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ORIGINAL COMMUNICATION

European muscle MRI study in limb girdle muscular dystrophy type R1/2A (LGMDR1/LGMD2A)

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Abstract

Background Limb girdle muscular dystrophy type R1/2A (LGMDR1/LGMD2A) is a progressive myopathy caused by deficiency of calpain 3, a calcium-dependent cysteine protease of skeletal muscle, and it represents the most frequent type of LGMD worldwide. In the last few years, muscle magnetic resonance imaging (MRI) has been proposed as a tool for identifying patterns of muscular involvement in genetic disorders and as a biomarker of disease progression in muscle diseases. In this study, 57 molecularly confirmed LGMDR1 patients from a European cohort (age range 7–78 years) underwent muscle MRI and a global evaluation of functional status (Gardner-Medwin and Walton score and ability to raise the arms).

Results We confirmed a specific pattern of fatty substitution involving predominantly the hip adductors and hamstrings in lower limbs. Spine extensors were more severely affected than spine rotators, in agreement with higher incidence of lordosis than scoliosis in LGMDR1. Hierarchical clustering of lower limb MRI scores showed that involvement of anterior thigh muscles discriminates between classes of disease progression. Severity of muscle fatty substitution was significantly correlated with *CAPN3* mutations: in particular, patients with no or one "null" alleles showed a milder involvement, compared to patients with two null alleles (i.e., predicting absence of calpain-3 protein). Expectedly, fat infiltration scores strongly correlated with functional measures. The "pseudocollagen" sign (central areas of sparing in some muscle) was associated with longer and more severe disease course.

Conclusions We conclude that skeletal muscle MRI represents a useful tool in the diagnostic workup and clinical management of LGMDR1.

Keywords LGMDR1/LGMD2A · Muscle MRI · Mercuri score · *CAPN3* mutations

Background

Calpainopathy, or limb–girdle muscular dystrophy type R1/2A (LGMDR1/LGMD2A), caused by mutations in the calpain-3 gene (*CAPN3*) [1], is one of the most common forms of LGMD, especially in Southern Europe, with a reported frequency ranging from 9 to 40% of LGMDs. In 2017 the ENMC International Workshop revised the former classifications of LGMDs, renaming "LGMD2A" as "LGMD R1 Calpain-3 related" [2]. The main clinical finding in calpainopathy is a progressive, proximal, and bilateral muscular weakness, but some patients may present just with high CK levels at onset [3]. Pelvi-femoral muscles are most commonly affected ("Leyden-Möbius" phenotype),

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primarily hip adductors and extensors, followed by hip and knee flexors. A minority of patients present with a primarily scapulohumeral ("Erb") phenotype, usually associated with milder progression. As the disease progresses, weakness and atrophy also involve the quadriceps and tibialis anterior in the lower extremities, and the triceps brachii and forearm muscles in the upper extremities [3, 4]. Paraspinal and abdominal muscles are also weak, causing a hyperlordotic posture. Facial, ocular, tongue, and neck muscles are usually spared [4–6]. The onset of symptoms usually occurs during adolescence, but varies between 2 and 50 years, and loss of independent ambulation ensues after 15–25 years, with a faster progression observed in patients with a juvenile onset [6].

To date, 496 unique pathogenic *CAPN3* variants have been reported, in addition to a smaller number of apparently neutral or unclassified variants. *CAPN3* mutations can be nonsense, splice site, frameshift, or missense; deep intronic or promoter mutations or large rearrangements are more rarely found [7–10]. Around 45% of identified mutations lead to the absence of the protein, indicating (together with the recessive pattern of inheritance) that LGMDR1 is due to a deficiency of calpain-3 function [7]. On the other hand, analysis of muscle biopsies shows that around 20–30% of patients have normal calpain-3 expression on Western blot [11]. These patients usually carry missense alleles leading to impaired autolytic activity, indicating a perturbation of calpain-3 function, and suggesting that LGMDR1 pathogenesis is associated with the loss of proteolytic activity [12]. On the other hand, the clinical outcome of missense mutations is hardly predictable, as they are associated with variable disease severity and protein amount. However, carrying two null mutations is considered a negative prognostic factor, while compound heterozygous and carriers of missense mutations seem to develop milder phenotypes [7, 13]. Recently, a dominant form of calpainopathy has been described in association with a heterozygous deletion of 21 base pairs in the *CAPN3* gene [14].

In the last decade, several studies have reported the value of MRI scans in identifying patterns of muscle involvement in genetically distinct muscle disorders [15–19]. In a previous study [3], seven patients affected by a particular clinical variant of LGMDR1 (with calf hypertrophy, rigidity of the spine and early contractures affecting multiple joints) were studied with muscle MRI of the lower limbs. The results showed a selective sparing of sartorius, gracilis, vastus lateralis, and gastrocnemius lateral head, opposed to a selective involvement of the medial head. This pattern may help in distinguishing LGMDR1 from other myopathies characterized by early contractures, such as Bethlem myopathy and Emery–Dreifuss dystrophy. Moreover, in a recent international study [13], 30 patients with calpainopathy underwent a CT scan of the lower limbs. The authors found significant hypodensity in hip adductors, followed by the hamstrings and medial head of the gastrocnemius, concordant with the manual muscle testing (MMT) scores. Another recent Chinese MRI study [20] proposed a diagnostic pattern consisting of severe substitution of biceps femoris together with sparing of extensor digitorum longus, with sensitivity of 76% and specificity of 90.48%.

We aimed to describe the pattern of fatty substitution in a cohort of European LGMDR1 patients featuring a wider mutational spectrum than in previously described cohorts.

Methods

Ethics statement

All evaluations were performed in the setting of "standard of care", and all the retrospective data analyzed were maintained completely anonymous.

Patient selection

This study included patients diagnosed with LGMDR1, recruited from ten European neuromuscular centers: Barcelona, Bern, Prague, Copenhagen, Munich, Newcastle Upon Tyne, Padova, Rome, Paris, and La Réunion. The inclusion criterion was a genetically proven LGMDR1 diagnosis, i.e., pathogenic *CAPN3* mutations on both alleles. We searched the Leiden Database [https://www.dmd.nl, curated by Johan den Dunnen and Jacqui Beckmann], for previous reports of each mutation. For splice site mutations, if experimental data about the assessment of RNA product were not available, we predicted the effects on splicing with the Human Splicing Finder [21] version 3.0. Novel missense mutations were evaluated with *in silico* predictors of pathogenicity (SIFT, PolyPhen-2, and Mutation Taster). The number of "null" alleles, i.e., predicting a loss of *CAPN3* open reading frame (frameshift, nonsense, etc.) and subsequent absence of calpain protein, was counted for each patient (0, 1, or 2).

MRI assessment

Patients underwent MRI (1.5 T or 3 T) with T1-weighted axial scans. Acquisition parameters are available on request. Images were acquired with a body coil and a simultaneous scan of both sides. Forty-eight skeletal muscles listed in Supplementary Table I were scored from 1 (normal) to 4 (end stage disease) according to the modified Mercuri scale [22]: normal (score 1), mildly/moderately moth-eaten appearance with occasional/numerous scattered hyperintense areas (< 30% of the muscle—score 2); severely moth-eaten appearance with confluent hyperintense areas (> 30% and < 60% of the muscle—score 3); confluent hyperintense areas (>

Table 1 Demographic and genetic features of the studied cohort

Gender	Male	Female	Total	
No. of patients	20	37	57	
Percentage	35	65	100	
Gardner-Medwin and Walton score (WGS)	Asymptomatic or minimal symptoms (GMW ≤ 3)	Moderate symptoms/walk with assistance (4 ≤ GMW ≤ 6)	Severe symptoms/unable to walk (GMW ≥ 7)	Total
No. of patients	17	30	10	57
Percentage	29.2	52.6	18.2	100
Genetic groups	0 null alleles	1 null allele	2 null allele	Total
No. of patients	22	27	8	57
Percentage (%)	39	47	14	100

60% of the muscle—score 4). The scans were assessed by one single observer.

Clinical assessments and clinic–radiological correlations

We cross-sectionally evaluated the ability to climb stairs, walk, to rise from floor, and raise arms above 90 degrees of abduction against gravity. We also assigned a global functional activity score, the Gardner-Medwin and Walton (GMW) score (0, normal; 1, unable to run; 2, altered gait; 3, climbs stairs with hand support; 4, cannot climb stairs; 5, cannot rise from chair; 6, walk only with aids; 7, cannot walk; 8, unable to move in a manual wheelchair; 9, unable to sit unsupported; 10, bedridden). Age at patient-reported onset of first symptoms was collected. We correlated Mercuri scores of each lower limb muscle with the GMW score, and Mercuri scores for each upper limb muscle with the ability/inability to raise arms.

Statistical analyses

MRI scores were by and large symmetrical (see "Results"), so data relative to right-side muscles were used for analyses. Age between groups was compared by Student's *t* test. Distribution of categorical variables between groups was compared by χ^2 or Fisher's exact test as appropriate. The effect of null mutation load on age at symptom onset was tested by Cox regression. Correlations between MRI scores and functional measures were evaluated with the Spearman method. Statistical significance was set at $p < 0.05$. Statistical analyses were performed with R version 3.4.3. Hierarchical clustering was used to identify group of participants with similar radiological features, and corresponding heatmaps were drawn with the "Pretty Heatmap" package for R, version 1.0.8.

Results

Participants

We recruited 57 LGMDR1 patients aged 7–78 years (mean 38.4 ± standard deviation 15.8), from 56 unrelated families. Distribution by gender, mutation and functional score (GMW) are reported in Table 1.

Mutational spectrum

Fifty-six unique *CAPN3* variants (summarized in Supplementary Table) were identified: 2 exon deletions (4%), 11 nonsense or frameshift mutations (19%), 4 splice site mutations (7%), 39 (70%) missense mutations. All patients were sporadic cases, except two siblings. Homozygosity for a pathogenic variant was found in 19 cases (33%). The frameshifting variant c.550delA was the most frequent (9 cases), followed by the missense mutation c.1468C>T, p.Arg490Trp in six cases. Both of these mutations have a founder effect in north-eastern Italy, especially in the city of Chioggia and in the Friuli-Venezia-Giulia region [2]. Also, as previously reported, c.550delA appears frequent in populations of Slavic ancestry [23–25]. Three mutations had not been previously reported ("private mutations"): c.776delG,c.2305C>T, p.Arg769Trp; c.2442G>C, p.Trp814Cys. The missense mutation p.Trp814Cys is not reported in gnomAD (gnomad.broadinstitute.org), while the c.2305C>T, p.Arg769Trp mutation was reported only three times (estimated allele frequency 0.0000122). This extreme rarity makes their pathogenicity very likely in the presence of a second mutation and a phenotype compatible with LGMDR1. Furthermore, both mutations were predicted *in silico* as very likely damaging/disease causing.

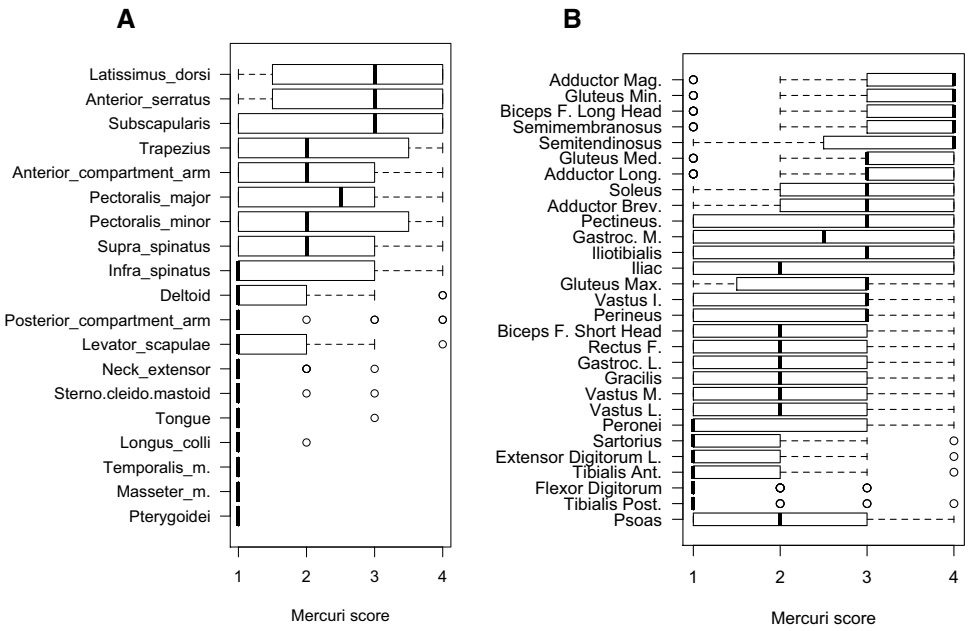

Fig. 1 **a**, **b** Box plots illustrating MRI scores for muscle groups of the upper limb (UL) and lower limb (LL). The Mercuri score is indicated on the horizontal axis. Muscles are ordered according to the median MRI score, in descending order from top to the bottom of the figure

Symmetric muscle involvement

Lower limb images were available for 52 patients (89.5%), while upper limb, trunk, neck, and head districts were available for 28 (49.1%). Minor asymmetry (1-point difference between contralateral muscles) was observed in two patients, while conspicuous asymmetry (2-point difference) was observed in six patients with asymmetric muscular involvement of the subscapularis, tibialis posterior, soleus, biceps femoris long head, gastrocnemius lateralis, tractus iliotibialis, pectineus, and rectus femoris. Despite being rather unusual, asymmetric muscle involvement has already been described in LGMDR1 [26].

Grading of fatty replacement

As shown in Fig. 1a, upper body muscles can be subdivided into three subgroups: the "severely affected", including the latissimus dorsi, serratus anterior, and subscapularis (median T1 score: 3); "intermediately affected", including trapezius, pectoralis major, pectoralis minor, supraspinatus, and anterior arm compartment (median score: 2); and "mildly affected", including deltoid, posterior arm compartment, levator scapulae, neck extensor, sternocleidomastoid, tongue, longus colli, temporalis, masseter, and pterygoidei (median score: 1). T1w signal was normal in the temporalis, masseter, and pterygoidei muscles.

Similarly (Fig. 1b), pelvic and lower limb muscles can be subdivided into "severely affected", including adductors, glutei, posterior thigh muscles with the exception of biceps femoris short head, and vastus intermedius (median score: 3–4); "intermediately affected", including the iliopsoas, biceps femoris short head, and other thigh muscles except the sartorius (median score: 2); and "mildly affected", including sartorius and leg muscles (including anterior and posterior compartment) (median score: 1). Scans from a representative patient in whom the "typical" LGMDR1 pattern may be recognized are shown in Fig. 3.

Hierarchical clustering of lower limb T1 scores

Detailed distribution and severity of fatty substitution for each patient are shown as a hierarchically clustered heatmap in Fig. 2. Scans from a representative patient, in whom the "typical" LGMDR1 pattern may be recognized, are shown in Fig. 3. Only one patient showed a normal MRI (age 12 years, 1 null and 1 missense allele). Hierarchical clustering of Mercuri scores (Fig. 2) showed that the studied cohort may be divided into three main clusters according to lower limb fatty replacement: severe (22 upper rows in the heatmap), intermediate (14 middle rows), and mild (16 bottom rows). The main differentiating factor in the three clusters appeared to be the involvement of the anterior compartment of the thigh. Looking at demographic, genetic, and clinical annotations of the heatmap, it appeared clearly that patients in the severe cluster were more frequently non-ambulant (9/22, 41%) than patients in the intermediate (2/14, 14%) and mild (0/16, 0%) clusters ($p = 0.005$). The load of null mutations in the severe cluster (17/44, 39%) was similar to that observed in the intermediate cluster (12/28, 42%), but somewhat higher than in the mild cluster (9/32, 28%; $p =$ n.s.). Surprisingly, for a progressive disorder such as LGMDR1, age did not different significantly between clusters: in fact, average age was highest in the mild cluster (43.7 ± 18.9 years, vs. 39.9 ± 15.2 in

Fig. 2 Hierarchically clustered heatmap of fatty substitution Mercuri scores in 25 right lower limb muscles, in MRIs from 52 LGMDR1 individuals. Rows, each corresponding to one individual, are hierarchically clustered (dendogram on the left) based solely on MRI data (Mercuri scores). Each column in the heatmap corresponds to one muscle, ordered left to right from cranial to caudal. A green–yellow–red gradient in the heatmap indicates increasing fatty substitution (legend to the right). The three columns on the left of the heatmap annotate individual clinical features (not used in the hierarchical clustering algorithm): sex, age, and mutation group

the intermediate and 39.8 ± 9.8 in the severe cluster). Distribution by sex (F/M) was 15/7 in the severe, 6/8 in the intermediate, and 12/4 in the mild cluster (p = n.s.), not indicative of any significant sex-related difference in this population.

Clinical–radiological correlations

A panel of correlations between lower limb muscle fatty substitution and GMW scores is shown in Supplementary Fig. 1. As expected, a negative correlation between T1 scores and functional status was observed for all muscles. The strongest correlation was observed in the iliacus muscle with a ρ coefficient of 0.81 and an infinitesimal p value. Correlation was very strong in almost all pelvic and thigh muscles, but weaker ($\rho < 0.5$) for the gluteus medius and minimus, and the peroneus. Correlations were weaker, although still statistically significant, in leg muscles, but notably higher in the peronei ($\rho = 0.74$, $p < 0.0001$). In upper limbs (Supplementary Fig. 2), stronger correlations between T1 scores and ability to raise arms were observed for the levator scapulae ($\rho = 0.52$, $p = 0.009$), trapezius ($\rho = 0.45$, $p = 0.03$), and deltoid ($\rho = 0.39$, $p = 0.042$).

Symptomatic onset and CAPN3 mutations

Cumulative incidence plots (Fig. 4) comparing age at onset between groups with 0 ($n = 21$), 1 ($n = 27$), or 2 null alleles ($n = 9$), showed earlier onset (median 10 years) in patients carrying two null alleles, as opposed to one or zero (median 14 and 20 years, respectively; p = n.s.).

Other MRI findings

In this cohort, 29/57 patients displayed a "pseudocollagen sign" characterized by a central area of high signal, previously described as 'central shadow' [3], due to a relative sparing of the central part of the muscle. This finding was already observed in collagen VI-related disorders, such as Bethlem myopathy [3, 17]. This sign was mostly present in moderately affected muscles of the lower limbs (Fig. 5). We correlated the presence or absence of "pseudocollagen

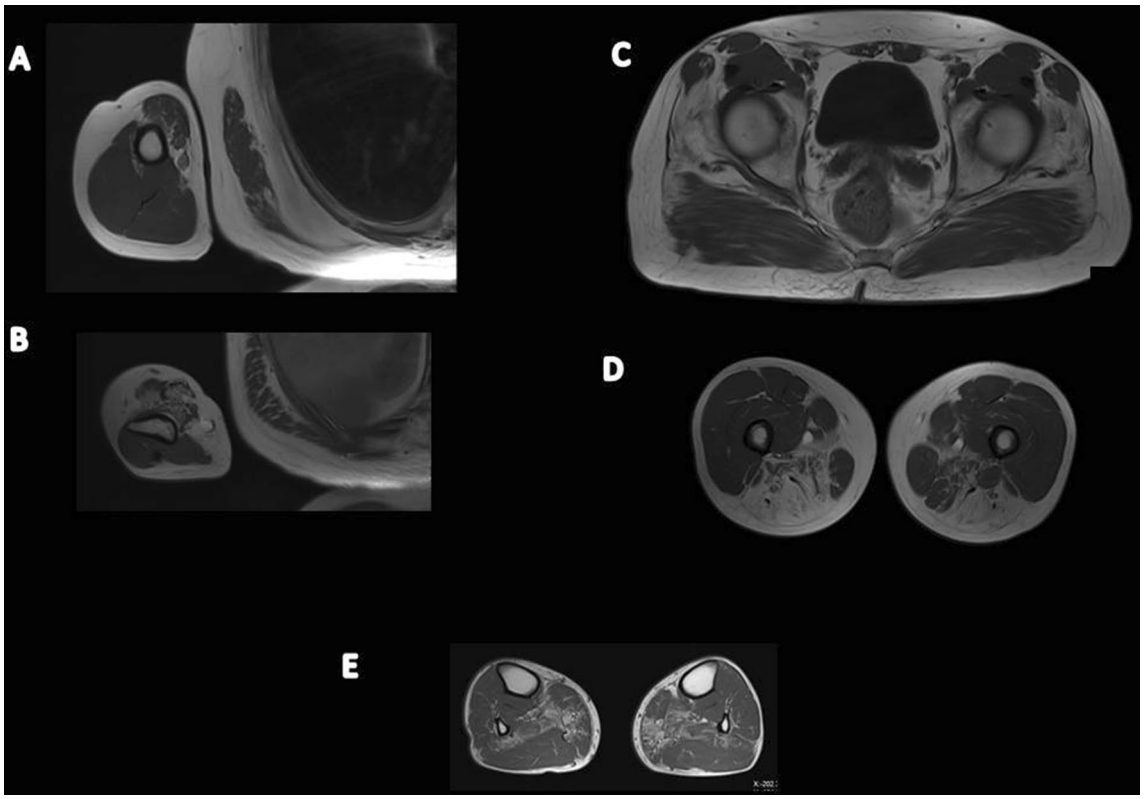

Fig. 3 Representative T1w MRI scan images. Upper and lower limb MRI scan (T1w sequence) of one LGMDR1 patient in his fifties with a moderate phenotype. **a** Arm and **b** forearm MRI scan showed moderate signs of fatty replacement of muscles belonging of the anterior compartment of arm and forearm respectively (Mercuri score 2). **c** Pelvis MRI scan showed advanced fatty replacement of gluteus minimus and medius (Mercuri score 3) and intermediate replacement of gluteus maximus (Mercuri score 2), along with a relative sparing of iliopsoas. **d** Thigh MRI scan showed a severe involvement of hamstrings and adductors (with a mild asymmetry, Mercuri score 3–4), along with a selective sparing of gracilis and quadriceps. **e** Leg MRI scan showed initial fatty replacement of soleus and gastrocnemius (Mercuri score 2) with a relative sparing of muscles belonging to the anterior compartment of the leg

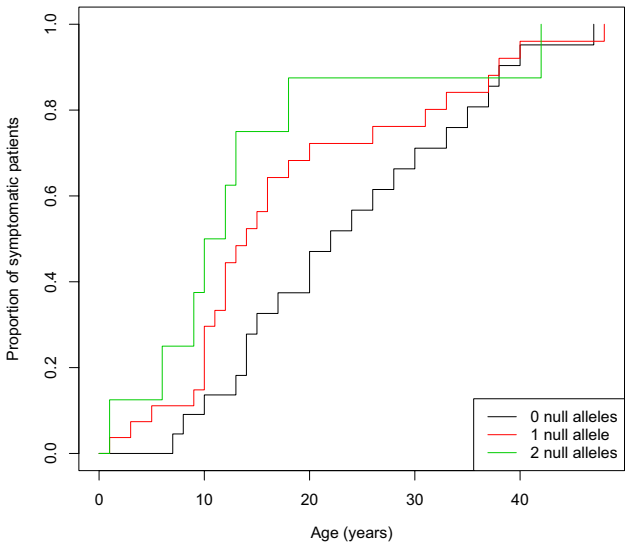

Fig. 4 Cumulative incidence plots of LGMDR1 symptom onset by genotypes. A trend toward an earlier onset in patients with two null alleles is observed

sign" with disease duration and GMW score, and we found that patients with a "pseudocollagen sign" had longer disease duration and worse disability (Supplementary Fig. 3). Moreover, when looking at scans of muscles of the posterior trunk, most patients showed a predominant involvement of extensor muscles (iliocostalis, longissimus dorsi, and spinalis dorsi), with a relative sparing of rotator muscles such as multifidus (Fig. 6). This finding agrees with the low incidence of scoliosis, and conversely high incidence of hyperlordosis in LGMDR1.

Discussion

A total of 57 genetically defined LMGDR1 patients were included in this international cohort from ten participating centers, nine belonging to the European continent and one overseas. The population studied here has several features that make it distinct from the few similar previous studies. First, two-thirds of the cohort consisted of female patients

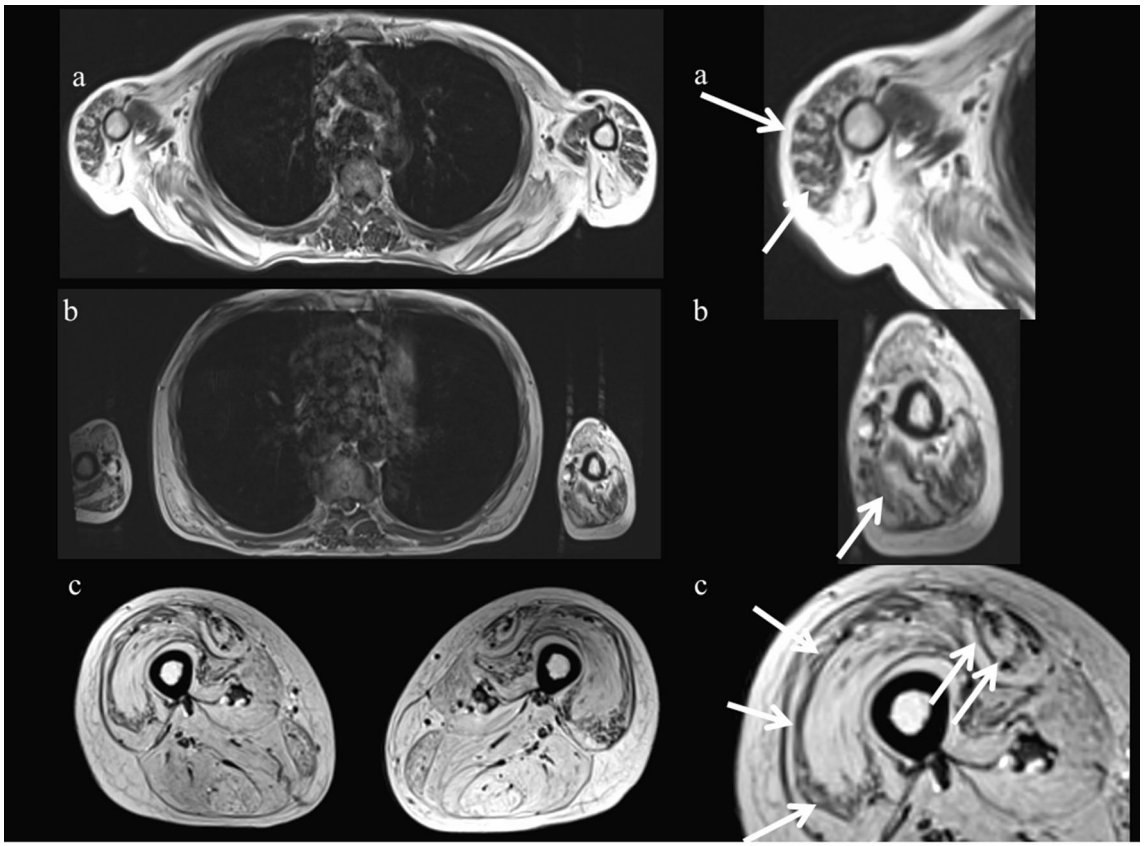

Fig. 5 T1w MRI scan images representative of the "pseudocollagen sign" in three T1-weighted axial views of a patient with LGMDR1 in his thirties. Scapular girdle (**a**), distal section of the arms (**b**) and midsection of the thighs (**c**) with zooms on the right side. Deltoid (**a**), like in collagen VI muscular dystrophy, shows a "fan"-like appearance (white arrows). In the triceps brachialis (**b**) the central part of the muscle is more "preserved" with a "band"-like appearance that has been described in collagen VI (white arrow). In the quadriceps (**c**) a band surrounded by fat at the central part of the muscle is visible in the vastus lateralis and rectus femoris, forming a "U" shape ("U-sign", white arrows)

(65%), while only one-third was male (35%). As calpainopathy is an autosomal disease, this situation is probably due to chance, but it may influence the characteristics of the population, as gender has been reported to influence the expressivity of LGMDs and especially calpainopathy [27]. Furthermore, the allelic composition of this multicenter cohort, collecting patients from different parts of Europe as well as from the genetic isolate of the La Reunion Island, is varied and peculiar. The percentage of null alleles (61%) was higher than previously reported [13], possibly contributing to the severity of clinical presentation and muscular involvement in our cohort. The most frequent mutation identified in our cohort was the frameshift mutation c.550delA, p.Thr184Argfs*36 in exon 4, which, as demonstrated previously [28–30], represents one of the most common *CAPN3* mutations in the European continent (in particular, in north-eastern Italy and Croatia) [2, 31]; the second most frequent mutation is the missense mutation c.1468C>T, p.Arg490Trp in exon 11. The latter mutation is situated in the calpain-3 domain III, and seems to impair its autolytic activity, possibly because of the charge variation in the residues involved in internal salt bridges, resulting in a reduced sensitivity to Ca^{2+} ions [27]. Homozygosity for a pathogenic variant was found in 19 cases (33%), 3 of these belonging to the "genetic isolate" of La Réunion, as typical of inbred populations.

Previous studies had demonstrated that the implementation of muscle MRI in the workup of patients affected by LGMDR1 shows several advantages compared to clinical evaluation alone [3]. Indeed, it can be used to address genetic testing in the presence of a compatible phenotype, and it can also identify a specific pattern of affected and non-affected muscles. We confirmed a predominant involvement of thigh muscles in the lower limbs, and mainly of the adductors (adductor magnus), hamstrings (semitendinosus, semimembranosus and biceps long head), and gluteus minimus, along with a relative sparing of sartorius, gracilis, quadriceps, biceps short head and iliopsoas. In the leg, we confirmed a predominant involvement of gastrocnemius medialis [14] with a lesser involvement of the lateral head,

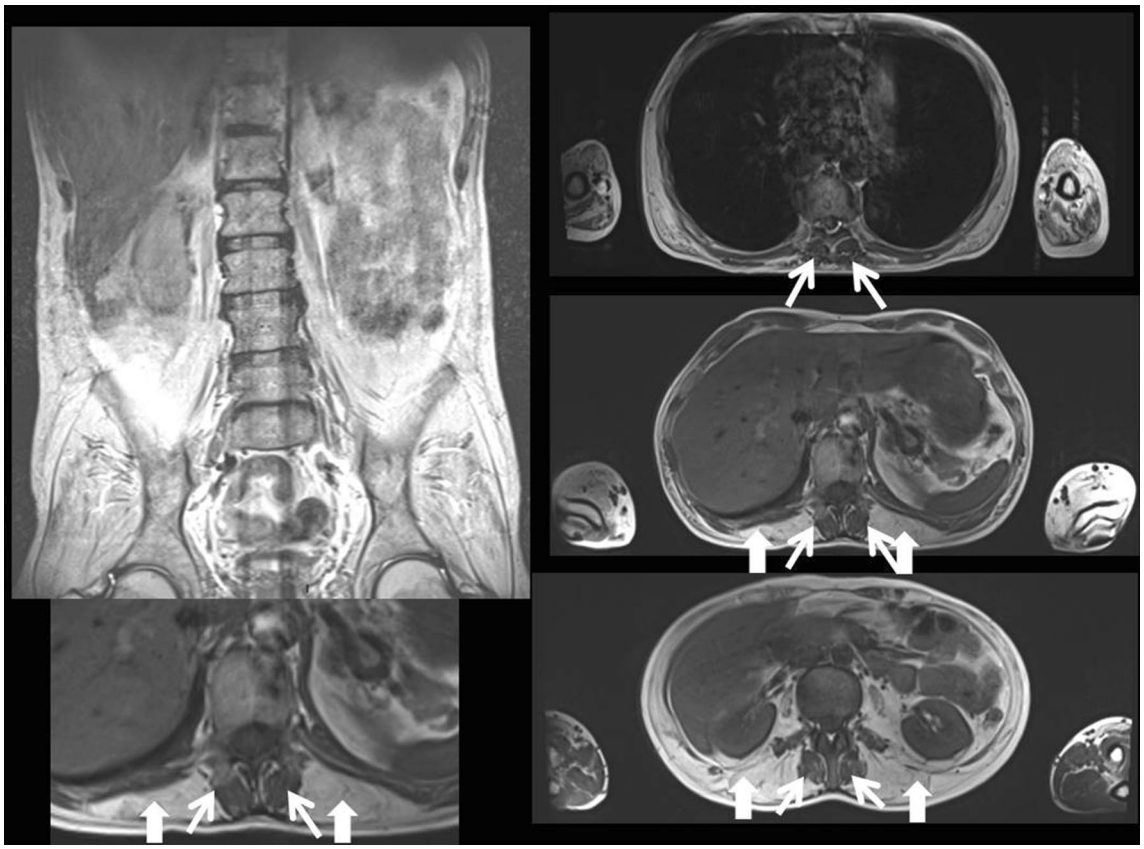

Fig. 6 Representative T1w MRI scan images of muscles of the back in T1 weighted axial and coronal views of patient with LGMDR1 in his 30s. Involvement of muscles of the back is more pronounced in extensors muscles (iliocostalis, longissimus dorsi and spinalis dorsi—thick arrows) than in rotators muscles (multifidus—thin arrows). Views in thoracic (right top), thoraco-lumbar junction (right middle) and lumbar (right bottom, zoom in left bottom). Involvement of muscles of the back is more pronounced in extensors muscles (iliocostalis, longissimus dorsi and spinalis dorsi) than in rotators muscles (multifidus). In the coronal scout view (left top) the symmetrical preservation of the multifidus muscles is associated with an absence of scoliosis

and a relative sparing of the muscles belonging to the anterolateral compartment of the leg (such as tibialis anterior and extensor digitorum longus), confirming findings from previous studies by the French and Chinese groups [13, 20].

A novel feature of this study was the MRI evaluation of the upper body districts (upper limbs, scapular girdle, neck and head), available for a number of patients of our cohort. As expected, absent or very mild muscular involvement was detected in the muscles of the head and neck. A significant involvement of the flexor muscles of the arm (biceps brachii) was also detected, with a relative sparing of the arm abductors such as deltoid and supraspinatus. Although some of these radiological findings had been already hinted at in the literature [13, 20], we confirmed this pattern in a relatively large and genetically varied LGMDR1 population, while being aware that the different MRI scanners used (1.5 T or 3 T) could have changed the sensitivity of the technique.

The recent advent of next-generation sequencing technique (NGS) has simplify the diagnostic workup of muscular dystrophies, however the recognition of this specific pattern of muscular involvement of the upper limbs could be helpful for differential diagnosis with other muscular dystrophies such as in facioscapulohumeral muscular dystrophy (FSHD) where there is a main involvement of trapezius, teres major and serratus magnus muscles [32] or in dysferlinopathy (LGMDR2/B2) which shows a predominant involvement of scapular muscles (subscapularis and latissimus dorsi) with a relative sparing of rhomboideus and levator scapulae [33]. Instead for the lower limbs the pattern of muscular involvement is rather similar with other LGMDs such sarcoglycanopathies (LGMD2C/R3-2F/R6) in which adductor and glutei muscles seem to be primarily affected (with a relative sparing of iliopsoas and muscles belonging to lower leg [19], or in LGMD2I/R9 where biceps femoris and internal adductor muscles are the first hamstring muscles involved, with a relative sparing of vastus intermedius and lateralis muscles [34].

Fatty substitution scores in lower limb muscles, which were available for the majority of this cohort, allowed an analytic approach based on hierarchical clustering, as shown in the heatmap in Fig. 2. The main observation was that

involvement of the anterior compartment of the thigh discriminated between severe vs. milder subphenotypes, and/or late vs. earlier stages of disease progression, adding to the clinical relevance of muscle MRI for staging, prognosis, and cohort stratification. When looking for causal factors driving this clustering, no obvious and major determinant emerged. Not even age, which would be naturally expected to be strongly correlated with fatty substitution severity in a progressive LGMD, differed significantly between clusters, suggesting the presence of other factors dictating phenotype. Null mutations, as previously suggested [4, 13, 27], appeared to be enriched in severely affected patients. This was confirmed by the time-to-event analysis of age at first clinical symptoms. However, the complex combinations of compound heterozygous alleles in our cohort impeded clear-cut conclusions, and several exceptions were observed which could partially justify the lack of significant correlation of number of null alleles with clinical severity and age of onset of symptoms. For instance, a group of missense mutations close to the catalytic domain (e.g., p.F80L, p P82L) seemed to be strongly associated with severe phenotypes, and more distal frameshifting or nonsense mutations (leading to a truncated, partially functional enzyme?) seemed to less consistently associate with severe disease, than proximal truncating variants. Additionally, due to a founder effect in the Venetian area, our population is characterized by a high incidence of the p.Arg490Trp mutation, associated with an upper limb-predominant "Erb" phenotype, with relative sparing of the pelvic girdle and lower limbs. These patients may complicate the interpretation of the heatmap represented in Fig. 2, which is solely based on lower limb data. Unfortunately, the score results for upper limb muscles were insufficient to perform a meaningful hierarchical clustering analysis. We plan to collect more whole-body MRI data to clarify this point. Finally, there might also be unknown environmental factors, as well as genetic modifiers (i.e., trans-acting polymorphisms in other genes), at play in determining the phenotype.

GMW scores showed a minimal disability in about one-third of patients (29.2%), moderate symptoms in about half the cohort (52.6%), and severe symptoms (wheelchair-bound patients) in a minority of patients (18.2%). T1 scores showed strong positive correlations with GMW scores. Vastus medialis, iliacus and psoas showed the strongest correlations with this score of global functionality of the lower limbs, probably due to the crucial role of these muscles in flexing the hip and blocking the knee during ambulation. Conversely, these correlations seemed to be weaker, albeit still significant, with muscles of the leg (except for gastrocnemius lateralis) and with muscles of the posteromedial compartment of the thigh (except for adductor brevis). Our findings suggest the possibility of application of the GMW score as a supportive criterion for cohort stratification not just in metabolic myopathies (such as Pompe disease) but also in LGMDR1 [35]. Usually, involvement of the upper limbs is not as early and frequent as that of lower limbs in LGMDR1. However, analysis of the motor function "raise arms" correlated to Mercuri scores of the upper limbs showed a strong correlation with some muscles, such as levator scapulae, latissimus dorsi, trapezius, deltoid, infraspinatus, and pectoralis major. While a tight correlation of muscle MRI with functional performance is anything but surprising [36], the most strongly correlated muscles may be taken as initial candidates for longitudinal studies based on quantitative MRI techniques.

Conclusion

We confirmed patterns of MRI muscle involvement in a large LGMDR1 cohort with a different allelic composition and geographic origin than previously described. We identified a crucial role of anterior thigh involvement in progression to severe disease stages in LGMDR1, and described a complex interaction of mutation type, gender, and age in dictating LGMDR1 phenotype. Peculiar radiological findings, such as pseudocollagen sign in severely affected individuals and multifidus sparing, shed light on some specific phenotypic features of calpainopathy in the LGMD scenario. As a follow-up of this study and as our future perspective, we plan to monitor the progression of muscle fatty substitution by quantitative MRI sequences such as three-point Dixon sequences, and to correlate these findings with longitudinal clinical data.

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Compliance with ethical standards

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References

1. Richard I, Broux O, Allamand V, Fougerousse F, Chiannilkulchai N, Bourg N, Brenguier L, Devaud C, Pasturaud P, Roudaut C et al (1995) Mutations in the proteolytic enzyme calpain 3 cause limb–girdle muscular dystrophy type 2A. Cell 81(1):27–40. https://doi.org/10.1016/0092-8674(95)90368-2
2. Straub V, Murphy A, Udd B (2018) LGMD workshop study group 229th ENMC international workshop: Limb girdle muscular dystrophies—Nomenclature and reformed classification Naarden, the Netherlands, 17–19 March 2017. Neuromuscul Disord 28(8):702–710. https://doi.org/10.1016/j.nmd.2018.05.007
3. Fardeau M, Hillaire D, Mignard C, Feingold N, Feingold J, Mignard D, de Ubeda B, Collin H, Tome FM, Richard I, Beckmann J (1996) Juvenile limb–girdle muscular dystrophy Clinical, histopathological and genetic data from a small community living in the Reunion Island. Brain 119(Pt 1):295–308. https://doi.org/10.1093/brain/119.1.295
4. Mercuri E, Lampe A, Allsop J, Knight R, Pane M, Kinali M, Bonnemann C, Flanigan K, Lapini I, Bushby K, Pepe G, Muntoni F (2005) Muscle MRI in Ullrich congenital muscular dystrophy and Bethlem myopathy. Neuromuscul Disord 15(4):303–310. https://doi.org/10.1016/j.nmd.2005.01.004
5. Urtasun M, Sáenz A, Roudaut C, Poza JJ, Urtizberea JA, Cobo AM, Richard I, García Bragado F, Leturcq F, Kaplan JC, Martí Massó JF, Beckmann JS, López de Munain A (1998) Limb–girdle muscular dystrophy in Guipúzcoa (Basque Country, Spain). Brain 121(Pt 9):1735–1747. https://doi.org/10.1093/brain/121.9.1735
6. Gallardo E, Saenz A, Illa I (2011) Limb–girdle muscular dystrophy 2A. Handb Clin Neurol 101:97–110. https://doi.org/10.1016/B978-0-08-045031-5.00006-2
7. Richard I, Roudaut C, Saenz A, Pogue R, Grimbergen JE, Anderson LV, Beley C, Cobo AM, de Diego C, Eymard B, Gallano P, Ginjaar HB, Lasa A, Pollitt C, Topaloglu H, Urtizberea JA, de Visser M, van der Kooi A, Bushby K, Bakker E, Lopez de Munain A, Fardeau M, Beckmann JS (1999) Calpainopathy—a survey of mutations and polymorphisms. Am J Hum Genet 64(6):1524–1540. https://doi.org/10.1086/302426
8. De Paula F, Vainzof M, Passos-Bueno MR, de Cássia M, Pavanello R, Matioli SR, Anderson L, Nigro V, Zatz M (2002) Clinical variability in calpainopathy: what makes the difference? Eur J Hum Genet 10(12):825–832. https://doi.org/10.1038/sj.ejhg.5200888
9. Sáenz A, Leturcq F, Cobo AM, Poza JJ, Ferrer X, Otaegui D, Camaño P, Urtasun M, Vílchez J, Gutiérrez-Rivas E, Emparanza J, Merlini L, Paisán C, Goicoechea M, Blázquez L, Eymard B, Lochmuller H, Walter M, Bonnemann C, Figarella-Branger D, Kaplan JC, Urtizberea JA, Martí-Massó JF, López de Munain A (2005) LGMD2A: genotype-phenotype correlations based on a large mutational survey on the calpain 3 gene. Brain 128(Pt 4):732–742. https://doi.org/10.1093/brain/awh408
10. Krahn M, Bernard R, Pecheux C, el Hammouda H, Eymard B, Lopez de Munain A, Cobo AM, Romero N, Urtizberea A, Leturcq F, Levy N (2006) Calpain Study Group of the French LGMD Network Screening of the CAPN3 gene in patients with possible LGMD2A. Clin Genet 69(5):444–449. https://doi.org/10.1111/j.1399-0004.2006.00603.x
11. Nascimbeni AC, Fanin M, Tasca E, Angelini C (2010) Transcriptional and translational effects of intronic CAPN3 gene mutations. Hum Mutat 9:1658–1669. https://doi.org/10.1002/humu.21320
12. Richard I, Roudaut C, Marchand S, Baghdiguian S, Herasse M, Stockholm D, Ono Y, Suel L, Bourg N, Sorimachi H, Lefranc G, Fardeau M, Sébille A, Beckmann JS (2000) Loss of calpain 3 proteolytic activity leads to muscular dystrophy and to apoptosis-associated IkappaBalpha/nuclear factor kappaB pathway perturbation in mice. J Cell Biol 151(7):1583–1590. https://doi.org/10.1083/jcb.151.7.1583
13. Richard I, Hogrel JY, Stockholm D, Payan CA, Fougerousse F; Calpainopathy Study Group, Eymard B, Mignard C, de Munain AL, Fardeau M, Urtizberea JA (2016) Natural history of LGMD2A for delineating outcome measures in clinical trials. Ann Clin Transl Neurol 3(4):248–265. https://doi.org/10.1002/acn3.287
14. Vissing J, Barresi R, Witting N, Van Ghelue M, Gammelgaard L, Bindoff LA, Straub V, Lochmüller H, Hudson J, Wahl CM, Arnardottir S, Dahlbom K, Jonsrud C, Duno M (2016) A heterozygous 21-bp deletion in CAPN3 causes dominantly inherited limb girdle muscular dystrophy. Brain 139(Pt 8):2154–2163. https://doi.org/10.1093/brain/aww133
15. Mercuri E, Pichiecchio A, Allsop J, Messina S, Pane M, Muntoni F (2007) Muscle MRI in inherited neuromuscular disorders: past, present, and future. J Magn Reson Imaging 25(2):433–440. https://doi.org/10.1002/jmri.20804
16. Tasca G, Iannaccone E, Monforte M, Masciullo M, Bianco F, Laschena F, Ottaviani P, Pelliccioni M, Pane M, Mercuri E, Ricci E (2012) Muscle MRI in Becker muscular dystrophy. Neuromuscul Disord 1(22 Suppl 2):S100–S106. https://doi.org/10.1016/j.nmd.2012.05.015
17. Quijano-Roy S, Avila-Smirnow D, Carlier RY, WB-MRI muscle study group (2012) Whole body muscle MRI protocol: pattern recognition in early onset NM disorders. Neuromuscul Disord 1(22 Suppl 2):S68–84. https://doi.org/10.1016/j.nmd.2012.08.003
18. Barp A, Bello L, Caumo L, Campadello P, Semplicini C, Lazzarotto A, Sorarù G, Calore C, Rampado A, Motta R, Stramare R, Pegoraro E (2017) Muscle MRI and functional outcome measures in Becker muscular dystrophy. Sci Rep 7(1):16060. https://doi.org/10.1038/s41598-017-16170-2
19. Tasca G, Monforte M, Díaz-Manera J, Brisca G, Semplicini C, D'Amico A, Fattori F, Pichiecchio A, Berardinelli A, Maggi L, Maccagnano E, Løkken N, Marini-Bettolo C, Munell F, Sanchez A, Alshaikh N, Voermans NC, Dastgir J, Vlodavets D, Haberlová J, Magnano G, Walter MC, Quijano-Roy S, Carlier RY, van Engelen BGM, Vissing J, Straub V, Bönnemann CG, Mercuri E, Muntoni F, Pegoraro E, Bertini E, Udd B, Ricci E, Bruno C (2018) MRI in sarcoglycanopathies: a large international cohort study. J Neurol Neurosurg Psychiatry 89(1):72–77. https://doi.org/10.1136/jnnp-2017-316736
20. Feng X, Luo S, Li J, Yue D, Xi J, Zhu W, Gao X, Guan X, Lu J, Liang Z, Zhao C (2018) Fatty infiltration evaluation and selective pattern characterization of lower limbs in limb–girdle muscular dystrophy type 2A by muscle magnetic resonance imaging. Muscle Nerve 58(4):536–541. https://doi.org/10.1002/mus.26169
21. Desmet FO, Hamroun D, Lalande M, Collod-Béroud G, Claustres M, Béroud C (2009) Human Splicing Finder: an online bioinformatics tool to predict splicing signals. Nucleic Acids Res 37(9):e67. https://doi.org/10.1093/nar/gkp215
22. Carlier RY, Laforet P, Wary C, Mompoint D, Laloui K, Pellegrini N, Annane D, Carlier PG, Orlikowski D (2011) Whole-body muscle MRI in 20 patients suffering from late onset Pompe disease: Involvement patterns. Neuromuscul Disord 21(11):791–799. https://doi.org/10.1016/j.nmd.2011.06.748
23. Pogoda TV, Krakhmaleva IN, Lipatova NA, Shakhovskaya NI, Shishkin SS, Limborska SA (2000) High incidence of 550delA

mutation of CAPN3 in LGMD2 patients from Russia. Hum Mutat 15(3):295. https://doi.org/10.1002/(SICI)1098-1004(200003)15:3%3c295:AID-HUMU15%3e3.0.CO;2-8
24. Milic A, Canki-Klain N (2005) Calpainopathy (LGMD2A) in Croatia: molecular and haplotype analysis. Croat Med J 46(4):657–663
25. Dorobek M, Ryniewicz B, Kabzińska D, Fidziańska A, Styczyńska M, Hausmanowa-Petrusewicz I (2015) The frequency of c.550delA mutation of the CANP3 gene in the Polish LGMD2A population. Genet Test Mol Biomark 19(11):637–640. https://doi.org/10.1089/gtmb.2015.0131
26. Matsubara E, Tsuchiya A, Minami N, Nishino I, Pappolla MA, Shoji M, Abe K (2007) A unique case of limb–girdle muscular dystrophy type 2A carrying novel compound heterozygous mutations in the human CAPN3 gene. Eur J Neurol 14(7):819–822. https://doi.org/10.1111/j.1468-1331.2007.01808.x
27. Fanin M, Nascimbeni AC, Angelini C (2014) Gender difference in limb–girdle muscular dystrophy: a muscle fiber morphometric study in 101 patients. Clin Neuropathol 33(3):179–185. https://doi.org/10.5414/NP300728
28. Fanin M, Nascimbeni AC, Fulizio L, Angelini C (2005) The frequency of limb girdle muscular dystrophy 2A in northeastern Italy. Neuromuscul Disord 15(3):218–224. https://doi.org/10.1016/j.nmd.2004.11.003
29. Richard I, Brenguier L, Dinçer P, Roudaut C, Bady B, Burgunder JM, Chemaly R, Garcia CA, Halaby G, Jackson CE, Kurnit DM, Lefranc G, Legum C, Loiselet J, Merlini L, Nivelon-Chevallier A, Ollagnon-Roman E, Restagno G, Topaloglu H, Beckmann JS (1997) Multiple independent molecular etiology for limb–girdle muscular dystrophy type 2A patients from various geographical origins. Am J Hum Genet 60(5):1128–1138
30. Dinçer P, Leturcq F, Richard I, Piccolo F, Yalnizoglu D, de Toma C, Akçören Z, Broux O, Deburgrave N, Brenguier L, Roudaut C, Urtizberea JA, Jung D, Tan E, Jeanpierre M, Campbell KP, Kaplan JC, Beckmann JS, Topaloglu H (1997) A biochemical, genetic, and clinical survey of autosomal recessive limb girdle muscular dystrophies in Turkey. Ann Neurol 42(2):222–229. https://doi.org/10.1002/ana.410420214
31. Canki-Klain N, Milic A, Kovac B, Trlaja A, Grgicevic D, Zurak N, Fardeau M, Leturcq F, Kaplan JC, Urtizberea JA, Politano L, Piluso G, Feingold J (2004) Prevalence of the 550delA mutation in calpainopathy (LGMD 2A) in Croatia. Am J Med Genet A 125A(2):152–156. https://doi.org/10.1002/ajmg.a.20408
32. Gerevini S, Scarlato M, Maggi L, Cava M, Caliendo G, Pasanisi B, Falini A, Previtali SC, Morandi L (2016) Muscle MRI findings in facioscapulohumeral muscular dystrophy. Eur Radiol 26(3):693–705. https://doi.org/10.1007/s00330-015-3890-1
33. Diaz-Manera J, Fernandez-Torron R, LLauger J, James MK, Mayhew A, Smith FE, Moore UR, Blamire AM, Carlier PG, Rufibach L, Mittal P, Eagle M, Jacobs M, Hodgson T, Wallace D, Ward L, Smith M, Stramare R, Rampado A, Sato N, Tamaru T, Harwick B, Rico Gala S, Turk S, Coppenrath EM, Foster G, Bendahan D, Le Fur Y, Fricke ST, Otero H, Foster SL, Peduto A, Sawyer AM, Hilsden H, Lochmuller H, Grieben U, Spuler S, Tesi Rocha C, Day JW, Jones KJ, Bharucha-Goebel DX, Salort-Campana E, Harms M, Pestronk A, Krause S, Schreiber-Katz O, Walter MC, Paradas C, Hogrel JY, Stojkovic T, Takeda S, Mori-Yoshimura M, Bravver E, Sparks S, Bello L, Semplicini C, Pegoraro E, Mendell JR, Bushby K, Straub V; Jain COS Consortium (2018) Muscle MRI in patients with dysferlinopathy: pattern recognition and implications for clinical trials. J Neurol Neurosurg Psychiatry 89(10):1071–1081. https://doi.org/10.1136/jnnp-2017-317488
34. Willis TA, Hollingsworth KG, Coombs A, Sveen ML, Andersen S, Stojkovic T, Eagle M, Mayhew A, de Sousa PL, Dewar L, Morrow JM, Sinclair CD, Thornton JS, Bushby K, Lochmuller H, Hanna MG, Hogrel JY, Carlier PG, Vissing J, Straub V (2014) Quantitative magnetic resonance imaging in limb–girdle muscular dystrophy 2I: a multinational cross-sectional study. PLoS ONE 9(2):e90377. https://doi.org/10.1371/journal.pone.0090377
35. Regnery C, Kornblum C, Hanisch F, Vielhaber S, Strigl-Pill N, Grunert B, Müller-Felber W, Glocker FX, Spranger M, Deschauer M, Mengel E, Schoser B (2012) 36 months observational clinical study of 38 adult Pompe disease patients under alglucosidase alfa enzyme replacement therapy. J Inherit Metab Dis 35(5):837–845. https://doi.org/10.1007/s10545-012-9451-8
36. Carlier PG, Marty B, Scheidegger O, Loureiro de Sousa P, Baudin PY, Snezhko E, Vlodavets D (2016) Skeletal muscle quantitative nuclear magnetic resonance imaging and spectroscopy as an outcome measure for clinical trials. J Neuromuscul Dis 3(1):1–28. https://doi.org/10.3233/JND-160145

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