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# 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  $\alpha$ -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

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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, GVDG 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 (Abcam) as initially described in [13], or an  $\alpha 3(\text{VI})$  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- childhood	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 <i>equinovarus</i> 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	<i>Pes cavus</i> , 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  $37.6 \pm 21.8$ ) 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

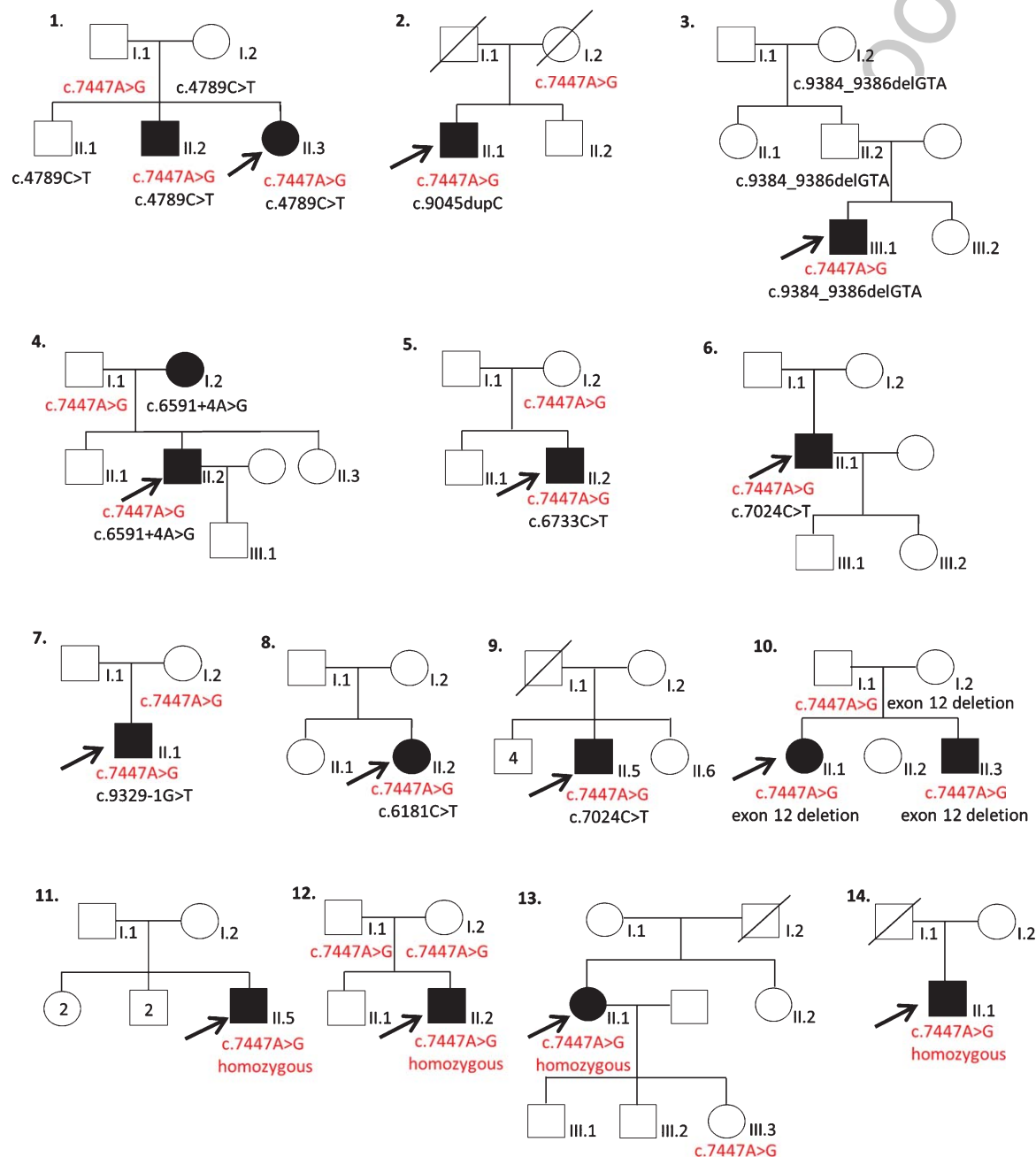


Fig. 1. Family pedigrees. Black filled symbols: affected individual. Crossed symbol: deceased individual.

190 studies were performed in eight families and  
 191 confirmed that the tested parents were healthy het-  
 192 erozygous carriers (Fig. 1).

193 Four patients (11-II.5, 12-II.2, 13-II.1 and 14-  
 194 II.1) were homozygous for the c.7447A>G *COL6A3*  
 195 variant. Patient 13-II.1's daughter was a healthy het-  
 196 erozygous carrier. No segregation studies could be  
 197 performed for patients 11-II.5, 12-II.2 and 14-II.1.  
 198 Patient 13-II.1 also harbored a heterozygous *TNXB*  
 199 variant c.4535\_4552del, p.(Asp1512\_Val1517del),  
 200 whose allelic frequency was found to be low in  
 201 gnomAD (13 allele count/275252) and reported as  
 202 probably pathogenic in one patient in the LOVD  
 203 database (reference #0000528445) associated with a  
 204 known pathogenic *TNXB* variant. No second *TNXB*  
 205 variant was identified in this patient.

206 Furthermore, no other variant that could affect  
 207 splice, were found in the *COL6A1-A2-A3* genes after  
 208 sequencing of the coding regions from cultured skin  
 209 fibroblasts for patients 12-II.2, 13-II.1 and 14-II.1  
 210 harboring the c.7447A>G at the homozygous state  
 211 (no skin biopsy was available for 11-II.5 patient) (data  
 212 not shown). Whole exome sequencing (WES) was  
 213 performed in three compound heterozygous patients  
 214 (7-II.1, 8-II.2, 10-II.1), thereby excluding additional  
 215 pathogenic variants in other disease-causing genes.

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

#### 221 *Clinical presentation of compound heterozygous* 222 *patients*

223 Eleven (92%) patients developed the first symp-  
 224 toms at birth or in early childhood, one around pub-  
 225 erty and one in adulthood. None of them had a  
 226 family history of neuromuscular disease. The most  
 227 commonly recognized presenting symptoms were  
 228 delayed motor development, waddling gait, poor spo-  
 229 rts performance and joint contractures. Decreased  
 230 fetal movements were noted in two patients. Gait  
 231 acquisition was delayed in two patients (5-II.2 and  
 232 10-II.3) who reached independent ambulation at 18  
 233 and 21 months, respectively. Two additional patients  
 234 (8-II.2 and 10-II.1) started walking at 14 months and  
 235 were noted to have a waddling gait. Muscle weakness  
 236 was present in all patients with predominant axial and  
 237 proximal involvement in 11 (92%), MRC grade 3-  
 238 4/5. Prominent distal weakness was observed in one  
 239 patient, affecting finger flexors and extensors in the

240 upper limbs and ankle dorsiflexion and eversion in  
 241 the lower limbs (MRC 3/5). Independent ambulation  
 242 was maintained in all patients although three patients  
 243 (25%) required assistance (Table 1).

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

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

257 Serum Creatine Kinase (CK) levels were mildly  
 258 to moderately elevated in all patients (range 120-  
 259 970 IU/L). Electromyography (EMG) was performed  
 260 in 10 patients, which revealed predominately myo-  
 261 pathic findings in seven patients. Patients 8-II.2 and  
 262 10-II.1 were found to have normal nerve conduction  
 263 studies (NCS). EMG was limited due to poor tol-  
 264 erance in patient 8-II.2. For patient 10-II.1, EMG  
 265 at age 5 years disclosed slightly increased duration  
 266 and amplitude and mildly reduced recruitment of the  
 267 left *vastus lateralis*. Lastly, for patient 9-II.5, NCS  
 268 showed absent peroneal motor response recorded at  
 269 the *extensor digitorum brevis* bilaterally and EMG  
 270 showed small motor units suggestive of a myopathic  
 271 pattern in the proximal upper limbs, but neurogenic  
 272 changes of the distal and proximal legs at age 55  
 273 years. Muscle MRI (Fig. 2A and Supplementary Fig-  
 274 ure 1) was performed in eight patients and showed  
 275 characteristic COL6-RM findings in seven, namely  
 276 fatty replacement starting around the fascia surround-  
 277 ing *vastus lateralis* ("outside in") and the so-called  
 278 "central cloud" pattern in the *rectus femoris*. Two  
 279 patients' MRI showed fatty replacement of pelvic  
 280 girdle and proximal leg muscles without the charac-  
 281 teristic previously mentioned findings. Eight patients  
 282 underwent a muscle biopsy, revealing prominent dys-  
 283 trophic features in seven (87.5%).

#### 284 *Clinical findings and ancillary tests in* 285 *homozygous patients*

286 Recognition of first symptoms was in childhood in  
 287 all four patients. Patient 11-II.5 presented with foot  
 288 deformities and steppage gait first recognized at age  
 289 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 atrophy without necrosis	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 ( <i>gastrocnemius</i> ), <i>peroneus lateralis</i>	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 ( <i>glutei</i> , <i>iliopsoas</i> , <i>quadriceps</i> )	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



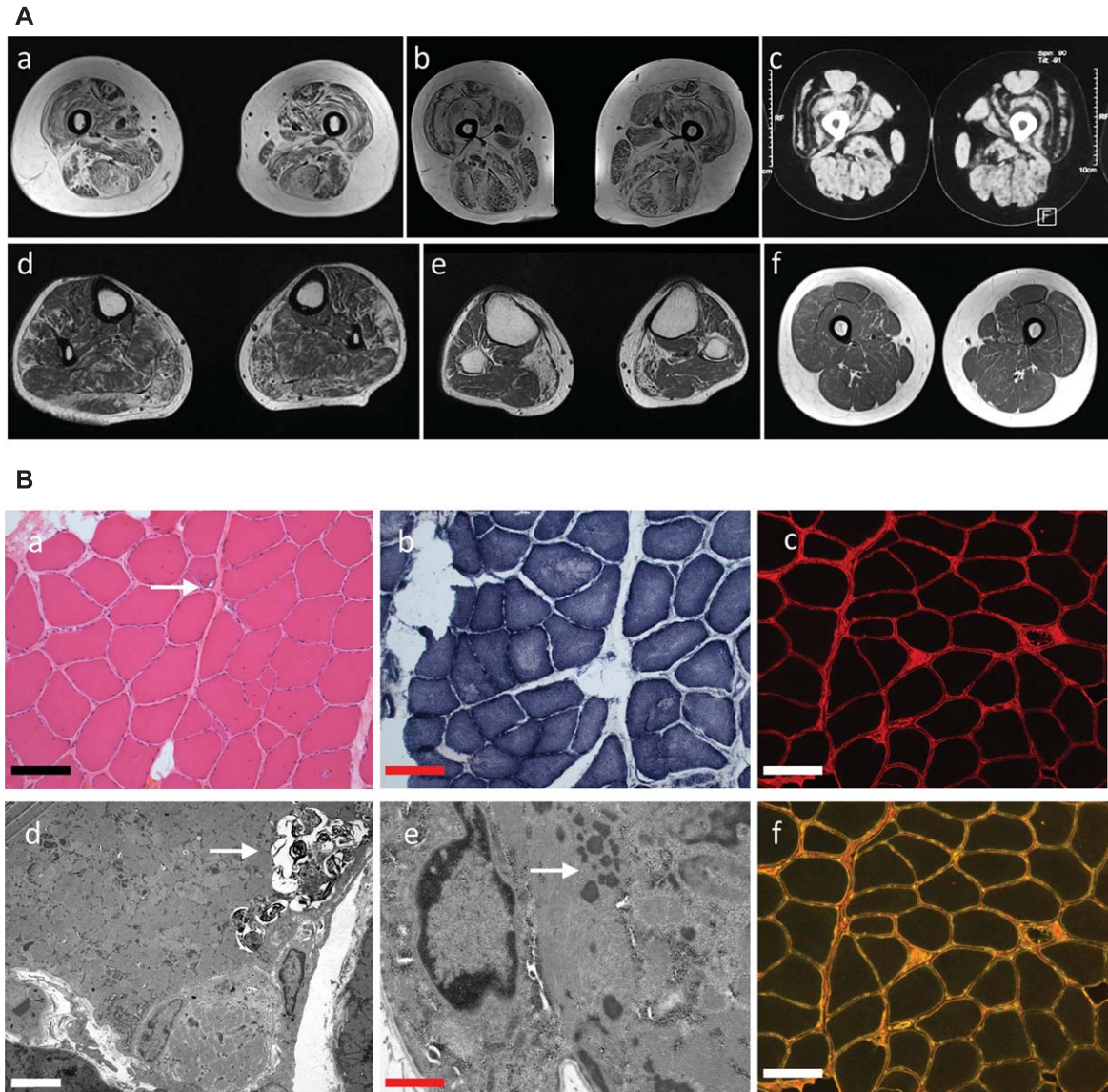


Fig. 2. A. Muscle imaging. Axial T1-weighted images. MRI from patients 1-II.1 (a) 6-II.1 (b) and 13-II.1 (c) show typical COL6-RM radiological findings, including fatty replacement starting around the fascia surrounding the muscle and the so-called “central-cloud” affecting the rectus femoris. (d,e) 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  $\mu$ m; (d) 15  $\mu$ m; (e) 1  $\mu$ m.

290 and toe-walking since early-childhood, patient 13-  
 291 II.1 presented with abnormal gait since childhood and  
 292 patient 14-II.1 presented since early childhood with  
 293 delayed motor milestones (delayed gait acquisition)  
 294 and difficulty running. The latter three patients were  
 295 found to have proximal weakness, while patient 11-  
 296 II.5 had predominant distal weakness and bilateral

297 *scapula alata*. Patient 14-II.1 had finger flexor and  
 298 Achilles contractures. Joint contractures were absent  
 299 in the remaining three patients, aside from mild ankle  
 300 contractures in patients 12-II.2 and 13-II.1 and none  
 301 of them had distal hyperlaxity, skin abnormalities  
 302 or respiratory involvement. Cardiac examination was  
 303 normal in patients 11-II.5, 13-II.1 and 14-II.1, while

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

308 CK levels were strikingly elevated in patients 11-  
 309 II.5 and 12-II.2, ranging from 700 to 2000 IU/L, while  
 310 patients 13-II.1 and 14-II.1 had normal CK levels.  
 311 Muscle MRI from the first two patients revealed non-  
 312 typical COL6-RM findings (Fig. 2A). Indeed, fatty  
 313 infiltration of posterior compartment of distal lower  
 314 limb muscles was observed in patient 11-II.5 and  
 315 mild fatty infiltration of proximal limb muscles was  
 316 noticed in patient 12-II.2. Muscle MRI from patients  
 317 13-II.1 and 14-II.1 showed typical COL6-RM find-  
 318 ings affecting *vastus lateralis* and *rectus femoris*.

319 Muscle biopsy from patient 11-II.5 showed inter-  
 320 nalized nuclei, myofibrillar disorganization, autoph-  
 321 agic vacuoles and nemaline rods (Fig. 2B), while  
 322 dystrophic changes were observed for patients 12-  
 323 II.2 and 13-II.1 and muscle biopsy from patient  
 324 14-II.1 disclosed only mild atrophy without necrosis  
 325 or regeneration and fiber size variation.

#### 326 Collagen secretion in cultured skin fibroblasts

327 Collagen VI immunolabelling was performed us-  
 328 ing two different antibodies, on fixed dermal fibro-  
 329 blasts derived from five compound heterozygous  
 330 patients (Table 2, Fig. 3 and Supplementary Figure 2),  
 331 and revealed reduced collagen VI secretion compared  
 332 to control cells, in which a dense network of deposited  
 333 collagen VI was detected. Since patients 1-II.2, 2-II.1  
 334 and 6-II.1 harbor a second mutation introducing a  
 335 premature termination codon that should lead to tran-  
 336 script degradation *via* the nonsense-mediated decay,  
 337 the COLVI staining detected most likely reflects the  
 338 protein synthesis sustained by the missense-bearing  
 339 allele. Similarly, the second mutation carried by pa-  
 340 tients 3-III.1 and 4-II.2 (delins and exon delet-  
 341 ion, respectively) should impair the assembly of  
 342 monomers and/or secreted microfibrils. These results  
 343 suggest that the missense variant does not signifi-  
 344 cantly prevent COLVI assembly and secretion. Inter-  
 345 estingly, COLVI immunolabelling of fibroblasts from  
 346 3 homozygous patients revealed a rather normal  
 347 secretion pattern (patients 11-II.5 and 13-II.1) or  
 348 reduced secretion (14-II.1) (Fig. 3 and Supplemen-  
 349 tary Figure 2). Accordingly, COLVI immunostaining  
 350 on muscle biopsy from patient 11-II.5 was also rather  
 351 preserved, with only focal points of discontinuous  
 signal (Fig. 2c and 2f).

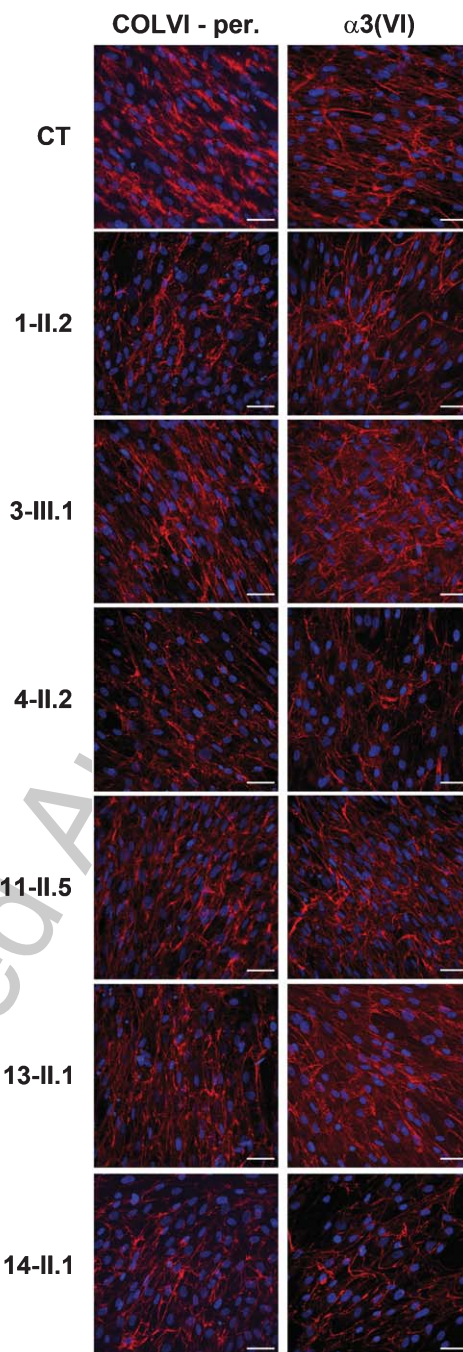


Fig. 3. Confocal imaging of COLVI secretion in fixed dermal fibroblasts from a control individual (CT) and 6 patients. COLVI (red) was detected with two polyclonal antibodies: Ab6588 (Abcam) on non-permeabilized cells (left panel) or an  $\alpha 3(\text{VI})$  chain specific on methanol-fixed cells (right 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 = 50  $\mu\text{m}$ .

## 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 HGMDPro-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  $34.7 \pm 21.5$  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  $\alpha 3(\text{VI})$  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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## 571 CONFLICT OF INTEREST

572 The authors have no conflict of interest to report.

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