Clinical and Molecular Spectrum Associated with COL6A3 c.7447A>G p.(Lys2483Glu) Variant: Elucidating its Role in Collagen VI-related Myopathies

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Abstract.
Background: Dominant and recessive autosomal pathogenic variants in the three major genes (*COL6A1-A2-A3*) encoding the extracellular matrix protein collagen VI underlie a group of myopathies ranging from early-onset severe conditions (Ullrich congenital muscular dystrophy) to milder forms maintaining independent ambulation (Bethlem myopathy). Diagnosis is based on the combination of clinical presentation, muscle MRI, muscle biopsy, analysis of collagen VI secretion, and *COL6A1-A2-A3* genetic analysis, the interpretation of which can be challenging.
Objective: To refine the phenotypical spectrum associated with the frequent *COL6A3* missense variant c.7447A>G (p.Lys2483Glu).
Methods: We report the clinical and molecular findings in 16 patients: 12 carrying this variant in compound heterozygosity with another *COL6A3* variant, and four homozygous.
Results: Patients carrying this variant in compound heterozygosity with a truncating *COL6A3*·variant exhibit a phenotype consistent with COL6-related myopathies (COL6-RM), with joint contractures, proximal weakness and skin abnormalities. All remain ambulant in adulthood and only three have mild respiratory involvement. Most show typical muscle MRI findings. In five patients, reduced collagen VI secretion was observed in skin fibroblasts cultures. All tested parents were healthy heterozygous carriers. Conversely, two out of four homozygous patients did not present with the classical COL6-RM clinical and imaging findings. Collagen VI immunolabelling on cultured fibroblasts revealed rather normal secretion in one and reduced secretion in another. Muscle biopsy from one homozygous patient showed myofibrillar disorganization and rimmed vacuoles.
Conclusions: In light of our results, we postulate that the *COL6A3* variant c.7447A>G may act as a modulator of the clinical phenotype. Thus, in patients with a typical COL6-RM phenotype, a second variant must be thoroughly searched for, while for patients with atypical phenotypes further investigations should be conducted to exclude alternative causes.

Keywords: Collagen VI-related myopathies, *COL6A3*, collagen type VI, neuromuscular disorders, limb-girdle muscular dystrophy (LGMD), congenital muscular dystrophy (CMD), muscular MRI, NGS

INTRODUCTION

Collagen VI is an extracellular matrix protein present in most tissues, notably in muscle, skin, tendon and blood vessels. Dominant or recessive autosomal mutations in each of the three "major" genes encoding the collagen VI α-chains (*COL6A1, COL6A2, COL6A3*) underlie collagen VI-related myopathies (COL6-RM), a heterogeneous group of disorders marked by a combined muscle and connective tissue involvement including joint laxity and contractures, as well as characteristic cutaneous abnormalities (*i.e.* keloid scars, keratosis pilaris and soft/velvety skin) in addition to muscle weakness.

COL6-RM clinical spectrum ranges from early-onset severe conditions (Ullrich congenital muscular dystrophy, UCMD) through phenotypes of intermediate severity to milder forms (Bethlem myopathy, BM) [1, 2]. Classically, UCMD patients present with congenital weakness and hypotonia, delayed motor milestones, associated with proximal joint contractures and concomitant marked distal hyperlaxity. Rigid spine, scoliosis, hip dislocation/dysplasia and prominent calcaneus are common features. Progressive weakness leads to early loss of ambulation in most patients, and restrictive respiratory involvement occurs in most severely affected patients during the first two decades of life [3]. Conversely, BM phenotype is marked by milder proximal weakness associated with contractures typically affecting Achilles tendons, elbows, pectoralis, long finger flexors and interphalangeal joints. Although a slowly-progressive condition, two-thirds of patients over the age of 60 years may need assistance with ambulation [4, 5].

Nonetheless, there is a wide clinical variability, and intermediate phenotypes are now well recognized. Interestingly, patients can present with predominantly proximal weakness and very few or absent contractures or distal hyperlaxity, more akin to a limb-girdle muscular dystrophy (LGMD) [6, 7]. Furthermore, recessive mutations in *COL6A2* have also been associated with severe and widespread contractures known as myosclerosis [8].

Diagnosis is based on the combination of clinical presentation supported by muscle MRI, muscle biopsy findings, immunohistochemical examination of collagen VI secretion and analysis of the *COL6A1-3* genes sequences. Characteristic muscle

MRI findings of COL6-RM include a peculiar fatty replacement starting around the fascia surrounding the muscle ("outside-in" picture) with the presence of the so-called "central-cloud or shadow" typically affecting the *rectus femoris* and *vastus lateralis* [9].

Although UCMD was initially described as an autosomal recessive condition, dominant, mostly *de novo* mutations, have also been identified. Conversely, rare autosomal recessive mutations have been reported in BM patients, although it is mostly inherited as a dominant condition [4, 10]. Interpretation of genetic variants can be challenging, since there are few mutational hotspots, and the *COL6A1-3* genes are highly polymorphic. Furthermore, the clinical consequence of the many *COL6A1-3* variants that affect residues of unknown impact on the heterotrimeric assembly of COLVI (in particular variants outside of the triple helical domain) may be difficult to interpret and to validate experimentally.

We report a total of 16 patients carrying the *COL6A3* missense variant c.7447A>G either as compound heterozygotes with another *COL6A3* variant (12 patients from 10 families) or in a homozygous state (4 unrelated patients). Upon extensive analysis of the clinical phenotype and ancillary tests including muscle imaging pattern, muscle biopsy histology and COLVI secretion in dermal fibroblasts, we discuss the potential pathogenicity of this variant.

MATERIAL AND METHODS

Patients

Through an international collaboration, we identified 16 patients from 14 families (Table 1) carrying the c.7447A>G variant in the *COL6A3* gene in heterozygosity with another *COL6A3* mutation ($n = 12$) or in homozygosity ($n = 4$). Clinical data and ancillary tests (including serum CK levels, EMG, muscle MRI and muscle biopsy) were retrospectively retrieved and analyzed. All patients were examined by at least one of the authors in specialized neuromuscular departments. Diagnostic skeletal muscle biopsies were obtained, processed for standard histological and immunochemical studies and fixed for electron microscopy as previously described [11].

Informed consent was obtained from all patients in agreement with local ethical committees and with the 1964 Helsinki declaration and its later amendments (NIH, National Institute of Neurological Disorders and Stroke (NINDS), Institutional Review Board (Protocol 12N0095))

Genetic analysis, bioinformatics analysis and variants interpretation

Details on genetic testing and bioinformatics analysis can be found in Supplemental data. Pathogenicity of variants was determined according to current ACMG guidelines [12]. Variants were filtered out according to their allele frequency ($\leq 1\%$) as reported in the GnomAD database (http://gnomad.broadinstitute.org/). We then evaluated each variant considering a review of the literature, the location of the variant in the gene and the resulting corresponding protein, the *in silico* prediction tools (Polyphen2, SIFT, GVGD and CADD for missense variants and SpliceSiteFinder-like, MaxEntScan, NNSPLICE, GeneSplicer and Human Splicing Finder for splicing variants) and functional studies when available. SuSPect method (http://www.sbg.bio.ic.ac.uk/suspect/) was also used for prediction. All variants considered as pathogenic and likely pathogenic have been confirmed by a second independent method (Sanger sequencing).

Collagen VI immunolabelling

Dermal fibroblasts from eight index patients and one control individual were cultured to confluency in DMEM (Gibco) supplemented with 10% FBS (Biosera), penicillin/streptomycin (5700U Pen/5700 μg Strep; Gibco), and 50 μg/ml L-Ascorbic acid-2-phosphate (Sigma). Fixed cells were immunostained using either the polyclonal antibody Ab6588 (Ab cam) as initially described in [13], or an α3(VI) specific antibody (HPA010080; Sigma-Aldrich). For the latter, cells were fixed with cold methanol prior to immunostaining. Confocal imaging was performed on a Nikon Ti2 microscope equipped with a motorized stage and a Yokogawa CSU-W1 spinning disk head coupled with a Prime 95 sCMOS camera (Photometrics). Z-stacks were obtained using a 0.15 μm steps, with a 40x/1.30 NA oil-immersion objective. Images were acquired with the same exposure setting, using the Metamorph software (Molecular Devices), and subsequently analyzed using Fiji [14]. Stacks were merged and Z projections (Sum slices) were obtained.

RESULTS

Genetics

Next Generation Sequencing (NGS) allowed the identification of the c.7447A>G p.(Lys2483Glu)

Table 1
Summarized clinical findings. DMM: delayed motor milestones; F : female; FH : follicular hyperkeratosis; FVC: forced vital capacity;LL : lower limbs; M : male; m : months; N/A : non-applicable; UL : upper limbs; y :years

Patient/ Gender	Onset	First symptoms	Age at last visit (y)	Weakness	Loss of ambulation	Contractures	Hyperlaxity Spine	Rigid involvement	Respiratory features	Skin
1-II.1/ M	Childhood	Poor sports performance	39	Axial+Proximal (UL 3/4, LL 3-4/5)	No	Wrists, elbows, knees, ankles	No	No	Yes(FVC 72%)	No
1-II.2/ F	Childhood	Difficulty in running, abnormal gait	31	Axial+Proximal (3/5 UL, 4/5 LL)	No. Walks with cane since 15y	Finger flexors, elbows, ankles	Yes	Yes	No	FH, prominent scars
2-II.1/ M	Childhood	Poor sports performance	62	Axial+Proximal (3/5 UL, LL)	No. Walks with cane since adulthood	Elbows, finger flexors, wrists, ankles, knees	No	Yes	No	No
3-III.1/ M	Early-childhood	Waddling gait, falls	18	Axial+Proximal (3/5UL,LL)	No	Finger flexors, ankles	Yes	Yes	Yes(FVC 68%)	FH
4-II.2/ M	Childhood	Difficulty running, joint contractures	51	Axial+Proximal	No	Elbows, fingers, wrists, ankles	No	No	No	No
5-II.2/ M	Early-chilhood	DMM, frequent falls, ankle contractures	21	Axial+Proximal (4/5 UL, LL)	No	Finger flexors, elbows, ankles	No	Yes	No	Velvety skin
6-II.1/ M	Early-childhood	Abnormal gait, toe walking	55	Axial+Proximal (3 + /5 UL, LL)	No. Walks with cane since adulthood	Fingers, elbows, knees, ankles	No	No	No	No
7-II.1/ M	Adulthood	Waddling gait, proximal weakness	64	Axial+Proximal (4/5 UL, LL)	No	Finger flexors, wrist flexors, triceps surae	No	No	No	No
8-II.2/ F	At birth	Congenital torticollis DMM,falls, waddling gait	3.5	Axial+Proximal (4/5 UL, LL)	No	Ankles	Yes	No	No	No
9-II.5/ M	Childhood	Proximal weakness	54	Axial+Proximal (4/5 UL, LL)	No	Finger flexors, elbows, ankles	No	No	Yes(FVC 60%)	FH
10-II.1/ F	At birth	Decreased fetal movements, DMM	7	Axial+Proximal (4/5UL, LL)+Distal (3-4/5)	No	Ankles	Yes	No	No	No
10-II.3/ M	Congenital	Decreased fetal movements, DMM	2	Axial+Proximal	No	Ankles	Yes	No	N/A	FH
11-II.5/ M	Childhood	Bilateral *equinovarus* steppage gait	49	Distal (3/5 LL)	No	No	No	No	No	No
12-II.2/ M	Early childhood	Foot deformities, toe-walking	13	Proximal	No	Ankles	No	No	No	No
13-II.1/ F	Childhood	*Pes cavus*, abnormal gait	60	Axial+Proximal	No	Ankles	No	No	No	No
14-II.1/ F	Early childhood	DMM, difficulty running	77	Proximal (3/5 UL, LL)+ Distal (4/5 LL)	No, walker-since 74y	Finger flexors, ankles	No	No	No	No

variant in 16 patients, 5 females and 11 males ranging from age 2 to 77 years (mean 37.6 ± 21.8) at last examination (Table 1).

The *COL6A3* c.7447A>G missense variant (rs139260335 in dbSNP) is located in exon 36. At the protein level, the substitution affects the C1/11th Von Willebrand factor A domain (VWA 11), located in a non-helical domain, and leads to an amino acid change with no significant post-translational modifications.

This variant was found either in compound heterozygosity associated with another exonic deletion, truncating or splicing *COL6A3* variant ($n = 12$) or in homozygous state ($n = 4$) (Table 2). Segregation

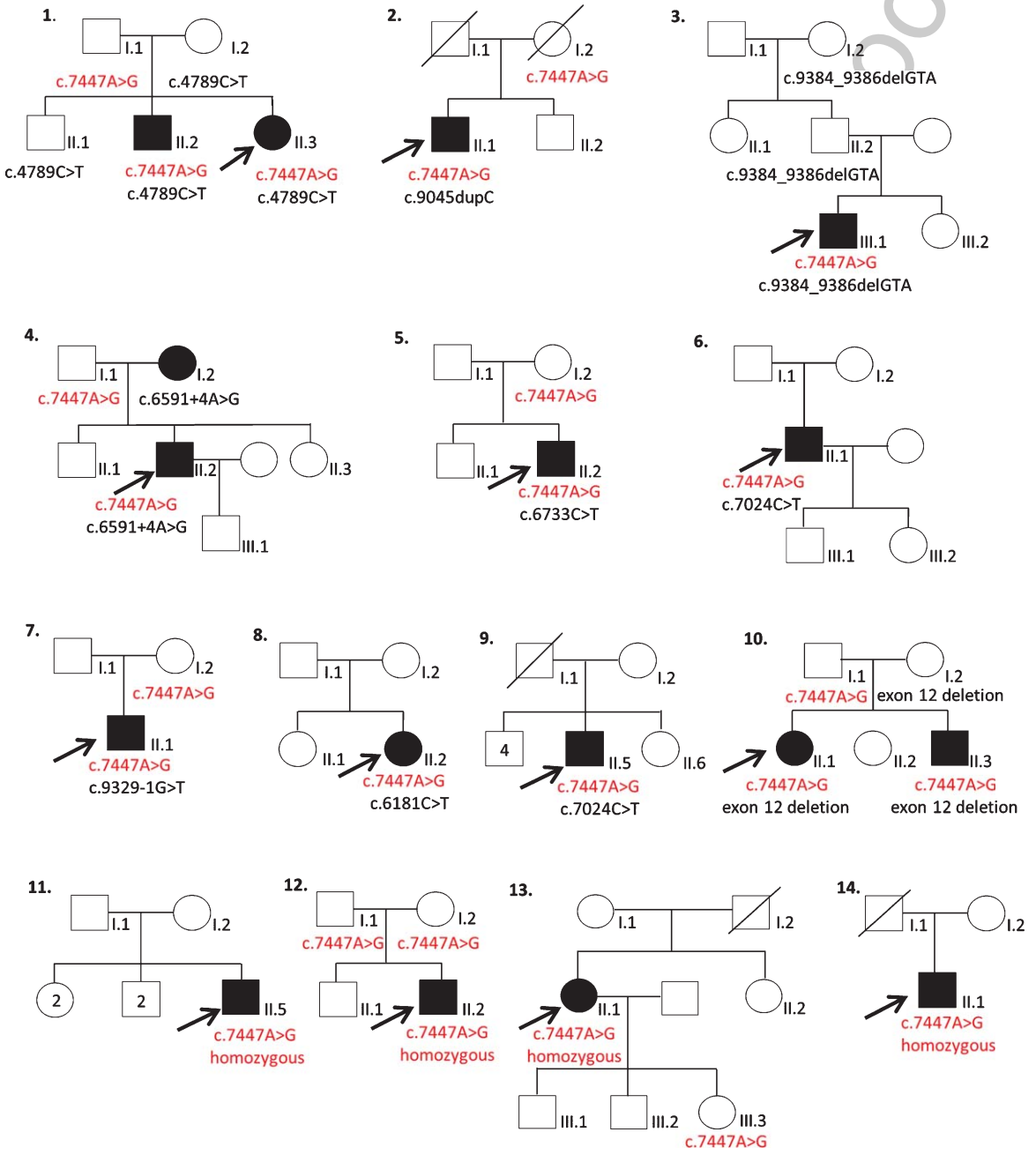

Fig. 1. Family pedigrees. Black filled symbols: affected individual. Crossed symbol: deceased individual.

studies were performed in eight families and confirmed that the tested parents were healthy heterozygous carriers (Fig. 1).

Four patients (11-II.5, 12-II.2, 13-II.1 and 14-II.1) were homozygous for the c.7447A>G *COL6A3* variant. Patient 13-II.1's daughter was a healthy heterozygous carrier. No segregation studies could be performed for patients 11-II.5, 12-II.2 and 14-II.1. Patient 13-II.1 also harbored a heterozygous *TNXB* variant c.4535_4552del, p.(Asp1512_Val1517del), whose allelic frequency was found to be low in gnomAD (13 allele count/275252) and reported as probably pathogenic in one patient in the LOVD database (reference #0000528445) associated with a known pathogenic *TNXB* variant. No second *TNXB* variant was identified in this patient.

Furthermore, no other variant that could affect splice, were found in the *COL6A1-A2-A3* genes after sequencing of the coding regions from cultured skin fibroblasts for patients 12-II.2, 13-II.1 and 14-II.1 harboring the c.7447A>G at the homozygous state (no skin biopsy was available for 11-II.5 patient) (data not shown). Whole exome sequencing (WES) was performed in three compound heterozygous patients (**7.II.1,** 8-II.2, 10-II.1), thereby excluding additional pathogenic variants in other disease-causing genes.

Regarding patient 11-II.5, NGS panel for analysis of genes related to LGMD, distal, myofibrillar panels including *NEB* and *ACTA1* genes (responsible for most forms of nemaline myopathy) disclosed no pathogenic variants.

Clinical presentation of compound heterozygous patients

Eleven (92%) patients developed the first symptoms at birth or in early childhood, one around puberty and one in adulthood. None of them had a family history of neuromuscular disease. The most commonly recognized presenting symptoms were delayed motor development, waddling gait, poor sports performance and joint contractures. Decreased fetal movements were noted in two patients. Gait acquisition was delayed in two patients (5-II.2 and 10-II.3) who reached independent ambulation at 18 and 21 months, respectively. Two additional patients (8-II.2 and 10-II.1) started walking at 14 months and were noted to have a waddling gait. Muscle weakness was present in all patients with predominant axial and proximal involvement in 11 (92%), MRC grade 3-4/5. Prominent distal weakness was observed in one patient, affecting finger flexors and extensors in the upper limbs and ankle dorsiflexion and eversion in the lower limbs (MRC 3/5). Independent ambulation was maintained in all patients although three patients (25%) required assistance (Table 1).

Joint contractures were present in all patients, mainly affecting finger flexors, elbows and ankles. Rigid spine was noted in four patients (33%). None of them had scoliosis. Distal hyperlaxity was observed in four patients (33%). Five patients (42%) were found to have cutaneous abnormalities including hyperkeratosis, keloid scars and velvety skin.

Forced Vital Capacity was measured in 15 patients and showed mild restrictive respiratory insufficiency in three of them (1-II.1, 3-III.1 and 9-II.5) but they did not need ventilatory support (Table 1). One patient (4-II.2) required a pacemaker due to left bundle branch block at age 48 years.

Serum Creatine Kinase (CK) levels were mildly to moderately elevated in all patients (range 120-970 IU/L). Electromyography (EMG) was performed in 10 patients, which revealed predominately myopathic findings in seven patients. Patients 8-II.2 and 10.II.1 were found to have normal nerve conduction studies (NCS). EMG was limited due to poor tolerance in patient 8-II.2. For patient 10.II.1, EMG at age 5 years disclosed slightly increased duration and amplitude and mildly reduced recruitment of the left *vastus lateralis*. Lastly, for patient 9-II.5, NCS showed absent peroneal motor response recorded at the *extensor digitorum brevis* bilaterally and EMG showed small motor units suggestive of a myopathic pattern in the proximal upper limbs, but neurogenic changes of the distal and proximal legs at age 55 years. Muscle MRI (Fig. 2A and Supplementary Figure 1) was performed in eight patients and showed characteristic COL6-RM findings in seven, namely fatty replacement starting around the fascia surrounding *vastus lateralis* ("outside in") and the so-called "central cloud" pattern in the *rectus femoris*. Two patients' MRI showed fatty replacement of pelvic girdle and proximal leg muscles without the characteristic previously mentioned findings. Eight patients underwent a muscle biopsy, revealing prominent dystrophic features in seven (87.5%).

Clinical findings and ancillary tests in homozygous patients

Recognition of first symptoms was in childhood in all four patients. Patient 11-II.5 presented with foot deformities and steppage gait first recognized at age 10 years. Patient 12-II.2 displayed equinovarus feet

Table 2
Genetics and ancillary tests. COL6-RM: Collagen VI related myopathies; FSV: fiber size variation; IN: internalized nuclei; LL : lower limbs; N/A : non-applicable; NCS: nerve conduction studies; ns: not shown;UL: upper limbs; WB : Western Blot

Patient	*COL6A3* variants	CK (IU/L)	Muscle biopsy	Muscle MRI	Collagen VI secretion	Compatible COL6-RM phenotype
1-II.1	c.7447A>G,p.Lys2483Glu+c.4789 C>T,p.Arg1597*	190	N/A	Typical COL6-RM features	N/A	Yes
1-II.2	c.7447A>G,p.Lys2483Glu+c.4789 C>T,p.Arg1597*	200	Dystrophic	Typical COL6-RM features	Reduced	Yes
2-II.1	c.7447A>G,p.Lys2483Glu +c.9045 dupC,p.Gly3016Argfs*6	185	Dystrophic	Typical COL6-RM features	Reduced (ns)	Yes
3-III.1	c.7447A>G,p.Lys2483Glu+c.9384_9386 delGTA, p.Trp3128_Tyr3129delinsCys	120	Dystrophic	Typical COL6-RM features	Reduced (ns)	Yes
4-II.2	c.7447A>G,p.Lys2483Glu +c.6538_6591del,p.Val2181_Gly2198del	700	Dystrophic	N/A	Reduced	Yes
5-II.2	c.7447A>G,p.Lys2483Glu +c.6733C>T,p.Gln2245*	500	Dystrophic	Fatty replacement of pelvic girdle and proximal LL muscles	N/A	Yes
6-II.1	c.7447A>G,p.Lys2483Glu+c.7024C>T,p.Arg2342*	219	Dystrophic	Typical COL6-RM features	Reduced	Yes
7-II.1	c.7447A>G,p.Lys2483Glu+c.9329-1G>T	600	Dystrophic	Typical COL6-RM features	N/A	Yes
8-II.2	c.7447A>G,p.Lys2483Glu+c.6181C>T, p.Arg2061*	470	N/A	N/A	N/A	Yes
9-II.5	c.7447A>G, p.Lys2483Glu+c.7024C>T, p.Asg2342*	500–900	Fibre atrophywithoutnecrosis	Typical COL6-RM features	N/A	Yes
10-II.1	exon 12 deletion [arr[GRCh37] 2q37.3 (238274212_238274975)x1]+c.7447A>G, p.Lys2483Glu	250	N/A	N/A	N/A	Yes
10-II.3	exon 12 deletion [arr[GRCh37] 2q37.3 (238274212_238274975)x1]+c.7447A>G, p.Lys2483Glu	170	N/A	N/A	N/A	Yes
11-II.5	c.7447A>G,p.Lys2483Glu homozygous	700	Mild FSV, IN, myofibrillar disorganization, autophagic vacuoles and *nemalin* rods	Atrophy and fatty infiltration of distal LL muscles (*gastrocnemius,*) *peroneus lateralis*	N/A	No
12-II.2	c.7447A>G,p.Lys2483Glu homozygous	1600-2000	Dystrophic. Diminished calpain 3 expression on WB	Mild fatty infiltration of proximal LL muscles*(glutei, iliopsoas, quadriceps)*	N/A	No
13-II.1	c.7447A>G,p.Lys2483Glu homozygous	200	Dystrophic	Typical COL6-RM features	Normal	Yes
14-II.1	c.7447A>G,p.Lys2483Glu homozygous	157	FSV, muscle fiber atrophy without necrosis or regeneration	Typical COL6-RM features	Reduced (ns)	Yes

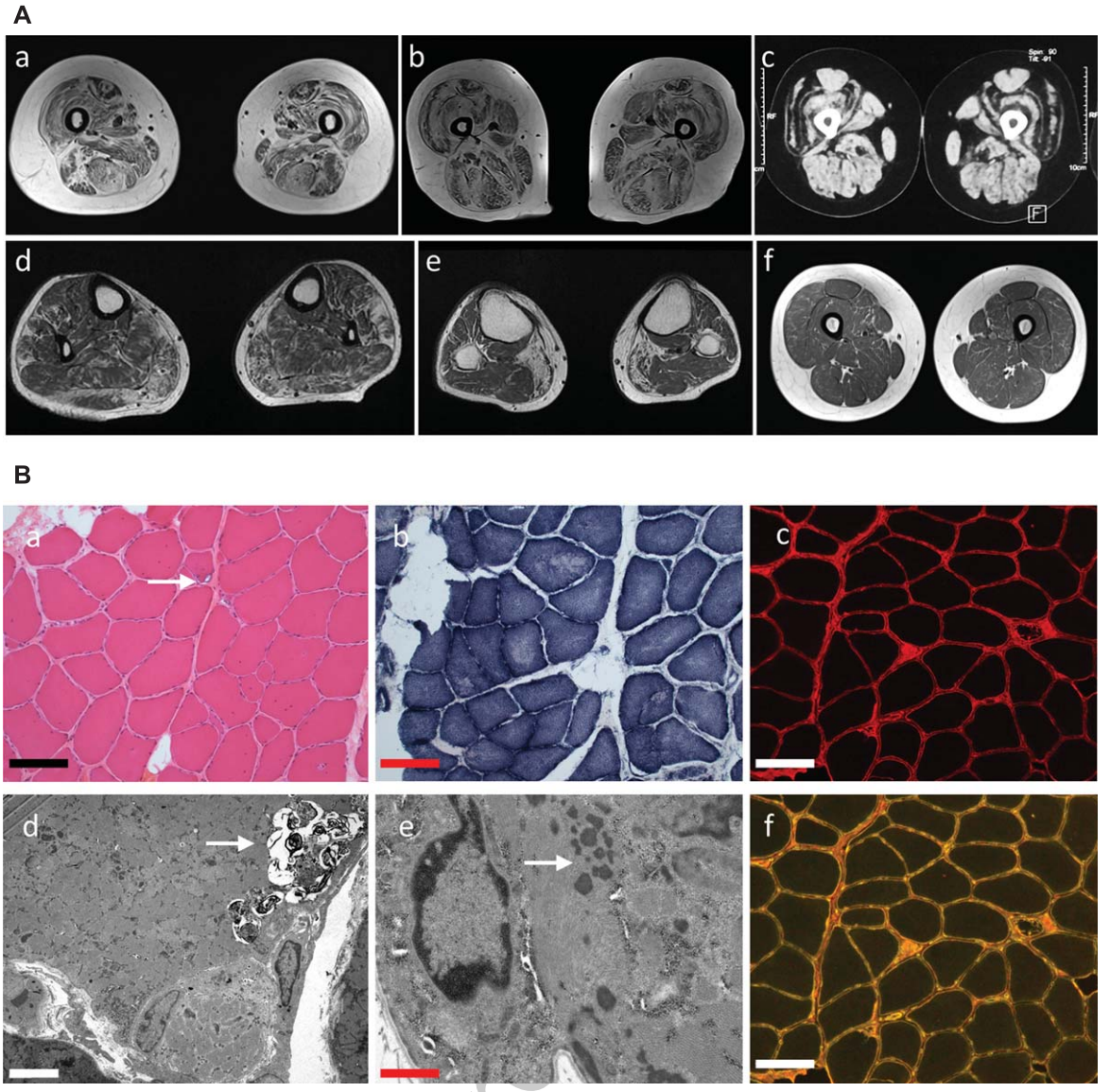

Fig. 2. A. Muscle imaging. Axial T1-weighted images. MRI from patients 1-II.1 (a) 6-II.1 (b) and 13-II.1 (c) show typical COL6-RM radiological findings, including fatty replacement starting around the fascia surrounding the muscle and the so-called "central-cloud" affecting the rectus femoris. (d,e) MRI from patient 11-II.5 reveals atrophy and fatty infiltration of distal lower limb muscles mainly affecting medial gastrocnemius and peroneus. (f) MRI from patient 12-II.2 showed no major muscle atrophy and mild fatty replacement of anterior and posterior thigh muscles. B. Muscle biopsy from patient 11-II.5. Hematoxylin and eosin (a) transversal frozen section show mild fiber size variation, numerous internalized nuclei and few rimmed vacuoles (arrow), while NADH-TR (b) reveal diffuse areas lacking oxydative activity. Note slightly irregular or discontinuous COLVI immunostaining (c). (d) Myofibrillar disorganization, autophagic vacuoles (arrow-d) and nemalin rods (arrow-e) are observed by electron microscopy (d,e). (f) COLVI and perlecan co-immunostaining. Scale bars: (a-c,f) 10 μm; (d) 15 μm; (e) 1 μm.

and toe-walking since early-childhood, patient 13-II.1 presented with abnormal gait since childhood and patient 14-II.1 presented since early childhood with delayed motor milestones (delayed gait acquisition) and difficulty running. The latter three patients were found to have proximal weakness, while patient 11-II.5 had predominant distal weakness and bilateral *scapula alata*. Patient 14-II.1 had finger flexor and Achilles contractures. Joint contractures were absent in the remaining three patients, aside from mild ankle contractures in patients 12-II.2 and 13-II.1 and none of them had distal hyperlaxity, skin abnormalities or respiratory involvement. Cardiac examination was normal in patients 11-II.5, 13-II.1 and 14-II.1, while

echocardiogram from patient 12-II.2 revealed mild aortic insufficiency at age 13 years. All patients maintained ambulation but patient 14-II.1, currently 77 years-old, requires assistance (walker) since age 74.

CK levels were strikingly elevated in patients 11-II.5 and 12-II.2, ranging from 700 to 2000 IU/L, while patients 13-II.1 and 14-II.1 had normal CK levels. Muscle MRI from the first two patients revealed non-typical COL6-RM findings (Fig. 2A). Indeed, fatty infiltration of posterior compartment of distal lower limb muscles was observed in patient 11-II.5 and mild fatty infiltration of proximal limb muscles was noticed in patient 12-II.2. Muscle MRI from patients 13-II.1 and 14-II.1 showed typical COL6-RM findings affecting *vastus lateralis* and *rectus femoris*.

Muscle biopsy from patient 11-II.5 showed internalized nuclei, myofibrillar disorganization, autophagic vacuoles and nemaline rods (Fig. 2B), while dystrophic changes were observed for patients 12-II.2 and 13-II.1 and muscle biopsy from patient 14-II.1 disclosed only mild atrophy without necrosis or regeneration and fiber size variation.

Collagen secretion in cultured skin fibroblasts

Collagen VI immunolabelling was performed using two different antibodies, on fixed dermal fibroblasts derived from five compound heterozygous patients (Table 2, Fig. 3 and Supplementary Figure 2), and revealed reduced collagen VI secretion compared to control cells, in which a dense network of deposited collagen VI was detected. Since patients 1-II.2, 2-II.1 and 6-II.1 harbor a second mutation introducing a premature termination codon that should lead to transcript degradation *via* the nonsense-mediated decay, the COLVI staining detected most likely reflects the protein synthesis sustained by the missense-bearing allele. Similarly, the second mutation carried by patients 3-III.1 and 4-II.2 (delins and exon deletion, respectively) should impair the assembly of monomers and/or secreted microfibrils. These results suggest that the missense variant does not significantly prevent COLVI assembly and secretion. Interestingly, COLVI immunolabelling of fibroblasts from 3 homozygous patients revealed a rather normal secretion pattern (patients 11-II.5 and 13-II.1) or reduced secretion (14-II.1) (Fig. 3 and Supplementary Figure 2). Accordingly, COLVI immunostaining on muscle biopsy from patient 11-II.5 was also rather preserved, with only focal points of discontinuous signal (Fig. 2c and 2f).

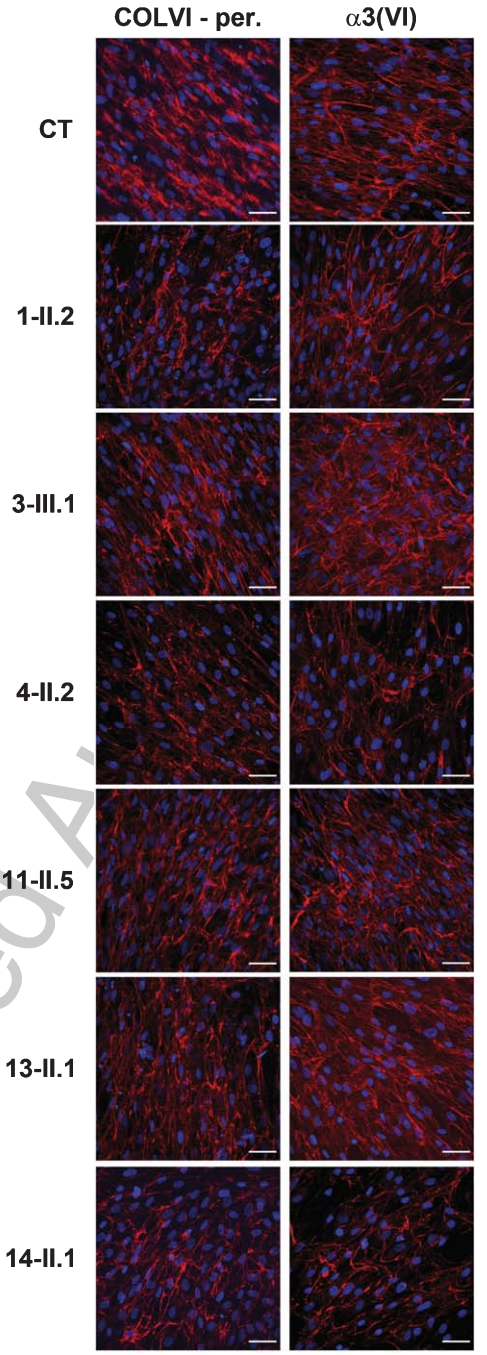

Fig. 3. Confocal imaging of COLVI secretion in fixed dermal fibroblasts from a control individual (CT) and 6 patients. COLVI (red) was detected with two polyclonal antibodies: Ab6588 (Abcam) on non-permeabilized cells (left panel) or an α3(VI) chain specific on methanol-fixed cells (right panel). Nuclei are identified by DAPI staining (blue). In three compound heterozygous patients (1-II.2, 3-III.1 and 4-II.2) reduced collagen VI secretion was observed. Immunolabelling of fibroblasts from homozygous patients 11-II.5 and 13-II.1 revealed a rather normal secretion, while it was clearly reduced in the culture from homozygous patient 14-II.1. Scale bars = 50μm.

DISCUSSION

COL6-RM diagnosis is based on the combination of clinical presentation (*i.e.* muscle weakness, prominent contractures, characteristic cutaneous abnormalities and variable respiratory involvement), muscular imaging, immunohistochemical examination of collagen VI on muscle biopsy and fibroblasts and analysis of the *COL6A1-3* genes.

Although the first mutations detected in UCMD were recessive null mutations, leading to an absence of collagen VI in muscle biopsy sections and in cultured dermal fibroblasts [15], *de novo* dominant mutations in the *COL6A1-3* genes are responsible for a large proportion of UCMD cases [1, 16–18]. In BM, most patients harbor heterozygous dominantly acting mutations, typically affecting glycine residues of the Gly-X-Y motif at the N-terminal end of the triple helical domain that exert a dominant-negative effect on the tetramer structure [6, 17, 19–21]. Nonetheless, recessive mutations in the *COL6A1-3* genes have also been detected in patients with typical BM [22, 23], most of them carrying a null mutation on one allele in heterozygosity with a missense mutation on the other.

Interpretation of genetic variants can be challenging and many variants in the *COL6A1-3* genes have not yet been fully characterized at the functional level. Such is the case of missense variants affecting regions outside of the triple helix. Furthermore, there is a number of patients with a compatible clinical and muscle imaging phenotype who have no detectable mutations in the three *COL6* genes coding sequence [24, 25]. In that sense, 5'/3' UTR regulatory elements or deep intronic splice mutations can go undetected by standard sequencing approaches, and muscle or fibroblasts RNA sequencing can be useful to detect these pathogenic variants [26].

Along these lines, the interpretation of the *COL6A3* c.7447A>G variant is complex. Its allelic frequency is 171/277005 and one homozygous individual is reported in GnomAD database. It is most prevalent in the non-Finnish European (0.001) and Latino (0.0007) populations. Its high allelic frequency raises questions about its pathogenicity, which remains unclear so far. Its predicted effect on SuSPect method (http://www.sbg.bio.ic.ac.uk/suspect/) [27] points to a low pathogenicity score (8 out of 100). The CADD score for this variant is 22.5 and it is classified as likely pathogenic or variant of unknown-signification in LOVD, Clinvar and likely pathogenic in the HG MDPro-database.

We report twelve patients carrying the c.7447A>G *COL6A3* variant in compound heterozygosity with a second *COL6A3* mutation. Most of these patients presented since childhood with proximal weakness associated with joint contractures, variable presence of rigid spine and skin abnormalities. All remained ambulatory at a mean age of 34.7 ± 21.5 and mild respiratory involvement was detected in three of them. This phenotype would be consistent with Bethlem myopathy (BM) [4, 7]. Muscle MRI showed typical COL6-RM findings in seven out of eight patients and collagen VI deposition in the extracellular matrix was reduced in dermal fibroblasts from five patients. WES analysis in three compound heterozygous patients excluded additional pathogenic variants in other neuromuscular disease-causing genes.

Conversely, homozygous patients exhibit strikingly different clinical features. Two out of four did not have a typical COL6-RM phenotype and presented with foot deformities, wing scapula and distal weakness or abnormal gait with toe-walking without major contractures, distal hyperlaxity, skin abnormalities or respiratory involvement. Moreover, CK levels of these two patients were strikingly high for COL6-RM, muscle MRI disclosed non-specific findings including only distal involvement in one, and muscle biopsy from the one patient showed myofibrillar disorganization, autophagic vacuoles and nemalin rods. Splicing defects were excluded after sequencing of the *COL6A1-A2-A3* coding regions in three patients harboring c.7447G>A at the homozygous state.

The *COL6A3* c.7447A>G variant has been previously reported in compound heterozygous patients [5, 28–31] with a clinical spectrum ranging from a mild phenotype when associated with a missense variant [30], to an intermediate phenotype with childhood onset and respiratory involvement [5] and a severe Ullrich-like phenotype [29], when associated with a second truncating *COL6A3* variant. To our knowledge, six homozygous patients have been reported so far [5, 29, 32]. Panadés-de Oliveira et *al*. [5] reported two homozygous siblings. The index case had proximal weakness, elevated CK levels (1000 IU/L) and dystrophic findings and rimmed vacuoles on muscle biopsy. The patient's sibling had asymptomatic significantly increased CK levels (4000 IU/L) detected at mid-age. Both had typical COL6-RM findings on muscle MRI findings. This study reported one additional homozygous patient who also carried a *COL6A1* c.2435-2A>G pathogenic splicing variant [5]. Interestingly, rimmed vacuoles were also found on the muscle biopsy from our patient 11-II.5.

The patient reported by Hunter et al. [29], presented with club feet, abnormal gait and lipoatrophy, developed scoliosis and hamstrings, ankles and feet contractures with overlapping toes. EMG studies revealed a chronic motor neuropathy and muscle biopsy disclosed abnormal myofibrillar architecture with fiber type grouping. Recently, Stavusis et al. [32] reported two siblings with mild proximal weakness and joint contractures carrying three *COL6A3* variants: the c.7447A>G variant in homozygosity combined with the heterozygous frameshift variant c.8074delT, p.(Tyr2692MetfsTer15). Strikingly, they also reported a third patient, carrying the *COL6A3* c.7447A>G variant in homozygosity, with proximal and distal weakness, diminished ankle reflexes and axonal polyneuropathy, but no further biological or genetic analysis regarding this polyneuropathy is reported. Interestingly, collagen VI is expressed in peripheral nerve [33–35]. Nonetheless, *COL6A1-A2-A3* mutations have not been found associated with hereditary neuropathies. On another note, muscle MRI of this patient showed diffuse and severe fatty infiltration without typical COL6-RM findings and CK levels were also elevated from two to four times the normal values. Unfortunately, no collagen VI immunolabeling on muscle biopsy or analysis of collagen VI production in dermal fibroblast cultures are reported for any of these homozygous patients.

Stavusis et al. [32] have also reviewed all the reported patients to date, and conclude that for compound heterozygous patients, the phenotype severity entirely depends on the second mutation. The authors speculate that this variant could perturb the binding properties of the protein and thus the stability of the heterotrimer and modulate the phenotype when present along with other mutations, as is the case for the patients with the c.8074delT variant, which leads to a premature termination of the protein. Nonetheless, the occurrence of another deep intronic mutation in the *COL6A1-3* genes cannot be excluded [32]. Strikingly, reported homozygous patients exhibit different clinical phenotypes such as peripheral neuropathy [29, 32], asymptomatic hyperCK [5] or limb-girdle myopathy with joint contractures [5, 32], associated in some cases with a non-typical COL6-RM histopathological presentation including rimmed vacuoles [32] or abnormal myofibrillar architecture with fiber type grouping [29].

At the functional level, the determinant role of the α3(VI) chain in the monomer assembly is well established [36]. However, the exact contribution of the VWA C1 domain, harboring the mutated Lysine residue at position 2483, remains poorly understood, although it has been shown to be important for chain recognition and assembly of triple helical molecules [37]. Immunostaining of muscle biopsy and fibroblast cultures show that the missense variant does not prevent COLVI deposition in the ECM, although further analyses, such as Western blotting, should be performed to obtain quantitative data. VWA domains being protein-protein interaction modules, the missense variant may alter interaction with binding partners as suggested by Stavusis et al. [32] but further investigations are needed to support this hypothesis experimentally.

In conclusion, this work expands the clinical and molecular spectrum of COLVI-related myopathies and is focused on the complex interpretation of the frequent *COL6A3* c.7447A>G variant and the associated clinical findings. Its high allele frequency and the different phenotypes observed in the homozygous patients reported so far, raise questions about the pathogenicity of this variant in homozygosity. Our results suggest that the *COL6A3* variant c.7447A>G may act as a modulator of the clinical phenotype. An in-depth analysis of clinical features and ancillary tests is mandatory in order to interpret the genetic analysis. For homozygous patients with a compatible COL6-RM phenotype, including clinical, MRI findings or altered collagen VI secretion, we recommend to thoroughly search for an additional genetic variant in any of the *COL6A1-3* genes, as in the cases reported by Panades et al. [5] and Stavusis et al. [32], including deep intronic variants leading to aberrant splicing that may go undetected in NGS based panels [26]. Thus, analysis of mRNA transcripts could be useful. In the event of a non-compatible phenotype such as atypical clinical findings, strikingly elevated CK levels, non-pathognomonic COL6-RM muscle MRI findings, further genetic analysis would be advisable to exclude alternative causes such as inherited neuropathies or other forms of myopathies. Further analysis of homozygous carriers with detailed clinical and ancillary tests data together with molecular and genetic studies would help to elucidate whether this variant is disease-causing or not. This would be of major importance to allow precise diagnosis, accurate management and genetic counseling for the affected patients and families.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

REFERENCES

[1] Bushby KMD, Collins J, Hicks D. Collagen Type VI Myopathies. Adv Exp Med Biol. 2014;802:185-99. doi:10.1007/978-94-007-7893-1_12

[2] Bönnemann CG. The collagen VI-related myopathies. Handb Clin Neurol. 2011;101:81-96. doi:10.1016/B978-0-08-045031-5.00005-0

[3] Yonekawa T, Nishino I. Ullrich congenital muscular dystrophy: Clinicopathological features, natural history and pathomechanism(s). J Neurol Neurosurg Psychiatry. 2015;86:280-7. doi:10.1136/jnnp-2013-307052

[4] Deconinck N, Richard P, Allamand V, Behin A, Lafôret P, Ferreiro A, et al. Bethlem myopathy: Long-term follow-up identifies COL6 mutations predicting severe clinical evolution. J Neurol Neurosurg Psychiatry. 2015;86:1337-46. doi:10.1136/jnnp-2013-307245

[5] Panadés-de Oliveira L, Rodríguez-López C, Cantero Montenegro D, Marcos Toledano M del M, Fernández-Marmiesse A, Esteban Pérez J, et al. Bethlem myopathy: A series of 16 patients and description of seven new associated mutations. J Neurol. 2019;266:934-41. doi:10.1007/s00415-019-09217-z

[6] Scacheri PC, Gillanders EM, Subramony SH, Vedanarayanan V, Crowe CA, Thakore N, et al. Novel mutations in collagen VI genes: Expansion of the Bethlem myopathy phenotype. Neurology. 2002;58:593-602. doi:10.1212/WNL.58.4.593

[7] Bönnemann CG. The collagen VI-related myopathies: Muscle meets its matrix. Nat Rev Neurol. 2011;7:379-90. doi:10.1038/nrneurol.2011.81

[8] Merlini L, Martoni E, Grumati P, Sabatelli P, Squarzoni S, Urciuolo A, et al. Autosomal recessive myosclerosis myopathy is a collagen VI disorder. Neurology. 2008;71:1245-53. doi:10.1212/01.wnl.0000327611.01687.5e

[9] Mercuri E, Lampe A, Allsop J, Knight R, Pane M, Kinali M, et al. Muscle MRI in Ullrich congenital muscular dystrophy and Bethlem myopathy. Neuromuscul Disord. 2005;15:303-10. doi:10.1016/j.nmd.2005.01.004

[10] Kim SY, Kim WJ, Kim H, Choi SA, Lee JS, Cho A, et al. Collagen VI-related myopathy: Expanding the clinical and genetic spectrum. Muscle Nerve. 2018. doi:10.1002/mus.26093

[11] Dubowitz V, Sewry CA. Muscle Biopsy: A Practical Approach. Philadelphia: Saunders Elsevier; 2007.

[12] Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: A joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med. 2015;17:405-24. doi:10.1038/gim.2015.30

[13] Hicks D, Lampe AK, Barresi R, Charlton R, Fiorillo C, Bonnemann CG, et al. A refined diagnostic algorithm for Bethlem myopathy. Neurology. 2008;70:1192-9. doi:10.1212/01.wnl.0000307749.66438.6d

[14] Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: An open-source platform for biological-image analysis. Nat Methods. 2012;9:676-82. doi:10.1038/nmeth.2019

[15] Vanegas OC, Bertini E, Zhang RZ, Petrini S, Minosse C, Sabatelli P, et al. Ullrich scleroatonic muscular dystrophy is caused by recessive mutations in collagen type VI. Proc Natl Acad Sci U S A. 2001;98:7516-21. doi:10.1073/pnas.121027598

[16] Dominant collagen VI mutations are a common cause of Ullrich congenital muscular dystrophy. - PubMed - NCBI n.d. https://www.ncbi.nlm.nih.gov/pubmed/?term=baker+2005+collagen+Vi (accessed April 22, 2020).

[17] Lampe AK, Bushby KMD. Collagen VI related muscle disorders. J Med Genet. 2005;42:673-85. doi:10.1136/jmg.2002.002311

[18] Lampe AK, Zou Y, Sudano D, O'Brien KK, Hicks D, Laval SH, et al. Exon skipping mutations in collagen VI are common and are predictive for severity and inheritance. Hum Mutat. 2008;29:809-22. doi:10.1002/humu.20704

[19] Jöbsis GJ, Keizers H, Vreijling JP, Visser M de, Speer MC, Wolterman RA, et al. Type VI collagen mutations in Bethlem myopathy, an autosomal dominant myopathy with contractures. Nat Genet. 1996;14:113-5. doi:10.1038/ng0996-113

[20] Pepe G, Giusti B, Bertini E, Brunelli T, Saitta B, Comeglio P, et al. A heterozygous splice site mutation in COL6A1 leading to an in-frame deletion of the α1(VI) collagen chain in an Italian family affected by Bethlem myopathy. Biochem Biophys Res Commun. 1999;258:802-7. doi:10.1006/bbrc.1999.0680

[21] Lucioli S, Giusti B, Mercuri E, Vanegas OC, Lucarini L, Pietroni V, et al. Detection of common and private mutations in the COL6A1 gene of patients with Bethlem myopathy. Neurology. 2005;64:1931-7. doi:10.1212/01.WNL.0000163990.00057.66

[22] Foley AR, Hu Y, Zou Y, Columbus A, Shoffner J, Dunn DM, et al. Autosomal recessive inheritance of classic Bethlem myopathy. Neuromuscul Disord. 2009;19:813-7. doi:10.1016/j.nmd.2009.09.010

[23] Gualandi F, Urciuolo A, Martoni E, Sabatelli P, Squarzoni S, Bovolenta M, et al. Autosomal recessive Bethlem myopathy. Neurology. 2009;73:1883-91. doi:10.1212/WNL.0b013e3181c3fd2a

[24] Tétreault M, Duquette A, Thiffault I, Bherer C, Jarry J, Loisel L, et al. A new form of congenital muscular dystrophy with joint hyperlaxity maps to 3p23-21. Brain. 2006;129:2077-84. doi:10.1093/brain/awl146

[25] Petrini S, D'Amico A, Sale P, Lucarini L, Sabatelli P, Tessa A, et al. Ullrich myopathy phenotype with secondary ColVI defect identified by confocal imaging and electron microscopy analysis. Neuromuscul Disord. 2007;17:587-96. doi:10.1016/j.nmd.2007.04.010

[26] Bolduc V, Reghan Foley A, Solomon-Degefa H, Sarathy A, Donkervoort S, Hu Y, et al. A recurrent COL6A1 pseudoexon insertion causes muscular dystrophy and is effectively targeted by splice-correction therapies. JCI Insight. 2019;4. doi:10.1172/jci.insight.124403

[27] Yates CM, Filippis I, Kelley LA, Sternberg MJE. SuSPect: Enhanced prediction of single amino acid variant (SAV) phenotype using network features. J Mol Biol. 2014;426:2692-701. doi:10.1016/j.jmb.2014.04.026

[28] Sframeli M, Sarkozy A, Bertoli M, Astrea G, Hudson J, Scoto M, et al. Congenital muscular dystrophies in the UK population: Clinical and molecular spectrum of a large cohort diagnosed over a 12-year period. Neuromuscul Disord. 2017;27:793-803. doi:10.1016/j.nmd.2017.06.008

[29] Hunter JM, Ahearn ME, Balak CD, Liang WS, Kurdoglu A, Corneveaux JJ, et al. Novel pathogenic variants and genes for myopathies identified by whole exome sequencing. Mol Genet Genomic Med. 2015;3:283-301. doi:10.1002/mgg3.142

[30] Briñas L, Richard P, Quijano-Roy S, Gartioux C, Ledeuil C, Lacène E, et al. Early onset collagen VI myopathies: Genetic and clinical correlations. Ann Neurol. 2010;68:511-20. doi:10.1002/ana.22087

[31] Fichna JP, MacIas A, Piechota M, Korostynski M, Potulska-Chromik A, Redowicz MJ, et al. Whole-exome sequencing identifies novel pathogenic mutations and putative phenotype-influencing variants in Polish limb-girdle muscular dystrophy patients. Hum Genomics. 2018;12. doi:10.1186/s40246-018-0167-1

[32] Stavusis J, Micule I, Wright NT, Straub V, Topf A, Panadés-de Oliveira L, et al. Collagen VI-related limb-girdle syndrome caused by frequent mutation inCOL6A3 gene with conflicting reports of pathogenicity. Neuromuscul Disord. 2020. doi:10.1016/j.nmd.2020.03.010

[33] Chen P, Cescon M, Megighian A, Bonaldo P. Collagen VI regulates peripheral nerve myelination and function. FASEB J. 2014;28:1145-56. doi:10.1096/fj.13-239533

[34] Chen P, Cescon M, Bonaldo P. The Role of Collagens in Peripheral Nerve Myelination and Function. Mol Neurobiol. 2015;52:216-25. doi:10.1007/s12035-014-8862-y

[35] Cescon M, Gregorio I, Eiber N, Borgia D, Fusto A, Sabatelli P, et al. Collagen VI is required for the structural and functional integrity of the neuromuscular junction. Acta Neuropathol. 2018;136:483-99. doi:10.1007/s00401-018-1860-9

[36] Fitzgerald J, Mörgelin M, Selan C, Wiberg C, Keene DR, Lamandé SR, et al. The N-terminal N5 subdomain of the α3(VI) chain is important for collagen VI microfibril formation. J Biol Chem. 2001;276:187-93. doi:10.1074/jbc.M008173200

[37] Lamandé SR, Mörgelin M, Adams NE, Selan C, Allen JM. The C5 domain of the collagen VI α3(VI) chain is critical for extracellular microfibril formation and is present in the extracellular matrix of cultured cells. J Biol Chem. 2006;281:16607-14. doi:10.1074/jbc.M510192200

[38] Krahn M, Biancalana V, Cerino M, Perrin A, Michel-Calemard L, Nectoux J, et al. A National French consensus on gene lists for the diagnosis of myopathies using next-generation sequencing. Eur J Hum Genet. 2019;27:349-52. doi:10.1038/s41431-018-0305-1