35% among patients referred from Western Europe, although a low number of samples submitted from some referral centers did not allow for a robust analysis of detection rate by geographic location. This likely reflects regional differences in expertise and infrastructure to sufficiently prescreen patients prior to submission for WES.
Our finding that eight genes – CAPN3, DYSF, ANO5, DMD, RYR1, TTN, COL6A2 and SGCA – accounted for one-quarter of muscle disease in our cohort suggests that testing these in a multi-gene panel is one prescreen strategy for patients suspected to have a genetic LGMD. That there was substantial phenotypic overlap between LGMDs and RYR1-associated myopathies, dystrophinopathies (including manifesting carriers), and collagen VI-related disorders in our study serve to remind clinicians to think broadly about differential diagnoses.

The identification of disease-causing mutations was our goal in so much as it enabled the ultimate genetic confirmation of a clinical diagnosis. We worked in close collaboration with the referring clinicians to ensure closed communication loops. For many MYO-SEQ patients, the benefits of WES extended beyond diagnosis. Fifteen percent of our cohort received a clinically meaningful outcome that lead to tailored management including therapeutic interventions, as well as family planning and counseling.

Nevertheless, 53% of MYO-SEQ patients remain without a genetic diagnosis despite the interrogation of the majority of protein-coding regions via WES. Many of these patients likely have unknown or partially uncharacterized diseases. A large number of disease-causing genes have only recently been identified [40–42]. Extending the gene list accordingly takes advantage of the fact that WES data can be repeatedly analyzed for pathogenic variants as novel disease genes are discovered. In cases where only variants of uncertain significance are detected, trio WES may enhance our capacity to further filter variants based on familial segregation of disease. We also recognize that in a minority of patients, WES may not be enough. A combination of orthogonal approaches – whole genome and transcriptome sequencing – would be better suited to detect genetic changes in intergenic or intronic regions that contribute to disease through transcription/splicing regulation and altered chromatin conformation, among other mechanisms [43].

Although established guidelines exist for the clinical interpretation of sequence variants, unambiguous assignment of pathogenicity is not always possible [44]. This is particularly the case for several very low-frequency variants underlying rare muscle diseases in our cohort. Understanding that variant-level features (e.g. absence of a variant in a control population, predictions from computational tools) support but do not strongly implicate pathogenicity, we struggled with the reporting of variants of uncertain significance (VUS) because of their unknown impact on the patient. VUS results represent a heavy burden on the clinician, who
must make the judgment call of how to counsel patients on these test results and whether to managed patient care as though the variant were disease causing. This highlights the significant need to develop curriculum for genetics and genomics for both trainees and established physicians. Many currently practicing physicians may not yet require genetics to perform their valuable role in health care. However, as patients become increasingly well-informed about the availability of genome-guided diagnosis and treatment, they will inevitably turn to their providers to help navigate the nuances and ambiguities that underlie genetic testing results.

In summary, we have shown the advantage of exome sequencing as a high-yield and reliable diagnostic tool in patients with a rare neuromuscular disorders. At the same time, we expanded the spectrum of disease that overlap with the limb-girdle muscle weakness phenotype. A significant portion of patients received prompt, appropriate treatment and follow-up after receiving their diagnosis. Applied in LGMDs and other neuromuscular conditions, our approach form a paradigm that could benefit research in other groups of rare diseases.

VI. Acknowledgments
We received funding from Sanofi Genzyme, Ultragenyx, LGMD2I Research Fund, Samantha J Brazzo Foundation, LGMD2D Foundation, and Kurt+Peter Foundation. We thank the patients for donating their tissue samples.
References


sequencing targeted to a cohort of 606 patients with unexplained limb-girdle muscle weakness. *Orphanet J Rare Dis.* 2017 Nov 17;12.


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### Supplemental Table 2

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Appendix A.
A novel recessive TTN founder variant is a common cause of distal myopathy in the Serbian population


Variants in the TTN gene have been associated with distal myopathies and other distinctive phenotypes involving skeletal and cardiac muscle. Through whole-exome sequencing we identified a novel stop-gain variant (c.107635C>T, p.(Gln35879Ter)) in the TTN gene, coding a part of the M-line of titin, in 14 patients with autosomal recessive distal myopathy and Serbian ancestry. All patients share a common 1 Mb core haplotype associated with c.107635C>T, suggesting a founder variant. In compound heterozygotes, nine other TTN variants were identified: four stop-gain, three frameshift, one missense and one splice donor variant. Patients homozygous for the common variant did not show significant clinical differences to the compound heterozygous patients. The clinical presentation of all patients was an adult onset distal myopathy with predominant lower limb involvement. In addition, most patients had normal to mildly elevated serum creatine kinase levels, myopathic electromyograms, normal cardiologic and respiratory tests and muscle pathology consistent with a dystrophic process. In this study, we describe a distinct phenotype for patients with distal myopathy associated with novel recessive TTN variants including a Serbian founder variant. Our results expand the phenotypic and genetic spectrum of titinopathies and will facilitate the diagnosis of this condition in patients of Serbian origin.

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INTRODUCTION

Distal myopathies are a group of progressive hereditary muscle disorders characterised by the onset of weakness and wasting in the lower legs, forearms, feet and/or hands.1 They are less frequent and less well studied than many muscular dystrophies affecting predominantly proximal muscles and therefore probably often underdiagnosed. There is a growing number of distinct and genetically defined distal myopathies with either autosomal dominant or recessive inheritance. It has been speculated that the selectivity of distal or proximal muscle involvement could be related to the subcellular localisation of the defective proteins: sarcomeric defects generally cause a more distal pattern of involvement, while sarcolemmal defects are more frequently responsible for proximal phenotypes.2

The TTN gene (OMIM 188840), located on chromosome 2q31, consists of 363 coding exons. It encodes titin, the largest protein known in humans. After myosin and actin, titin is the third most common filament in both skeletal and cardiac muscle. The molecular structure of titin is divided into four regions: the N-terminal Z-disc, the I-band and A-band regions and the C-terminal M-line, encoded by the last six exons of the TTN gene (358–363, or Mex1 to Mex6). Disorders caused by variants in the TTN gene can be classified into three groups: cardiomyopathies, diseases of skeletal muscles and congenital disorders affecting both types of muscles.3 Before the application of next-generation sequencing technologies, it was very difficult to diagnose conditions associated with TTN variants because of the size of the gene.4,5 Even now it is difficult to determine the pathological variants because of the complex and repetitive gene structure and the fact that almost every individual carries at least one rare, mostly non-pathogenic TTN variant.

Several distinctive phenotypes involving skeletal muscles have been associated with variants in the TTN gene. Tibial muscular dystrophy (TMD; OMIM 600334) is a late-onset, autosomal dominant disorder characterised by weakness and wasting predominantly in the anterior compartment of the distal lower limbs.1 It was first described in Finland where it represents the most common muscle disease.6 The FINmaj variant is the most prevalent TTN variant in the Finnish population (~2:10 000) due to a founder effect. It is a complex 11-bp insertion/deletion resulting in a 4-amino-acid exchange in the last exon (Mex6, exon 363) of the TTN gene.7 Several other variants localised in the second to last (Mex5, exon 362) and the last exons of TTN have been described in TMD patients from Belgian, French, Spanish and Italian populations.8–11 Homozygosity of the FINmaj allele has been associated with a more distal muscle involvement, while sarcolemmal defects generally cause a more proximal pattern of involvement, including distal lower limbs.8

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variant is rare and causes limb-girdle muscular dystrophy 2J (OMIM 608807), a phenotype with earlier onset and more proximal muscle involvement.\textsuperscript{1,6,10} Recently, atypical, more complex or severe phenotypes have been explained by a second TTN variant in several TMD patients from different European countries and in LGMD2J patients being compound heterozygous for the FINmaj variant and an additional TTN variant.\textsuperscript{12} Hereditary myopathy with early respiratory failure (OMIM 605689), an autosomal dominant disease involving proximal, distal and respiratory muscles, represents yet another entity caused by TTN variants.\textsuperscript{13} The spectrum of titinopathies is constantly being broadened.

In the present study, we identified recessive TTN variants as a molecular cause of distal myopathy resembling TMD in a well-defined cohort of patients with Serbian ancestry through whole-exome sequencing (WES). A stop-gain TTN variant (c.107635C>T, p.(Gln3587Ter)) was found in all patients (hereafter referred to as the 'common variant') – 3 patients being homozygotes and 11 compound heterozygotes. Additionally, we showed that the common variant was part of a haplotype block that likely originated from a single founder event. Our findings suggest that screening for the identified founder TTN variant should be the first step in the diagnostic algorithm of distal myopathies in patients with Serbian ancestry.

**MATERIALS AND METHODS**

**Clinical assessment**

Patients were recruited at the Neurology Clinic, Clinical Centre of Serbia, School of Medicine, University of Belgrade and the Clinic for Neurology and Psychiatry for Children and Youth, Belgrade, Serbia as a part of the international MYO-SEQ project. This project provides WES for 1000 index patients with unexplained limb-girdle muscle weakness and elevated serum CK levels. Initial clinical details were submitted through PhenoTips,\textsuperscript{14} a tool developed for sharing phenotypic and genetic data in a safe and ethical way using the Human Phenotype Ontology.\textsuperscript{15} Appropriate informed consent was provided by all the patients after the Ethics Committee of the School of Medicine, University of Belgrade approved this study – no. 29/VI-18. The initial analysis included 91 unrelated index patients, with 19 having a predominantly distal phenotype. In this group with a distal phenotype, 14 patients from 14 unrelated Serbian families carried a specific TTN founder variant and were analysed further in this study. Among the five remaining patients with distal phenotype, three have been diagnosed with dysferlinopathy, one with desminopathy, and one remained without a conclusive genetic diagnosis.

The majority of patients (12/14) were sporadic, while two pedigrees suggested autosomal recessive inheritance. Consanguinity was probably present in one family (patient no. 02). First-degree relatives of patients were also assessed where possible. Clinical assessment included a detailed medical and family history and neurological examination, performed by an experienced neurologist. Muscle strength was scored using the Medical Research Council Muscle Grading Scale.\textsuperscript{16} Follow-up visits were regularly scheduled for all patients. Electromyography (EMG) and nerve conduction studies were performed in all patients. Serum CK levels were measured in all patients, and where possible in available family members. Respiratory function was assessed by spirometry annually, while regular cardiac examinations included ECG each year and echocardiogram every second year. Muscle biopsies were performed in seven patients.

Muscle magnetic resonance imaging (MRI) was performed in six patients in axial and coronal planes of the lower limbs using the following sequences: T1-weighted (T1w), T2-weighted, proton-density weighted and three-point Dixon.\textsuperscript{17,18} Images were assessed on an individual muscle basis and graded according to the five-point scale published by Mercuri et al.\textsuperscript{19}

**Whole-exome sequencing**

DNA was extracted from blood using standard techniques at the Neurology Clinic, Clinical Centre of Serbia, School of Medicine, University of Belgrade, Serbia. WES was performed at the Broad Institute of Harvard and MIT (Cambridge, MA, USA), using Illumina exome capture, 38 Mb baited target, and their in-solution hybrid selection process with >250 ng of input DNA (at >2 ng/ul). The sequencing pipeline included sample plating, library preparation (2-plexing of samples per hybridisation), hybrid capture, sequencing (76 bp paired reads), sample identification quality control check and data storage. The hybrid selection libraries cover >80% of targets at 20x, with a mean target coverage of >80x. The exome sequencing data was demultiplexed and each sample’s sequence data was aggregated into a single Picard BAM file. Alignment was carried out against the human reference hg19, build 37 using Burrows-Wheeler Aligner and variants were called using the Genome Analysis Toolkit software (Broad Institute, Cambridge, MA, USA). Data were analysed using the Broad Institute open source platform seqr (https://seqr.broadinstitute.org). The selection of potential variants from the whole-exome analysis was restricted to 169 genes that are known to be associated with neuromuscular disorders. Filtering criteria of potential variants included population frequency of <1% and moderate to high impact on protein structure (ie, missense, stop-gain, splice site, frameshift and in-frame indels). TTN variants and exons were annotated according to transcript ENS00000589042.1 and protein sequence ENSP00000467141.1 (RefSeq NM_001267550 and NP_001254479).

**Validation of identified genetic variants**

Sanger sequencing was used to confirm the presence of variants identified by WES in 13 patients (DNA sample from patient no. 04 was not available), and to perform segregation analysis in eight families, involving 18 first-degree relatives. Sanger sequencing was carried out with the BigDye Terminator v.1.1 Cycle Sequencing Kit (Life Technologies, Grand Island, NY, USA) on the Applied Biosystems 3130 Genetic Analyser (Applied Biosystems, Warrington, UK) at the Centre for Human Molecular Genetics, Faculty of Biology, University of Belgrade, Serbia. Additionally, 103 control individuals of Serbian origin were screened for the common c.107635C>T variant at the John Walton Muscular Dystrophy Research Centre, Institute of Genetic Medicine, Newcastle University, UK. All identified variants were submitted to the European Genome-Phenome Archive (https://www.ebi.ac.uk/) and the TTN Leiden Open Variation Database (http://www.lovd.nl/TTN; patient IDs 87088, 87240–87245 and 87247–87255).

**Haplotypic analysis**

The size of the haplotype associated with the common c.107635C>T variant was first estimated using WES data for the homoygous patients and a homozygous block was identified by running the full Variant Caller Format files on Homozygosity Mapper (http://www.homozygosimapper.org/). In addition, an Illumina Infinium array containing ~250 000 markers allowed the identification of homozygosity blocks based on non-coding single-nucleotide polymorphisms (SNPs). From this, a 2.8 Mb window surrounding the TTN gene, containing 230 SNPs with an allele frequency >1%, was analysed in all patients. As no parental data were available, haplotypes of compound heterozygous cases were phased by inferring the genotype based on the common founder haplotype observed in the homoygous cases. The haplotype segregating with the c.107635C>T variant was also reconstructed by genotyping analyses of eleven polymorphic microsatellites located upstream (D2S1776, D2S2188, D2S2314, D2S138, D2S148 and D2S300), within (D2S385) and downstream (D2S384, D2S2310, D2S364 and D2S152) of the TTN gene. Microsatellite haplotypes were phased by family segregation analysis, except for patient nos 05, 08 and 13 in whom the haplotypes were phased as described above for SNPs haplotypes. Genotyping was performed by fragment analysis on Applied Biosystems 3130 Genetic Analyser (Applied Biosystems) and data were analysed with GeneMapper ID v.3.2.1 software (Applied Biosystems).

The age of the most recent common ancestor of c.107635C>T variant in the analysed sample (‘mutation age’) was estimated by the single locus method based on the expected exponential decay of linkage disequilibrium between the variant and nearby polymorphic loci (D2S314, D2S138, D2S148, D2S310 and D2S364) through recombination over time.\textsuperscript{20,21} Microsatellite alleles segregating with the variant and their frequencies were determined from haplotype data of 14 disease chromosomes allowing ±1 repeat unit, while allele frequencies in the Serbian population were obtained from 56 normal chromosomes. To estimate the recombination fraction (\(\theta\)) between a given marker and the

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The missense variant is found in exons encoding the I-band regions of titin and others were in exons and one splice donor variant, were found in the rest of the patients. Only two additional frameshift variants, four stop-gain, one missense patients (nos 01, 08 and 14) had the same frameshift variant origin. The common variant was absent, suggesting that it is not a

ExAC, we screened a group of 103 control individuals of Serbian population might not be well represented among the Exome Aggregation Consortium (ExAC) data set. As the Serbian population were homozygous for the variant (Table 1). Two heterozygous

TTN variants are a frequent cause of distal myopathy in Serbia. Through WES we identified a novel stop-gain variant (c.107635C>T, p.(Gln35879Ter)) in 14 patients (out of 19) with distal myopathy of Serbian ancestry. This variant is located in exon 362 (Mex 5), coding a part of the M-line region of titin. Three patients (nos 02, 03 and 13) were homozygous for the variant (Table 1). Two heterozygous carriers of European (non-Finnish) origin are reported in the Exome Aggregation Consortium (ExAC) data set. As the Serbian population might not be well represented among the >60 000 individuals of ExAC, we screened a group of 103 control individuals of Serbian origin. The common variant was absent, suggesting that it is not a frequent benign variant in the Serbian population.

In all 11 patients heterozygous for the common variant, a second variant in the TTN gene was detected (Table 1). Three of those patients (nos 01, 08 and 14) had the same frameshift variant c.103360delG; p.(Glu34454AsnsTer3), located in Mex1 (exon 358). Only two additional frameshift variants, four stop-gain, one missense and one splice donor variant, were found in the rest of the patients. Among those variants, two were located in Mex1, one was in the TTN exons encoding the I-band regions of titin and others were in exons encoding the A-band (Table 1). The missense variant is found in ExAC (MAF = 8.3 × 10^{-6}, allele count is 1 out of 120 160).

Sanger sequencing confirmed the presence of the WES-identified TTN variants in 13 patients (DNA sample of one patient was not available). According to the family segregation analysis, all parents were heterozygous carriers for one of the TTN variants identified in patients, and among other tested first-degree relatives, six out of seven were heterozygous carriers (Supplementary Table 1). All relatives who were heterozygous carriers of those variants were asymptomatic, which is in accordance with the presumed autosomal recessive mode of inheritance (Figure 1).

Two identified TTN variants are founder variants

Initial homozygosity analysis based on exonic variants of patient nos 02 and 05, both homozygous for the common variant, indicated that the size of a common haplotype associated with the c.107635C>T variant was ~4.8 Mb. Further detailed analysis of genome-wide SNP array data, including non-coding SNPs, delineated the size of the core haplotype (the part of the ancestral haplotype shared by all the TTN chromosomes) to just over 1 Mb between rs334613: A>G (hg19 chr2:179166242A>G) and rs7591863:A>G (hg19 chr2:180196356A>G). The common haplotype was also confirmed by fine mapping of polymorphic microsatellites and some of them were in strong linkage disequilibrium with the common variant (Supplementary Table 2). Taking all these data together the size of the core haplotype was estimated to be 1 Mb (Figure 2). In addition, microsatellite analysis also showed a common haplotype of at least 7.4 Mb for the c.103360delG variant, which was identified in three patients (data not shown). These results support a common origin for both variants, c.107635C>T and c.103360delG, in Serbian patients.

### Table 1 TTN variants identified in 14 patients with distal myopathy and Serbian ancestry

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Abbreviations: Alt, alteration; chr, chromosome; HGVS, human genome association variation; HGVSs, the HGVS coding sequence name; HGVSp, the HGVS protein sequence name; ref, reference.
with c.107635C>T possibly representing a major Serbian TTN founder variant.

The majority of c.107635C>T chromosomes shared a haplotype that exceeded the size of the core haplotype, indicating that recombination events shaped the ancestral haplotype in the past and thus enabling the estimation of the ‘mutation age’. The mean age of the most recent common ancestor of the c.107635C>T variant in our sample was estimated at 40.9 ± 11.2 generations (95% CI: 29.7–52.1). This corresponds to 820 ± 223 (95% CI: 625–1015) or 1023 ± 280 (95% CI: 778–1268) years assuming an average rate of 20 and 25 years per generation, respectively (Supplementary Table 2).

Patients with the common c.107635C>T TTN variant have a homogenous phenotype

We analyzed phenotypic data of the 14 patients harbouring the c.107635C>T TTN variant in our cohort. Eight patients were female (57%) and six were male (43%) with an average age at the time of the last examination of 36.8 ± 10.9 years. The mean disease duration was 7.8 ± 6.1 years, not taking into account patient no. 04, for whom the age of onset was unknown. The clinical presentation associated with the common c.107635C>T variant was distal myopathy resembling TMD. The age of onset ranged between 14 and 44 years (mean 28.0 ± 9.4 years, median 25 years; Tables 2 and 3). The youngest patient (no. 14) was oligosymptomatic, with mild hyperCKemia, Achilles tendon (AT) contractures and a tendency of toe walking as the only clinical signs. Since the age of 9 years, patient no. 14 complained of calf pain following extensive physical activity, but the patient is still engaged in sports at the age of 16 years. Cramps, myalgia and fatigue were not common among the other patients. The symptoms typically started with tripping caused by foot dorsiflexor weakness, which over years progressed to foot drop. In addition, difficulties when walking uphill or standing up were also noted at disease onset. While the majority of patients had disease onset in their 20s, it was noted that all three patients homozygous for the common variant (nos 02, 05 and 13) had an onset of symptoms in their 40s (Table 3). On the other hand, the three patients compound heterozygous for both founder variants (nos 01, 08 and 14) were among those with the earliest onset of symptoms (Table 3).

The facial and bulbar muscles were not affected. Scapular winging and weakness of the shoulder girdle muscles were present in about half of the patients, and were usually mild. Other muscles of the upper limbs were rarely affected, with the exception of mild weakness of the elbow extensors that was noted in four patients (Table 2). Predominant lower limb involvement was a distinctive characteristic of the disease in our patient cohort (Figure 3). A common feature was prominent muscle wasting of the anterior compartment of the lower legs. This was rarely the only region involved (4/14), more often it was combined with wasting of the posterior compartment of the lower legs and of the hamstring muscles (9/14). Two of those patients showed general wasting of the lower limbs (Table 2). Muscle hypertrophy was not detected. Muscle strength was most severely diminished in the distal muscles of the lower limbs, with foot and toe dorsiflexors being most severely affected. Plantar flexors were only mildly affected in the majority of patients, while more than half of the patients had proximal weakness of their lower limbs, mainly affecting the hip muscles (8/14) and the knee flexors (7/14) and extensors (5/14; Table 2). Contractures of the AT were present in all patients (Table 2). Contractures or hypermobility in other joints were not noted. Asymmetric muscle involvement was present in 5/14 patients. All patients but one, who

![Figure 1 Segregation analysis of TTN variants in the family of patient no. 01. Both unaffected parents carry one of the variants identified in the patient, which is in accordance with an autosomal recessive mode of inheritance. The unaffected brother is a carrier of the wild-type alleles. The pedigree symbol for the patient is shown in black.](image-url)
started using a wheelchair at the age of 46 years (no. 04), remained ambulant, with the oldest patient being 53 years of age (Table 2).

There was no notable difference in the pattern or severity of muscle wasting and weakness between patients homozygous for the common variant and compound heterozygous patients. In addition, the only patient carrying the common variant in combination with a missense variant (no. 10) presented a similar clinical presentation to the other null compound heterozygous cases, suggesting that this variant is likely pathogenic.

Normal serum CK levels were noted in six patients, and mildly elevated levels in eight patients (normal value <150 IU/l). EMG showed a myopathic pattern and nerve conduction velocities (NCVs) were normal in all 14 patients. Regular cardiologic examinations were normal in all but one patient (no. 07), who had a mild dilated cardiomyopathy diagnosed at the age of 46 years. Echocardiography showed borderline diastolic and normal systolic dimension of the left ventricle and global hypocontractility of the left ventricle with ejection fraction of 45%. The patient has been treated with bisoprolol 2.5 mg each day and furosemide 40 mg every third day. The last cardiac ultrasound was performed at the age of 53 years – progression was not observed. The respiratory function as measured by spirometry was normal in all patients.

Two patients (nos 06 and 07) reported family members with muscle symptoms. Sister of patient no. 06 has walking difficulties without cardiac symptoms, but she declined clinical examination and genetic analysis. Brother of patient no. 07 had leg weakness and died from cardiac disease at the age of 34 years, but we were not able to obtain more detailed data. There were no muscle and cardiac diseases in family members of other patients, and we did not observe any abnormalities on neurological examination in all those who accepted to be examined.

Muscle biopsy

Seven patients underwent a muscle biopsy. Most biopsies showed end-stage findings with atrophic fibres, lots of fibrous tissue, fatty replacement and an increase in internal nuclei. There were no neurogenic findings. The muscle biopsy of the youngest patient (no. 14) with a short course of the disease showed milder changes with muscle fibre size variation (hypotrophy and occasional atrophy), increase in connective tissue and visible macrophages. Findings in all patients were compatible with muscular dystrophy (Figure 4). No specific immunohistochemical changes were detected and no signs of protein accumulation.

Muscle MRI

Muscle MRI of the thighs and lower legs was performed in six patients (Figure 5). The youngest patient (no. 14) had a completely normal muscle MRI in all applied sequences. The other five patients showed severely affected anterior compartments of the lower legs (5/5), with fatty replacement of muscle tissue. In all but one patient this region was more severely affected than the posterior compartment. The most
<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (years) and sex</th>
<th>Onset</th>
<th>UL proximal/which muscles</th>
<th>UL distal</th>
<th>LL proximal/which muscles</th>
<th>LL distal</th>
<th>Pattern of muscle involvement</th>
<th>Scapular winging</th>
<th>AT contr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>34, F 21</td>
<td>Normal</td>
<td>Yes</td>
<td>S.Abd 4</td>
<td>Normal</td>
<td>HF 4, HE 4, H.Abd 4, H.Add 4, KF 4</td>
<td>2 4 3</td>
<td>Anterior distal LL</td>
<td>Mild</td>
</tr>
<tr>
<td>02</td>
<td>49, F 44</td>
<td>Normal</td>
<td>Yes</td>
<td>S.Abd 4</td>
<td>Normal</td>
<td>HF 4</td>
<td>1 5 3</td>
<td>Global in LL (especially anterior distal LL)</td>
<td>Mild</td>
</tr>
<tr>
<td>03</td>
<td>30, M 25</td>
<td>Increased (up to 3x)</td>
<td>Yes</td>
<td>S.Abd 4, EE 4</td>
<td>Small hand muscles 3</td>
<td>HF 4, HE 4, H.Abd 4, H.Add 4, KF 4, KE 4</td>
<td>2 4 2</td>
<td>Anterior distal LL/hamstring mm.</td>
<td>Mild</td>
</tr>
<tr>
<td>04</td>
<td>48, M N/A</td>
<td>Increased (2-5x)</td>
<td>No (46 years)</td>
<td>S.Abd 4, EE 4</td>
<td>Normal</td>
<td>HF 4, HE 4, H.Abd 4, H.Add 4, KF 4, KE 4</td>
<td>1 4 2</td>
<td>Anterior distal LL/hamstring mm.</td>
<td>Yes</td>
</tr>
<tr>
<td>05</td>
<td>44, M 40</td>
<td>Normal</td>
<td>Yes</td>
<td>Normal</td>
<td>Normal</td>
<td>HF 4</td>
<td>1 4 3</td>
<td>Anterior distal LL/hamstring mm.</td>
<td>No</td>
</tr>
<tr>
<td>06</td>
<td>47, F 25</td>
<td>Increased (up to 2x)</td>
<td>Yes</td>
<td>S.Abd 3, EE 4</td>
<td>Normal</td>
<td>HF 3, HE 4, H.Abd 4, H.Add 3, KF 4, KE 3</td>
<td>1 4 2</td>
<td>Anterior distal LL</td>
<td>Mild</td>
</tr>
<tr>
<td>07</td>
<td>44, F 39</td>
<td>Normal</td>
<td>Yes</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>1 4 2</td>
<td>Anterior distal LL</td>
<td>No</td>
</tr>
<tr>
<td>08</td>
<td>25, M 22</td>
<td>Increased (up to 3x)</td>
<td>Yes</td>
<td>S.Abd 4, EE4, EF4</td>
<td>Normal</td>
<td>HF 4, HE 4, H.Abd 4, H.Add 4, KF 4, KE 4</td>
<td>0 4 1</td>
<td>Generalised in LL/ mild in UL</td>
<td>Yes</td>
</tr>
<tr>
<td>09</td>
<td>43, F 28</td>
<td>Increased (up to 2x)</td>
<td>Yes</td>
<td>Normal</td>
<td>Normal</td>
<td>HF 4, HE 4, H.Abd 4, H.Add 3, KF 3, KE 4</td>
<td>2 4 2</td>
<td>Distal LL/hamstring mm.</td>
<td>No</td>
</tr>
<tr>
<td>10</td>
<td>32, M 28</td>
<td>Increased (up to 2x)</td>
<td>Yes</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>2 4 2</td>
<td>Distal LL</td>
<td>No</td>
</tr>
<tr>
<td>11</td>
<td>27, F 19</td>
<td>Normal</td>
<td>Yes</td>
<td>Normal</td>
<td>Normal</td>
<td>KF 4</td>
<td>2 4 2</td>
<td>Distal LL</td>
<td>No</td>
</tr>
<tr>
<td>12</td>
<td>29, F 25</td>
<td>Increased (up to 2x)</td>
<td>Yes</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>2 4 2</td>
<td>Distal LL</td>
<td>No</td>
</tr>
<tr>
<td>13</td>
<td>53, F 40</td>
<td>Normal</td>
<td>Yes</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>2 4 1</td>
<td>Anterior distal LL</td>
<td>No</td>
</tr>
<tr>
<td>14</td>
<td>15, M 14</td>
<td>Increased (up to 2-5x)</td>
<td>Yes</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>4 5 5</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

Abbreviations: AS, asymmetry; AT, Achilles tendon; contr., contractures; DF, dorsal flexors; EE, elbow extensors; EF, elbow flexors; F, female; H.Abd, hip abductors; H.Add, hip adductors; HE, hip extensor; HF, hip flexor; KE, knee extensors; KF, knee flexors; LL, lower limbs; M, male; NA, data not available; PF, plantar flexor; S.Abd, shoulder abductor; UL, upper limbs; y, years; ‘+’, present; ‘−’, absent.
Table 3 Summary of characteristics of 14 patients with distal myopathy and Serbian ancestry

<table>
<thead>
<tr>
<th>Main findings</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTN c.107635C&gt;T, p.(Gln35879Ter)</td>
<td>14/14</td>
</tr>
<tr>
<td>Adult onset (second to fourth decade)</td>
<td>13/14</td>
</tr>
<tr>
<td>Patients remaining ambulant through follow-up period</td>
<td>13/14</td>
</tr>
<tr>
<td>Creatine kinase normal or mildly elevated (up to five times)</td>
<td>14/14</td>
</tr>
<tr>
<td>Distal or proximodistal myopathy</td>
<td>13/14</td>
</tr>
<tr>
<td>Muscle weakness</td>
<td></td>
</tr>
<tr>
<td>Distal muscles of LL most affected (DF of foot and toes most affected)</td>
<td>13/14</td>
</tr>
<tr>
<td>Foot drop after several years</td>
<td>13/14</td>
</tr>
<tr>
<td>Proximal weakness of LL</td>
<td>9/14</td>
</tr>
<tr>
<td>Mild proximal weakness of UL</td>
<td>6/14</td>
</tr>
<tr>
<td>Muscle wasting</td>
<td></td>
</tr>
<tr>
<td>Most prominent in anterior compartment of LL</td>
<td>13/14</td>
</tr>
<tr>
<td>Hamstring muscles</td>
<td>6/14</td>
</tr>
<tr>
<td>Severe global wasting of LL</td>
<td>2/14</td>
</tr>
<tr>
<td>Scapular winging (mild to moderate)</td>
<td>6/14</td>
</tr>
<tr>
<td>Achilles tendons contractures</td>
<td>14/14</td>
</tr>
<tr>
<td>Respiratory function normal(^a)</td>
<td>14/14</td>
</tr>
<tr>
<td>Cardiomyopathy absent(^b)</td>
<td>13/14</td>
</tr>
<tr>
<td>Dilatative cardiomyopathy</td>
<td>1/14</td>
</tr>
<tr>
<td>MRI compatible with dystrophy process</td>
<td>6/6</td>
</tr>
<tr>
<td>Muscle biopsy compatible with dystrophic process</td>
<td>7/7</td>
</tr>
<tr>
<td>EMG: myopathic</td>
<td>14/14</td>
</tr>
</tbody>
</table>

Abbreviations: DF, dorsal flexors; EMG, electromyography; LL, lower limbs; MRI, magnetic resonance imaging; UL, upper limbs.
\(^a\)Respiratory function was performed annually.
\(^b\)Electrocardiography was performed annually and echocardiography every second year (more details are given in the text).

severely affected muscle in the anterior compartment was the tibialis anterior (5/5) followed by the extensor digitorum longus muscle (3/5). The peroneal muscles were involved in three patients. Fatty replacement in the posterior compartment was far less prominent and was only seen in three of the six patients, affecting the gastrocnemius muscles, the soleus and also the tibialis posterior muscle (1/3). One patient (no. 01) showed very notable asymmetry of calf muscle involvement, with the tibialis anterior, peroneus longus and gastrocnemius muscles being affected on one side, while only the tibialis posterior was unaffected on the other. The hamstring muscles were the most frequently involved muscles of the thighs. Fatty replacement was most commonly seen in the biceps femoris (4/5), followed by semimembranosus (3/5), and semitendinosus muscle (3/5). Several patients also showed involvement of the femoral quadriceps muscles, while a lesser degree of degeneration was observed in the adductor longus of two patients and in the sartorius muscle of another patient. In conclusion, both proximal and distal muscle involvement was seen on T1w axial MR images. The hamstring muscles were the most severely affected muscles of the thighs, while the anterior compartment of the lower legs showed more severe pathology than the posterior compartment.

DISCUSSION

We report on a cohort of patients with distal myopathy of Serbian ancestry. Our results show that recessive variants in the TTN gene, notably the common founder variant c.107635C>T, p.(Gln35879Ter), are responsible for the fairly uniform and recognizable phenotype resembling that of TMD.\(^6\)

Our findings support an autosomal recessive mode of inheritance for the distal myopathy associated with the common TTN variant c.107635C>T. Segregation analysis within families of our patients showed that a heterozygous state of the c.107635C>T variant is insufficient to cause the disease. We showed that novel TTN variants found in a compound heterozygous state are pathogenic in combination with the common variant. The common variant c.107635C>T is located in the Mex5 (362) exon, encoding a part of the M-line region of titin, while other observed variants are located in the Mex1 (358) exon, encoding also a part of the M-line region, and in exons encoding either A- or I-band regions of titin (Table 1). To our knowledge, only one Mex5 variant associated with TMD has been reported in two generations (mother and son) of French family C.\(^9\) Originally it was described as a dominant variant probably arising \emph{de novo} in the mother,\(^9\) but later two different second TTN variants, located in exons encoding the A-band region of titin, were identified in these patients.\(^12\) Only a few variants outside the Mex5 and Mex6 exons have been associated with TMD or LGMD2J, and all have been described as a second variant in patients bearing Mex5 or Mex6 variants and presenting with more severe, complex or unusual phenotypes,\(^12\) although many of them have been reported in other titinopathies.\(^3\) It has been suggested that sequencing of the last six TTN exons (Mex1–6), encoding the M-line part of titin, should be performed in patients with a TMD phenotype,\(^1\) which is in accordance with the identification of the common variant in Mex5 and the three variants in Mex1 in our cohort.

The observed common haplotype shared by the chromosomes harbouring the c.107635C>T variant, with the 1 Mb core haplotype, favours the hypothesis of a single founder event for this variant. The estimated age of the c.107635C>T variant in our sample is about 800–1000 years. Although speculative, this estimation suggests that the most recent common ancestor probably lived in Medieval Serbia. As all analysed patients are of Serbian origin from present-day countries of Serbia and Bosnia and Herzegovina, it can be speculated that the founder variant might have arisen around the time when the majority of the Medieval Serbian principalities were united in a state called Raška (Rascia), which then resulted in the establishment of Serbia in the Balkans. Considering a broad 95% CI and inability to estimate the true variant age by the applied single locus method, it may not be excluded that the founder event might have happened earlier, but probably after the South Slavs (including Serbs) settled in the Balkan during the Early Middle Age. The chromosomes with the second founder variant c.103360delG share the more extended common haplotype (at least 7.4 Mb) in comparison with the common c.107635C>T variant, suggesting that it is a more recent founder event. It can be expected for both founder variants, particularly for the common one, to be found elsewhere in the Balkan countries and possibly also in other populations.

The apparent high frequency of the founder c.107635C>T variant, associated with a rare disease resembling TMD in a country with a small population size, probably makes it the most common cause of distal myopathy in Serbian patients. This finding, together with the better described phenotype, may facilitate genetic testing, which should be incorporated at an early stage in diagnostic algorithms for Serbian patients with distal myopathy.

Salient phenotypical features in our cohort are predominant lower limb involvement and prominent weakness of distal muscles, especially the ankle and toe dorsiflexors. Despite the predominance of distal involvement, mild to moderate weakness of proximal leg
muscles was observed in most patients and should be added as one of the hallmarks of the disease. Asymmetry was not an uncommon feature. Sparing of hand muscles despite the disease duration is worth mentioning as well. Involvement of facial or bulbar muscles was not observed. Our results did not suggest gender predominance or differences in disease course or severity between female and male patients.

Clear similarities with TMD suggest that the disease in our patients represents one part of the spectrum of the same disorder. On the other hand, some differences should be highlighted. TMD is an autosomal

Figure 3. Lower limb involvement in patients with TTN variants. Muscle atrophy is most pronounced in the anterior compartment of the lower legs, but can also be seen in the posterior compartment of the lower legs and in the hamstring muscles. The two upper images show the legs of patient no. 01 and the lower images the legs of patient no. 11.

Figure 4. Histological findings in muscles biopsies of patients with TTN variants. Haematoxylin and eosin staining of muscle tissue from patient nos 01, 10 and 14. Biopsies showed an increase in internal nuclei (a), dystrophic changes with significant fibrous and fat tissue replacement, but without predominance of muscle fibre types (b). For comparison, the biopsy of the youngest patient shows minor fibre size variation and an increase in connective tissue only (c).
dominant disorder, and homozygosity for the variant shows a different, more severe phenotype of LGMD2J. Our homozygous patients for the common variant c.107635C>T did not show specific phenotypical features or differences in disease severity. They did show later onset of the disease, in their 40s, which is similar to the onset described for TMD. For the majority of our patients disease onset was in their 20s. In addition, affection of the hip and shoulder girdle, not typically seen in TMD, was one of the prominent phenotypical features in our cohort.

Previously atypical TMD phenotypes were explained by compound heterozygosity in patients with the FINmaj TTN variant. There is a lot more to be understood on how and why different TTN variants contribute to the phenotype. The molecular mechanisms underlying distal myopathies caused by TTN variants are far from fully understood, which makes interventional therapy strategies more challenging. All but one of our patients remained ambulant throughout the follow-up period. This form of titinopathy is unlikely to result in wheelchair dependency before the sixth decade in the majority of patients.

Figure 5 Thigh and calf muscles of six patients with titinopathy assessed by T1w MRI. For all patients one axial image of the lower legs and two images of the thigh muscles are shown. (a) In patient no. 01, the hamstring muscles were severely affected (asterisk) and there was notable asymmetry in pathology of the calf muscles (arrow). (b) Patient no. 03 showed both hamstring (asterisk) and femoral quadriceps muscle involvement (arrow). Both anterior and posterior compartments of the lower legs were asymmetrically affected. (c) There was marked atrophy in an obese patient. (d) In patient no. 09, the semimembranosus muscle was mildly affected (arrow), while in the lower legs the tibialis anterior, extensor digitorum longus and peroneus muscles were severely affected (asterisk) and the posterior compartment spared. (e) Patient no. 13 showed a typical pattern of affected hamstring (asterisk) and tibialis anterior muscles (arrow). (f) The youngest patient, no. 14, with short disease course and normal MRI of muscles.

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patients, but bearing in mind that the oldest patient was 53 years old when last seen, further follow-up will be needed. Life expectancy seems not to be diminished, considering that no heart or respiratory muscle involvement has been observed, except for cardiomypathy in one patient.

In addition to the clinical diagnosis, a normal to mildly elevated serum CK level, a myopathic EMG, normal NCVs, normal cardiac and respiratory assessments and a dystrophic muscle biopsy are all helpful, although unspecific tools, in establishing the diagnosis.

Muscle MRI could be a helpful diagnostic tool. The selective pattern of involvement can vary between patients, but we found prominent muscle pathology in the anterior compartment of the lower legs together with involvement of the calf and of the hamstring muscles. This pattern is similar to the MRI pattern described in patients with autosomal dominant TMD. However, we noted that the extent and localisation of muscle pathology was variable depending on the stage of the disease and that fatty replacement of muscle tissue was also seen in some muscles not typical for TMD, such as the peroneal muscles.

In this study, we presented a distinct distal myopathy phenotype found in a Serbian patient cohort, which may facilitate the diagnosis of this condition in other patients. Population studies are still to be performed, but it is already clear that the common TTN founder variant explains a sizable portion of distal myopathy patients from Serbia and may represent the most common single cause of distal myopathy in patients of Serbian origin. It is now important to establish prevalence data of this disease in Serbia, as well as in other surrounding Balkan countries to adjust the diagnostic algorithm. One of the earliest diagnostic steps in patients with distal myopathy should be variant screening of the common founder TTN variant.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on European Journal of Human Genetics website (http://www.nature.com/ejhg)
Appendix B.
A homozygous DPM3 mutation in a patient with alpha-dystroglycan-related limb girdle muscular dystrophy

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Abstract

Defects of O-linked glycosylation of alpha-dystroglycan cause a wide spectrum of muscular dystrophies ranging from severe congenital muscular dystrophy associated with abnormal brain and eye development to mild limb girdle muscular dystrophy. We report a female patient who developed isolated pelvic girdle muscle weakness and wasting, which became symptomatic at age 42. Exome sequencing uncovered a homozygous c.131T > G (p.Leu44Pro) substitution in DPM3, encoding dolichol-P-mannose (DPM) synthase subunit 3, leading to a 50% reduction of enzymatic activity. Decreased availability of DPM as an essential donor substrate for protein O-mannosyltransferase (POMT) 1 and 2 explains defective skeletal muscle alpha-dystroglycan O-glycosylation. Our findings show that DPM3 mutations may lead to an isolated and mild limb girdle muscular dystrophy phenotype without cardiomyopathy.

Keywords: Limb girdle muscular dystrophy; Alpha-dystroglycan; Dolichol-P-mannose synthase; DPM

1. Introduction

Limb girdle muscular dystrophies (LGMD) represent an increasingly large and heterogeneous group of autosomal dominant and recessive disorders. In many patients, the molecular origin remains unknown and next generation sequencing has become a very important tool to hasten the genetic diagnosis and to identify variants and mutations in genes not previously associated with LGMD. Here, we report an adult female patient with autosomal recessive LGMD (LGMD2), in whom exome sequencing by inclusion in the MYO-SEQ project (Newcastle upon-Tyne, UK) revealed a homozygous substitution in DPM3, encoding dolichol-P-mannose (DPM) synthase subunit 3.

2. Case report

The patient’s medical history was uneventful. Early motor developmental milestones normally acquired and the patient started walking at the age of 1 year. At age 30 years, the patient presented with right-sided painful brachial plexopathy. Otherwise, the clinical neurological examination was normal. The neurological work-up was indicative of an inflammatory origin and the patient was diagnosed with neuralgic amyotrophy. She recovered within a few weeks. Surprisingly, serum creatine kinase (CK) activity was elevated at 4310 IU/L (N < 200). A deltoid muscle biopsy only showed mild nonspecific myopathic changes. At age 42, the patient had difficulty rising from a chair and developed an unsteady gait with tendency to fall. Because of persistingly high CK levels (2732 IU/L), a quadriceps muscle biopsy was performed, which showed a dystrophic pattern and alpha-dystroglycan (aDG) deficiency as demonstrated by immunoblotting with a IIH6C4 antibody at 1:500 dilution (Millipore SA, Overijse, Belgium).
This led to the diagnosis of aDG-related LGMD. No mutations in FKRP were found. At age 57, proximal lower limb weakness had clearly progressed. The patient could not get up from a chair without using her hands and had difficulty going upstairs. Manual muscle testing (MRC grades) showed the following abnormalities: gluteus maximus 0/5; iliopsoas and quadriceps 3/5; gluteus medius and adductors 2/5; hamstrings 4/5, tibialis anterior, tibialis posterior, peroneus longus, and triceps surae 3/5. Gowers sign was positive. Deep tendon reflexes, cranial nerves and sensation were normal. Brain MRI, respiratory, cardiac and ophthalmologic work-up were unremarkable. Nerve conduction studies were normal. EMG showed brief small amplitude polyphasic motor unit action potentials with early recruitment in the iliopsoas muscle. Muscle MRI results are shown in Fig. 2. There is no family history of neuromuscular disease. The mother died at age 94 of pancreas carcinoma. The father is healthy at age 95. One brother died at age 58 of intestinal cancer, one sister at age 39 of a brain tumour. Two other sisters and 2 sons are healthy. Serum CK levels were normal in the father and the 2 sisters.

3. Molecular analysis

Exome sequencing by inclusion into MYO-SEQ (Newcastle University, Newcastle upon-Tyne, UK) was performed at the Broad Institute’s Genomics Platform, using Illumina exome capture. A homozygous c.131T > G (p.Leu44Pro) substitution was identified in DPM3 (gene coverage 95%). DPM3 encodes DPM synthase subunit 3. This change is extremely rare in the control population (MAF = 0.00084%) and only found in the heterozygous state. Leu44 is an evolutionary highly conserved amino acid and a change to proline is predicted to be pathogenic by in silico tools (Mutation Taster, PolyPhen, UMD-Predictor and SIFT). Segregation studies identified heterozygosity in the father and in one of the two sisters. Maternal DNA was not available. Transferrin isoelectric focusing as well as mass spectroscopy of transferrin N-glycans in serum [1] were normal, indicating that N-glycosylation was well preserved in liver and serum. DPM synthase activity was analysed according to Barone et al [2] by measuring the formation of radio-active DPM in cultured fibroblasts and was reduced by 50% as compared to a healthy control.

4. Discussion

We report a patient with mild LGMD2 and without central nervous system involvement, caused by a homozygous substitution in DPM3. This gene encodes DPM synthase subunit 3 and we found that the enzymatic activity of DPM synthase was reduced by 50%. DMP synthase is an enzyme complex composed of 3 protein subunits, DPM1, DPM2, and DPM3. Whereas DPM2 stabilises the complex, DPM3 tethers...
the catalytic subunit (DPM1) to the endoplasmic reticulum membrane [3]. DPM synthase catalyses the synthesis of DPM from GDP-mannose and dolichol phosphate. As DPM is an essential donor substrate required in different glycosylation pathways (N-glycosylation, C- and O-mannosylation, GPI-anchor formation) [4,5], it is not surprising that O-mannosylation of aDG, catalysed by protein O-mannosyltransferase (POMT) 1 and 2, is compromised when one of the subunit-encoding genes is mutated. This was first reported in an 11-year-old female patient with mild LGMD2 and a 254T>C (p.L85S) mutation in DPM3 [6]. At age 20, she developed dilated cardiomyopathy and at age 21, she had a stroke-like episode involving the right temporo-parietal region with normal brain MRI. In addition to abnormal N-glycosylation, deficient O-mannosylation of aDG was confirmed in a muscle biopsy and the disorder was classified as DPM3-CDG. Later, Barone et al [2] reported 3 children from 2 families with DPM2 mutations with profound developmental delay, intractable seizures, microcephaly, and early fatal outcome. The patients had aDG-deficient congenital muscular dystrophy. DPM1 mutations have been reported in 7 cases with various degrees and combinations of early onset encephalopathy, seizures, microcephaly, dysmorphic features, developmental delay, optic atrophy, and cerebellar dysfunction [7–10]. These were classified as Congenital Disorders of Glycosylation (CDG) type I (DPM1-CDG) due to abnormal N-glycosylation. In 5 of these patients, CK levels were elevated but evidence of muscular dystrophy was not reported. In 2013, Yang et al [11] reported an infant with DPM1 mutations and showed deficient O-mannosylation of aDG as well, presenting with aDG-deficient congenital muscular dystrophy with seizures but otherwise minimal central nervous system involvement on MRI only. Our findings show that abnormal aDG O-mannosylation related to DPM3 mutations may lead to LGMD2 phenotype without cardiomyopathy or central nervous system involvement and with presumably normal N-glycosylation.

References


Appendix C.
Identification of GAA variants through whole exome sequencing targeted to a cohort of 606 patients with unexplained limb-girdle muscle weakness

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Abstract

Background: Late-onset Pompe disease is a rare genetic neuromuscular disorder caused by a primary deficiency of α-glucosidase and the associated accumulation of glycogen in lysosomal vacuoles. The deficiency of α-glucosidase can often be detected using an inexpensive and readily accessible dried blood spot test when Pompe disease is suspected. Like several neuromuscular disorders, Pompe disease typically presents with progressive weakness of limb-girdle muscles and respiratory insufficiency. Due to the phenotypic heterogeneity of these disorders, however, it is often difficult for clinicians to reach a diagnosis for patients with Pompe disease. Six hundred and six patients from a European population were recruited onto our study. Inclusion criteria stipulated that index cases must present with limb-girdle weakness or elevated serum creatine kinase activity. Whole exome sequencing with at least 250 ng DNA was completed using an Illumina exome capture and a 38 Mb baited target. A panel of 169 candidate genes for limb-girdle weakness was analysed for disease-causing variants.

Results: A total of 35 variants within GAA were detected. Ten distinct variants in eight unrelated index cases (and four siblings not sequenced in our study) were considered disease-causing, with the patients presenting with heterogeneous phenotypes. The eight unrelated individuals were compound heterozygotes for two variants. Six patients carried the intronic splice site c.-13 T > G transversion and two of the six patients also carried the exonic p.Glu176ArgfsTer45 frameshift. Four of the ten variants were novel in their association with Pompe disease.

Conclusions: Here, we highlight the advantage of using whole exome sequencing as a tool for detecting, diagnosing and treating patients with rare, clinically variable genetic disorders.

Keywords: Whole exome sequencing, Sequence variants, Pompe disease

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Background
Pompe disease (OMIM 232300) is a rare autosomal recessive lysosomal storage disorder that most prominently affects muscle tissue. The disease is generally classified into two broad categories: an infantile- and a late-onset form. Patients with the infantile-onset form of the disease typically present with generalised weakness, hypotonia, respiratory distress and cardiomyopathy, and without intervention do not survive beyond 12 months of age [1]. Late-onset Pompe disease is more clinically heterogeneous [2], yet often displays a central characteristic manifestation of a slowly progressive proximal myopathy. This occurs with respiratory weakness and elevated serum creatine kinase activity, while there is normally no clinically relevant cardiac involvement [3]. Estimates of the frequency of all cases of Pompe disease vary, but have been reported to be as high as approximately 1:40,000 [4, 5]. The late-onset classification of the disease is even rarer at a frequency of 1:57,000 [5].

The varied clinical spectrum can be attributed to the many different genetic mutations that are associated with Pompe disease; to date, the associations of over 522 variants have been reported and collated by the Pompe Disease Mutation Database [6]. Such mutations occur within the GAA gene and result in a deficiency of the encoded lysosomal enzyme, acid α-glucosidase, which is essential for glycogen hydrolysis. The accumulation of glycogen within lysosomes subsequently impairs the correct functioning of the organelles and the affected tissue, primarily skeletal and cardiac muscle [7], and results in the clinical presentation of Pompe disease.

Considering the rarity and variability of the disorder, a correct clinical diagnosis is often difficult to achieve, and so many patients are therefore not treated with an efficacious disease-modifying enzyme replacement therapy (ERT) in a timely manner. ERT, a recombinant human acid α-glucosidase termed alglucosidase alfa, has been reported to extend survival in infantile Pompe disease [8] and ameliorate disease progression of the late-onset form [9]. As prompt diagnosis and treatment is beneficial to patient survival [10], a more robust investigatory approach is required to detect and diagnose affected individuals in the early stages of the disease.

Exome sequencing is a useful tool to interrogate the proportion of the genome that is enriched for functional coding variants, specifically those that are able to disrupt protein structure and function [11]. This unbiased analysis enables the detection of distinct mutations in patients with overlapping phenotypes, overall expanding existing genotype-phenotype correlations. Exome sequencing, therefore, has the benefit of furthering the understanding of disease pathology, offering accurate diagnoses where the traditional methodologies of clinical examinations failed to do so, and in some cases also enabling a prompt intervention in the disease progression.

The MYO-SEQ project was established in 2014 and aimed to use whole exome sequencing to (i) contribute to the diagnostic pathway of patients affected by limb-girdle muscular weakness, (ii) improve the diagnostic awareness of rare genetic neuromuscular diseases, and (iii) speed up the integration of next-generation sequencing technologies into healthcare [12]. We screened 606 patients with unexplained limb-girdle weakness for potentially pathogenic variants in 169 genes that are known to be associated with muscle disease. Here, we report on the characterisation of twelve European patients (eight index cases and four siblings) who harbour disease-associated variants within GAA as identified through the MYO-SEQ project.

Methods
Patient recruitment and inclusion criteria for whole exome sequencing
Ethical approval was granted by the Newcastle and North Tyneside research ethics committee (REC reference number 09/H0906/28) and by the local ethical committees of the participating centres. A standardised form for collecting detailed phenotypic information was created using the PhenoTips online software tool [13]: this was completed by the referring clinician for each patient enrolled onto the project. Informed written consent was given by the patients, who were anonymised by the collaborating centres by using unique MYO-SEQ patient identification codes. The fundamental requirement for inclusion in the project was that of unexplained limb-girdle muscle weakness and/or elevated serum creatine kinase activity.

Lysis of whole blood cells
Blood samples were taken from each patient using an EDTA Vacutainer™ Safety-Lok™ system (BD Biosciences, UK). Two buffers were prepared prior to cell lysis: Buffer A (pH 8.0; 10 mM Tris-HCl, 320 mM sucrose, 5 mM MgCl₂, 1% Triton X-100) and Buffer B (pH 8.0; 400 mM Tris-HCl, 0.5 M EDTA pH 8.0, 150 mM NaCl, 1% SDS). Five millilitres (ml) of whole blood was mixed with 40 ml of Buffer A for 4 minutes (min) at room temperature before centrifugation at 3000 revolutions per minute (rpm) for 10 min. The cell pellet was resuspended in 20 ml Buffer A before the mixing and centrifugation steps repeated once more. The pellet was resuspended in 2 ml Buffer B, mixed for 10 min at room temperature with 500 µl sodium perchlorate, and incubated at 65 °C for 25 min with regular vortexing.

Nucleic acid extraction from lysed whole blood cells
The preparation of lysed cells was mixed for 10 min at room temperature with 2 ml ice cold chloroform followed by centrifugation at 4 °C for 10 min at 3000 rpm. The
The detected variants were matched to the patient’s phenotype and those that were most likely to be disease-causing were reported back to the referring clinician. A positive diagnosis of Pompe disease was sought by the clinicians by quantifying α-glucosidase activity using dried blood spots (DBS) or fibroblasts, and/or performing Sanger sequencing to independently confirm the detected variants.

Results

Detection of variants within GAA

Of the 606 patients whose exomes were sequenced, 268 (44%) were female and 338 (56%) were male; their ages ranged from 4 years to 88 years (mean 40.1 years, median 40.0 years). In the first instance, a search of the GAA exons of all 606 patients was performed to identify those who harboured rare (<1%) coding variants. A total of 34 distinct coding variants were identified (Table 1) in 35 unrelated individuals. Of these variants, ten were synonymous and so were not considered potentially disease-causing. Of the remaining 24 variants, 16 were missense, three were frameshift, one created a stop codon and four affected the splice site regions of the gene. Eight of the 24 variants were novel, meaning they did not occur in the ExAC control population [14]. Finally, six of the 24 variants, three of which were novel, have been previously listed in the Pompe Disease Mutation Database [6] and so have a known association with the disease.

Since there were no homozygous coding variants that could in isolation account for the phenotype of the patients, and in fact an extremely common GAA mutation in Pompe disease is intronic [23], we extended the search to include flanking regions that were captured by the exome sequencing. As a result, the common intronic c.-32-13 T > G variant was detected and was considered potentially pathogenic when in combination with either a coding or an already reported pathogenic variant (Table 1).

Analysis of GAA variants and classification of disease-causing mutations

It was next necessary to interrogate the 22 patients that accounted for the 24 coding and one intronic GAA variants that were potentially disease-causing. Patients 3, 5, 7, 8, 18, and 32 all harboured the intronic c.-32-13 T > G variant in addition to a coding variant within GAA, which generated a compound heterozygous haplotype that could be responsible for α-glucosidase deficiency. Patients 17 and 35 carried two coding variants: patient 17 carried one frameshift and one missense, both of which were absent in the ExAC control population [14], and patient 35 harboured two missense variants, one of which was absent in the ExAC population [14] and the other previously reported in the Pompe Disease Mutation Database [6]. Patient 21 harboured a missense variant that was predicted to be damaging, while a second
### Table 1: GAA variants detected in the patients sequenced by the MYO-SEQ project

<table>
<thead>
<tr>
<th>Patient</th>
<th>Chromosome</th>
<th>Coding</th>
<th>Protein</th>
<th>Reported</th>
<th>Polymorphism</th>
<th>Variant</th>
<th>Predicted deleteriousness</th>
<th>ClinVar clinical significance</th>
<th>ExAC v3 allele frequency</th>
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<tr>
<td>17*</td>
<td>chr17:78,092,521 c.2716G &gt; A</td>
<td>p.Val906lle</td>
<td>No</td>
<td>–</td>
<td>Missense</td>
<td>Tolerated</td>
<td>Benign</td>
<td>Disease-causing</td>
<td>Damaging</td>
</tr>
<tr>
<td>22</td>
<td>chr17:78,092,562 c.2757C &gt; T</td>
<td>–</td>
<td>No</td>
<td>–</td>
<td>Synonymous</td>
<td>No data</td>
<td>No data</td>
<td>No data</td>
<td>No data</td>
</tr>
<tr>
<td>3, 5, 7, 8, 18, 32</td>
<td>chr17:78,078,341 c.-32-13 T &gt; G</td>
<td>–</td>
<td>Yes</td>
<td>–</td>
<td>Intronic</td>
<td>No data</td>
<td>No data</td>
<td>No data</td>
<td>No data</td>
</tr>
</tbody>
</table>

Rows 1-34: all rare (< 1%) coding variants detected. Those highlighted with an asterisk (*) were classified as disease-causing and occurred in combination with the c.-32-13 T > G intronic variant for all but patients 17 and 35. Row 35: intronic c.-32-13 T > G transversion considered to contribute to disease pathology. Reported variants are listed in the Pompe Disease Mutation Database [6].
variant affected a splice site, neither of which were novel: this was not considered a likely cause of the patient’s phenotype. The ten variants considered disease-causing are detailed in Fig. 1, and the summarised patient information is detailed in Table 2. The remaining 14 patients only carried one heterozygous variant, meaning it was highly probable that α-glucosidase deficiency was not the cause of their disorder.

**GAA variants were associated with a highly varied clinical spectrum**

There was a wide variability in the presenting symptoms between each of the eight patients, despite the variants all affecting the same gene. This was particularly notable for patients 18 and 32 who both carried the same GAA variants, c.525delT and c.-32-13 T > G. Patient 18 had a slowly progressive phenotype after the onset of symptoms in her fifth decade of life, displaying proximal muscle weakness and a serum creatine kinase activity of 599 U/L (N < 170 U/L). The patient also showed a myopathic electromyogram (EMG). A muscle biopsy was indicative of a myofibrillar myopathy, with numerous vacuolated, degenerated and atrophic fibres confirmed by NADH-tetrazolium reductase staining. The periphery of the vacuoles had an abnormal immunoreactivity for desmin and p62. The fibre type proportions and distributions appeared normal; however an ultrastructural examination displayed Z-band streaming with an accumulation of granular material. Glycogen accumulation in the vacuoles was noted, but the overall appearance was not suggestive of a glycogenosis, while a DBS test revealed α-glucosidase activity to be within the normal to lower ranges. Following our report of the GAA variants to the collaborating centre, this patient has since been referred for ERT.

In contrast, patient 32 had a non-progressive phenotype after the onset of his symptoms in childhood. His serum creatine kinase activity was persistently elevated; when first investigated at age 15 years of age it was 662 U/L (N < 190 U/L). The patient had no cardiac involvement nor exhibited extreme respiratory distress, although a 13% reduction between sitting and supine forced vital capacity was observed. The patient described only slight dyspnoea following physical exertion, but otherwise considered himself healthy. Both a muscle biopsy and an EMG were mildly myopathic. A DBS test displayed reduced enzymatic activity, while Sanger sequencing of the variants confirmed Pompe disease. Additionally, the reduction in α-glucosidase activity and carriage of the variants were confirmed in his two siblings. Both, however, are currently asymptomatic with elevated serum creatine kinase levels only (563 U/L and 1331 U/L for his 10 year old sister and 23 year old brother, respectively). ERT is scheduled for the index case, and all three of these family members will be closely monitored.

A further example to highlight the varied clinical presentations was seen in patient 3. This individual displayed a slowly progressive proximal lower limb and axial weakness with the onset of symptoms in his third decade of life. There was no respiratory insufficiency and an EMG showed no abnormalities. Despite normal cardiac function, the patient was previously diagnosed with Brugada syndrome, a genetic cause for abnormal electrocardiogram findings that are associated with an increased risk of sudden cardiac death. Serum creatine kinase activity was measured at 1729 U/L (N < 190 U/L). The two GAA variants were confirmed by the collaborating centre using Sanger sequencing, while a reduced enzymatic activity was confirmed using a DBS test. Since the diagnosis of Pompe disease in the index case, he was started on ERT and his asymptomatic brother, with only an elevated serum creatine kinase activity, was also diagnosed with the disease. This clearly demonstrates that

**Fig. 1** Ten distinct variants within GAA were identified as disease-causing. One variant was intronic and nine were exonic. Six patients were heterozygous for the intronic c.-32-13 T > G variant in addition to an exonic variant and two patients were heterozygous for two exonic variants.
Table 2 Demographic information and reported clinical presentations of eight patients with causal variants in GAA

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Ethnicity</th>
<th>Variant 1</th>
<th>Variant 2</th>
<th>Pace of progression</th>
<th>Onset</th>
<th>Serum creatine kinase</th>
<th>Weakness</th>
<th>Respiratory insufficiency</th>
<th>Referred for treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Male</td>
<td>German</td>
<td>c.2331 + 2 T &gt; A</td>
<td>c.-32-13 T &gt; G</td>
<td>Slow progression</td>
<td>Young adult</td>
<td>Increased less than 10x</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>Male</td>
<td>Romanian</td>
<td>c.2066_2070dupAGCCG</td>
<td>c.-32-13 T &gt; G</td>
<td>Slow progression</td>
<td>Middle age</td>
<td>Increased more than 10x</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>7</td>
<td>Female</td>
<td>Serbian</td>
<td>c.2269C &gt; T</td>
<td>c.-32-13 T &gt; G</td>
<td>Slow progression</td>
<td>Middle age</td>
<td>Increased less than 10x</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>8</td>
<td>Female</td>
<td>Serbian</td>
<td>c.2051C &gt; G</td>
<td>c.-32-13 T &gt; G</td>
<td>Slow progression</td>
<td>Late onset</td>
<td>Normal</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>17</td>
<td>Male</td>
<td>Caucasian</td>
<td>c.1192delC</td>
<td>c.2716G &gt; A</td>
<td>Progressive</td>
<td>Young adult</td>
<td>Normal</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes (now ceased)</td>
</tr>
<tr>
<td>18</td>
<td>Female</td>
<td>White British</td>
<td>c.525delT</td>
<td>c.-32-13 T &gt; G</td>
<td>Slow progression</td>
<td>Middle age</td>
<td>Increased more than 10x</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>32</td>
<td>Male</td>
<td>Caucasian</td>
<td>c.525delT</td>
<td>c.-32-13 T &gt; G</td>
<td>Non-progressive</td>
<td>Childhood</td>
<td>Increased less than 10x</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>35</td>
<td>Male</td>
<td>Caucasian</td>
<td>c.569G &gt; A</td>
<td>c.2020C &gt; G</td>
<td>Progressive</td>
<td>Young adult</td>
<td>Increased less than 10x</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>
related individuals diagnosed with the same genetic disease can present with a varied phenotypic spectrum.

Patient 5 initially presented with back pain and elevated serum creatine kinase levels (1500 U/L), and retrospectively recognised mild proximal upper limb weakness when raising a load. The disease course progressed over the following nine years, with asymmetrical proximal weakness becoming more obvious. At hospitalisation, a reduced enzyme activity was observed in the patient and also in his daughter. Only two patients were not started on ERT: patient 7 lives in another country to where she was enrolled onto the project and patient 8 is in her mid-70s and is not ambulant.

Comparison of compound heterozygous patients at two exonic GAA variants
Patient 17 harboured two novel coding variants within GAA, c.1192delC and c.2716G > A, neither of which have been listed in the Pompe Disease Mutation Database [6]. Importantly, however, c.1192delC results in a frameshift mutation at the same position as a reported pathogenic duplication [24]. The patient presented with progressive proximal muscle weakness, pain and fatigability in his fourth decade of life, but with normal levels of serum creatine kinase activity. He also displayed weakness of the back muscles and had notable respiratory dysfunction: his forced vital capacity was reduced to 2.41 (47% predicted normal value) in the sitting and to 2.0 l (39% predicted normal value) in the lying position. An EMG detected mild myopathic changes and the muscle biopsy showed increased variability in fibre diameter without any abnormal glycogen accumulation. The patient had two DBS tests: the first suggested a reduction in α-glucosidase activity, while the second detected normal levels of enzyme activity. A subsequent analysis of α-glucosidase in lymphocytes and fibroblasts revealed borderline and low normal activity, respectively. It is likely, therefore, that c.2716G > A may be a weaker mutation, and so only slightly affects enzyme activity. The variants were independently confirmed by the collaborating centre before ERT was initiated for the patient. At the start of ERT, the patient achieved 100 m in a six minute walk test, which marginally increased to 105 m after 9 months of alternating weekly administrations of 20 mg/kg Myozyme®. Measures of respiratory muscle strength similarly remained unchanged; from a vital capacity of 45% to 47%, a maximal inspiratory pressure of 5.6 kPa to 5.8 kPa and a maximal expiratory pressure of 6.1 kPa to 5.4 kPa. The referring clinician observed a negligible effect on muscle pain and weakness – as might be expected with borderline enzymatic activities treated over such a short period – and so the patient was withdrawn from the regime. Eighteen months after ERT cessation, the status of the patient remained stable with a six minute walk test of 95 m.

Similarly, patient 35 carried two coding variants within GAA: c.569G > A and c.2020C > G, with the former recognised in the Pompe Disease Mutation Database [6] and the latter completely absent in the ExAC unaffected control population [14]. The patient presented in his fourth decade of life with rapidly progressive proximal lower limb weakness, difficulty in ascending stairs and an inability stand unaided from a supine position. His older sister is also affected with an earlier onset of her symptoms at 21 years of age. The patient now has scoliosis, fatigability, paraspinal muscle atrophy, and proximal upper limb atrophy and weakness. Paraclinical examinations revealed a myopathic EMG, a dystrophic biopsy and mildly elevated serum creatine kinase levels. A calpain deficiency was detected through immunoblot analysis and could be attributed to two heterozygous CAPN3 variants (p.Gly333Asp [c.998G > A] and p.Ala726Ser [c.2176G > T]). This suggested that the patient is likely to be affected by both LGMD2A and Pompe disease. The two GAA variants were independently confirmed and in contrast to patient 17, the unambiguous absence of glycogen was confirmed in the patient and in his sister. ERT is now being administered to both individuals.

Discussion
Whole exome sequencing is emerging as an affordable technology to investigate rare, monogenic diseases. Coding and functional regions account for only 1% of the entire human genome, yet harbour 85% of known disease-causing variants [25]. On this basis, we sequenced the exomes of a cohort of 606 patients with unexplained limb-girdle weakness. We examined a panel of 169 genes that were known to be associated with muscle diseases with Mendelian patterns of inheritance in a large cohort of European patients with unexplained limb-girdle weakness. We identified eight unrelated index cases and four siblings that had compound heterozygous mutations in GAA: these patients were likely to be affected by both Pompe disease, a rare lysosomal storage disorder that is commonly characterised by proximal muscle weakness [2]. The remaining 168 genes are currently under analysis and we are yielding similarly positive results for many other muscle diseases; so far the overall diagnostic rate for the project is 49%.

We have shown that next-generation sequencing is advantageous in the healthcare and diagnosis of patients suffering from unexplained limb-girdle muscle weakness. Other studies have shown similar benefits [26], achieving a correct diagnosis for patients with overlapping, and therefore indistinguishable, clinical phenotypes [27]. In a recent publication that interrogated the exomes of 504 patients of European descent, variants in GAA were considered pathogenic in 10 patients (1.9% of the cohort).
[28]. This is highly comparable to the 12 patients with GAA variants detected by MYO-SEQ. While such an approach helps better understand rare neuromuscular disorders, it was especially important in this study for the patients that suffered from treatable conditions such as Pompe disease. It is most beneficial to the affected individuals that ERT is initiated as soon as possible as the therapy acts to ameliorate disease progression [29]. Despite DBS tests being widely available, inexpensive and sensitive to changes specifically in α-glucosidase activity [30], they can sometimes fail to robustly detect subtle changes in enzyme activity. This is considered more common in later onset forms of the disorder, where the level of enzyme activity may be proportional to the age of onset [29]. Although reduced enzymatic activity was detected by DBS tests in 7.6% of patients in a cohort presenting with elevated serum creatine kinase and/or limb-girdle muscular weakness, late-onset Pompe disease was only confirmed in 2.4% of the patients [31]. DBS tests may therefore be inconclusive for many patients with a proximal muscle weakness phenotype. Such a discrepancy was exemplified in the clinical assessment of patient 18 who presented with the onset of symptoms in her fifth decade of life and normal to slightly lower α-glucosidase activity. Despite the borderline enzymatic activity levels detected, the individual was subsequently found to carry two reported pathogenic GAA variants. Therefore, exome sequencing offers an alternative, accurate and reliable methodology for rare disease diagnosis where traditional detection techniques may not be as efficient. We suggest that DBS should still be used as a first-tier diagnostic step when Pompe disease is suspected, however, diagnostic yields are influenced by the type and level of pre-screening, and so it is inaccurate to compare the outcomes of different approaches.

As Pompe disease is an autosomal recessive disorder, single heterozygous variants are unlikely to result in a depletion of α-glucosidase activity that is sufficient to cause a clinical phenotype. Accordingly, it has been found that many affected individuals carry compound heterozygous mutations rather than isolated heterozygous or homozygous variants [32–35]. This immediately allowed our analysis to be focussed on the patients that harboured two variants: of course, a second variant may still be missed if it resides in a deep intronic region not covered by WES. Nevertheless, of the nine compound heterozygous patients that we identified, we considered eight to carry variants that were sufficiently severe to cause Pompe disease. Four of the ten likely pathogenic variants that were identified in this study had never been previously reported in Pompe disease [6] and did not occur in the ExAC control population [14]. These variants that we present here – c.1192delC, c.2020C > G, c.2051C > G and c.2716G > A – are therefore novel in the understanding of Pompe disease.

As would be expected based on previous findings [23, 35], the intronic c.-32-13 T > G variant was the most frequent in this largely Caucasian population, and occurred in six compound heterozygous index cases. It is therefore essential that analyses of GAA gene sequences should be extended to flanking regions in order to capture such pathogenic intronic variants. This variant affects a splice site of the gene, and so the result is the production of alternative transcript isoforms and low levels of α-glucosidase activity. This can give rise to the typical spectrum of Pompe disease phenotypes depending on the haplotype it occurs in [36]. Overall, the specific mutations, their haplotypes, and their genetic positions are all key in determining enzymatic activity and thus, the clinical phenotype of patients affected by Pompe disease.

Conclusions

In summary, we have identified twelve individuals (eight index cases and four siblings) with compound heterozygous mutations in the GAA gene; four of the ten variants have not been previously reported. This study has expanded the existing genotype-phenotype correlations; aiding a deeper understanding of Pompe disease, the underlying genetic variations and the associated varied clinical presentation. We have shown the advantage of using next-generation sequencing in the diagnosis of a rare, treatable neuromuscular condition. As a result, patients have benefitted from a swifter administration of appropriate disease management. Our data suggest that exome sequencing is a reliable and accurate diagnostic tool and is able to detect pathogenic variants in patients for whom traditional clinical methodologies have failed.

Abbreviations

DBS: dried blood spot; EMG: electromyogram; ERT: enzyme replacement therapy; ExAC: Exome Aggregation Consortium; GAA: α-glucosidase encoding gene; min: minute; ml: millilitre; rpm: revolutions per minute

Acknowledgements

We thank the patients for donating their tissue samples.

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Availability of data and materials

The datasets generated and analysed during the current study are not publicly available in order to provide secure protection of personal material, but are available from the corresponding author on reasonable request.
Authors’ contributions
All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. VS had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study conception and design: VS. Funding: VS. Acquisition of data: KJ, AT, MB, LP, KGK, VRS, SP, AH, PM, EA, AEB, AL, AKL, ML, LX. Analysis and interpretation of data: KJ, AT, MB, LP.

Ethics approval and consent to participate
Full ethics approval was granted for the Newcastle MRC Centre Biobank for Rare and Neuromuscular Diseases by the Newcastle and North Tyneside research ethics committee (REC reference number 09/H0906/28). All patients provided informed consent for the storage and use of their biomaterial in the Newcastle MRC Centre Biobank for Rare and Neuromuscular Diseases.

Consent for publication
Not applicable.

Competing interests
VS is or has been a principal investigator for trials sponsored by Sanofi Genzyme, GSK, Prosensa/BioMarin Pharmaceuticals, Ionis Pharmaceuticals and Sarepta Therapeutics. VS received speaker honoraria from Sanofi Genzyme. For Genzyme, GSK, Prosensa/BioMarin Pharmaceuticals, Ionis Pharmaceuticals and VS is or has been a principal investigator for trials sponsored by Sanofi Genzyme, GSK, Prosensa/BioMarin Pharmaceuticals, Ionis Pharmaceuticals and VS is or has been on advisory boards for Audentes Therapeutics, Sarepta Therapeutics. VS received speaker honoraria from Sanofi Genzyme. For Genzyme, GSK, Prosensa/BioMarin Pharmaceuticals, Ionis Pharmaceuticals and VS is or has been on advisory boards for Audentes Therapeutics, Sarepta Therapeutics. VS is or has been on advisory boards for Audentes Therapeutics, Sarepta Therapeutics, Summit the last 3 years VS is or has been on advisory boards for Audentes Therapeutics, Sarepta Therapeutics. VS received speaker honoraria from Sanofi Genzyme. For Genzyme, GSK, Prosensa/BioMarin Pharmaceuticals, Ionis Pharmaceuticals and VS is or has been on advisory boards for Audentes Therapeutics, Sarepta Therapeutics. VS received speaker honoraria from Sanofi Genzyme. For Genzyme, GSK, Prosensa/BioMarin Pharmaceuticals, Ionis Pharmaceuticals and VS is or has been on advisory boards for Audentes Therapeutics, Sarepta Therapeutics.

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References


Appendix D.
RESEARCH PAPER

Limb girdle muscular dystrophy due to mutations in POMT2

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ABSTRACT

Background Mutations in the gene coding for protein O-mannosyl-transferase 2 (POMT2) are known to cause severe congenital muscular dystrophy, and recently, mutations in POMT2 have also been linked to a milder limb-girdle muscular dystrophy (LGMD) phenotype, named LGMD type 2N (LGMD2N). Only four cases have been reported so far. ClinicalTrials.gov ID: NCT02759302

Methods We report 12 new cases of LGMD2N, aged 18–63 years. Muscle involvement was assessed by MRI, muscle strength testing and muscle biopsy analysis. Other clinical features were also recorded.

Results Presenting symptoms were difficulties in walking, pain during exercise, delayed motor milestones and learning disabilities at school. All had some degree of cognitive impairment. Brain MRIs were abnormal in 3 of 10 patients, showing ventricular enlargement in one, periventricular hyperintensities in another and frontal atrophy of the left hemisphere in a third patient. Most affected muscle groups were hip and knee flexors and extensors on strength testing. On MRI, most affected muscles were hamstrings followed by paraspinal and gluteal muscles. The 12 patients in our cohort carried 11 alleles with known mutations, whereas 11 novel mutations accounted for the remaining 13 alleles.

Conclusion We describe the first cohort of patients with LGMD2N and show that unlike other LGMD types, all patients had cognitive impairment. Primary muscle involvement was found in hamstring, paraspinal and gluteal muscles on MRI, which correlated well with reduced muscle strength in hip and knee flexors and extensors. The study expands the mutational spectrum for LGMD2N, with the description of 11 novel POMT2 mutations in the association with LGMD2N.

Clinical trial registration NCT02759302.

INTRODUCTION

Recessive mutations in the gene coding for protein O-mannosyl-transferase 2 (POMT2) are known to cause severe congenital muscular dystrophies (CMD), such as Walker-Warburg syndrome and muscle–eye–brain disease. These conditions are characterised by structural brain and muscle involvement at birth and a low chance of survival past childhood. In 2007, mutations in POMT2 were linked to a milder limb-girdle muscular dystrophy (LGMD) phenotype, named LGMD type 2N (LGMD2N).1 LGMD is a group of heterogeneous diseases characterised by wasting and weakness of the muscles of the shoulder and hip region.2

The POMT2 protein forms a complex with protein O-mannosyl-transferase 1 (POMT1), and catalyses the first step in the synthesis of O-mannosyl glycan, located on the extracellular protein, α-dystroglycan. Alpha-dystroglycan (α-DG) is part of the dystrophin-associated glycoprotein complex at the sarcolemma. Here, it forms an essential link between the subsarcolemmal cytoskeleton and the extracellular matrix of the muscle cells, and plays an important role in membrane integrity and force transmission.3

Only four young patients (age 4–18 years) with a LGMD2N phenotype have been reported as separate cases so far.4–6 Thus, detailed knowledge about the disease characteristics is lacking for LGMD2N. Through an international collaboration between seven clinical centres, and facilitated by the use of next-generation sequencing (NGS) in unclassified myopathies with limb girdle weakness, we have identified a group of 12 patients affected by LGMD2N. We report on the specific clinical features of this rare form of LGMD, extend the mutational spectrum and describe findings on MRI of brain and muscle as well as immunohistochemical analyses on muscle biopsies.

METHODS

All patients consented to participate.

Subjects

Patients affected by LGMD2N were identified primarily by NGS in cases with undiagnosed limb girdle and in a few cases by direct Sanger sequencing. Twelve patients with genetically verified LGMD2N were included in the study (table 1). Two of the patients (cases 1 and 2) were siblings of consanguineous parents, and cases 9 and 10 were siblings of non-consanguineous parents. All other patients were unrelated.
<table>
<thead>
<tr>
<th>Case 1*</th>
<th>Case 2*</th>
<th>Case 3</th>
<th>Case 4</th>
<th>Case 5</th>
<th>Case 6</th>
<th>Case 7</th>
<th>Case 8</th>
<th>Case 9†</th>
<th>Case 10†</th>
<th>Case 11</th>
<th>Case 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender/age (years)</td>
<td>Woman/25</td>
<td>Man/20</td>
<td>Woman/51</td>
<td>Man/52</td>
<td>Man/54</td>
<td>Man/41</td>
<td>Woman/18</td>
<td>Woman/18</td>
<td>Woman/18</td>
<td>Man/29</td>
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</tr>
<tr>
<td>Phenotype</td>
<td>LGMD</td>
<td>LGMD</td>
<td>LGMD</td>
<td>LGMD</td>
<td>CMD/LGMD</td>
<td>LGMD</td>
<td>CMD/LGMD</td>
<td>LGMD</td>
<td>LGMD</td>
<td>LGMD</td>
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</tr>
<tr>
<td>Disease onset (years)</td>
<td>2</td>
<td>5</td>
<td>4</td>
<td>8</td>
<td>From birth</td>
<td>From birth</td>
<td>From birth</td>
<td>From birth</td>
<td>4</td>
<td>2</td>
<td>55</td>
</tr>
<tr>
<td>Presenting symptoms</td>
<td>Difficulties walking and running</td>
<td>Difficulties walking and running</td>
<td>Learning difficulties, oriented to special school</td>
<td>Difficulties in learning mathematics and science</td>
<td>Delay in cognitive development</td>
<td>Delay in cognitive development</td>
<td>Congenital, delay in development</td>
<td>Congenital, delay in development</td>
<td>Difficulties walking and running</td>
<td>Difficulties walking and running</td>
<td>Weakness of lower legs</td>
</tr>
<tr>
<td>Age at muscle MRI (years)</td>
<td>25</td>
<td>20</td>
<td>49</td>
<td>49</td>
<td>54</td>
<td>38</td>
<td>17</td>
<td>18</td>
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<td>Arab (DK)</td>
<td>CA (FR)</td>
<td>CA (FR)</td>
<td>CA (DK)</td>
<td>CA (AZ)</td>
<td>CA (SP)</td>
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<td>CA (BE)</td>
<td>CA (UK)</td>
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<tr>
<td>Brain MRI</td>
<td>Central atrophy, ventricular enlargement</td>
<td>Normal</td>
<td>Normal</td>
<td>T2-weighted periventricular hyperintensities</td>
<td>Frontal atrophy in left hemisphere</td>
<td>ND</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
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<td>Normal</td>
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<td>25</td>
<td>19/10</td>
<td>20</td>
<td>26/30</td>
<td>51</td>
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<td>ND†</td>
<td>ND‡</td>
<td>76%</td>
<td>51%</td>
<td>ND‡</td>
<td>76%</td>
<td>71.7%</td>
<td>42.2%</td>
<td>72%</td>
<td>ND</td>
<td>83%</td>
</tr>
<tr>
<td>ECG</td>
<td>Single premature ventricular contraction</td>
<td>Sinus bradycardia with right bundle branch block</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Non-specific repolarisation abnormalities</td>
<td>ND</td>
<td>Normal</td>
</tr>
<tr>
<td>Cardiac echo</td>
<td>ND</td>
<td>Normal</td>
<td>Normal</td>
<td>LVEF = 67%</td>
<td>Normal</td>
<td>LVEF = 65%</td>
<td>LVHF = 50%</td>
<td>Normal</td>
<td>Dilated cardiomyopathy, LVEF = 43%–50%</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>10m walk test</td>
<td>10.98 s</td>
<td>6.26 s</td>
<td>16 s</td>
<td>16 s</td>
<td>70.67 s</td>
<td>Cannot walk</td>
<td>&lt;6 s</td>
<td>&lt;6 s</td>
<td>Cannot walk without support</td>
<td>7.41 s</td>
<td>ND</td>
</tr>
<tr>
<td>Walking aids</td>
<td>None</td>
<td>None</td>
<td>Two canes</td>
<td>One cane</td>
<td>Walking frame with wheels</td>
<td>Wheelchair</td>
<td>None</td>
<td>None</td>
<td>Wheelchair</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>CK (U/L)</td>
<td>1590</td>
<td>5410</td>
<td>4201</td>
<td>398</td>
<td>51B</td>
<td>2792–4000</td>
<td>2840</td>
<td>2000–4000</td>
<td>7646</td>
<td>5086</td>
<td>3000</td>
</tr>
</tbody>
</table>

* and † siblings. ‡ ND, Not done. § Cognitive development such as language and motor milestones
Arab, Arabic; AZ, Azerbaijan; BE, Belgian; CA, Caucasian; CK, creatine kinase; CMD, congenital muscular dystrophy; DK, Danish; Fr, French; PVC, forced vital capacity; IR, Iranian; LGMD, limb-girdle muscular dystrophy; LVEF, left ventricular ejection fraction; MMSE, Mini-Mental Status Examination; ND, not done; SP, Spanish; UK, British.
Clinical evaluation
A thorough medical history was taken and focused on presenting symptom, age at disease onset, muscle cramps and myalgia. Sometimes disease onset and presenting symptom had to be obtained from parents or medical records. Walking capability was examined by the 10 m walk test and by recording the use of walking aids. Limb muscle strength was evaluated at the current ages indicated in Table 1 by manual muscle testing (Medical Research Council scale). Respiratory function was assessed by a spirometry (forced vital capacity (FVC)) and cardiac function by ECG and echocardiography. Cognitive function was screened by using the Minimal Mental Status Examination (MMSE).

MRI
Whole-body muscle MRI was performed in all patients and brain MRI in 10 of the 12 patients. The MRI scanners differed among the participating clinical centres, but only axial T1-weighted images were assessed. Four cross-sectional slices at the level of calves, thighs, L4 and pelvis were chosen for the evaluation of muscle involvement (Figure 1). Replacement of muscle by fat was graded according to the Mercuri scale.

Muscle biopsies
Muscle biopsies were obtained from the tibialis anterior, gastrocnemius or deltoid muscles and stained with H&E for general histopathological evaluation. For immunohistochemical evaluation of α-DG glycosylation, muscle sections were stained with the antibodies VIA4-1 and IIH6C (Merck-Millipore, Temecula, California, USA) and goat anti-mouse Alexa Fluor 594 antibodies (ThermoFisher, Waltham, Maryland, USA) using standard protocols. Biopsies for immunohistochemistry were only available for cases 1, 5, 9 and 10.

Molecular findings
Blood samples were drawn for determination of plasma creatine kinase (CK) concentration and for extraction of DNA from leucocytes, according to standard procedures. Mutations in POMT2 were identified by whole exome sequencing of leukocyte DNA at the Broad Institute’s Genomics Platform, using Illumina exome capture, 38 Mb baited target and the Broad’s in-solution hybrid selection process. The mutations were confirmed by Sanger sequencing. In two cases (3 and 4), mutations were found directly by Sanger sequencing. Mutation frequencies were estimated using Exome Aggregation Consortium database with 60,706 unrelated individuals as control population (Table 2).
Neuromuscular

Figure 2  Muscle strength evaluation by using the MRC scale. Values range from 0 to 5, including plus and minus for 4 and 5 (4+ equals 4.33 and 5− equals 4.66). Boxplots show the distribution of MRC scores for each motion, including a median line. Dots representing minimal and maximal values. ext, extension; flx, flexion, L, left; MRC, Medical Research Council; R, right.

RESULTS
Clinical evaluation
Disease onset varied from birth to 55 years (table 1). Three participants had disease onset at birth and were classified as CMD/LGMD, because they had a typical LGMD phenotype as adults. Two with onset at birth still walked unassisted at age 18 years and one was still ambulatory with assistance at age 54 years. Similar reasoning to classify patients as LGMD2N, despite disease onset at birth, was also applied in two of the four previously reported young cases of LGMD2N.15 Presenting symptoms were in most cases related to ambulatory function, showing either as a delay in the ability to walk, or troubles in walking, climbing stairs or running. Ten patients were ambulatory while one was wheelchair users, and one could only walk when assisted. Some patients presented with learning difficulties in school, especially case 3, who attended a special school. Cases 3 and 4 also experienced pain during mild exercise which preceded muscle weakness in childhood. Delay in cognitive function in case 5 was followed by delayed motor milestones due to muscle weakness. The MMSE score was decreased relative to normal in most patients (table 1), and all were described by their physicians as having some degree of cognitive impairment, although no formal neuropsychological tests were performed.

Two cases had reduced left ventricular ejection fraction (LVEF) (table 1). Case 7 was diagnosed with dilated cardiomyopathy at age 18 years and treated with an angiotensin-converting enzyme inhibitor. The echocardiography of case 5 revealed a mild reduction of LVEF without any cardiac symptoms. Case 3 had hypertension and was treated with an antihypertensive drug. FVC was measured in eight patients and was reduced in all with an average FVC of 66% of the predicted value. Cases 1, 2 and 5 could not cooperate during pulmonary function tests, because of poor intellectual capacity, and case 10 was unavailable for FVC measurements, because he was lost to follow-up.

Muscle strength examination showed reduced force in all patients (figure 2), especially in hip and knee flexors and extensors. Knee flexor muscles were weaker than the extensors and the opposite pattern was seen across the hip. As apparent from figure 2, muscle weakness was mostly symmetric, except in case 6, who had a much weaker left relative to right leg. Muscle strength of forearm and finger muscles showed normal force in all patients.

Proximal muscle atrophy was observed in most patients, and atrophy of the shoulder girdle was found in cases 9, 10 and 12, who had significant scapular winging (figure 3). CK levels were highly elevated in all participants, except in two cases (cases 4 and 5) in whom it was slightly elevated (table 1).

MRI
Brain MRIs were abnormal in 3 of the 10 patients in whom brain imaging was carried out. Mild ventricular enlargement due to central and cortical atrophy was found in case 1, periventricular hyperintensities in case 4 and frontal atrophy of the left hemisphere in case 5 (figure 4).

Muscle MRI revealed a pattern of selective muscle involvement, most strikingly affecting the hamstring, paraspinal and gluteal muscles (figure 1). Consistent with the evaluation of muscle strength, the hamstring muscles were more severely affected (average 3.8 on the Mercuri scale) than the anterior thigh muscle group (average 3.3 for the quadriceps).

In the muscles of the lower leg, both degree of fatty infiltration and muscle involvement differed among the participants (figure 1), but there was a predilection for selective involvement of the muscles of the posterior compartment; on average, the gastrocnemius muscles (average 3.2 on Mercuri) were more severely affected compared with the tibialis anterior muscle (average 1.7 on Mercuri).

Muscle biopsy
A dystrophic pattern with fibre size variation and central nuclei was present in muscle biopsies from six cases. Immunohistochemical staining of α-DG glycosylation demonstrated a clear signal reduction (figure 5). Glycosylation was also absent or severely reduced in muscle biopsies of cases 1, 9 and 10 (data not shown).

In the last two patients (cases 3 and 4) for whom a muscle biopsy was available, immunohistochemistry was performed using only the VIA-4 antibody, because IIHC6 antibodies were not routinely used in that lab. The stains showed reduced glycosylation of α-DG in cases 3 and 4 with the VIA-4 antibody. The four other cases showed highly reduced glycosylation using both antibodies,
except case 1, in whom glycosylation was significantly reduced using the IIHC6 antibody, but only mildly reduced using the VIA-4 antibody.

Molecular findings
The patients carried 11 novel mutations in POMT2 (table 2). Case 3 carried the known mutation, c. 1997A>G, which previously has been linked to a CMD phenotype.6 9

DISCUSSION
We describe the first cohort of patients affected by LGMD2N due to mutations in the POMT2 gene, which so far has only been described in a few single-case reports. The major new findings of the study are: (1) patients with LGMD2N, unlike other recessively inherited LGMDs, are cognitively impaired; (2) the disease primarily affects hamstring, paraspinal and gluteal muscles; (3) the mutational spectrum of LGMD2N is expanded by the addition of 11 new mutations in POMT2, adding to the 11 known mutations causing LGMD2N and (4) patients with LGMD2N seem to have a wide range of disease onset. The muscle involvement and clinical presentation of patients with autosomal-recessive LGMD differ highly among the various subtypes. In this study, the degree of muscle involvement also varied among the 12 patients. One patient had an asymmetric appearance of muscle involvement, although generally the pattern on strength testing and MRI showed consistent involvement, preferentially of the hamstring, paraspinal and gluteal muscles in a symmetrical pattern. This pattern of muscle involvement was similar to that found in the worldwide most prevalent form of recessive LGMD, LGMD2A, which is caused by mutations in the CAPN3 gene. Besides the pattern of leg involvement, prominent scapular winging, as seen in three of our patients with LGMD2N, and asymmetry are also occasionally encountered in LGMD2A.10 The same pattern of muscle involvement is also seen in another glycosylation defect of α-DG, LGMD2I, caused by mutations in FKRP (fukutin-related protein gene). Patients with LGMD2I present with a similar, preferential involvement of the posterior thigh. When calf muscles are involved in LGMD2I, the medial gastrocnemius and soleus muscles are affected first, which was also seen in our patients with LGMD2N together with a rather hypertrophic appearance of the calf muscles.11 Assessment of calf muscle hypertrophy was based on the clinical examination performed by an experienced myologist at each centre; cross-sectional area measurements of different muscle groups could aid in reliably judging muscle bulk. The combination of different MRI features can, however, yield valuable clues, as for example, LGMD2I is typically associated with muscle hypertrophy, contrasting with the atrophic phenotype of LGMD2A. If imaging is performed in relatively early disease stages, a similar pattern seems to be observed in different dystroglycanopathies. The other way around, MRI imaging can aid in the interpretation of candidate sequence variants obtained by NGS techniques. Dystroglycanopathies comprising >18 separate disorders and more remain to be discovered.15 The disorders affect glycosylation of α-DG, and in the majority of cases result in CMD associated with brain abnormalities. POMT2 deficiency was first reported to cause CMD,13 and only later was the defect associated with a rare phenotype compatible with LGMD.14–6 Only in

Figure 4  T1-weighted brain MRI with sagittal and transverse slices showing central and cortical atrophy and mild ventricular enlargement in case 1 and frontal atrophy of left hemisphere in case 5.

Figure 5  Muscle biopsy from a healthy control and case 5, displaying myopathic features (increased internalised nuclei and increased variation of muscle fibre diameter). Staining, using α-dystroglycan glycosylation-specific antibodies IIHC6 and VIA4-1, demonstrates loss of glycosylation. Bar is 50 μm. α-DG, alpha-dystroglycan.
rare cases have patients with a LGMD phenotype been systematically associated with brain involvement. First, LGMD2I has been proposed to have occasional involvement of the frontal and posterior parietal lobes of the brain, as suggested by brain MRI scans from 10 patients with LGMD2I.14 Patients were reported to have mild problems in graphic element integration, but this cognitive impairment was not related to the MRI findings.14 Thus, although the general clinical experience is that patients with LGMD2I have normal cognitive function, there is evidence to suggest a mild impairment.14 15 Patients with LGMD2M, caused by mutations in the FKTN gene, have been reported to have normal cognitive function and brain structure.16-17 A recent study of two siblings with POMT1 deficiency and a LGMD phenotype (LGMD2K) were reported to have intellectual disability and focal cortical dysplasia on brain MRI.18 The same pattern of cognitive and structural brain involvement has been found in a cohort of five patients with LGMD2K.19 POMT1 and POMT2 dimerize to attach the initial O-linked mannose onto α-DG. It can be argued that the mutations in POMT1 and POMT2 generally cause more severe phenotypes due to the requirement of this initial mannose for extracellular matrix anchoring. However, most of the enzymes responsible for the transfer of sugar moieties to the initial mannose, and subsequent expanding glycan, fukutin, FKRP, POMTGT1 and LARGE, may result in CMD, muscle-eye-brain disease or Walker-Warburg syndrome when the genes are mutated.16-21 The absence of phenotypes without some brain involvement in patients with POMT2 deficiency suggests that minor changes to the structure of POMT2, which has nine transmembrane helices and requires N-glycosylation at multiple sites, are likely to affect function or binding to POMT1 significantly.22 23 Cognitive function was not quantitatively assessed in our patients, due to practical difficulties in doing so across seven centres in six countries. However, the cognitive impairment was evident from MMSE and the patients’ inability to succeed in school. Cognitive function in our patients was not quantitatively assessed using a neuropsychological examination, due to practical difficulties in coordinating this effort across seven centres in six countries. The use of the MMSE score is not validated for this cohort, but was used as an instrument to gain access to an index of cognitive function, which together with information on performance at school allowed a general judgement of the intellectual capabilities. Three of our patients showed abnormalities on brain MRI, but no uniform brain changes across their scans. The patients with MRI abnormalities were the ones with the lowest MMSE scores, suggesting a link with cognitive impairment. However, cognitive impairment was also present in patients with normal brain MRI scans (table 1). Abnormal brain MRI findings could also have other causes than LGMD2N. As shown in figure 4, case 5 had frontal atrophy of the left hemisphere, which could relate to the patient’s hypoxia at birth. T2-weighted periventricular hyperintensities as found in case 4 are often linked to cerebrovascular diseases, and this patient was also treated for hypertension. On the other hand, learning difficulties had been present since age 13 in school, which suggests that the periventricular lesions likely played no role in the cognitive function. The finding of diffuse central and cortical atrophy in case 1, a 25-year-old woman with no history of other organic diseases, is likely related directly to the POMT2 deficiency. Although our study is the first to suggest consistent cognitive dysfunction in a LGMD subtype, case reports of patients with LGMD affected by glycosylation defects of α-DG suggest that brain abnormalities may be present in some LGMD subtypes, especially caused by POMT1 and POMT2 mutations. Attention to cognitive aspects should therefore be exercised when diagnosing patients with dystroglycanopathies and an LGMD phenotype.

Alpha-DG is also glycosylated in cardiac muscle cells, which might account for the frequent occurrence of cardiac affection in CMD and LGMD caused by glycosylation defects of α-DG. Dilated cardiomyopathy has been reported in patients with mutations in the FKTN gene,24 25 and a third of patients with LGMD2I develop cardiomyopathy.26-28 Two patients with POMT1 mutations and a LGMD phenotype have also been reported with ventricular dilatation of the heart.29 In the present study, two cases had reduced LVEF (cases 5 and 7), suggesting that patients with LGMD2N are at risk of developing pump failure. In accordance with this, Martinez et al recently reported three siblings with a CMD phenotype and a homozygous c.1997A>G mutation in POMT2, which was also present in case 3 in our study. The three siblings had reduced LVEF, dilatation of the aortic root and/or left ventricular wall motion abnormalities.30 These and our findings suggest that regular cardiac investigations should be carried out in patients with LGMD2N.

Our study disclosed 11 new mutations in POMT2, which were either inherently pathogenic, because they were frame-shift mutations, or predicted by various in-silico prediction tools (PolyPhen, Mutation Taster and FATHMM) to be pathogenic. No prediction was given for the mutation c.1654–5T>G in case 12, as it was predicted to be located at an extended splice site. These new mutations were all absent or extremely rare in the background population. As mutations affect wide range of sites in the POMT2 gene, including the transmembrane helices, N-glycosylation sites and variable effects on hydrophobic/hydrophilic changes, there appears to be little resilience in the POMT2 structure before mutations become pathogenic (table 2).

In conclusion, we demonstrate the clinical features in the first cohort of patients with LGMD2N, showing a pattern of muscle affection similar to other LGMDs, most notably LGMD2A and 2I, and a consistent cognitive affection, which has not been described as a signature feature of other LGMD types. Our study quadruples the number of cases of LGMD2N reported in the world, and therefore suggests that the condition may be more prevalent than hitherto considered.

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Contributors  S10: design of study, analysis, acquisition and interpretation of data, and drafting the manuscript. KE, TS, PDeU, JB, KGC, RF-T, LP, AI, JC, WDeR, SN, SJ-O, CB-S, FL, DGMeA, ML, LX, IN and VS: acquisition of data and revision of manuscript. TK: acquisition and interpretation of data, and revision of manuscript. Jvd: design of study, acquisition and interpretation of data, and revision of manuscript.

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Patient consent  Obtained.

Ethics approval  Danish National Committee on Health Research Ethics (H-3-2012-163 withamendment #41665, #43449 and #50556) and the local Ethical Review Boards of the participating centers.

Provenance and peer review  Not commissioned; externally peer reviewed.

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Limb girdle muscular dystrophy due to mutations in *POMT2*

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Appendix E.
Case report

Title: A novel compound heterozygous mutation in the POMK gene causing limb-girdle muscular dystrophy-dystroglycanopathy in a sib pair

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Keywords: Protein-O-mannosyl kinase; *POMK*; Dystroglycanopathy; Limb-girdle muscular dystrophy-dystroglycanopathy type 12 C; MDDGC12; LGMD12C; Muscular dystrophy

**Highlights**

- We report two novel compound heterozygous mutations in the protein-O-mannosyl kinase (*POMK*) gene, causing limb-girdle muscular dystrophy-dystroglycanopathy (type C12; MDDGC12).
- The probands are two Finnish siblings who have a phenotype consistent with mild limb-girdle muscular dystrophy.
- At the age of 10 and 13 years, they are mildly affected and the disease course seems slow or even non-progressive.
Manuscript

Abstract

We describe two Finnish siblings in whom an incidentally detected elevated creatine kinase activity eventually led to a diagnosis of limb-girdle muscular dystrophy-dystroglycanopathy (Type C12; MDDGC12). When diagnosed at age 10 and 13 years, they were mildly affected with a slow or non-progressive disease course. The main symptoms comprised infrequent hip cramps triggered by flexion, neck cramps triggered by yawning, transient growing pains, calf hypertrophy and mild proximal muscle weakness. Their cognitive and motor developments were unremarkable and they were physically active. Whole-exome sequencing revealed compound heterozygous mutations, both of which were novel, in the protein O-mannosyl kinase (POMK) gene in both siblings; a missense mutation, p.Pro322Leu (c.965C>T), and a nonsense mutation, p.Arg46Ter (c.136C>T). The results were confirmed by Sanger sequencing, showing that the parents were heterozygous carriers of one mutation each. This report adds to the literature by providing phenotype and genotype data on this ultra-rare POMK-related dystroglycanopathy.
1. Introduction

Dystroglycanopathies are a group of congenital muscular dystrophies characterised by considerable clinical and genetic heterogeneity. They are caused by disruption in the interactions between the transmembrane protein dystroglycan and extracellular matrix components. These interactions are necessary for normal muscle and brain development [1]. In these interactions, glycosylation plays a crucial role. Most known dystroglycanopathy mutations are located in genes related to the glycosylation process rather than in the dystroglycan gene itself [1].

The POMK gene (MIM 615247), located on chromosome 8p11, encodes protein O-mannose kinase which is necessary for proper glycosylation and function of the dystroglycan complex [2]. Homozygous mutations in the POMK gene cause limb-girdle muscular dystrophy-dystroglycanopathy (type C12; MDDGC12) (MIM 616094). To our knowledge, two such families have been described; one in which two Jordanian sibs presented with limb-girdle muscular dystrophy (LGMD) and cognitive impairment, and another in which a child born to consanguineous parents presented with LGMD, mild learning difficulties and congenital mirror movements [1,3]. POMK mutations can also cause the more severe Walker-Warburg syndrome (type A12; MDDGA12) (MIM 615249), encompassing brain and eye abnormalities in addition to the muscular dystrophy. To our knowledge, three such families have been described in previous literature [1, 2, 4]. Due to the small number of cases described, little is known about the clinical presentation of disorders caused by POMK mutations.

We report clinical, molecular, histological, neurophysiological and imaging data on a sib pair with limb-girdle muscular dystrophy-dystroglycanopathy type C12, caused by a novel compound heterozygous mutation in the POMK gene. The incidental finding of elevated serum creatine kinase (CK) activity in the probands in infancy, which eventually led to the
diagnosis, allowed for observation of the onset and for longitudinal follow-up of the progression of the disease.

2. Case Report

2.1 Clinical phenotype

The probands comprise a Finnish sib pair born to non-consanguineous parents (Figure 1a). The sister was born at term with birth asphyxia due to placental abruption. She suffered a mild asphyxia-related kidney injury for which she was on follow-up. At 6 years of age, while being investigated for elevated liver enzymes, she presented with elevated CK values of 1000-4000 U/L. Her motor and cognitive development as well as her growth were normal. She started walking at age 15 months. In childhood, she suffered from transient growing pains. Since school age, she has experienced infrequent hip cramps triggered by hip flexion when doing for instance sit-ups, and by cramps in the neck region triggered by yawning. She has, at times, suffered from exercise-induced knee ache. Testing of muscle strength at 10 years of age showed muscle power below average for hip adductors and flexors, as well as for knee extensors and ankle plantar flexors. Subsequent evaluations showed muscle strength within normal limits.

Her electromyography (EMG) findings were normal, however, only one muscle (right gastrocnemius) was examined due to her fear of needles. Neurography of the median, tibial peroneal and sural nerves gave normal results. A muscle biopsy at 7 years of age was paraffin-embedded and showed normal findings. Echocardiography and spirometry showed no signs of cardiac or respiratory involvement. Magnetic resonance imaging (MRI; 1.5 Tesla scanner) of muscles in shoulders, upper arms, thighs and pelvic region was normal (not shown).
The younger brother was born preterm by caesarean section due to placenta praevia. His motor and cognitive developments as well as his growth were unremarkable. He started walking at age 13 months. As a consequence of his sister’s incidentally detected CK elevation, his CK level was determined, showing markedly increased levels of up to 7000 U/L. In childhood, he suffered from transient nocturnal growing pains. He also suffered from thigh stiffness and infrequent pain and cramps in the thighs and groins triggered by hip flexion. Like his sister, he experienced infrequent neck region cramps triggered by yawning. Muscle strength in hip abductors and adductors as well as upper limbs was reduced, making it, at times, difficult for him to do sit-ups and arm push-ups.

His EMG was normal (neurography of tibial, peroneal and sural nerve, as well as myography of vastus lateralis, tibialis anterior and biceps). A muscle biopsy taken from vastus lateralis at age 6 showed moderate chronic myopathic changes, single inflammatory cells, mild α-dystroglycan deficiency and slight up-regulation of major histocompatibility complex 1 (MHC1) protein. Echocardiography revealed mild enlargement and borderline decreased function of the left ventricle. There were no signs of respiratory involvement. MRI (1,5 Tesla) findings in the muscles of the shoulder region, upper arms, thighs and pelvic region was within normal limits (not shown).

On clinical examination at age 13 and 10 years respectively, the sister and brother had calf hypertrophy (Figure 2), mild lumbar lordosis and slightly winged scapulae, brisk tendon reflexes in lower extremities, weakened but positive tendon reflexes in upper extremities, absent Babinski signs and negative Gowers´ signs. They had no mirror movements, which have been related to POMK mutations in a previous report [4]. The brother had difficulty in walking on heels. Both sibs were physically active and considered themselves symptom free and as physically fit as their peers.
Family history of muscle disease was non-contributory, and the parents’ CK levels were normal.

2.3 Molecular findings

The patients were enrolled into the MYO-SEQ project (myo-seq.org), an international research collaboration that applies whole exome sequencing (WES) to patients with undiagnosed limb-girdle muscle weakness. At least 250 ng of DNA (>2 ng/µl) for each sibling was used as a template for WES. The resulting data were processed by the Genomics Platform at the Broad Institute of Harvard and MIT (Boston, MA, USA) as described previously [5]. The variant call set was uploaded onto the Broad Institute of Harvard and MIT’s seqr platform (https://www.seqr.broadinstitute.org). POMK was analysed for biologically relevant variants by considering the (i) population frequency detailed by the Exome Aggregation Consortium (ExAC) [6], (ii) deleteriousness of the variant predicted by PolyPhen-2 [7], MutationTaster2 [8] and FATHMM [9], (iii) ClinVar reports of pathogenicity and (iv) published literature on POMK.

Both siblings were shown to harbour two rare variants in POMK. The first was a stop-gained nonsense mutation, p.Arg46Ter (hg19 chr8:42958827, c.136C>T), and the second was a missense mutation, p.Pro322Leu (hg19 chr8:42977932, c.965C>T). Sanger sequencing confirmed the presence of the variants in trans in the siblings. Each unaffected parent was only heterozygous for one of the variants each (Figure 1b).

3. Discussion
The *POMK* gene (synonym SGK196) is a 350 amino acid protein comprising 5 exons [4]. The full-length wild-type *POMK* has a predicted molecular weight of 40.15 kDa. The gene is conserved in mammals and amphibia [2]. Supporting the presumption that *POMK* mutations cause muscle disease, Di Costanzo and colleagues observed high expression of *POMK* in muscle and brain tissues during fetal development, especially during myocyte differentiation. In zebrafish embryos, knockdown of *POMK* led to muscle dystrophy, stressing the importance of *POMK* for early muscle development [2]. Moreover, protein analysis studies by von Renesse et al. showed absence of POMK protein signals in fibroblasts and muscle cells derived from patients with homozygous truncating *POMK* mutations, whereas such signals were present in controls [2].

The novel mutations in our patients comprised one nonsense mutation (p.Arg46Ter) in exon 4, resulting in a premature termination of the amino acid sequence, and another, a missense mutation (p.Pro322Leu), quite late in the gene in exon 5. *In silico* prediction tools suggest both variants are damaging to the protein. Interestingly, all cases with *POMK*-related dystroglycanopathies reported to date have at least one mutation in exon 5 [1-4]. Despite the lack of functional studies of the specific mutations identified in our patients, the phenotype, consistent with LGMD, the elevated CK activity as well as the α-dystroglycan deficiency in the muscle biopsy of the brother strongly argues for pathogenicity of the mutations. Hence, the current report supports previous studies claiming a causative role of *POMK*-mutations in dystroglycanopathies.

POMK-related dystroglycanopathies are ultra-rare autosomal recessive disorders. To our knowledge, when taking into account the current study, there are, to date, 9 published cases.
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from 6 different families [1-4]. As with dystroglycanopathies in general, their phenotypic presentations vary widely. On comparison with the published cases, we were unable to pinpoint any clear-cut genotype-phenotype correlation. Whether this lack of correlation is explained by chance due to the small sample, or by a factual lack of correlation, remains to be seen as new cases are identified in the future. Our two patients, who had a childhood onset of mild LGMD with normal cognition, represent the least severe phenotype among the published POMK-cases. The mild cardiac involvement observed in the boy, however, has not been reported previously and may either represent a new phenotypic feature of POMK-related dystroglycanopathies or be unrelated to it. The two Jordanian sibs with MDDGC12 had a clinical presentation of infancy-onset, fairly slowly progressive LGMD with cognitive deficit, caused by a severe protein-truncating homozygous missense mutation in POMK [1]. Interestingly, the same allelic variant was identified in two more severely affected Lebanese siblings diagnosed with MDDGA12, who had infancy-onset muscle weakness and hypotonia, developmental delay, wheel-chair dependency, affected vision and brain MRI abnormalities - again illustrating the wide phenotypic variation [2]. Furthermore, the adolescent boy reported by Ardicli et al. had childhood-onset slowly progressive LGMD with congenital mirror movements and brain abnormalities on MRI, caused by homozygous missense mutation in POMK [3]. Finally, an Italian boy diagnosed with MDDGA12 had a severe infancy-onset phenotype of hypotonia, cognitive impairment, epilepsy, brain and eye abnormalities. He deceased at the age of 4 years, and had a compound heterozygous POMK mutation; one frameshift mutation causing a premature termination, and another mutation causing protein substitution of a highly conserved amino acid in exon 5 [1].

In summary, we report novel compound heterozygous POMK mutations in two siblings with a childhood onset of LGMD, who are mildly affected with a slow or non-progressive disease
course. They were cognitively unaffected and seem to have an isolated muscle affection, however, the cardiac involvement in the brother may represent a new phenotypic feature of MDDGA12C. This report adds to the literature by providing phenotype data on this ultra-rare disease. As with rare diseases in general, further case reports are needed to elucidate the entire phenotypic spectrum of POMK-related dystroglycanopathies.

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Declarations of interest: none.
References


FIGURE LEGENDS

Figure 1. a) Pedigree showing the affected siblings and their unaffected, non-consanguineous carrier parents. b) Sequencing chromatograms of POMK in the affected siblings showing compound heterozygous mutations. Both parents are heterozygous carriers of either mutation.

Figure 2. Photographs of the probands showing calf hypertrophy, slightly accentuated lumbar lordosis and slightly winged scapulae.