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ARTICLE

# *CRTAP* mutations in lethal and severe osteogenesis imperfecta: the importance of combining biochemical and molecular genetic analysis

Fleur S Van Dijk*[,1], Isabel M Nesbitt[2], Peter GJ Nikkels[3], Ann Dalton[2], Ernie MHF Bongers[4], Jiddeke M van de Kamp[1], Yvonne Hilhorst-Hofstee[5], Nicolette S Den Hollander[5], Augusta MA Lachmeijer[1], Carlo L Marcelis[4], Gita MB Tan-Sindhunata[1], Rick R van Rijn[6], Hanne Meijers-Heijboer[1], Jan M Cobben[7] and Gerard Pals[1]

[1]*Department of Clinical Genetics, VU University Medical Cen, Amsterdam, The Netherlands;* [2]*Sheffield Molecular Genetics Service, Sheffield Children's Hospital NHS Foundation Trust, Sheffield, United Kingdom;* [3]*Department of Pathology, University Medical Centre, Utrecht, The Netherlands;* [4]*Department of Human Genetics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands;* [5]*Department of Clinical Genetics, Leiden University Medical Centre, Leiden, The Netherlands;* [6]*Department of Pediatric Radiology, Academic Medical Centre, Amsterdam, the Netherlands;* [7]*Department of Pediatrics, Academic Medical Centre, Amsterdam, The Netherlands*

**Autosomal recessive lethal and severe osteogenesis imperfecta (OI) is caused by the deficiency of cartilage-associated protein (CRTAP) and prolyl-3-hydroxylase 1 (P3H1) because of *CRTAP* and *LEPRE1* mutations. We analyzed five families in which 10 individuals had a clinical diagnosis of lethal and severe OI with an overmodification of collagen type I on biochemical testing and without a mutation in the collagen type I genes. *CRTAP* mutations not described earlier were identified in the affected individuals. Although it seems that one important feature of autosomal recessive OI due to *CRTAP* mutations is the higher consistency of radiological features with OI type II-B/III, differentiation between autosomal dominant and autosomal recessive OI on the basis of clinical, radiological and biochemical investigations proves difficult in the affected individuals reported here. These observations confirm that once a clinical diagnosis of OI has been made in an affected individual, biochemical testing for overmodification of collagen type I should always be combined with molecular genetic analysis of the collagen type I genes. If no mutations in the collagen type I genes are found, additional molecular genetic analysis of the *CRTAP* and *LEPRE1* genes should follow. This approach will allow proper identification of the genetic cause of lethal or severe OI, which is important in providing prenatal diagnosis, preimplantation genetic diagnosis and estimating recurrence risk.**
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**Keywords:** osteogenesis imperfecta; recessive; CRTAP; collagen type 1

## Introduction

Osteogenesis imperfecta (OI) was first described by the Swedish surgeon, Olaus Jakob Ekman, in a family with hereditary bone fragility (1788).[1] On account of the extreme variability in the presentation of OI, a classification into four types (OI type I (mild), II (lethal), III (severe) and IV (moderate) was introduced by Sillence *et al* (1979) (the 'Sillence classification')). Type II OI was further subdivided into type A, B and C on the basis of radiological features. The earlier assumption based on pedigree findings that OI type II was inherited in an autosomal recessive way shifted toward the view that OI type II was primarily

***Correspondence: Dr FS van Dijk, Department of Clinical Genetics, De Boelelaan 1117, 1081 HV, Amsterdam, The Netherlands.**
**Tel: +31 20 4440150; Fax: +31 20 4440769;**
**E-mail: fs.vandijk2@vumc.nl**
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inherited as an autosomal dominant disease[2–5] with the discovery of heterozygous mutations in the collagen type I genes, *COL1A1* and *COL1A2,* in all types of OI. In addition, it was recorded that most recurrences in OI type II and III were caused by gonadal mosaicism in one of the parents. The empirical recurrence rate of lethal OI was estimated at 6%.[6–10] However, it seemed that not all patients with lethal or severe OI had a collagen type I mutation.[11,12] A hypomorphic loss-of-function of *CRTAP*-encoding cartilage-associated protein (CRTAP) was shown to cause the rhizomelic form of moderate–severe recessive OI, originally described as OI type VII.[13,14] Furthermore, complete loss-of-function mutations of *CRTAP* and of *LEPRE1*, which encode CRTAP and prolyl-1-hydroxylase (P3H1), seemed to cause autosomal recessive lethal and severe OI.[14,15,16] CRTAP, P3H1 and cyclophilin B (CyPB) encoded by *PPIB* form a protein complex in the rough endoplasmic reticulum (rER), which is responsible for 3-hydroxylation of proline at position 986 in the $\alpha$1 chain of collagen type I. In individuals affected with lethal or severe OI due to mutations in *COL1A1*, *COL1A2*, *CRTAP* and *LEPRE1*, comparable delayed migration of collagen type I is evident, which is visible as backstreaking, increased band width or baseline shift on electrophoresis (see Figure 1). This phenomenon is due to posttranslational overmodification and can be explained by the structure of collagen type I, which is first synthesized as procollagen type I consisting of two pro$\alpha$1(I) chains and one pro$\alpha$2(I) chain, containing N- and C-terminal propeptides. Posttranslational modification necessary to reach intertwining of the chains resulting in triple-helical conformation takes place in the rER. Currently, two posttranslational modification systems of collagen type I are known, namely, (i) hydroxylation of multiple lysine and proline residues by lysyl hydroxylase and prolyl-4-hydroxylase and (ii) 3-hydroxylation of a single residue in the $\alpha$1 chain, proline at position 986 by the protein complex in the rER, consisting of P3H1, CRTAP and CyPB. Triple-helical folding occurs in the C-to-N direction, making lysines and prolines inaccessible to posttranslational modification as soon as the chain in which they are located is folded. Posttranslational overmodification takes place when a delay in triple-helical folding results in overprocessing of the constituent collagen type I chains by the enzymes involved in posttranslational modification. This delay can be caused by collagen type 1 mutations disturbing the primary structure of collagen type I or by deficiency in two of the components of the prolyl-3-hydroxylation complex, CRTAP and P3H1 LEPRE1.[17–20] Here, we describe 10 individuals affected with lethal or severe OI due to novel *CRTAP* mutations.

## Materials and methods

### Patients

We selected 13 individuals from seven families for screening, on the basis of clinical diagnosis of OI type II/

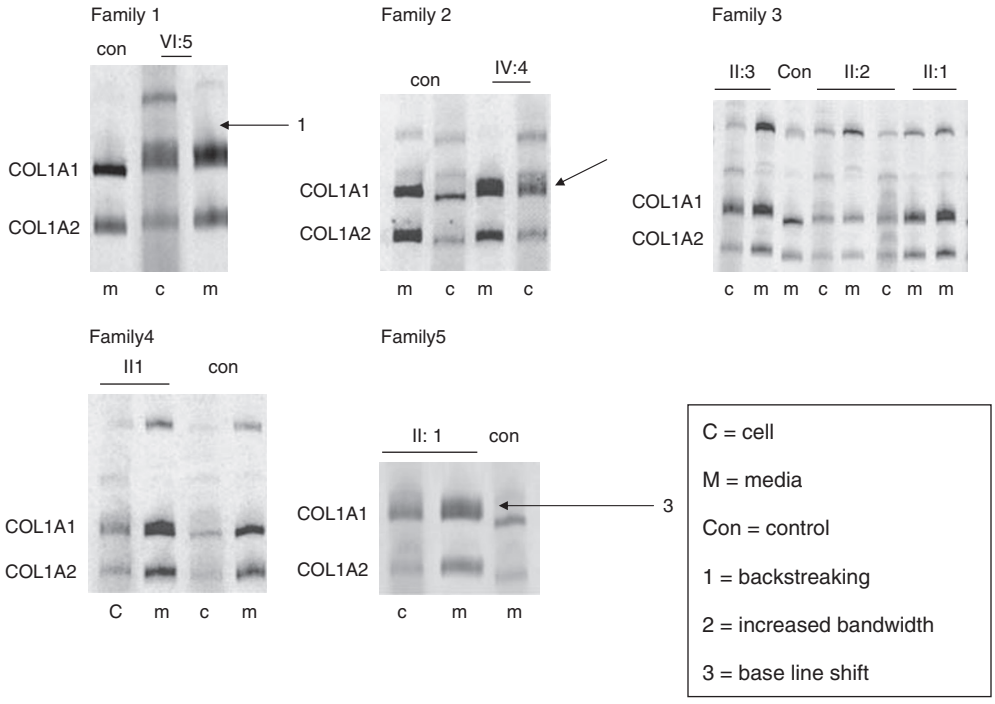


**Figure 1** Biochemical testing in families 1–5. Overmodification on electrophoresis is evident by either backstreaking and/or baseline shift and/or increased band width. Three examples of these biochemical features are numbered in the electrophoresis images of the families.

III, collagen type I overmodification on electrophoresis and absence of collagen type I mutations.

### Protein analysis

Skin biopsies were obtained from the affected individuals, and fibroblast cultures were established under standard conditions. Cells were seeded at 35 000 cells/cm$^2$. Labeling of the fibroblasts and purification of the collagen molecules were carried out as reported elsewhere, excluding the carrying out of SDS electrophoresis without 3% stacking gel and exposing the dried gels to a phosphor imager screen (Molecular Dynamics, Sunnyvale, CA, USA).[21]

### DNA analysis of the collagen type 1 genes

cDNA of the *COL1A1* and *COL1A2* genes was prepared from total RNA from cultured fibroblasts. RNA was isolated using the RNA Isolation Minikit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Full-length single-stranded cDNA was prepared with oligo-dT-primer and Superscript II RT Reverse Transcriptase (Invitrogen, San Diego, CA, USA). The complete coding sequences of the *COL1A1* and *COL1A2* genes (reference sequence NM_000088 and NM_000089) were both analyzed by direct sequencing in 11 overlapping PCR fragments (primer sequences available on request).

### Molecular analysis of the *CRTAP* and *LEPRE1* genes

Molecular analysis of *CRTAP* (reference sequence NM_006371), *LEPRE1* (reference sequence NM_022356) and *PPIB* was carried out as reported elsewhere.[14,15,21]

## Results

In all 13 individuals from seven families, posttranslational overmodification on electrophoresis was evident. In 2 individuals from one family, *LEPRE1* mutations were detected, in 1 individual no mutations in *CRTAP*, *LEPRE1* and *PPIB* were detected and 10 individuals from five families appeared to have novel *CRTAP* mutations. Here, we describe the clinical features and mutation analysis of patients with OI due to *CRTAP* mutations only.

### Family 1

***Clinical findings*** The parents were consanguineous (Caucasian) and the mother was pregnant with male monozygotic twins (Figure 2). An advanced ultrasound screening at 21 weeks of gestation showed bowed and short femura <p5 in both fetuses (VI:3 and VI:4). In one fetus, a possible fracture of the femur was seen. Owing to the strong suspicion of OI type II/III, the pregnancy was terminated at 23 + 2 weeks of gestation. No permission was given for autopsy. On the babygram, fractures of the

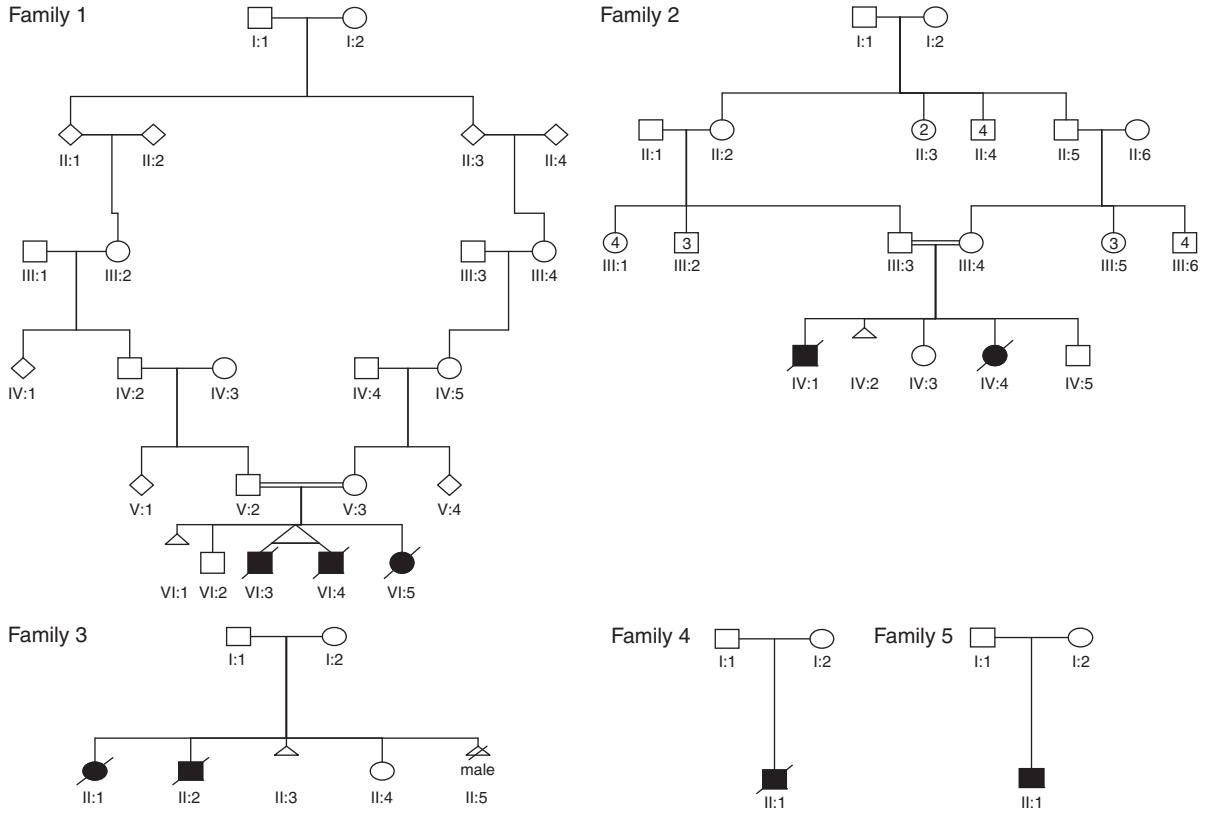


**Figure 2** Pedigree families 1–5.

femora were detected in both fetuses, with no apparent rib fractures (Supplementary Appendix Figures 3 and 4). The fetuses were diagnosed with OI type II-B.

In the following pregnancy, an advanced ultrasound in the 19th week of gestation again showed skeletal abnormalities consisting of a fracture of the femur, a bowed tibia and short osseous elements. The parents decided to proceed with the pregnancy, and a daughter (VI:5) was born by cesarean section at 39 weeks of gestation, with an Apgar score of 9, 9 and 10 after 1, 5 and 10 min, respectively. Birth weight was 3245 g. The neonate had normocephaly, a round face with shallow orbits, white sclerae, long philtrum, small thorax, rhizomelic shortening of the upper and lower extremities and abducted position of the legs (Figure 3). A skeletal overview showed evidence of multiple fractures (Figure 4). The girl was diagnosed with OI type III and is alive at the age of 2 years.

***Mutation analysis*** In the twin, monozygosity was confirmed by marker studies. Mutation screening of the *COL1A1* and *COL1A2* genes in the fetuses, VI:3 and VI:4, identified a variant, c.613 C>G; p.pro205Ala, in the *COL1A1* gene. This variant has been reported in literature before and is probably associated with osteopenia.[23] Mutation analysis of the *ALPL* gene responsible for hypophosphatasia was negative. In the third affected child, no mutations in the *COL1A1*, *COL1A2* and *LEPRE1* genes were found.

Sequencing of the *CRTAP* gene in the three affected children revealed homozygosity for the mutation, c.21_22dupGG, introducing a premature termination codon at p.Ala8fs in exon 1. The parents were heterozygous for this mutation.

**Family 2**

***Clinical findings*** The parents were consanguineous (Moroccan). In the third trimester of pregnancy, extensive skeletal abnormalities were observed in the fetus. A boy (IV:1) was born with multiple fractures, consistent with the diagnosis of OI type II/III. He died 1 month after birth (Figure 2).

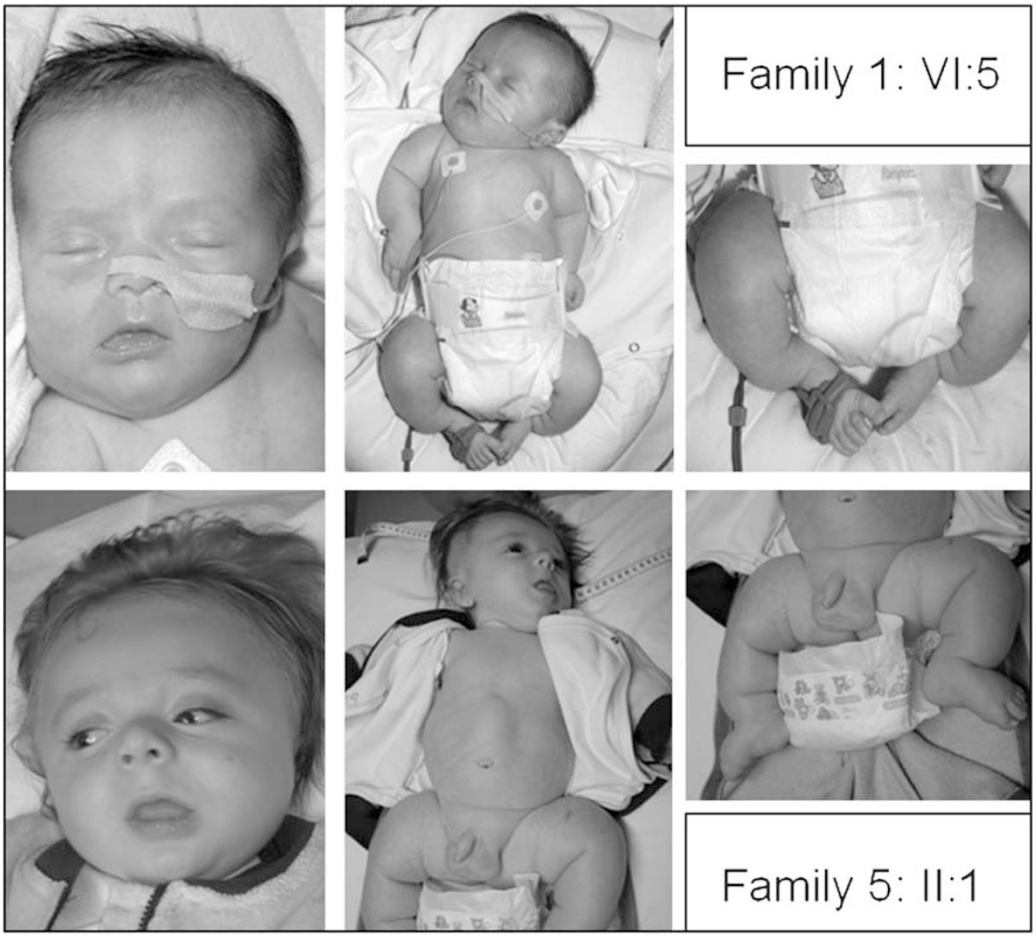


**Figure 3** Clinical assessments of family 1 VI: 5 and family 5 II:1. Note round face with shallow orbits, white or grayish sclerae, long philtrum, small thorax, rhizomelic shortening of the upper and lower extremities and abducted position of the legs.

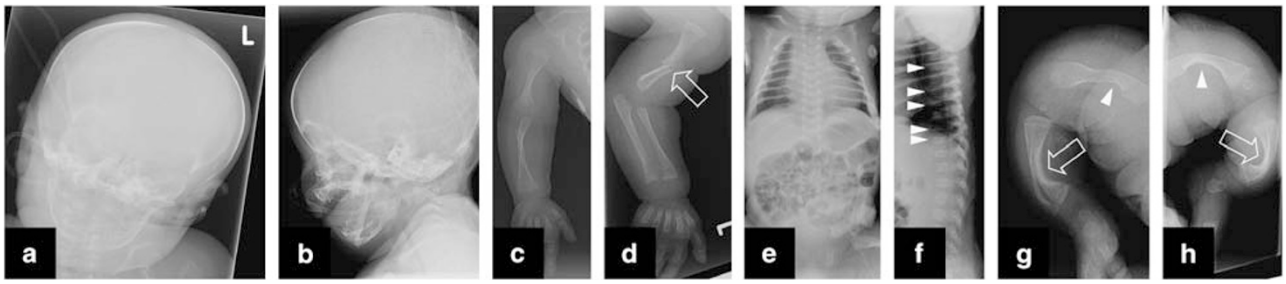


**Figure 4** Radiographs of individual VI:5 from family 1. (**a**) Apparent normal mineralization of the skull, no Wormian bones. (**b**) Mild brachycephaly. (**c**) Normal aspect of the right arm. A rhizomelic shortening may exist that may also be because of projection, given the normal length of the left humerus. (**d**) Oblique mid diafyseal fracture of left humerus (arrow). (**e**) Normal chest radiograph without rib fractures. (**f**) Platyspondyly of thoracic vertebrae, 5, 7, 8, 10 and 11 (arrowheads). (**g**) Bowing of femora (arrowhead), tibiae and fibulae (arrow). (**h**) Bowing of femora (arrowhead), tibiae and fibulae (arrow).

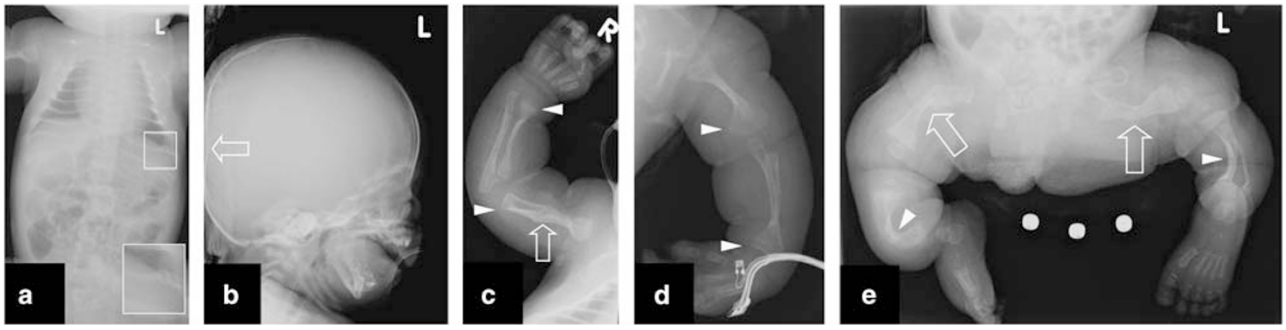


**Figure 5** Radiographs of individual IV:4 from family 2. (**a**) Small thorax. Slender ribs. Lateral fracture of the eighth left rib (see insert). Asymmetric platyspondyly, Th5 and Th7 (visible on original radiograph). (**b**) Slight flattening of the skull (arrow). (**c**) Consolidation of the right humerus fracture (arrow). Humerus measures 4.38 cm (normal range = 6.0–7.7 cm.) and the ulna 4.9 cm (normal range = 5.8–7.2 cm.). Broad distal metaphyses of the left humerus and radius (arrowhead). Possible fracture of the left radius. (**d**) Broad distal metaphyses of the left humerus and ulna (arrowhead). (**e**) Bilateral proximal femur fracture, with consolidation and angulation (arrows). Bilateral bowing of tibia and fibula (arrowheads).

In the fourth pregnancy, ultrasound images around the 20th week of gestation displayed skeletal abnormalities suggestive of OI. At 23 + 2 weeks of gestation, shortened and deformed extremities, a fracture of the left femur and a bell-shaped thorax were observed on ultrasound screening. A girl (IV:4) was born at 38 + 3 weeks. A skeletal overview showed gracile bones and multiple fractures with callus formation (Figure 5). The girl died 1 week later because of respiratory insufficiency. A femur was taken for histological examination (Figure 6).

***Mutation analysis*** Fibroblast cultures of the first child became infected, and no mutations in the *COL1A1*, *COL1A2* and *LEPRE1* genes were identified in the second affected child. Sequencing of the *CRTAP* gene showed homozygosity for the mutation, c.404delG, introducing a premature termination codon at p.Ser135fs in exon 1 of the *CRTAP* gene. The parents were both heterozygous for this mutation.

**Family 3**

***Clinical findings*** A caucasian girl (II:1) was born at 40 + 6 weeks of gestation with Apgar scores of 7 and 9 at 1 and 5 min, respectively. On physical examination, fragmentation of the skull bones was observed and a short thorax with short and bowed extremities was noticed, suggesting OI. The child had no overt blue sclerae. A skeletal overview showed multiple rib fractures and fractures of both the upper and lower extremities. The neonate was diagnosed with OI type II-C/III. At the age of 2.5 months, she died due to respiratory failure.

In the following pregnancy, an ultrasound in the 17th week showed no evidence of fractured or bowed extremities. However, at 18 + 4 weeks of gestation, fractures of both femora were observed. A recurrence of OI was suspected and the pregnancy was terminated at 20 + 2 weeks. Multiple fractures were evident (Figure 7) and the fetus (II:2) was diagnosed with OI type II (Figure 2).

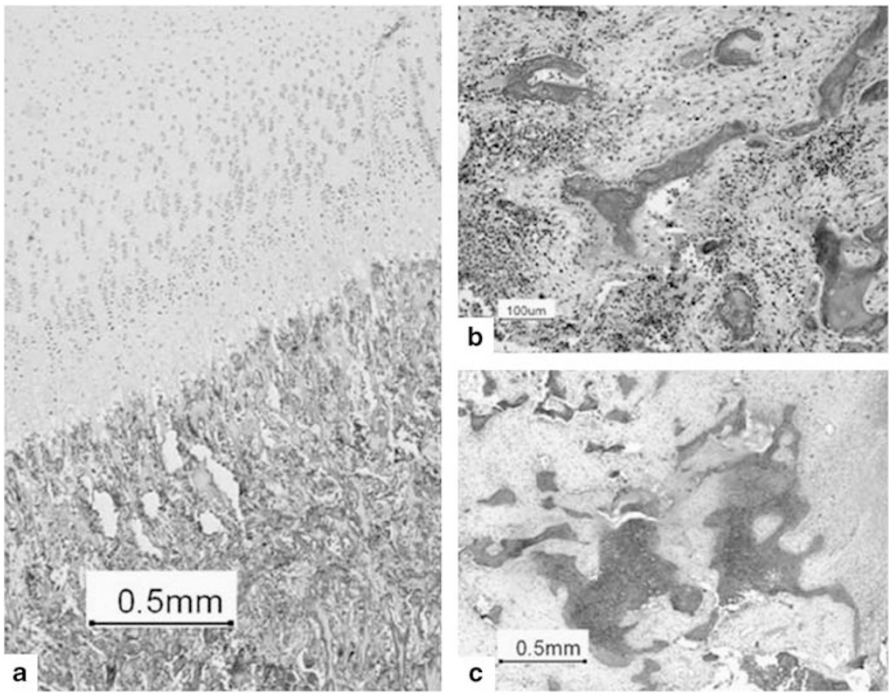


**Figure 6** Bone histology of family 2 IV:4. (**a**) H and E stain of the epiphysial growth zone of femur. Note the sharp demarcation between cartilage and primary spongiosa. The epiphysial growth zone and primary spongiosa are normal. The resting cartilage shows no abnormalities. (**b**) H and E stain of the metaphysis for OI, typical small, slender and hypercellular bony trabeculae and fibrotic medulla. The bony trabeculae consist of woven bone. (**c**) Low-power PAS acian blue stain of the midshaft of the femur, with metaplastic cartilage formation (blue) in the area of a fracture with fibrotic marrow. This formation of cartilage is quite typical for the severe forms of OI.[26]

The fifth pregnancy (II:5) was terminated because of an evident posttranslational overmodification of collagen type 1 in cultured chorion villi.

***Mutation analysis*** Mutations in the *COL1A1*, *COL1A2* and *LEPRE1* genes were not detected in the three affected children. Sequencing of the *CRTAP* gene showed that they were all compound heterozygous for a c.198C>A mutation predicted to result in a p.Tyr66X amino acid change in exon 1 and a c.471+2 C>A splice-site change in intron 1 of the *CRTAP* gene. The mother was heterozygous for the c.471+2 C>A mutation and the father was heterozygous for the c.198C>A mutation.

**Family 4**

***Clinical findings*** At a pregnancy duration of 20 weeks, ultrasound abnormalities were observed consisting of short and bowed extremities and a short thorax. Suspecting OI, a cesarean section was carried out at 39+4 weeks of gestation. A caucasian boy (II:1) was born with Apgar scores of 2–3 and 8–9 after 1 and 5 min, respectively. He had a large anterior fontanel, short thorax, short and bowed extremities and glandular hypospadia. A skeletal overview showed wormian bones, as well as multiple fractures of ribs and of both the upper and lower extremities (Figure 8). He was diagnosed with OI type III and died 5 days after birth (Figure 2).

***Mutation analysis*** Mutations in the *COL1A1*, *COL1A2* and *LEPRE1* genes were not detected in the affected child. Sequencing of the *CRTAP* gene showed that the affected child was compound heterozygous for a splice-site mutation, c.923-2A>G, in intron 4 and for two mutations on the other allele in exon 1, namely, c.38C>A and c.469A>G. The mother was heterozygous for the c.38C>A and c.469A>G mutations in exon 1. The father was not available.

**Family 5**

***Clinical findings*** In the first pregnancy, abnormalities of the extremities were observed in the fetus at 23 weeks of gestation. At the 24th week of gestation, short upper and lower extremities were observed, indicating severe skeletal dysplasia. At a gestation period of 40+4 weeks, a caucasian boy (II:1) was born. He had a round face with shallow orbits, grayish sclerae, small thorax, rhizomelic shortening of the upper and lower extremities, abducted position of the legs and normocephaly (Figure 9). A skeletal overview showed multiple fractures, suggesting OI type II/III. The child is alive at the age of 4 years (Figure 2).

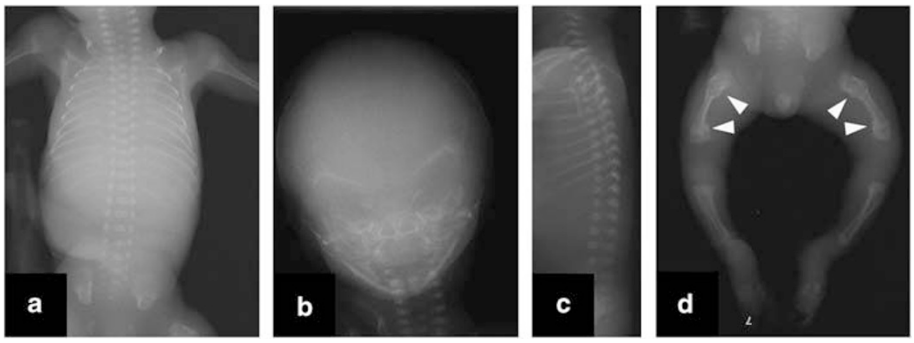


**Figure 7** Radiographs of II:2 from family 3. (**a**) Slender ribs. No rib fractures, two fractures of right femur with callus formation. (**b**) Underexposed radiograph, which makes evaluation for mineralization difficult. The calvaria seem to be thin for gestational age. (**c**) No vertebral abnormalities. (**d**) Two fractures of both femora with callus formation (arrowheads).

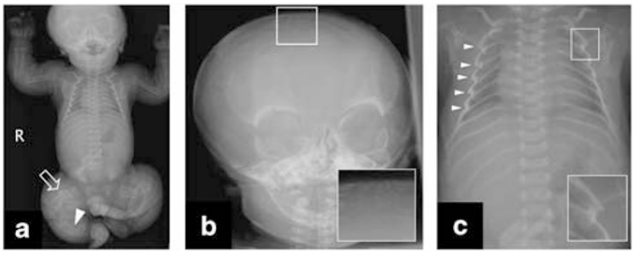


**Figure 8** Radiological images of II:2 from family 4. (**a**) No fractures of humeri, radii and ulnae. Multiple fractures of the femora, with loss of modeling (arrow). Fracture and bowing of right tibia (arrowhead). (**b**) Wormian bones (see insert), broad skull. (**c**) Slender ribs, lateral rib fractures of the left rib 2 (see insert) and right ribs 2–6 (arrowheads).

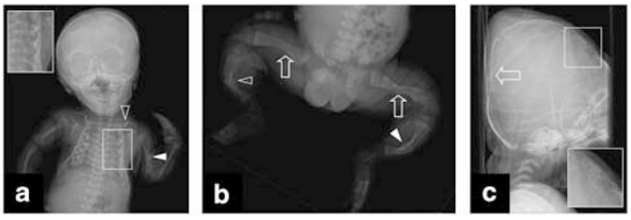


**Figure 9** Radiological images of II:1 from family 5. (**a**) Fracture of the left clavicula (arrowhead), small thorax, normal vertebral column, fractures of left ribs 4–7 (see insert). Fractures of the left humerus (solid arrowhead) and radius. Consolidated fracture of right humerus. (**b**) Fracture of the right femur (arrow) and tibia (arrowhead), severe bowing of right fibula and tibia. Multiple fractures of the left femur (arrow), bowing and fracture of left tibia (solid arrowhead). (**c**) Wormian bones (see insert) and flattening of the occiput (arrow).

***Mutation analysis*** Mutations in the *COL1A1*, *COL1A2* and *LEPRE1* genes were not detected in the affected child. Sequencing of the *CRTAP* gene showed homozygosity for the c.471 + 2C > A mutation in intron 1 of *CRTAP*.

## Discussion

We describe 10 individuals with lethal or severe OI on the basis of clinical (Table 1 Supplementary Appendix), radiological (Table 1) and biochemical (Figure 1) findings, in whom novel *CRTAP* mutations (Table 2) were found. So far, six probands with lethal or severe OI due to *CRTAP* mutations have been reported in literature. An important question is whether the phenotype of patients with lethal OI due to *CRTAP* mutations differs from that of patients with OI due to mutations in the collagen type I genes.

Barnes *et al*,[15] described in three individuals with OI due to *CRTAP* mutations, some clinical features in *CRTAP*-related OI, which differed from the clinical features in individuals with OI due to collagen type I mutations, namely, (i) no relative macrocephaly, (ii) a round face with shallow orbits, (iii) white or grayish sclerae and (iv) rhizomelic shortening of the upper and lower extremities. Indeed, our patients, VI:5 and II:1 from family 1 and 5 depicted in Figure 3, show no relative macrocephaly, a round face and no blue sclerae. The clinical assessments of VI:5 suggest rhizomelic shortening of the arms. However, because of the absence of sufficient clinical details of the other liveborn children with *CRTAP* mutations, the specificity of these clinical differences is uncertain. Therefore, a distinction between autosomal dominant OI due to collagen type I mutations and autosomal recessive OI due to *CRTAP* mutations on clinical features proves difficult.

Radiologically, bowing of extremities and/or fractures could be observed in the prenatal and neonatal period in all our patients with *CRTAP* mutations. The earliest appearance of features of OI was noted in the 18th week of pregnancy (Table 1 Supplementary Appendix). However, this pregnancy was monitored extensively because of an affected sib. Recently, 'popcorn' epiphyses have been suggested to be a distinguishing radiological feature of autosomal recessive OI, reflecting a cartilaginous dysplasia at the developing growth plate.[22] However, histology of

**Table 1** Revised radiological features of probands with *CRTAP* mutations.

| *-Family -(Pedigree nr) -Age at radiological imaging* | *Skull* | *Ribs and vertebrae* | *Upper extremities* | *Lower extremities* | *Original Sillence classifcation* | *Revised Sillence classification* |
|---|---|---|---|---|---|---|
| 1 (VI:3) AD 23+2 weeks | NA (No abnormalities) | No rib fractures. Mild platyspondyly vertebrae 4–10. | NA | Fractures of both femora with minor loss of modeling | II-B | III |
| 1 (VI:4) AD 23+2 weeks | Slight flattening of the skull | No rib fractures. | Normal aspect of right arm. Possible fracture of proximal left ulna. | Two fractures in left and right femora, minor loss of femoral shape. Irregularity of the distal tibiae. | II-B | III |
| 1 (VI:5) full term | Mild brachycephaly | No rib fractures. Platyspondyly of thoracic vertebrae 5,7,8,10,11. | Normal aspect of the right arm. Oblique mid diaphyseal fracture of left humerus. | Bowing of femora, tibiae and fibulae. | III | III |
| 2 (IV:1) 2.5 weeks | U (Unknown) | Thin ribs with distal flaring. Fractures of left third and possible fourth rib. | Fracture of right humerus. | U | II/III | II-B/III |
| 2 (IV:4) 1 week | Slight flattening of the skull | Small thorax. Slender ribs. Lateral fracture of eighth rib. Asymmetric platyspondyly Th5, Th7. | Consolidation of right humerus fracture. Broad distal metaphyses of left humerus and radius. Possible fracture of left radius. | Bilateral proximal femur fracture with consolidation and angulation. Bilateral bowing of tibia and fibula. | II/III | III |
| 3 (II: 1) full term | U | U | U | U | II-C/III | U |
| 3 (II: 2) AD 18+2 weeks | The calvaria seem to be thin for gestational age | No rib fractures. | No evaluation possible. | Two fractures in right femur and two fractures in left femur with callus formation. | II-B | III |
| 3 (II: 3) | U | U | U | U | U | U |
| 4 (II: 1) full term | Wormian bones, broad skull. | Gracile ribs. Lateral rib fractures of left rib 2 and right ribs 2–6. | No fractures of humeri, radii and ulnae. | Multiple fractures of femora with loss of modeling. Fracture and bowing of right tibia. No evaluation possible of right fibula, left tibia and fibula. | III | III |
| 5 (II: 1) full term | Wormian bones and flattening of the occiput. | Fracture of left clavicula. Small thorax. Normal vertebral column. Fractures of left ribs 4–7. | Fracture of left humerus and radius. Consolidated fracture of right humerus. | Fracture of right femur and tibia, severe bowing of right fibula and tibia. Multiple fractures of left femur, bowing and fracture of left tibia. | III | III |

**Table 2** *CRTAP* mutations identified in affected individuals

| *Family (pedigree nr)* | *Exon/intron* | *Mutation* | *Effect of mutation on mRNA* | *Effect of mutation on protein* |
|---|---|---|---|---|
| 1 (VI:3,VI:4, VI:5) | Exon 1 | c.21_22dupGG<br>c.21_22dupGG | Not tested | p.Ala8GlyfsX6<br>p.Ala8GlyfsX6 |
| 2 (IV:4) | Exon 1 | c.404delC<br>c.404delC | Not tested | p.Ser135ThrfsX39<br>p.Ser135ThrfsX39 |
| 3 (II:1, II:2, II:5) | Exon1 | c.198C>A | No effect | p.Tyr66X |
| | Intron1 | c.471+2 C>A | Splice error resulting in nonsense-mediated decay | 0-allele |
| 4 (II:1) | Intron 4 | c.923-2A>G | Loss of acceptor site and use of kryptic site resulting in c.923-936del | p.Leu308X |
| | Exon 1 | c.38C>A[a] | No effect | p.Ala13Glu |
| | Exon 1 | c.469A>G[a] | No effect | p.Lys157Glu |
| 5 (II:1) | Intron 1 | c.471+2C>A<br>c.471+2C>A | Splice error resulting in nonsense-mediated decay | 0-allele |

[a]The c.38 C>A mutation and the c.469 A>G both reside on the maternal allele.

the femoral epiphysis in proband IV:4 from family 2 did not show a primary cartilaginous dysplasia (see Figure 4). Furthermore, popcorn epiphysis has been reported in a patient with OI due to a *COL1A2* mutation, and recently in other patients with OI type III and IV due to *COL1A1* and *COL1A2* mutations.[24,25] Popcorn epiphyses were absent in the 4-year-old proband II:1 from family 5 (see Figure 5 Supplementary Appendix). Considering the above, we hypothesize that popcorn calcifications reflect a disturbance of enchondral ossification, which can occur in *CRTAP*-related OI as well as in *COL1A1/2*-related OI.

Importantly, on revision of the available radiographs by a pediatric radiologist, the classification of OI differed in three of the eight cases (Table 2), which stresses the need for evaluation of radiographs by a pediatric radiologist in all cases of OI. It is interesting that a radiological diagnosis of OI type II-B or III was evident in all the affected individuals with *CRTAP* mutations in the literature,[15,22] as well as in all our patients. One important difference between OI type II-A, -B and -C is the extent and manner of rib involvement.[3] In OI II-A, the ribs are very broad with contiguous fractures (contiguous beading), whereas in OI II-B, the ribs are rather small, with an irregular course and few fractures, and in OI II-C, which is very rare, varying thickness and discontinuous beading of the ribs are noted. In OI III, rib fractures are less commonly observed. Interestingly, a recurrence rate from 0% in OI II-A patients[4] to 7.7% in OI II-B patients[5] has been described. It seems that *CRTAP* mutations cause OI type II-B/III, and it might be that recurrence in OI type II-A is because of gonadal mosaicism of *COL1A1/2* mutations only. However, OI type II-B/III can also be caused by *COL1A1/2* mutations, which makes differentiation of *CRTAP*-related OI on the basis of radiological features difficult.

On biochemical testing by electrophoresis, full overmodification of collagen type I was observed comparable with predominantly inherited lethal and severe OI due to collagen type I mutations. However, in some instances, it is possible to discriminate between autosomal recessive and predominantly inherited lethal and severe OI, as in the latter, some normal molecules can be made and chains with normal mobility can be visualized.

In the affected individuals, genomic and complementary DNA sequencing of the *COL1A1* and *COL1A2* genes was negative. Mutation analysis of *CRTAP* and *LEPRE1* genes identified seven new *CRTAP* mutations in five families and one new homozygous *LEPRE1* mutation in one family. An overview of (new) *CRTAP* mutations is given in Table 2 and Figure 6 of the Supplementary Appendix. Pathogenicity of the identified mutations was supported by *in silico* analysis. The c.21_22dupGG and c.404delC mutations in exon 1 are frameshift mutations, leading to a stop codon 6 and 39 amino acids downstream, respectively. The c.471 + 2C>A mutation is a splice donor-site mutation that changes the second nucleotide in intron 1, which is the unusual nucleotide, C. (see Supplementary Appendix Figure 1). cDNA sequencing proves that this mutation causes a null allele (see Supplementary Appendix Figure 2). The 198C>A mutation replaces tyrosine at position 66 with a premature termination codon. It is interesting to note that II:1 from family 4 has three mutations. The c.923-2A>G mutation in intron 4 is a splice acceptor site mutation leading to disruption of the normal splicing of exon 5. The c.469A>G mutation in exon 1 and the c.38 C>A mutation reside on the same allele. The c.469A>G mutation is a non-conservative change resulting in a p.Lys157Glu amino acid change and could therefore be considered pathogenic. However, the c.38C>A missense mutation resulting in p.Ala13Glu might also be pathogenic, as it disturbs the charge distribution in the signal peptide and could lead to retainment of CRTAP in the rER. Currently, functional studies are being conducted to investigate the pathogenicity of these mutations.

In conclusion, although a diagnosis of OI type II-A seems to point to autosomal dominant OI, in OI types II-B and III, clinical, radiological and biochemical differentiation between autosomal dominant OI caused by collagen type 1 mutations and autosomal recessive OI caused by *CRTAP* mutations proves difficult. Therefore, it is important to carry out biochemical analysis in combination with preferably genomic and complementary DNA sequencing of the collagen type I genes in individuals affected with lethal or severe OI. Posttranslational overmodification without collagen type I mutations should prompt *CRTAP* or *LEPRE1* mutation analysis. This approach is important for providing accurate recurrence risk, prenatal diagnosis and preimplantation genetic diagnosis. Moreover, therapeutic options might differ in the future. Furthermore, knowledge of the proportion of cases of lethal and severe OI with recurrence due to *CRTAP* and *LEPRE1* mutations will lead to a more accurate estimation of recurrence risk due to germline mutations in the collagen type I genes. It may very well be that the greater part of the empirical recurrence risk in OI type II and III, currently estimated at 6% for OI type II,[9] can be attributed to autosomal recessive causes.

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