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► **To cite this version:**

Sergey N Bardakov, Roman V Deev, Raisat M Magomedova, Zoya R Umakhanova, Valérie Allamand, et al.. Intrafamilial Phenotypic Variability of Collagen VI-Related Myopathy Due to a New Mutation in the COL6A1 Gene. *Journal of Neuromuscular Diseases*, 2020, pp.1-12. 10.3233/JND-200476 . hal-03094479

HAL Id: hal-03094479

<https://hal.sorbonne-universite.fr/hal-03094479>

Submitted on 4 Jan 2021

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Intrafamilial Phenotypic Variability of Collagen VI-Related Myopathy Due to a New Mutation in the *COL6A1* Gene

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Abstract. A family of five male siblings (three survivors at 48, 53 and 58 years old; two deceased at 8 months old and 2.5 years old) demonstrating significant phenotypic variability ranging from intermediate to the myosclerotic like Bethlem myopathy is presented. Whole-exome sequencing (WES) identified a new homozygous missense mutation chr21:47402679 T>C in the canonical splice donor site of the second intron (c.227 + 2T>C) in the *COL6A1* gene. mRNA analysis confirmed skipping of exon 2 encoding 925 amino-acids in 94–95% of resulting transcripts. Three sibs presented with intermediate phenotype of collagen VI-related dystrophies (48, 53 and 2.5 years old) while the fourth sibling (58 years old) was classified as Bethlem myopathy with spine rigidity. The two older siblings with the moderate progressive phenotype (48 and 53 years old) lost their ability to maintain a vertical posture caused by pronounced contractures of large joints, but continued to ambulated throughout life on fully bent legs without auxiliary means of support. Immunofluorescence analysis of dermal fibroblasts demonstrated that no type VI collagen was secreted in any of the siblings' cells, regardless of clinical manifestations severity while fibroblast proliferation and colony formation ability was decreased. The detailed genetic and long term clinical data contribute to broadening the genotypic and phenotypic spectrum of *COL6A1* related disease.

Keywords: Ullrich congenital muscular dystrophy, fibroblasts, contractures, myosclerotic phenotype of Bethlem myopathy, collagenopathy, type VI collagen, leaky splicing, *COL6A1*

INTRODUCTION

Collagen VI-related myopathies are among the most frequently identified forms of congenital muscular dystrophies, including Bethlem myopathy (BT

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HLM1, Bethlem myopathy 1, OMIM # 158810), Ullrich myopathy (UCMD 1, Ullrich congenital muscular dystrophy 1, OMIM # 254090) and a number of intermediate clinical phenotypes [1, 2]. Collagen VI-related muscular dystrophies are the second most common form of congenital muscular dystrophy in Europe, Japan and Australia. For instance, the prevalence rate of UCMD and Bethlem myopathy in northern England is 0.13 and 0.77 per 100,000 population, respectively [3, 4]. Collagen VI-related myopathies form a single continuum, the most severe type of which is UCMD [5–7]. UCMD is characterized by an early onset, often from birth, manifesting as muscle weakness and hypotonia, proximal joint contractures, distal joint hypermobility, as well as a number of bone deformities such as kyphoscoliosis and torticollis. The mildest form of collagen VI-related myopathies is classical BTHLM characterized by distal as well as proximal contractures, proximal muscle weakness and remaining ambulation into adulthood [8, 9]. In addition, two opposing variants for BTHLM have been described: limb-girdle phenotype is characterized by late or no contractures [6] and myosclerotic myopathy is characterized by early, diffuse, progressive contractures resulting in severe limitation of movement of axial, proximal and distal joints [7, 10]. The CK activity level is either unchanged, or 1.5 – 2-fold increased [11].

Collagen VI-related myopathies are caused by dominant as well as recessive mutations in *COL6A1*, *COL6A2* (21q22.3) and *COL6A3* (2q37) genes, encoding for the corresponding subunits of type VI collagen: $\alpha 1$ (VI), $\alpha 2$ (VI), $\alpha 3$ (VI) [9, 12, 13].

In skeletal muscles type VI collagen provides interaction between muscle basement membranes and the extracellular matrix and is known to bind biglycan, types I and IV collagen and decorin [14]. In addition, disrupted induction of autophagy and the development of mitochondrial mediated apoptosis with a *d* in myofibers has been reported in a mouse model of deficiency of $\alpha 1$ (VI) [15, 16].

UCMD may be caused by recessive inheritance due to homozygous or compound heterozygous mutations; however, dominantly acting mutations in *COL6A1*, *COL6A2* and *COL6A3* have also been described and may be more common, in particular *de novo* mutations. [17, 18]. Although the typical genetic mechanism of inheritance for Bethlem myopathy is dominant, recessive mutations have also been seen in patients [19–21]. An autosomal recessive inheritance mode has also been described specifically for the rare myosclerotic variant of BTHLM [7].

Inter-, intrafamilial, intergenerational phenotypic variability and disease progression in collagen VI-related myopathies are common. One of the reasons described for phenotypic variability is sporadic and parental mosaicism of dominantly acting collagen VI heterozygous mutations [22–24]. However, no significant phenotypic variability among siblings with homozygous mutations in the *COL6A1* gene has been reported previously. We present the results of a familial observation of five siblings (aged 8 months, 2.5, 43, 53, 58 years) with distinct phenotypic variability (intermediate phenotype and BTHLM with spine rigidity) due to a new homozygous mutation in *COL6A1*. We undertook an in-depth comparison of phenotypic differences in three sibs (43, 53, 58 years old). We also describe remarkable adaptive changes at the late stage of the disease which allowed two siblings (43 and 53 years old) to continue ambulation throughout life on fully bent legs.

PATIENTS AND METHODS

Examinations

Examinations and tests were performed after informed consent was obtained. There was documented consanguinity of the parents in generation II in this Avarian family in Dagestan. Three out of five affected male siblings in generation III (Patient III:1, 48 years old, Patient III:2, 53 years old, and Patient III:3, 58 years old) with clinical manifestations of collagen VI-related myopathy were examined between 2016 and 2018. The two other affected siblings were not personally examined (Patient III:11, presented with floppy baby syndrome and died of respiratory failure at 8 months old, Patient III:12 presented with early-onset severe proximal joint contractures and distal joint hypermobility and died as a result of a head injury at 2.5 years old) (Fig. 1). We present here the results of the most current examination of the three surviving siblings along with data from medical records.

Laboratory tests and investigations

Serum CK level were determined in all patients, X-rays of knee and ankle joints, spirometry, ECG, echocardiography, EMG, MRI of pelvic and lower extremity muscles (T1-, T2-WI, T2-FatSat) were performed in all patients.

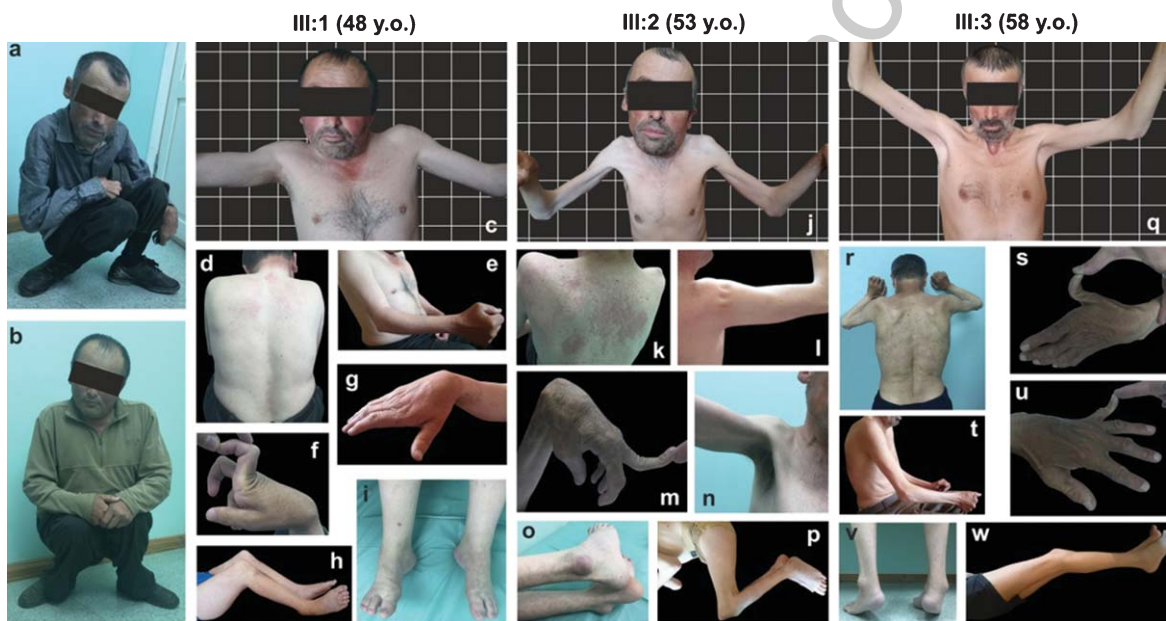
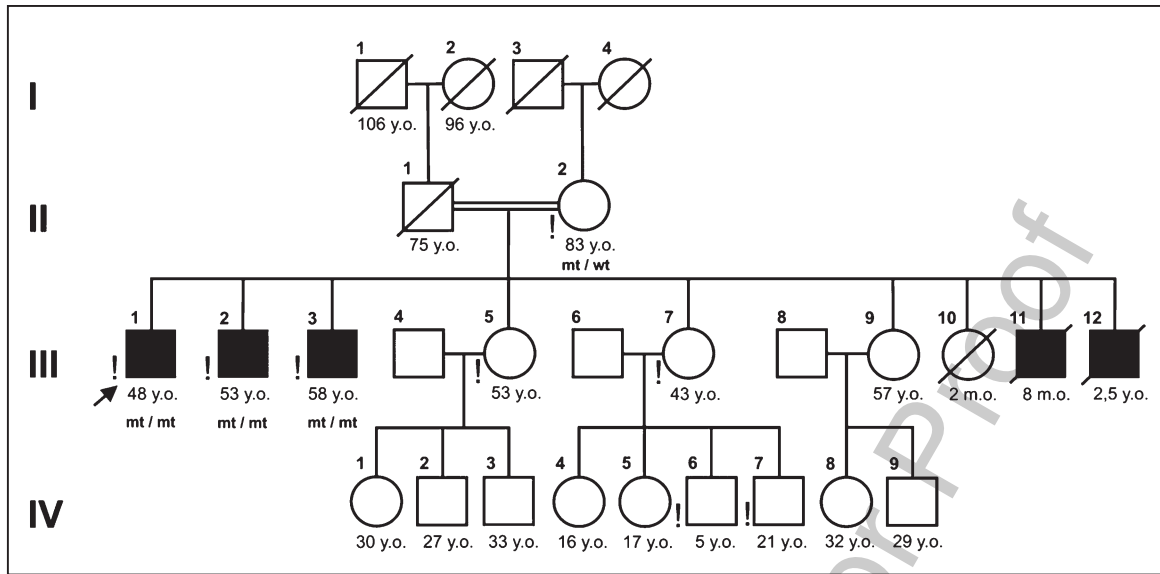


Fig. 1. Pedigree of the siblings' family. Symbols in black represent sick family members; exclamation mark - family members personally examined by the author; mt – mutant allele; wt – wild type allele. The position of siblings when walking (a – Patient III:1, 48 years old, b – Patient III:2, 53 years old). The phenotype of siblings (Patient III:1, 48 years old – c, d, e, f, g, h, i; Patient III:2, 53 years old – J, k, l, m, n, o, p; Patient III:3, 58 years old – q, r, s, t, u, v, w). Written informed consents of patients are on file.

Genetic testing

Whole-exome sequencing (WES) was performed on DNA samples obtained from Patient III:1, III:2, III:3 using a pair-end method (2×90 base pairs) on the Illumina HiSeq 2000 platform using the SureSelectV4 target sequences enrichment system (51M).

The average reading depth was $85 \times$ in all samples. Potentially pathogenic variants were identified in comparison with reference human genome databases and the changes detected were confirmed by standard methods (Sanger sequencing and PCR). Segregation analysis of the identified mutations was performed in the mother (II:2) of the siblings.

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Primary dermal fibroblast culture

Biopsy samples (4 mm³) were taken from the skin behind-the-ear of patients III:1, III:2, and III:3 under local anesthesia (2% lidocaine solution). The biopsy samples were treated with 0.05% type II collagenase (Sigma, USA) at 37° C for 12 hours, then centrifuged at 200 g for 10 min. The cell pellet was resuspended and cultured in DMEM (Sigma, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, USA), and 20 µg/ml gentamicin (Sigma, USA) at 37° C 5% CO₂. Skin fibroblasts of a 35-year-old healthy male were used as the control.

Fibroblast colony-formation efficacy (CFE-F)

CFE-F was evaluated at passage 1 under the standard procedure. The cell suspension was split into three 100 mm Petri dishes to obtain a clonal inoculum density of 3–4 cells/cm² in DMEM (Sigma, USA) supplemented with 10% FBS (HyClone, USA), and 20 µg/ml gentamicin (Sigma, USA). The Petri dishes were incubated in a CO₂-incubator under saturated humidity conditions at 37° C in the 5% CO₂ atmosphere for 14 days. Thereafter, the culture dishes with the pre-formed colonies were washed three times with PBS (pH 7.4) and fixed with 70% alcohol for 15 minutes at room temperature. Then alcohol residuals were removed by triple washing with distilled water, and the colonies were stained with a KaryoMAX® Giemsa Stain Stock Solution (Gibco, USA) at 37° C for 20 minutes. The dishes with stained colonies were thoroughly washed from excessive stain and dried at room temperature for 5–7 hours. Formed colonies were counted, the number and shape of their constituent cells determined. A total number of explanted cells in counted colonies only was more than 20. CFE-F was calculated according to the Fridenshtein's equation for stromal progenitor cells: a pre-formed colonies to explanted cells ratio was multiplied by 100%. CFE-F values reflect individual fibroblast progenitor cell counts. When the colony formation efficacy ranges from 45 to 49% in men and from 36 to 45% in women, the regenerative potential is considered normal [25–27].

Fibroblast proliferative potential

All fibroblasts colonies were classified as dense, diffuse and mixed ones. The fibroblast proliferative potential (a proliferative index) was calculated by a colony distribution (dense, diffuse, mixed) using

the formula: $PI = [1 (DC) + 2 (MC) + 3 (DC1)] / 100\%$ [26, 27], where PI is the proliferative index, DC – diffuse colonies, (%), MC – mixed colonies, (%), DC1 – dense colonies, (%). The proliferative potential depends on the number of mitotically active cells in the culture, which determines the rate of regenerative processes in the dermis. The proliferative potential of dermal fibroblasts is higher in denser colonies and less in diffuse ones. When the proliferative index ranges from 2.0 to 2.4 in men and from 1.8 to 2.0 in women, the proliferative potential is considered normal.

Immunofluorescent staining of skin-derived fibroblasts

Collagen VI was stained on confluent cells cultured with 0.25 mM ascorbic acid as previously reported by Hicks et al. (2008), and triton X-100 to permeabilize the cells in order to detect intracellular retention of COLVI [28]. Fibroblasts were incubated with collagen VI polyclonal antibodies (abcam, Ab6588, dilution – 1:1000). Fluorescence was detected under an Axioplan 2 microscope (Zeiss, Germany) equipped with the HBO 100 mercury lamp (Zeiss). Single channel images and overlays were captured using the Metavue software (Molecular Devices).

Transcript analysis

Total RNA was extracted from fibroblast cultures using the ReliaPrep™ RNA Cell Miniprep System kit (Promega, France) and reverse transcribed into cDNA with the Superscript RTIII kit (Life Technologies SAS, France) following the manufacturer's recommendations. cDNA samples were subjected to PCR amplification using primers located in exons 1 and 3 of the *COL6A1* gene (5'-ACCGTTAGTATGC GAGTTTCTGGCTGGGAGCAGGA-3' and 5'-TC GGATAGTCAGTCGTTTATAGCGCAGTCGGTGT A-3', respectively). *RPLP0* was used as reference gene (5'-ATGTGGGCTTTGTGTTCCACCPCR-3' and 5'-TCCAGTCTTGATCAGCTGCA-3'). PCR products were analysed on 2% agarose gel and semi-quantification was performed using the ImageJ software.

Ethics statement

All procedures were performed after the patients signed an informed consent form as required by the

Table 1
Clinical characteristics and pulmonary function parameters of the sibs (Patient III:1, III:2, III:3)

Characteristic (age of onset, years)	Patients		
	III:1	III:2	III:3
Beginning of independent walking	3–4	3	2,5
Running and jumping	Impossible	Impossible	Delay
Muscular weakness	3 (m. quadr. femor. 3/5 MRC)	3 (m. quadr. femor. 3–4/5 MRC)	6–7 (m. quadr. femor. 4/5 MRC)
Difficulty in stair climbing	3–4	3–4	33
Difficulty in standing up from a sitting position	6	7	40
Means of support	7–13 (crutches)	10–13 (crutches)	Did not use
Muscle atrophy	Shoulder girdle, upper limbs	Diffuse, shoulder girdle mainly	Shoulder girdle and upper limbs
Knee joint contractures	3–4	3–4	7–8
Hip joints contractures	3–4	3–4	7–8
Elbow joint contractures	13–14	13	18–19
Shoulder joint contractures	16–18	15–16	23–25
Wrist joint contractures	23–25	18	30–32
Achilles tendon contractures	13	13	16–17
Joint hypermobility	Does not persist	Persists	Persists
Dyspnea, years	No	40	40
Pulmonary function parameters (at the last visit)			
Age at last visit, y.o.	48	53	58
Tidal volume, ml ($\Delta\%$ of N)	200 (50)	100 (25)	150 (37.5)
VC, ml ($\Delta\%$, of proper VC)	1400 (39.2)	1100 (33.8)	1800 (52.3)
Proper VC, ml.	3572	3252	3444
Inspiratory reserve volume, ml ($\Delta\%$ of N)	800 (57)	700 (50)	1000 (71)
Expiratory reserve volume, ml ($\Delta\%$ of N)	400 (40)	300 (30)	650 (65)

Note: 1. VC is the vital capacity of the lungs; 2. Proper VC of the lungs = height (cm) \times 0.052 – age (years) \times 0.028 – 3.2; 3. N expiratory reserve volume (ERV) – 1000–1400 ml; 4. N inspiratory reserve volume (IRV) – 1400 ml (IRV = VC – (RV + ERV)).

237 Declaration of Helsinki (2013) and the study proto-
238 col (#AC-2348-082018) approved by the local Ethics
239 Committee of the Dagestan State Medical Academy
240 (Russia). All patients signed an informed consent
241 form for publication their medical data and pho-
242 tographs.

243 Description of clinical cases

244 All patients were born following normal pregnan-
245 cies. The parents were second cousins and clinically
246 unaffected. The proband's father (II:1) died of stom-
247 ach malignancy at the age of 75 years. The family
248 history is otherwise negative for neuromuscular dis-
249 eases (Table 1).

250 Review of history and medical records data for
251 siblings III:1 and III:2 shows evidence of a similar
252 disease onset in 3–4 years old and progression corre-
253 sponding to the intermediate phenotype of collagen
254 VI-related dystrophies while maintaining the ability
255 to walk with bent knees and thigh joints from the age
256 of 10–13 years (Fig. 1). The eldest sibling (Patient
257 III:3) had a significantly lower rate of progression,
258 the minimal severity of contractures, with a Trendelenburg gait retained and classified as BTHLM.

Clinical neurological examination (at the last visit)

259 Patient III:2 (53 years old) was extremely thin
260 with a BMI of only 14.9 (less than 15.0: severely
261 underweight). He had the most severe contractures,
262 diffuse muscle atrophy with a tight feel to the mus-
263 cles (the mid-upper arm circumference (MUAC) –
264 16/15 cm; normal value for adult men greater than
265 23 cm [29]), that was a manifestation of interme-
266 diate phenotype of collagen VI-related dystrophies
267 with a prominent diffuse contractural component.
268 The youngest sibling (Patient III:1, 48 years old) had
269 a normal trophic status with a BMI 23.31, but with a
270 similar degree of prominent contractures, he had an
271 earlier and more pronounced proximal muscle weak-
272 ness and corresponded to intermediate phenotype of
273 collagen VI-related myopathy with the predominance
274 of a myopathic syndrome (MUAC – 22.5/22.0 cm).
275 The eldest sibling (Patient III:3, 58 years old) was
276 very thin with a BMI being 17.01 (16.0–18.49),
277 but with minimal limb contractures except for
278 spinal (axial) rigidity and milder muscle weakness
279 despite atrophic muscles (MUAC – 19/18 cm), over-
280 all consistent with a Bethlem phenotype with spinal
281 rigidity.
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Distribution of contractures

All siblings examined had torticollis, cervicothoracic scoliosis, cervical and lumbar spine rigidity due to asymmetric contractures in mm. scalenii, and m. sternocleidomastoideus, which were most pronounced in Patient III:2 (Fig. 1).

All siblings examined had contractures of large joints predominantly in the lower extremities which were most pronounced in Patient III:2. Flexion contractures in shoulder joints from 40 to 130° were caused by sclerotic shortening of mm. pectorales, and m. latissimus dorsi. Significant elbow flexion contractures up to 40–70° were due to m. brachioradialis and m. biceps brachii shortening. Wrist joints were characterized by flexion contractures due to sclerotic shortening of mm. flexor digitorum profundus et superficialis, m. and flexor carpi ulnaris, limiting the extension of the flexion position from 45° (Patient III:2), to 45° of the extensor position (Patient III:3), and hand supination to 15–60° and the wrist joint ulnar deviation to 10–45°. There were hip flexion contractures from 10 to 70° of the flexion position. Knee flexion contractures were most pronounced in siblings III:1 and III:2 (up to 60–110° of the flexion position), while being 10–15° in sibling III:3. All patients had equinovarus foot deformity (from 15 to 30°), most evident in sibling III:3. Patients III:2 and III:3 had a specific calcaneal prominence (Fig. 1).

Hypermobility of small joints of the upper limbs persisted throughout the life. Patients III:2 and III:3 had hyperextension up to 45° in distal interphalangeal joints of II–V fingers and up to 80–90° in interphalangeal joints of the thumbs (Fig. 1). Thus, hypermobility was minimal in Patient III:1 and most pronounced in Patient III:2.

Distribution of muscle strength and atrophy

All examined siblings had muscle weakness predominantly in the flexors of the neck, levators of the scapula, elbow extensors, digit flexors, thigh adductor muscles, lower leg extensors, and foot adductor muscles (3/5 MRC). Muscle weakness predominated in distal parts of the upper limbs (3–4/5 MRC), and in proximal ones of the lower extremities (3–4/5 MRC). It was most pronounced in Patient III:1, and least pronounced in Patient III:3 (Fig. 1)

Muscle atrophy and contractural changes were most intense in Patient III:2 and Patient III:3.

Upper extremity muscle atrophies were diffuse involving deltoid muscles to a least degree, where sclerotic changes resulted in the formation of 3–4 separate bundles (Fig. 3). Muscle atrophy was mainly observed in distal parts of the lower limbs.

Tendon reflexes could not be elicited.

Skin findings

Type I follicular hyperkeratosis was most pronounced in Patient III:1, to a lesser extent in Patient III:2, while none observed in Patient III:3. In addition, all siblings had signs of seborrheic dermatitis and did not have abnormal keloid formation (Fig. 3).

Blood biochemistry

Serum CK activity was slightly increased (235–280 U/l) in the patients.

MRI of the lower extremities

A MRI pattern showed typical signs of perifascial pronounced fatty infiltration, resulting in increased signal on T1 weighted images along the muscle periphery and around the central fascia (in m. rectus femoris), as well as more diffuse involvement of other muscles [30–32].

Fatty replacement was more pronounced in thigh and pelvic girdle muscles than in the calf ones predominantly involving the gluteus and thigh posterior muscles (stage 2B–3, according to E. Mercuri, 2002) [33]. Patients III:1 and III:2 had the most pronounced fatty infiltration. Yet, Patients III:1 and III:2 had a relatively hypertrophic appearance of m. rectus femoris, m. vastus lateralis, m. sartorius, m. semimembranosus, m. semitendinosus and m. biceps femoris caput longus, which thus appeared to be less involved, potentially related or contributing to ambulation with bent hip and knee joints. Patient III:3 had moderate atrophy of m. rectus femoris and m. vastus lateralis (Fig. 2).

Spirometry

Spirometry demonstrated a decrease in all basic pulmonary function parameters. The tidal volume was decreased by 50% (200 ml), 75% (100 ml) and 62% (150 ml) in patients III:1, III:2 and III:3, respectively (Table 1). Patients III:2 and III:3 had

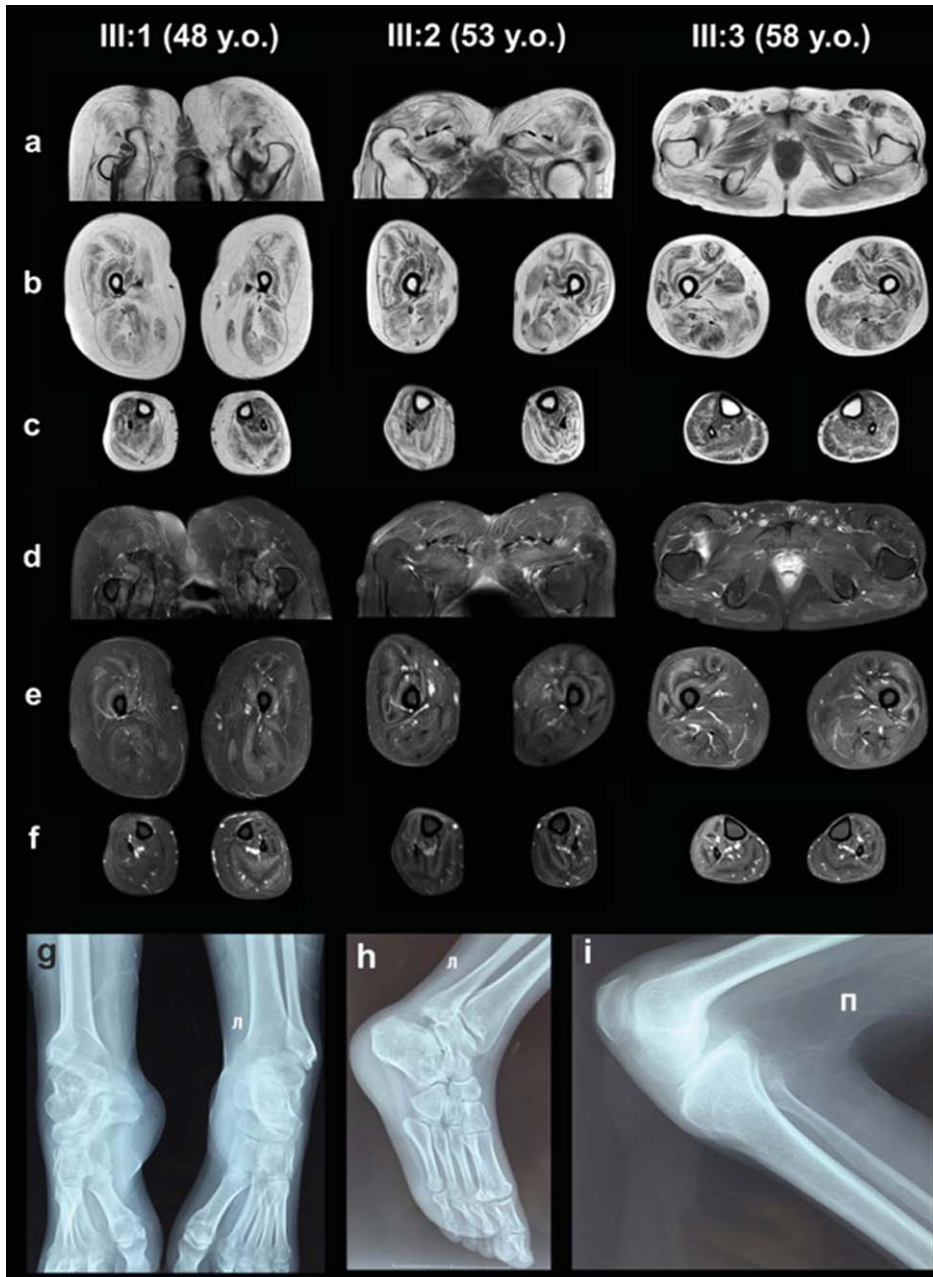


Fig. 2. MRI of the pelvic girdle and lower extremities muscles (a, b, c – T1-WI, d, e, f – T2-WI FatSat). X-ray examination of the ankles (g, h – Patient III:1, 48 years old) and knee joint (i – Patient III:2, 53 years old).

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complained of dyspnea independent of body position from the age of 40. However, because of limited access to medical technology none of the patients had initiated non-invasive ventilatory support. Despite the untreated hypoventilation and living at high altitude (about 4100 m above sea level) they were able to maintain physical activity.

X-ray examination of knee and ankle joints

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The results of X-ray scans showed chronic, knee-joint overloads and elevated patellae (an Insall-Salvati ratio of 1.4) in patients III:1 and III:2. The scans also uncovered signs of osteoarthritis in the ankle joints of patients III:1 and III:2. Lateral

386 subluxations of the talus bones were combined with
387 wedge-shaped deformations at the tibial-talus articu-
388 lar surfaces of patients III:1 and III:2 (Fig. 2).

389 *Echocardiography*

390 Patient III:2 had Stage 1 aortic valve insufficiency.
391 All patients (III:1 – 65%, III:2 – 66%, III:3 – 69%)
392 had normal left ventricular ejection fraction as deter-
393 mined by Teichholz and normal blood pressure.

394 *Electromyography*

395 Needle electromyography demonstrated a myo-
396 pathic pattern.

397 *Genetic testing*

398 Sequencing of Patient III:1, III:2 and III:3 DNA
399 samples established a new homozygous mutation
400 chr21:47402679T>C, leading to a change in the sec-
401 ond intron canonical splice site (c.227 + 2T>C) in the
402 *COL6A1* gene. Sanger sequencing showed that the
403 proband's mother (II:2) is a carrier of heterozygous
404 (c.227 + 2T>C) in the *COL6A1* gene.

405 *Dermal fibroblast culture*

406 All patients had an abnormal collagen VI secre-
407 tion by dermal fibroblasts, with severe to completely
408 absent assembled matrix, associated with stained
409 single extracellular microfibrils and intracellular
410 retention of collagen VI precursors. (Fig. 3).

411 There was a reduced CFE-F in all patients – by 30%
412 in Patient III:1, by 35% in Patient III:2 and by 32% in
413 Patient III:3 when compared to the control (45–49%)
414 [26]. Reduced CFE-F indicates decreased fibroblast
415 precursor cell counts in the patient's fibroblasts and
416 decreased regenerative potential of the dermal fibrob-
417 last population.

418 Patients III:2 and III:3 had a normal proliferation
419 index, while it being slightly below the normal in
420 Patient III:1. A decreased proliferative index indi-
421 cates a low mitotically active cell count in the culture,
422 and hence a decreased regeneration rate in Patient
423 III:1.

424 *mRNA analysis*

425 mRNA analysis in all patients revealed exon 2
426 skipping containing 130 bp, coding for the begin-
427 ning of the N1-domain. The skip results in an out-of-

428 frame transcript. We also detected full length tran-
429 scripts at low percentage (III:1 – 5.4%; III:2 – 6.6%;
430 III:3 – 6.5%). Thus, the homozygous mutation in the
431 *COL6A1* gene leads to a «leaky» out-of-frame skip of
432 mRNA with synthesis allowing for the generation of
433 a small percentage of full length transcript expected
434 to generate wild type protein.

435 **DISCUSSION**

436 Two out of three siblings (48 and 53 years old)
437 had severe contractures of the proximal joints and
438 might most closely corresponded to an intermediate
439 phenotype of collagen VI-related dystrophies (or the
440 milder end of the “moderate progressive” phenotype
441 of Brinas et al. [11], because both patients were able
442 to walk independently up to 7–8 years, followed by
443 the ineffective use of crutches and the beginning of
444 walking on bent legs due to progressive contractures,
445 but relatively better preserved muscle strength [34].
446 The eldest one had BTHLM with spinal rigidity, in
447 some aspects reminiscent of the myosclerosis pheno-
448 type described previously as caused by a homozygous
449 nonsense *COL6A2* mutation (p.Q819X) [7]. The
450 coexistence of divergent phenotypes in siblings of
451 the same family has been previously recognized in
452 the COL6-related dystrophies [8]. Genetic analysis
453 was not possible for patient III:12 (2.5 y.o.) due to
454 his early death; however, he probably also had colla-
455 gen VI-associated dystrophy, as he had an early-onset
456 pronounced proximal joint contracture and distal
457 hypermobility. Patient III:11 (8 m.o.) cannot be clas-
458 sified due to his early death from a respiratory failure,
459 but hypotonia and distal hypermobility suggest the
460 same disease.

461 It is noteworthy to highlight that patients aged 48
462 (III:1) and 53 (III:2) years could walk with bent knee
463 and hip joints for 35–40 years, indicating that upright
464 ambulation was predominantly impaired by contrac-
465 tures rather than diffuse weakness and attesting to
466 considerable adaptive capabilities of these patients
467 and their musculoskeletal system.

468 This family also highlights the considerable vari-
469 ability in the degree of joint contractures even with the
470 same underlying collagen VI mutation. The mecha-
471 nism of joint contractures in the collagen VI-related
472 dystrophies is still not fully explored and might
473 reside within the muscle connective tissue, but also
474 in tendon and joint capsules. Clearly there must be
475 additional genetic modifiers determining the extend
476 of such contractures.

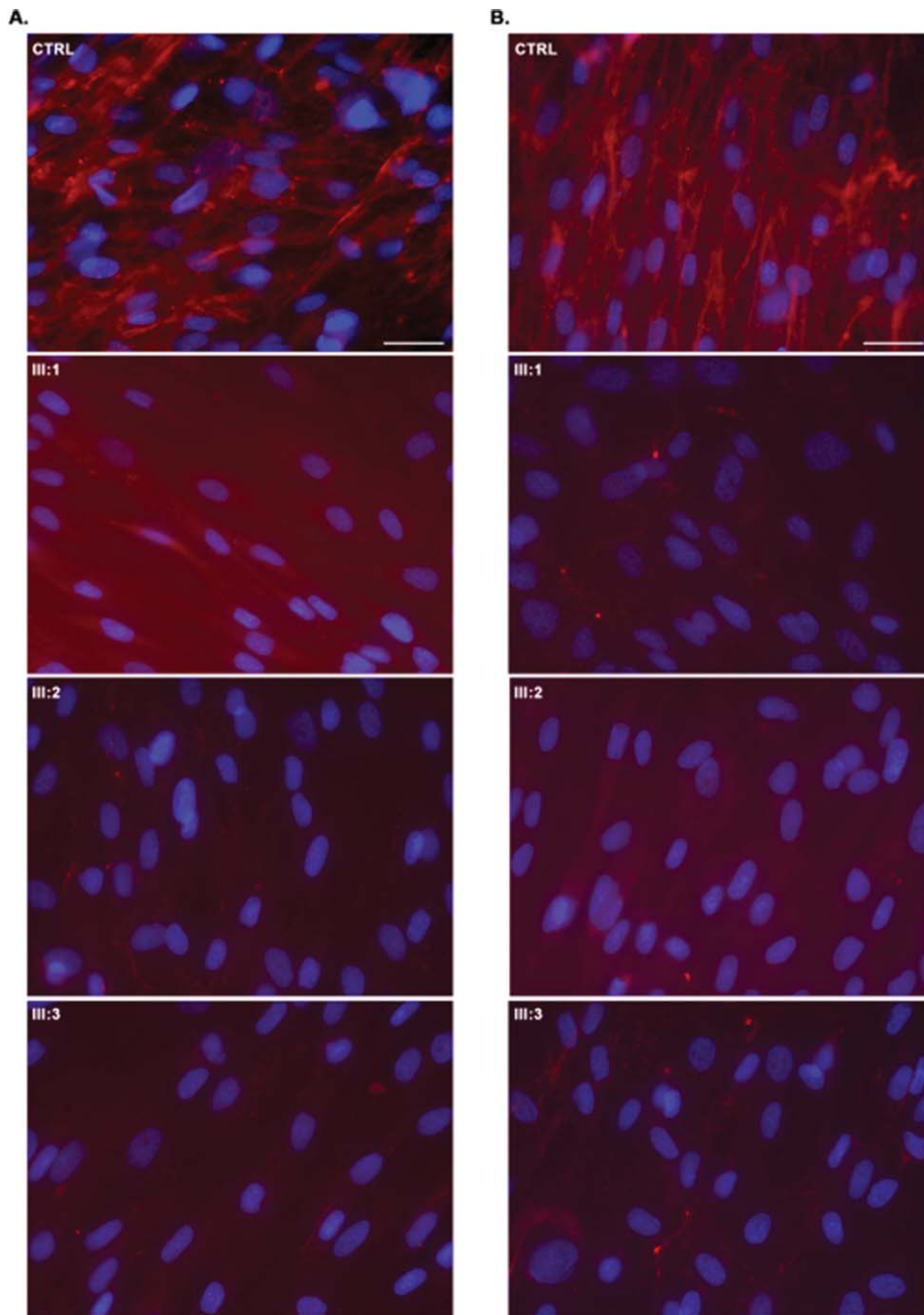


Fig. 3. Collagen VI immunofluorescence in UCMD dermal fibroblasts (permeabilised (A) and non-permeabilised (B) conditions according to Hicks et al. (2008); CTRL – normal control, which has an abundance of well-organized collagen VI microfibrils showing a linear and unidirectional trend; III:1; III:2, III:3 – significant collagen VI rarefaction with stained single extracellular microfibrils and intracellular protein retention were noticed in most cells. Dermal fibroblasts immunostained for matrix-deposited collagen VI (red) and with the nuclear stain DAPI (blue). Scale bar 50 μ m.

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Despite considerable muscle atrophies in the upper limbs of siblings moving with the bent knee and hip joints (Patients III:1, III:2), there was hypertrophy of the posterior thigh muscles and some anterior thigh

muscles (m. vastus lateralis, m. rectus femoris) in the lower extremities. Findings on muscle MR were consistent with a collagen VI related dystrophy (perifascial involvement) and there were diffuse lesions

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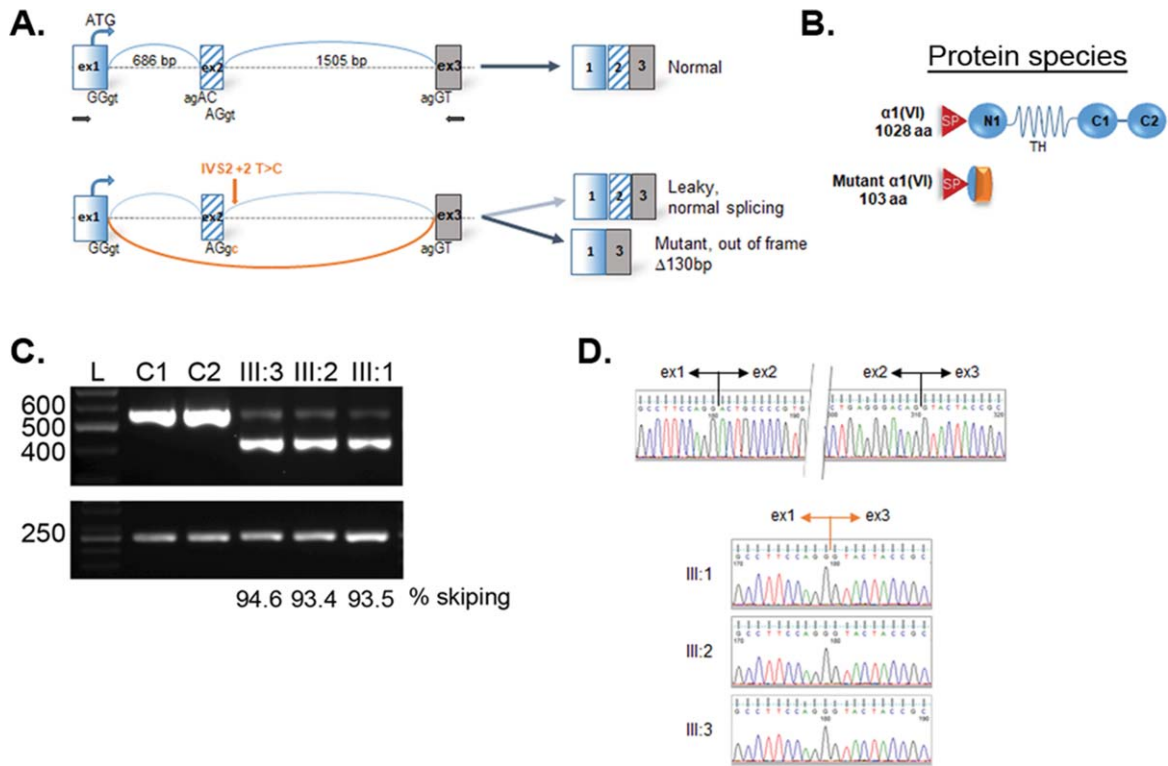


Fig. 4. Skipping of exon 2 in *COL6A1*. Schematic representation of mutation effect on “leaky” splicing, which allows generating some normal transcripts and significant amount of mutant transcript with deletion of 2 exon having a size of 130 bp in patient (A); scheme of normal full-length (1028 aa) and mutant protein $\alpha 1$ (VI) 103 aa; SP – signal peptide; TH – triple helical domain; C1–C2 –terminal domains (B); RT-PCR gel showing an additional faster migrating band in the patient lanes (III:1; III:2; III:3), but not in the normal controls (C1, C2) (C); sequencing chromatogram of cDNA representing the upper normal band (top) and lower band in patient III:1, III:2, III:3 revealing the loss of sequence corresponding to exon 2. *RPLP0* was used as reference gene (bottom) (D).

in muscles that appeared hypertrophied [30, 32]. Partial sparing of the contractile properties of myofibrils within an otherwise affected muscle might help account for these adaptive capabilities of skeletal muscles. In context of an otherwise severely contractural phenotype [35], this may be allowing for the functional compensation seen in the siblings.

It is of interest that in this family the pulmonary function was relatively better preserved, compared to the functional severity of the skeletal muscle involvement. If the patients were within the UCMD phenotype one would have predicted earlier need for non-invasive ventilation latest at beginning of the second decade of life as previously reported [34, 36]. This supports our functional classification of the family in the intermediate to Bethlem range with less pulmonary involvement but with a considerable contractural phenotype. Therefore, restrictive changes of the chest, may be in part independent on the severity of proximal joint contractures [37]. Three patients described did not obviously require non-invasive

ventilation as he has adapted to physical activity without a wheelchair in high altitudes. However, a nocturnal polysomnography will be needed to fully assess his sleep oxygenation and possibility for CO₂ retention.

The newly described homozygous chr21:4740 2679T>C mutation in the *COL6A1* gene results in a change in the canonical splice donor site of the second intron (c.227 + 2T>C), is likely to lead to the destruction of the splice site and the formation of an aberrant transcript with an out-of-frame skip of exon 2. In all patients described semi-quantitative PCR of fibroblast mRNA found a significant predominance of this aberrant transcript (93.4, 93.5 and 94.6%) which did not undergo a nonsense-mediated decay, but also confirmed the presence of full length wild type transcripts, presumably allowing for the production of some normal collagen VI. However, the predominant synthesis of severely truncated $\alpha 1$ (VI) results in severe rarefication of extracellular microfibers and diffuse intracellular accumulation of type VI collagen

precursors, detected in an immunofluorescence assay of permeabilised dermal fibroblasts. Thus, the mutation is "leaky" and therefore consistent with the overall milder presentation in this family compared to a full "null" situation, which would be predicated to result in a severe UCMD phenotype. However, we found no significant differences in the percentage of normal transcript formation among patients, to account for the milder phenotype in Patient III:3. In addition, all siblings had equally reduced fibroblast colony formation efficacy, which might suggest a genetically determined decrease of fibroblast progenitor cell counts, irrespective of the phenotype. While our findings appears to be in contrast to a previously reported correlation between a disease severity, the mutation type and the degree of decrease in collagen synthesis by fibroblasts [18, 38] it is possible that there are variations in the levels of normal collagen VI matrix formation *in vivo* in muscle and tendons that are not reflected in our *in vitro* assays.

CONCLUSION

This family observation demonstrates intrafamilial phenotypic and functional variability of collagen VI-related dystrophy presenting on the intermediate to Bethlem spectrum in patients with particularly pronounced differences in the contractural manifestations of the disease. It is caused by a newly described homozygous *COL6A1* out-of-frame exon 2 skipping splice mutation. We demonstrate its leakiness allowing for the generation of some normal transcript. While the analysis on mRNA, collagen VI matrix secretion and proliferative ability did not reveal obvious differences that would explain the cause of intrafamilial phenotypic variability, more subtle differences in the degree of normal matrix deposition *in vivo* as well as the presence of yet to be identified genetic modifiers may be responsible for the variability observed in this family.

ACKNOWLEDGMENTS

The authors are grateful to E.A. Pomerantseva, the head of «GeneticO» laboratory (Moscow), and her employees (genetic testing).

CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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