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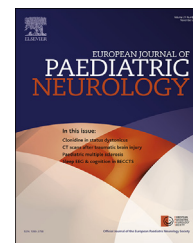
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Original article

Somatic mosaicism represents an underestimated event underlying collagen 6-related disorders



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ABSTRACT

Background: Collagen VI-related disorders (COL6-RD) are a group of heterogeneous muscular diseases due to mutations in the COL6A1, COL6A2 and COL6A3 genes, encoding for collagen VI, a critical component of the extracellular matrix. Ullrich congenital muscle disorder and Bethlem myopathy represent the ends of a clinical spectrum that includes intermediate phenotypes of variable severity. UCMD are caused by recessive loss of function mutations or *de-novo* dominant-negative mutations. The intermediate phenotype and BM are more commonly caused by dominantly acting mutations, and less commonly by recessive mutations. Recently parental mosaicism for dominant mutations in COL6 have been reported in four COL6-RD families and germinal mosaicism has been also identified in a family with recurrence of UCMD in two half-sibs.

Methods and results: Here we report three unrelated patients affected by a COL6-RD who carried *de novo* mosaic mutations in COL6A genes. These mutations, missed by Sanger sequencing, were identified by next generation sequencing.

Conclusions: This report highlights the importance of a complete diagnostic workup when clinical and histological findings are consistent with a COL6-RD and strengthen the impression that mosaicisms are underestimated events underlying COL6-RD.

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1. Introduction

Collagen type VI-related dystrophies (COL6-RD) are a spectrum of conditions ranging from the severe Ullrich congenital muscular dystrophy (UCMD; MIM# 254090) to the intermediate phenotype and milder Bethlem myopathy (BM; MIM# 158810).¹

COL6-RD can be caused by mutations in each of the three genes encoding the collagen 6 (colVI) α chains, $\alpha 1$ (COL6A1), $\alpha 2$ (COL6A2), and $\alpha 3$ (COL6A3). Approximately 10% of patients with a clinical diagnosis of COL6-RD have been reported to lack mutations in COL6A1, COL6A2 and COL6A3, suggesting that either a subset of mutations in these genes escape detection or the presence of other still unrecognized disease genes implicated in these conditions.²

Recently germinal and somatic mutations in COL6 genes have been reported in few cases of COL6-RD.^{2,3} Parental mosaic dominant mutations with subsequent full inheritance and penetrance of the mutation in the heterozygous offspring were documented in three COL6-RD families with marked inter-generational phenotypic heterogeneity conditions.² An additional case of COL6-RD related to a de novo mutation in COL6A2 gene has been also reported in the same article.²

Furthermore, in a family with recurrence of COL6-RD in two half-sibs the occurrence of paternal germinal mosaicism was documented.³

These finding may explain, in part, the inter-familial clinical variability that is frequently observed in this group of diseases, but also suggests that mosaicism may represent an underestimated event responsible for a number of cases.

Here we report a detailed clinical, immuno-histochemical and molecular characterization of three unrelated patients affected by a COL6-RD who carried mosaic COL6A gene mutations. All patients had clinical and histological findings that were highly suggestive of a Col6-related myopathy notwithstanding Sanger sequencing failed in revealing any mutation in the relevant genes. In all these cases, the use of second generation sequencing (SGS), either targeted resequencing or whole exome sequencing (WES), provided evidence for the occurrence of a somatic COL6 gene mutation, allowing molecular confirmation of the diagnosis based on clinical and histological evaluation.

2. Patients and methods

All patients were diagnosed and followed at the Unit of Neuromuscular and Neurodegenerative Disorders of the Bambino Gesù Children's Hospital. Skin and muscle biopsy were performed for diagnostic purposes. Biological samples including blood, hair, urine and saliva and additional muscle sample of patients were obtained for investigational purposes after signed informed consent was secured. DNA was obtained from circulating leukocytes, fibroblasts, muscle, hair and saliva using standard procedures. Human studies related to the studies of muscle biopsies and molecular genetic analysis have been approved by our ethics committee.

3. Muscle biopsy

Muscle biopsies, performed on quadriceps muscle, were obtained using standard techniques, and cryostat muscle sections were stained using standard histological and histochemical protocols.

4. Skin biopsy and fibroblast cultures

Cultured fibroblasts were obtained from skin biopsies. Cells were grown in Dulbecco's modified minimum essential medium (D-MEM) supplemented with 20% fetal calf serum (Life Technologies, Gaithersburg, MD), penicillin and streptomycin, as previously reported.⁴ Confluent cultures were treated 5 days with 0.25 mM L-ascorbic acid, then processed for immunofluorescence analysis (see below).

5. Immunofluorescence labeling of muscle biopsies and dermal fibroblasts

Confluent cells were incubated with monoclonal mouse anti-ColVI (MAB1944, Millipore) antibody, followed by AlexaFluor488 conjugated anti-mouse IgG (Life Technologies), as previously described.⁵ In another series of experiments, cells were formaldehyde fixed, permeabilized with 0.15% Triton X-100, then processed for ColVI staining as described above. Nuclei were counterstained using 1 μ g/ml Hoechst 33342 (Life Technologies).

Skeletal muscle cryosections (5 μ m) were double stained with monoclonal mouse anti-ColVI (MAB1944) and rat anti-300kD $\alpha 2$ laminin (Alexis) antibodies, which were revealed with goat AlexaFluor488 anti-mouse and AlexaFluor555 anti-rat immunoglobulins (Life Technologies), respectively. Slides were mounted with Prolong antifade reagent (Life Technologies).

Images were acquired by Olympus Fluoview FV1000 confocal microscope using an immersion oil 60 \times objective (numerical aperture 1.42). Fluorochromes unmixing was performed by acquisition of automated-sequential collection of multi-channel images, and Z reconstructions were obtained with a scanning mode of 1024 \times 1024 pixels, sampling speed of 20 μ s/pixel and z stack of 0.3 μ s/slice. Z-stacks were imported into IMARIS (Bitplane, Zurich, CH) software to obtain their 3D surface rendering. Images were processed using Photoshop software (Adobe Systems Inc., CA).

6. Muscle MRI

All the patients underwent muscle imaging by MRI of pelvic and lower limbs on 1.5T scanners according to a standard protocol.⁶ Patient 3 was also studied with an MRI protocol for the upper girdle.⁷

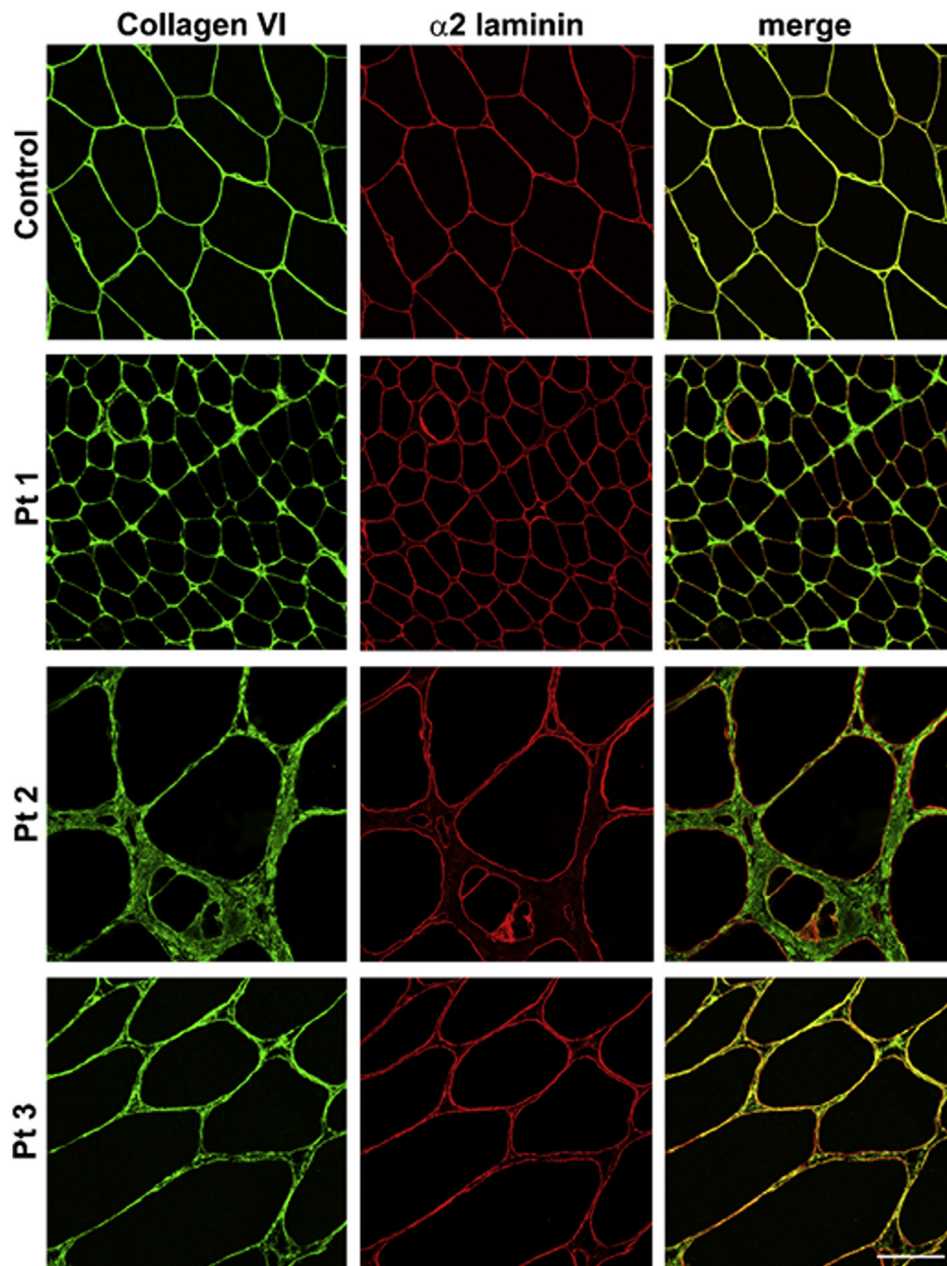


Fig. 1 – Immunostaining of colVI in skeletal muscle. Confocal images of Collagen VI (green) and $\alpha 2$ laminin (red) immunofluorescence in control and patients' muscles. ColVI was normally distributed around myofibers and blood vessels in control muscles and fully colocalized (yellow signal in overlapped images) with the basal lamina marker (red). In Pt1 muscle, ColVI was weakly reduced around several myofibers, as highlighted by its superimposed image on $\alpha 2$ laminin. In Pt2 muscle, the ColVI distribution was uniformly reduced around the basal lamina of myofibers, whereas it resulted weakly thinned around Pt3 myofibers. Bar: 50 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

7. Molecular diagnostic investigations

The three patients had previously been screened by Sanger sequencing using cDNA obtained from fibroblasts (Pt1) or genomic DNA extracted from circulating leukocytes (Pt2 and Pt3) according to standard methods, without identifying any pathogenic *COL6A* gene mutations.

A trio-based WES approach was conducted on patients 1 and 3 by using genomic DNA extracted from circulating leukocytes. -Exome capture was carried out using SureSelect Human All Exon V4 capture kit (Agilent), and sequencing was performed using a HiSeq2000 platform (Illumina). WES data analysis used an in-house implemented pipeline. Paired-end reads (90bp) alignment (UCSC GRCh37/hg19), and variant calling and

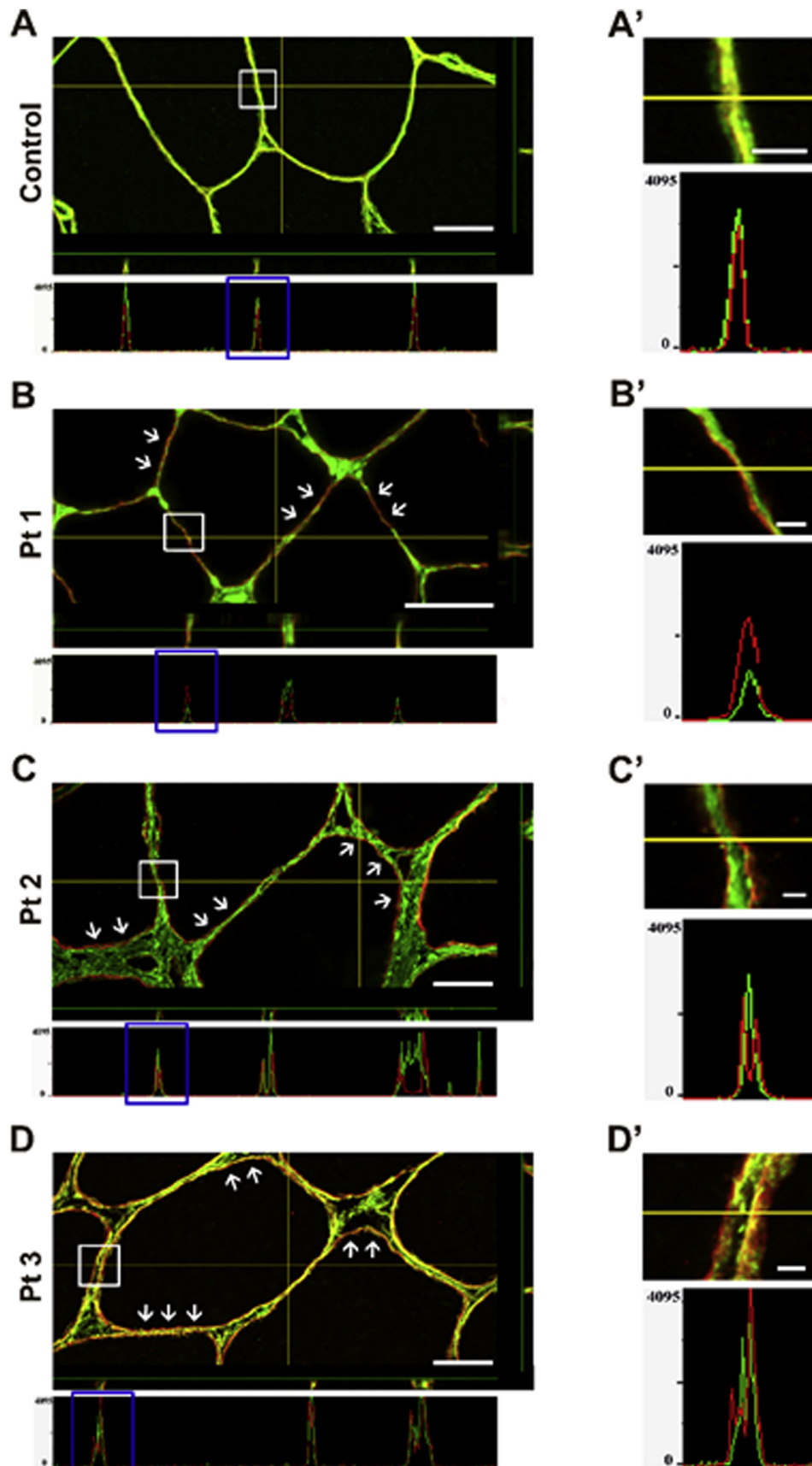


Fig. 2 – XYZ stack profiles. ColVI and $\alpha 2$ laminin co-distributions investigated in confocal Z-reconstructions, XZ- and YZ-axis projections (A–D), and in intensity XZ stack profiles (A'–D'). ColVI and laminin co-localization was clearly highlighted in normal muscle (A) and a complete co-distribution of their signals was noticed in orthogonal projections (A, lower panel

filtering were attained as previously described.^{8–11} Somatic SNPs and small INDELS variants were detected using muTect2 algorithm.¹² SnpEff toolbox v4.2 was used to predict the functional impact of variants, and retain missense/nonsense/frameshift changes, coding indels, and intronic variants at exon–intron junctions. Functional annotation of variants was performed by using snpEff v4.2 and dbNSFP2.9.^{13,14} Variants were prioritized on the basis of the functional relevance of genes, taking into account X-linked, autosomal dominant, and autosomal recessive inheritance models.

Pt2 was screened by targeted parallel sequencing using a dedicated gene panel (BU lab). SeqCap EZ Choice Library (Roche NimbleGen) designed to target the coding exons of 236 myopathy-related genes as described.¹⁵

All putative disease-causing variants were confirmed by Sanger sequencing.

8. Quantitative mRNA analyses

SNaPshot analysis was used to confirm each mutation and quantify the proportion of the mutated mRNA in the three patients with suspected mosaicism.^{16–18} Briefly, exon 16 of COL6A3, and exons 7 and 8 of COL6A2, which encompassed the mutations identified in patients 1, 2 and 3, respectively, were amplified with primers used in the diagnostic setting, and an aliquot of the PCR product was subjected to SNaPshot analysis according to the manufacturer's recommendations (ABI Prisms SNaPshot™ Multiplex Kit; Applied Biosystems). Primer sequences used for quantification of mosaic mutations are available on request. The relative proportion of normal and mutant mRNA species was quantified by using the GeneMapper version 3.0 program (Applied Biosystems), and expressed as percentage of mutant allele quantity versus that of the wild-type allele.

Targeted sequencing performed on a MiSeq platform using a custom gene panel containing the COL6A1, COL6A2 and COL6A3 genes (Nextera® Rapid Capture, Illumina, San Diego, California, USA) was performed also using genomic DNA extracted from muscle and fibroblasts of the three patients to more accurately define the percentage of the mutated allele in different tissues.

9. Results

9.1. Patient 1

This patient was previously described as an Ullrich phenotype, in which mutations in the three COL6A genes were

excluded by direct sequencing analysis performed on retro-transcribed RNA.⁴ Briefly, the girl had a clinical phenotype characterized by early onset proximal muscle weakness and a clear joint laxity, skin hyperlaxity, follicular hyperkeratosis and cheloid formation at the site of the surgical biopsy. Serum CK levels were normal. The ColVI defect was demonstrated by immunofluorescence studies on muscle biopsy in which the protein label was reduced and discontinuously distributed around myofibers (Fig. 1). In the deeper study of ColVI alterations by confocal microscopy, we found clear differences between patient and control muscles in ColVI distribution and expression by analysis of XZ stack intensity and colocalization profiles in ColVI- α 2 laminin double-stained samples (Fig. 2, B and B'); further 3D rendering of confocal z-stacks pointed out the discontinuous distribution of ColVI observed around several myofibers (Fig. 3). However, by immunofluorescence and electron microscopy analyses we previously observed that secretion, assembly and structure of the ColVI microfibril network appeared normal in fibroblast culture, as well as immunoprecipitation of cell layer and culture media showed an apparently normal amount of ColVI.⁴ Muscle MRI performed at the age of 4 years showed diffuse replacement at thigh level and relative sparing of rectus femoris, gracilis and adductor longus, consistent with a UCMD myoimaging phenotype (Fig. 4).¹⁹

At last examination (15 years-old), she is still able to walk but has a severe thoracolumbar scoliosis, multiple joint contractures and restrictive respiratory insufficiency (Forced vital capacity (FVC) 1.5 l, 54% of predicted value).

9.2. Patient 2

He is a 14 years-old boy with an intermediate COL6-RD phenotype. He is the second child of non-consanguineous parents, and was born by caesarean delivery after uncomplicated pregnancy. Poor fetal movements were reported and mild hypotonia, bilateral hip-dysplasia and talipes were present at birth. He acquired autonomous ambulation at the age of 18 months and had always a waddling gait with difficulties in raising from the floor, jumping and running. At the age of 17 months a first muscle biopsy was performed elsewhere showing a mild collagen VI reduction and hypotrophy of type 1 myofibers (samples not available for re-evaluation). Our first neurological examination, performed at 4 years, showed slight waddling gait, difficulties in standing up and inability to jump. He presented marked distal hyperlaxity (finger and ankles), proximal contractures (hips and knees), and prominent calcaneus. Over the years the disease had a slight progressive course. At the age of 13 years, a second muscle biopsy

and blue inset), in high magnification of myofiber periphery (yellow signal, white inset in A') as well as in the distribution of intensity XZ stack profile (high magnification of the blue inset in A'). Confocal Z stacks of Pt1 muscle (B–B') showed a reduced colocalization pattern between ColVI and α 2 laminin around several myofibers (arrows in B, high magnification of the white inset in B'), as visualized by altered distribution profiles of intensity XZ stack (low panels in B and B'). ColVI distribution was uniformly reduced around myofibers' basal lamina in Pt2 muscle (C) and did not overlap with α 2 laminin (arrows in C, top panel in C'), as confirmed by intensity XZ stack profiles (low panel in C, high magnification of the blue inset in C'). In Pt3 muscle, ColVI immunolabeling was weakly reduced and thinned around myofibers at some points (arrows in D, high magnification of the white inset in D') showing a partial co-localization with the basal lamina marker (low panels in D and D'). Bars: 20 μ m (A–D) and 2 μ m (A'–D'). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

on quadriceps was performed showing marked dystrophic features. Confocal imaging confirms a reduced ColVI distribution and colocalization with $\alpha 2$ laminin around the basal lamina of myofibers (Fig. 1), as highlighted by 3D reconstruction (Fig. 3) and pointed out by intensity XZ stack profiles (Fig. 2, C and C'). In dermal fibroblasts, a reduced microfibrillar ColVI deposition as well as an intracellular protein retention in most cells were documented (Fig. 5). Muscle MRI performed at the age of 13 years showed a pattern of muscle involvement suggestive of UCDM – like phenotype, characterized by involvement of thigh's muscles with relative sparing of the rectus femoris, gracilis adductor longus and sartorius, and typical subfascial deposition in the gastrocnemii (Fig. 4).¹⁸ At present, the boy is still able to walk but he presents a tip-toe waddling gait and inability to get up from floor and to climb stairs. He has multiple joint contractures (including finger and ankles). FVC is slightly reduced (2.10 l; 80% of predicted value).

9.3. Patient 3

He is a 23 year-old boy with a Bethlem phenotype. He is the only child of non-consanguineous healthy parents. Motor milestones were achieved normally but since early infancy he was clumsy and frequent falls associated to early fatigability were reported. At the age of 16 years, he came to medical attention for the incidental finding of high CK (600 UI/L, about 3 times \times n.v.). Neurological examination showed mild axial and neck flexor muscles weakness, elbows and finger contractures and generalized muscle hypotrophy. Both muscle hypotrophy and contractures were more pronounced on the right side. First muscle biopsy performed at the age of 16 years showed fiber size variability, an increase of connective tissue in the absence of myonecrosis or inflammatory infiltrates. ColVI immunolabeling was faintly reduced and thinned around myofibers at some points as observed by confocal microscopy and 3D rendering analyses (Figs. 1 and 3) showing a partial co-localization with the basal lamina marker (Fig. 2, D and D'). In dermal fibroblasts, the amount of microfibrillar ColVI matrix was weakly reduced and ColVI retention was documented in several cells (Fig. 5). Muscle MRI performed at the age of 22 years showed a strikingly asymmetric involvement with significant right-sided hypotrophy and partial fatty replacement at thigh level and asymmetrical subfascial fat deposition in the gastrocnemii. The last clinical examination at the age of 23 years, documented stable muscle and respiratory functions.

9.4. Genetic results

In all patients scanning of the entire coding sequence of the COL6A1, COL6A2 and COL6A3 genes by direct sequencing analysis did not reveal any pathogenetic mutation. WES analysis was performed on Pt1 and Pt3 but failed in identifying any functionally relevant putative disease causing variant. Based on clinical and histological evidence pointing to a COLVI defect the WES data were reanalyzed by means of muTect2 using the annotated variant and genotype attained by the Haplotype Caller-based analysis as reference to explore the possible occurrence of low-frequency variants compatible with a mosaicism state. This approach allowed the

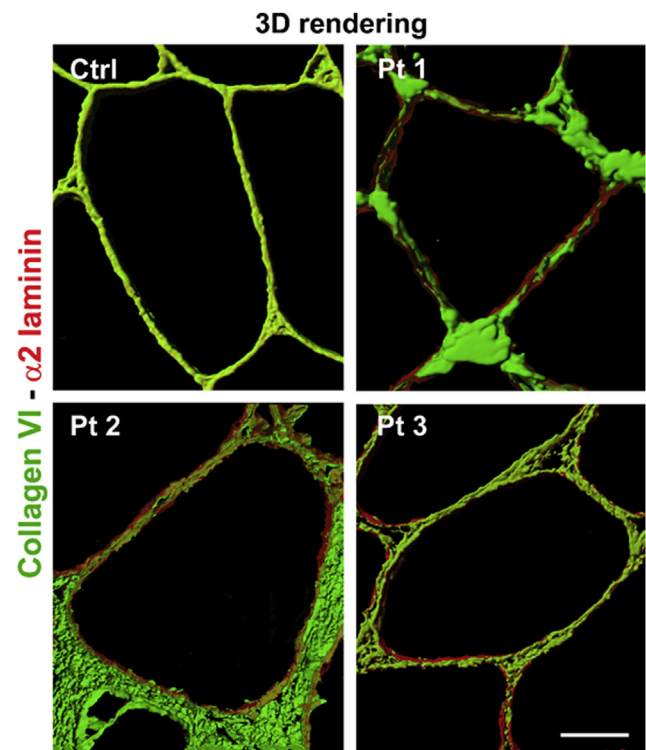


Fig. 3 – 3D muscle. Three dimensional rendering of representative control and patients' myofibers double-stained with ColVI and $\alpha 2$ laminin antibodies. In normal myofiber a full overlap of the two distributions (yellow signal) is visualized, whereas in patients' myofibers the ColVI isosurface (green) showed different degrees of discontinuous distribution around basal lamina (red). Bar: 20 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

identification of a splice site variant in COL6A3 (c.6210 + 1G > C) and a missense COL6A2 change (c.902G > A, p.Gly301Asp) affecting a highly conserved glycine residue within the TH domain in Pt1 and Pt3, respectively. Both variants represented a small proportion of total reads (<10% in both cases), which was suggestive of a low grade somatic mosaicism. Similarly, a COL6A2 missense change predicting the substitution of a conserved glycine residue within the TH domain (c.893G > A, p.Gly298Glu) was identified by targeted resequencing in Pt2. In addition, in this case, reads encompassing the variant were found to account for a minority of reads.

Sanger sequencing of the relevant exons was repeated, providing evidence of the presence of the identified changes, which were fairly detectable and identifiable on one strand only. For Pt1, RT-PCR analysis was performed on retro-transcribed RNA from muscle biopsy, documenting a mutated transcript lacking exon 16 at low levels.

9.5. Quantification of the mutant versus normal allele

To assess the degree of mosaicism, the relative abundance of the mutant allele was analyzed and quantified by SNaPshot

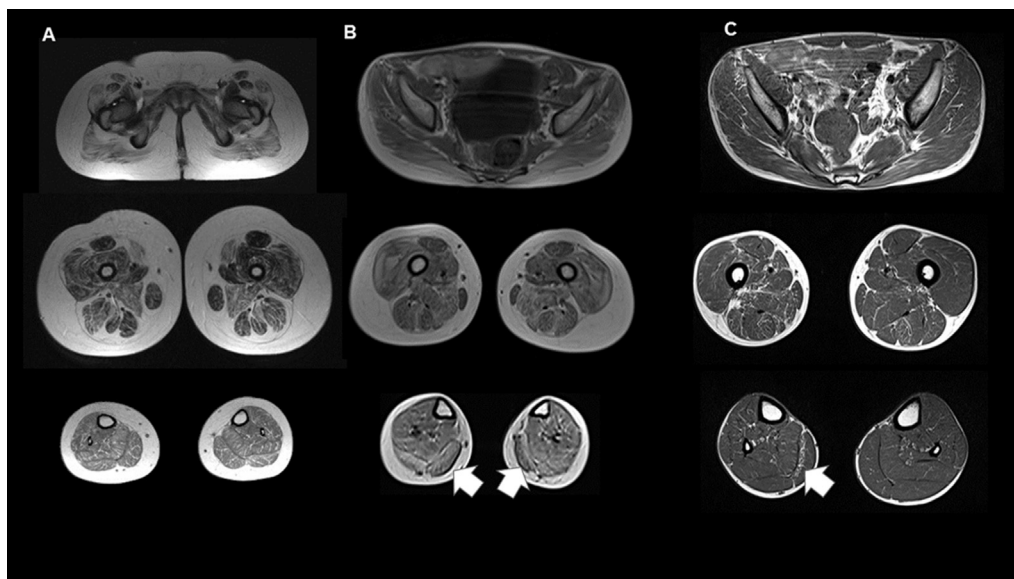


Fig. 4 – Muscle MRI. T1-weighted axial muscle MRI slices at pelvis, thigh and lower leg level of patients X (A), Y (B) and Z (C). A and B show diffuse fatty replacement at thigh level and relative sparing of rectus femoris, gracilis, adductor longus and, in patient Y, also sartorius, a pattern reminiscent of Ullrich CMD (Mercuri, *annals* 2010). Patient C shows strikingly asymmetric involvement, with significant right-sided hypotrophy and partial fatty replacement at thigh level. The arrows point to the rim of subfascial fat deposition in the gastrocnemii, typical of Bethlem myopathy, evident in B and asymmetrically in C.

technology. Compared to the expected 0.50 ratio, genomic DNA obtained from saliva, hair, urine, blood, muscle and fibroblasts of Pt1 documented a relative abundance of the COL6A3 c.6210 + 1G > C mutation equal to 9%, 10%, 23%, 12%, 13% and 17%, respectively. Similarly, the percentage of mosaicism of the COL6A2 c.893G > A (p.Gly298Glu) mutation in Pt2 ranged from 8% (leukocytes) to 19% (saliva), and was estimated as 18% in muscle. A comparable picture was also documented for the COL6A2 c.902G > A (p.Gly301Asp) missense change in saliva, hair, urine, blood, muscle and fibroblasts of Pt3, with a ratio of 13%, 11%, 10%, 7%, 11% and 13% of the mutant allele, respectively. All these data are summarized in Fig. 6.

Results obtained from a custom targeted sequencing panel using genomic DNA extracted from fibroblasts and muscle of the three patients provided similar results, with ratios of 17% and 18% for the variant affecting COL6A3 (Pt1) and 16% and 21%, and 10% and 9% for the two variants identified in COL6A2 (Pt2 and Pt3, respectively) (Fig. 7).

10. Discussion

Somatic and germline mosaicisms for COL6A genes have recently been reported.^{2,3} Parental mosaicisms for dominant mutations in COL6A genes were reported in familiar cases of COL6-RD in which the inter-generational phenotypic heterogeneity mimicked genetic anticipation. Postzygotic origin of a COL6A2 mutation was also demonstrated in a patient who manifested an intermediate phenotype of COL6-RD.² More recently, Armaroli et al. reported a case of paternal germline COL6A1 mutation, which lead to the recurrence of UCMD in two half-sibs born from healthy parents.³

In the first families reported by Donkervoort, 3 of the 5 carriers of somatic mutations were almost asymptomatic exhibiting mild clinical signs that were only noticed by clinicians. In each patient the degree of mosaicism was analyzed in different somatic tissues and the percentages of mutation in fibroblasts (considering the full heterozygote as 100%) ranged from 24% to 42%, whilst, in some patients, in blood and saliva it was undetectable. No correlation was found between the mutation load and disease severity, albeit in cultured dermal fibroblasts, the collagen VI matrix deposition was less organized when the mutation was highly represented.

In the last decade, in our Neuromuscular Unit we performed 13 diagnosis of COL6-RD, based on clinical and histological finding. In all but 4 patients, Sanger sequencing allowed the identification of the pathogenic mutations in COL6A genes. Consistent with their clinical features, WES analysis performed in two patients excluded the occurrence of putative disease-causing mutations in known or novel disease genes. Based on these findings, possible somatic origin of COL6A mutations was considered, and mosaic mutations in COL6A2 and COL6A3 genes were identified by second generation sequencing in three of them.

Pt1, who presents an intermediate phenotype, harbors a somatic splice-site c.6210 + 1G > C mutation in COL6A3. The mutation load (considering the full heterozygote as 50%) ranged from 9% to 23% in different tissues, being 13% and 17% respectively in muscle and fibroblasts. This patient displayed a ColVI deficiency at the level of the basal lamina of myofibers and blood vessels whilst cultured dermal fibroblasts did not show any significant defect in secretion, assembly and structure of the ColVI microfibril network. The c.6210 + 1G > C splicing mutation, which leads to skipping of exon 16, has been commonly reported in UCMD.^{20–22} Exon skipping in the

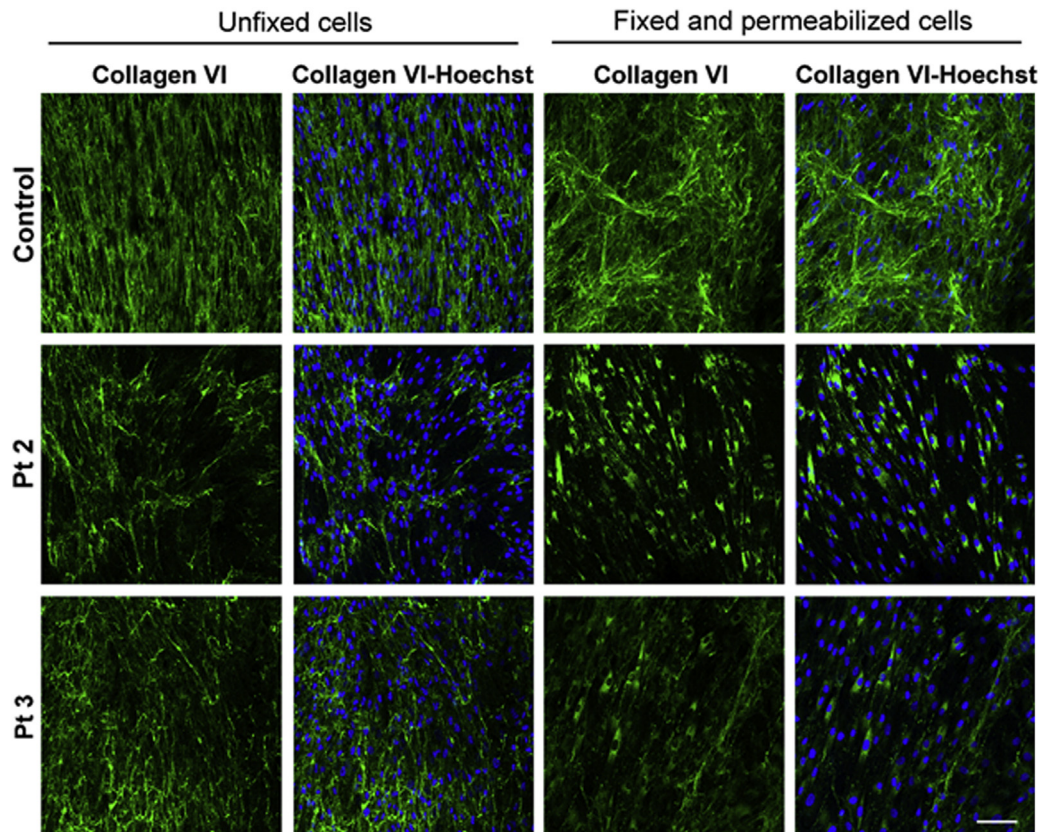


Fig. 5 – ColVI immunoreactivity in dermal fibroblast. Confocal laser microscopy of the ColVI matrix in control and patients' dermal fibroblasts. ColVI immunofluorescence (green) was performed in unfixed (left panel) and fixed-permeabilized cells (right panel) to point out, respectively, the microfibrillar network deposition and the intracellular protein retention in patients' cells. In control fibroblasts (top panel) a normal quantity, alignment and assembly of ColVI microfibrils were observed, and no intracellular retention was detected. In Pt2 fibroblasts, a reduced deposition and assembly of microfibrillar ColVI as well as an intracellular protein retention were noticed in most cells. In Pt3 fibroblasts, the amount of microfibrillar ColVI was weakly reduced while ColVI retention was observed in several cells. Cell density was checked by counterstaining with Hoechst (blue). Bar: 100 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

N-terminal end of the TH domain does not appear to disrupt the formation of triple helical monomers, but it seems to disrupt the microfibrils formation at the level of the basal lamina of myofibers, thus resulting in a dominant negative mode of action.²³ As in the cases reported by Donkervoort et al., who were mosaics for a COL6A2 splice site affecting an exon encoding part of the TH domain of the protein, skipping of exon 16 in Pt1 was associated with an intermediate COL6-RD phenotype even at very low mutation load.²

In Pt2, who manifested an intermediate phenotype that was however more severe than the former, a clear defect of ColVI expression in muscle and fibroblasts was well documented. In this patient, the percentage of the COL6A2 c.893G > A (p.Gly298Glu) mutation in muscle was 18%. This mutation affects the 15th Gly-X-Y triplets in the TH domain of COL6A2 which is clustered in a short segment of the triple helical domain (including Gly-X-Y triplets 10–15). Substitution of glycine residues in TH domain has been demonstrated that compromises the intracellular assembly and disulfide bonding of the ColVI tetramers, leading to severe clinical phenotypes.^{23,24} The COL6A2 p.Gly301Asp mutation, that we

found in Pt3, was already reported in a patient manifesting a UCMD phenotype.²² Our patient had a Bethlem phenotype characterized by mild elbow and finger contractures and very mild and non-progressive muscle axial weakness. The milder clinical phenotype may be related to the very low percentage of mutation that was documented in several examined tissues. Of note, although this mutation affects the 16th Gly-X-Y triplet within the TH domain, it does not cluster within the critical region absolutely required for assembly, which possibly explains its minor clinical impact.

It should be noted that, unlike previous reported mosaic patients, who were almost asymptomatic, our patients complained clear clinical phenotypes of COL6-RDs, and the muscle MRI was confirmatory for the diagnosis. Of note, in pt3 the presence of clearly asymmetric involvement both clinically and on imaging was an additional clue to suspect the somatic mosaicism. All had glycine substitutions or a small deletions in the triple helical domain in COL6 genes, which are predicted to severely affect collagen assembly and have been associated so far to a severe phenotype. However, in our patients the percentage of mutation in several tissues, including muscle

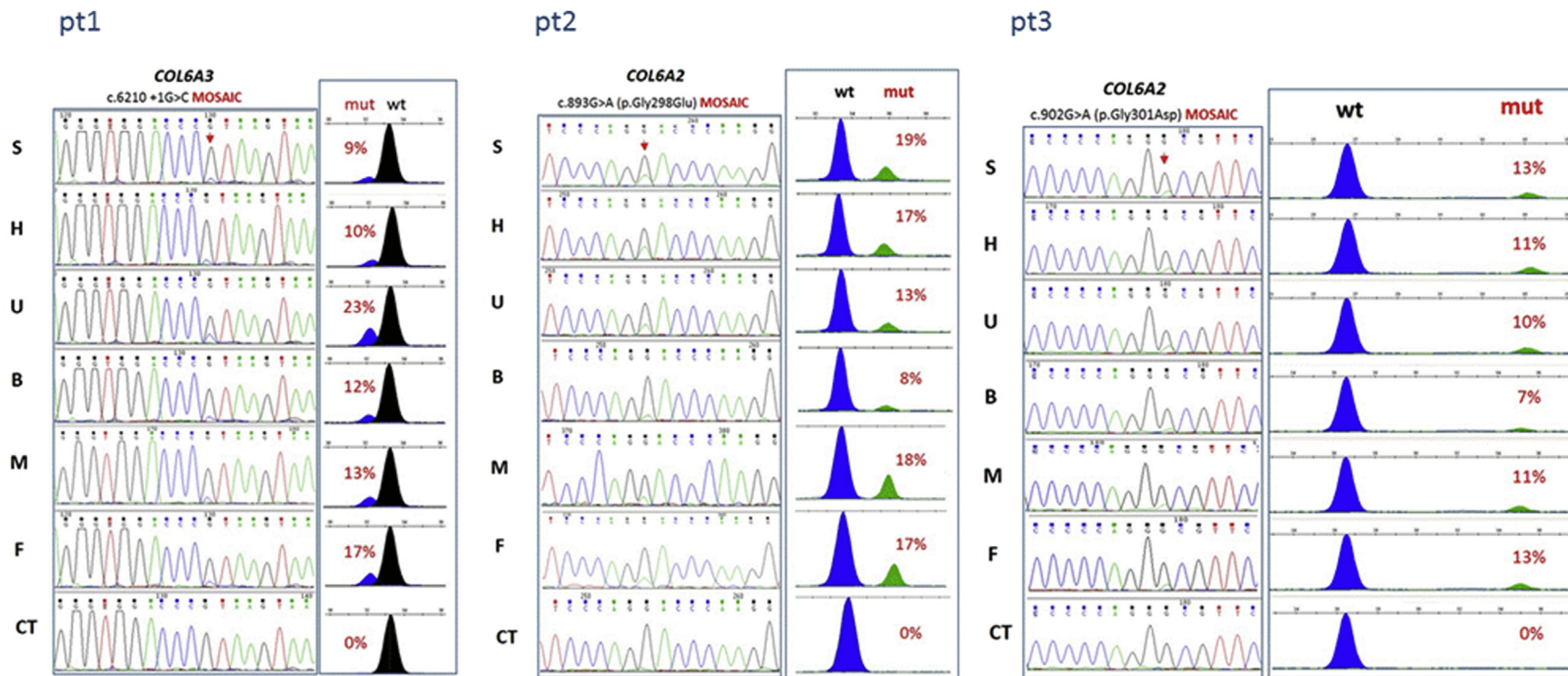


Fig. 6 – Sequencing analysis and SNaPshot results. Sequencing analysis and SNaPshot show the somatic mutations in COL6A3 c.6210 + 1G > C (Pt1), COL6A2 c.893G > A (p.Gly298Glu) (Pt2) and COL6A2 c.902G > A (p.Gly301Asp) (Pt3). The percentages of mutations detected in different tissues of patients are also shown.

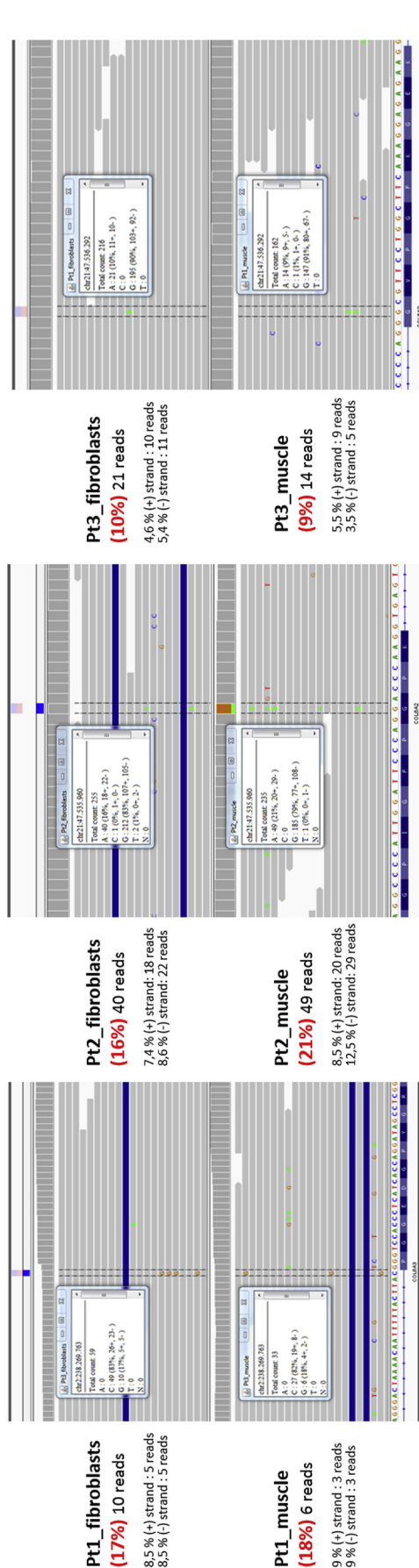


Fig. 7 – Sequence read alignment data. IGV alignment of Bam and VCF files, generated using a targeted NGS panel, in DNA from fibroblasts and muscles, shows the COL6A2 (pt2 and pt3) and COL6A3 (pt1) mutations in mosaic state.

and fibroblasts, is relatively low, thus explaining the mildest symptomatology from what was reported in patients harboring constitutional mutations. Despite previous reports documented that the percentage of mutation does not always correlate with the severity of the disease, a suggestive association between disease severity and mutation load was noticed in the present cases, as illustrated by Pt2 who has the highest percentage of mutation in muscle and shows the most severe phenotype. While a higher number of cases are required to confirm this finding, we anticipate that this correlation could be possibly explained by the similar functional impact of the identified mutations on assembly.

Although the mutations in our mosaic cohort are in different chains, $\alpha 2$ (VI) in Pt2 and Pt3 and $\alpha 3$ (VI) in Pt1, they are all clustered in the N-terminal side from the cysteine residue of the triple helical domain. The cysteine residue, located in the THD in position 89 and 50 respectively in $\alpha 2$ (VI) and $\alpha 3$ (VI) chains, are involved in stabilizing ColVI dimer (COL6A2) and are known to link to the scissor-like connections used in the formation of tetramers.²² This molecular mechanism can explain why in our patients the formation of the microfibrillar network is impaired even with mosaic distribution of COL6 mutation.

11. In conclusion

In our experience, somatic mosaic mutation in COL6A genes are recurrent events underlying COL6-RD, representing around 20% of the patients of our cohort.

The somatic occurrence of COL6A gene mutations should be considered in all cases clinically fitting a condition within the COL6-RD phenotypic spectrum, in absence of any mutation in COL6A gene mutation. Asymmetrical muscle involvement is a features that may also suggests a mosaic condition.

In these cases, deep parallel sequencing to evaluate the possible occurrence of somatic events in COLA6 genes should be warranted.

Contributors

AD and FF contributed equally planned the study and interpreted the results. AD and GT led patients' recruitment, clinical phenotyping and RMI characterization. FF and FG performed genetic analysis, AB, MN and BU provided NGS and WES. RC, SP, MV, VD collected samples and performed histological analysis; AF, MT and ER contributed to the critical reading of the manuscript. All the authors reviewed and contributed to the editing of manuscript.

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Competing interests

The authors have no conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ejpn.2017.07.009>.

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