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## **The role of PTHrP in chondrocyte differentiation.**

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Novel mutations in the PTHR1  
causing Blomstrand  
Osteochondrodysplasia  
type I and II

2



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JCEM, provisionally accepted



## **Abstract**

**Context:** The parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor type 1 (PTHr1) plays a key role in endochondral ossification, which is emphasized by diseases resulting from mutations in the PTHr1 gene. Among these diseases is the human variant of the PTHr1 knockout mice, Blomstrand osteochondrodysplasia (BOCD).

**Objective:** BOCD can be divided into two types, depending on the severity of the skeletal abnormalities. The molecular basis for this heterogenic presentation is unknown.

**Design and patients:** We performed mutation analysis in 2 families with type I BOCD and in 3 families with the less severe form, type II BOCD. The latter include 1 case that has not been described before.

**Results:** In one of the type I BOCD cases a nonsense mutation (R104X) was found, resulting in a truncated PTHr1, while in the second type I BOCD case no receptor mutation was identified. A splicing defect (intronM4+27C>T) was demonstrated in one of the type II BOCD cases. The use of the aberrant splice site resulted in a truncated protein. Although at low levels, the wild type transcript remained present. In the other 2 families with type II BOCD a previously identified missense P132L mutation was found. Functional analysis demonstrated a near complete inactivation of the PTHr1 with low residual activity.

**Conclusions:** In combination with data presented in literature, we conclude that type I BOCD is caused by a complete inactivation of the PTHr1, whereas low levels of residual activity due to a near complete inactivation of the PTHr1 result in the relatively milder presentation of type II BOCD.

## **Introduction**

The parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor type 1 (PTHr1) is a member of a subclass of the G-protein-coupled receptor family. The typical structure of these receptors is characterized by an extracellular N-terminus for ligand binding, a seven-transmembrane domain, and an intracellular C-terminus. The intracellular parts of the transmembrane domains and the C-terminus are responsible for the activation of the two dominant signalling cascades, the adenylate (AC) / protein kinase A (PKA) and the phospholipase C beta (PLC $\beta$ ) / protein kinase C (PKC) pathways.

Signalling through the PTHr1 plays a key role in calcium and phosphorus homeostasis in foetal and adult life<sup>(1)</sup>. Recent studies have demonstrated the essential role of PTHr1 signalling in cartilage and bone development in mice. Mice lacking the PTHrP gene die perinatally and show accelerated chondrocyte differentiation, leading to dwarfism<sup>(2)</sup>. A more severe bone phenotype is caused by loss of the PTHr1 gene. Depending on the genetic background these mice die at various embryonic ages<sup>(3)</sup>.

The essential role for PTHr1 signalling in bone and cartilage development has also been shown in human diseases. Mutations in the PTHr1 can be divided into dominant and recessive mutations. Dominant mutations are found in Jansen's metaphyseal chondrodysplasia (JMC) and Ollier disease<sup>(4;5)</sup>. Four different heterozygous mutations have been identified in JMC patients, causing a constitutively activation of the PTHr1, leading to skeletal malformations and dwarfism<sup>(4;6-8)</sup>. In a paper by Hopyan et al. in two out of 6 patients with enchondromatosis (Ollier and Maffucci diseases), which are common benign tumours of cartilage and bone, a

heterozygous mutation in the PTHR1 gene was identified<sup>(5)</sup>. The mutation is thought to result in upregulation of the Indian Hedgehog/PTHrP pathway<sup>(5)</sup>. However, in another study with a larger panel of patients, no mutations were identified in the PTHR1 gene, suggesting that the PTHR1 gene is not the main culprit for enchondromatosis<sup>(9)</sup>.

Recessive mutations have been identified in Eiken syndrome and in Blomstrand osteochondrodysplasia (BOCD)<sup>(10-14)</sup>. Eiken syndrome, an extremely rare syndrome presently reported in only 1 family, is caused by a homozygous nonsense mutation. This mutation resulted in a truncated protein, only missing a small part of the C-terminus, possibly leading to a disbalance between the activation of the AC/PKA and the PLC $\beta$ /PKC signalling pathways<sup>(10;15)</sup>. BOCD is a rare autosomal recessive disorder characterized by advanced skeletal maturation and premature ossification. In addition, BOCD is also characterized by extra-skeletal manifestations, like hypoplastic lungs, tooth abnormalities, aortic coarctation, and absence of breast development<sup>(16;17)</sup>. The skeletal and extra-skeletal abnormalities resemble the defects observed in PTHR1 knockout mice<sup>(3;18)</sup>. It has been proposed, that BOCD presents in two forms, type I and type II<sup>(16)</sup>. Type I BOCD is the classical and most severe form, characterized by extremely short and malformed bones. Although also lethal, the skeletal manifestations in type II BOCD are less severe compared to type I.

The first case of BOCD was described by Blomstrand et al. in 1985<sup>(19)</sup>. Until now, 10 other families have been described with the same disorder<sup>(16;20-26)</sup>. In the majority of the cases parental consanguinity was noted. Only 2 reports have been published in which the affected foetuses were born to non-consanguineous parents<sup>(16;23)</sup>. In three BOCD cases a mutation in the PTHR1 gene has been described. Jobert et al. reported in 1998 for the first time an inactivating mutation in the PTHR1 gene<sup>(13)</sup>. In the same year a missense mutation in another BOCD case was reported<sup>(11;12)</sup>. Thereafter, another study revealed that a frame shift mutation in the PTHR1 gene, resulting in a truncated protein, was the cause of BOCD in the case described by Den Hollander et al.<sup>(14;21)</sup>.

In this study, we address the molecular basis for the heterogenic presentation of BOCD by performing mutation analysis in 2 type I and 3 type II BOCD cases.

## Materials and Methods

### *Cases included in this study*

Case 1 is a type I BOCD and was the first reported case of BOCD described in detail by Blomstrand et al.<sup>(19)</sup>. The female foetus was born after 29 weeks of gestation to consanguineous parents. The second case, a female foetus born to consanguineous Caucasian parents at 32 weeks of gestation, is described in detail elsewhere and is classified as type I BOCD (case I in<sup>(16)</sup>). Case 3, classified as a type II BOCD, is described for the first time in the present study (for detailed description see next paragraph). Case 4 is one of the three affected siblings born to Asian parents who were first-degree cousins and who had no family history of skeletal dysplasia (case II in<sup>(16)</sup>). The first two foetuses were described in detail before<sup>(16)</sup>. Another pregnancy of the same parents resulted in a third affected foetus, showing the same characteristics as its sibs. All three foetuses were classified as type II BOCD. In the fifth case the foetus was obtained from a terminated pregnancy at 26 weeks of gestation (case III in<sup>(16)</sup>). The Asian parents were non-consanguineous. Post-mortem radiography and osteochondral

histopathology classified the skeletal dysplasia as type II BOCD. Parental consent was obtained for this study.

### **Case 3 Clinical report and family history**

Case 3 was a male infant whose mother presented with polyhydramnion at 32 weeks. Foetal ultrasound at 32 weeks revealed furthermore a relatively large head, a small thorax, lung hypoplasia and very short dense tubular bones. Because of the gross polyhydramnion and the probable lethal prognosis labour was initiated. At birth the infant did not breathe and died within minutes. Birth weight was 1800 gram. The boy showed a short stature (Crown-Rump Length (CRL) 28.5 cm), a large head (Head Circumference (HC) 32 cm) and a hypoplastic viscerocranium (fig. 1A). The face showed typical abnormalities: severe micrognathia with a protruding tongue, a hypoplastic nose, and low-set ears were noted. The infant also had a narrow thorax and no nipples. The limbs were symmetrically shortened. Autopsy revealed hypoplastic lungs and a pre-ductal aortic coarctation. Abdominal and pelvic organs showed no abnormalities. Radiography showed generalised osteosclerosis and advanced skeletal maturation, a small viscerocranium, short ribs, ossification of laryngeal cartilage and patella (fig. 1A). The tubular bones were short with metaphyseal broadening. Most carpal and tarsal bones were ossified. Histology of tubular bones showed a reduction of the resting and proliferative zone of the growth plates (fig. 1B). The epiphysis showed irregular columnization of the hypertrophic chondrocytes. The overall picture resembles the clinical manifestations of BOCD. Given the relatively well-developed long bones, a recognizable epiphysis and a normal diaphyseal bone marrow space this case was classified as type II BOCD.

The parents both originated from a 250-person village in Turkey, but they deny any consanguinity. This foetus was the 7th pregnancy of these parents. Two sons are healthy, one child died one week old of unknown cause, one child had been healthy until the age of death at 11 months of unknown cause, one earlier pregnancy had ended in the birth of a stillborn boy at 28 weeks pregnancy and one pregnancy ended in a spontaneous miscarriage at 12 weeks. No medical information on these deceased children is available.

### ***Sequence analysis and site-directed mutagenesis***

From cases 1 and 2 only tissue blocks embedded in paraffin were present. Sections from these blocks were deparaffined and hydrated through graded ethanols. Subsequently, DNA was extracted by proteinase K (Invitrogen) digestion and ethanol precipitation. From cases 3, 4 and 5 genomic DNA was isolated from cultured fibroblasts by sequential proteinase K treatment and high salt precipitation.

Primer sets used for the amplification of the coding exons of hPTH1R were previously described<sup>(14;27)</sup>. PCR products were sequence verified using an ABI thermal sequencer (PE Applied Biosystems, Foster City, CA, USA).

A mutant PTHR1 receptor cDNA was created by PCR based site directed mutagenesis, using the wild type receptor as a template. The mutant construct was sequence verified and cloned in the pCDNA3.1 expression vector. Primer sets to check for the expression of wild type and mutant PTHR1 transcripts in case 3 are indicated in table 1.

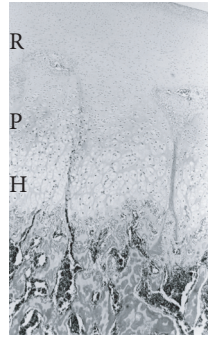
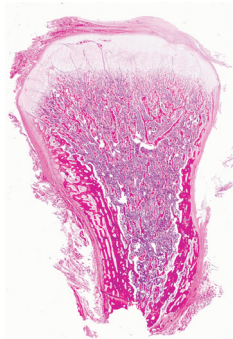
**Cell culture, transient transfection assays and cAMP production**

At autopsy of cases 3, 4, and 5 a skin biopsy was taken and dermal fibroblast were cultured in  $\alpha$ MEM, containing 100 U/ml penicillin (Invitrogen, Breda, The Netherlands), 100 U/ml streptomycin (Invitrogen), and 10 % fetal calf serum (FCS; Integro BV, Zaandam, The Netherlands). Cells were seeded at a density of 15000 cells/cm<sup>2</sup> in a 24-wells tissue culture plate. After 4 days cells were used for intracellular cAMP determination after a challenge with

1A



1B



**Figure 1. Case 3: phenotypic presentation of type II BOCD.**

A) Left panel: external view of a foetus delivered 32 week after gestation, showing short stature and micromelia. Right panel: whole body radiograph, showing advanced skeletal maturation, a small viscerocranium, short ribs, ossification of laryngeal cartilage and patella. The tubular bones are short with metaphyseal broadening. Most carpal and tarsal bones are ossified. B) Histology of a femoral head, showing a reduction in size of the epiphysis. High magnification shows a decrease in resting chondrocytes (R) and a near complete absence of column-wise orientated proliferating chondrocytes (P). Note the irregular border between the growth plate and the primary spongiosum. H: Hypertrophic zone.

| Name | Sequence               |
|------|------------------------|
| S8   | TGTGGGGCTTCACAGTCTTCG  |
| AS8  | AAGTCCCAGCACCCGGTG     |
| AS9  | GGATGAAGTTGAGCACAAATGG |

Table 1: Primer sets used for PCR analysis in case 3.

hPTHrP(1-34) or bNle<sup>8,18</sup>PTH(1-34) (Bachum Holding AG, Bubendorf, Switzerland), using an enzymimmunoassay (Amersham, Freiburg, Germany), according to the manufacturers protocol, as described before<sup>(14)</sup>. COS-7 cells were cultured in bicarbonate-buffered DMEM, containing 7.5 % FCS, 100 U/ml penicillin, and 100 U/ml streptomycin. For transient transfection assays, cells were seeded in a 75 cm<sup>2</sup>-disk. At 80 % confluence, cells were overnight transfected with 6 µg of the pcDNA3.1 expression vector (Invitrogen), containing either the wild type or mutant human PTHR1 cDNA or no insert (mock) using Fugene-6 (Roche, Indianapolis, IN). The following day, cells were trypsinized and seeded at a density of 15000 cells/cm<sup>2</sup> in a 24-wells tissue culture plate. After 2 days cells were used for determination of intracellular cAMP as described before<sup>(14)</sup>.

### **RT-PCR analysis**

Dermal fibroblasts were cultured as described above. Cells were seeded at a density of 15000 cells/cm<sup>2</sup> in a 56 cm<sup>2</sup> tissue culture disk. After confluence, total RNA was isolated according to the method of Chomciznsky and Sacchi<sup>(28)</sup>. Next, total RNA was reverse transcribed into cDNA using random hexamer primers (Amersham, Freiburg, Germany). To correct for variations in RNA content and cDNA synthesis between the different samples, cDNAs were equalized on the basis of their content of the housekeeping gene beta-2-microglobulin as described in detail elsewhere<sup>(29)</sup>. Semi-quantitative PCR was performed for the PTHR1 or for various parts of the PTHR1 under the following conditions: cDNA was denatured at 94°C for 5 minutes, followed by cycles of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C, and final extension at 72°C for 10 minutes.

## **Results**

### **Case 1**

Sequence analysis of the coding exons and the flanking exon-intron boundaries of the PTHR1 gene revealed a homozygous point mutation (338C>T), causing a premature stop codon at position 104 (R104X) (fig. 2A). The presence of the mutation was verified by restriction fragment length analysis (fig. 2B). DNA corresponding to exon 1 from the foetus was amplified by PCR and was, as expected, resistant to KpnI restriction. Due to the substitution a truncated protein was formed, only consisting of the signal peptide and the first 79 amino acids. This mutant protein lacks all functional domains of the PTHR1, including a large part of the extracellular N-terminus, the transmembrane domains and the intracellular C-terminus, and is, therefore, completely inactivating.



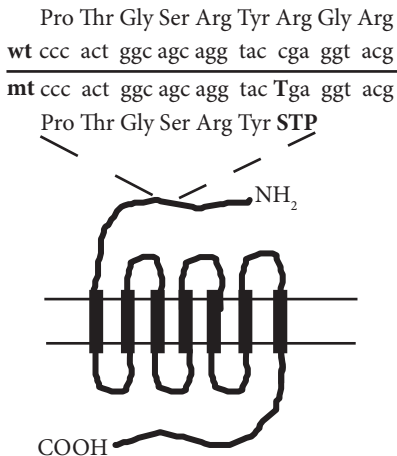
**Case 2**

Sequence analysis of the PTHR1 gene did not reveal a mutation in the coding exons and flanking exon-intron boundaries (data not shown).

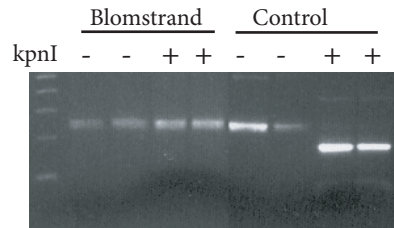
**Case 3**

The dermal fibroblast of the affected foetus showed no cAMP accumulation after a challenge with a high dose of PTH or PTHrP (fig. 3A). To establish whether the abnormalities were indeed caused by a mutation in the PTHR1, all coding exons and flanking exon-intron boundaries of the PTHR1 gene were sequenced. In none of the coding exons a mutation was found. However, a homozygous point mutation was identified in the intron between exon M4 and exon EL2 (intronM4+27C>T) (fig. 3B). This mutation creates a perfect match with the consensus sequence of an exon-intron boundary with higher homology than the native splice site. RT-PCR analysis of the PTHR1 gene revealed decreased expression and a larger transcript in the fibroblasts from the patient compared to control samples (fig. 3C). To test whether or not the splicing machinery can still use the native splice site, RT-PCR was performed using a reverse primer, spanning the wild type exon M4 and EL2 splice site. This primer can only result in amplification of wild type, but not in mutant transcripts. As shown in figure 3D still a small amount of wild type PTHR1 mRNA was expressed in fibroblasts of the patient compared to control samples, indicating the preferential but not exclusive use of the aberrant splice site. The formation of an aberrant splice site and subsequently the extension of exon M4 resulted in a premature stop codon, thereby creating a truncated protein, lacking the 5<sup>th</sup>, 6<sup>th</sup>, and 7<sup>th</sup> transmembrane domains, the intervening intra- and extracellular domains, as well as the cytoplasmic tail (fig. 3E).

2A



2B

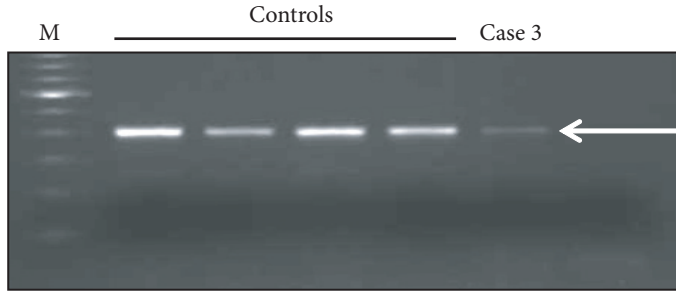


**Figure 2. Case 1: premature stopcodon in PTHR1.**

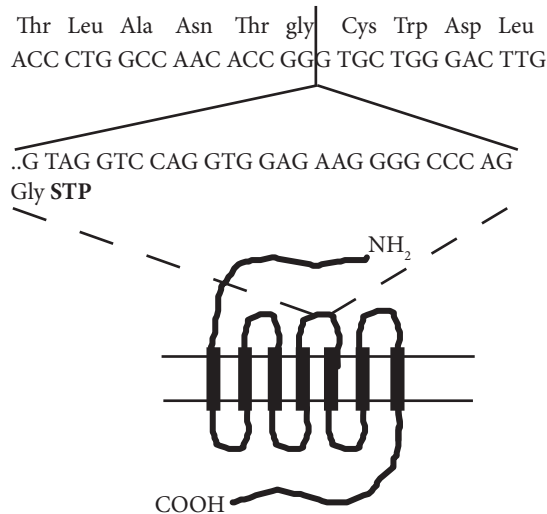
A) Comparison of the DNA and amino acid sequences of the wild type (wt) and the mutant (mt) receptors. Sequence analysis of PTHR1 gene revealed a 338C>T substitution (capital and bold), resulting in a premature stop codon in the extracellular part of the receptor. The position of the mutation is shown in a schematic representation of the structure of the PTHR1. B) Exon E2 was amplified and subjected to restriction enzyme analysis. The PCR product of the patient but not of a control was resistant to KpnI restriction.



3D



3E



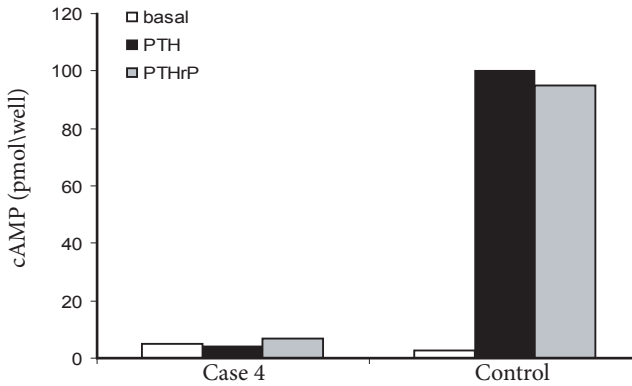
**Figure 3. Case 3: Introduction of a novel splice site.**

A) Dermal fibroblast of the affected foetus and control dermal fibroblasts were treated with a high dose (10<sup>-7</sup> M) PTH(1-34) or PTHrP(1-34) and cAMP accumulation was measured. In contrast to control, dermal fibroblasts of the affected foetus showed no cAMP accumulation. B) A homozygous point mutation was identified 27 bp downstream of exon M4 (intronM4+27C>T), creating a new exon-intron boundary with higher homology to the consensus sequence than the wild type splice site. Native and mutated exon/intron boundaries are underlined. C) RT-PCR of the PTHR1 using a sense primer (S8) located in exon M4 and an antisense primer (AS9) located in the downstream exon EL2 revealed a bigger transcript in case 3 compared to control samples, which is indicative for aberrant splicing. D) RT-PCR was performed with a sense primer (S8) specific for exon M4 and an antisense primer (AS8) overlapping the wild type exon-exon boundary of exon M4 and EL2. Only in the case of wild type mRNA this primer combination results in an amplicon. A small amount of wild type PTHR1 mRNA was expressed in the affected sample compared to control samples, indicating the preferential but not exclusive use of the aberrant splice site. E) Comparison of the DNA and amino acid sequences of the wild type (wt) and the mutant (mt) receptors. When the alternative splice site is used, the last amino acid of exon M4 will be followed by a stop codon. This results in a truncated protein, lacking the transmembrane domains 5, 6 and 7 and the cytoplasmatic C-terminus. STP: stop codon.

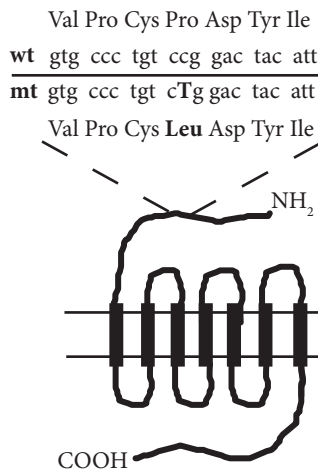
**Case 4**

Dermal fibroblasts of case 4 showed no cAMP accumulation after a challenge with a high dose of PTH or PTHrP (fig. 4A), indicating abrogated PTHrP signalling. We subsequently sequenced the coding exons and flanking exon-intron boundaries of the PTHrP gene. A homozygous point mutation (423C>T) in exon E3 was identified, resulting in a missense mutation at position 132 (P132L) (fig. 4B). The presence of the mutation was verified by restriction fragment length analysis (fig. 4C). DNA corresponding to exon 3 from the foetus was amplified by PCR and was, as expected, resistant to MspI enzymatic activity. The same mutation was identified in the two other sibs with BOCD. Functional analysis by transient transfections of wild type and mutant receptors in COS-7 cells revealed that the mutant receptor could still accumulate cAMP after receptor activation, although to a lesser extent compared to the wild type receptor (fig. 4D).

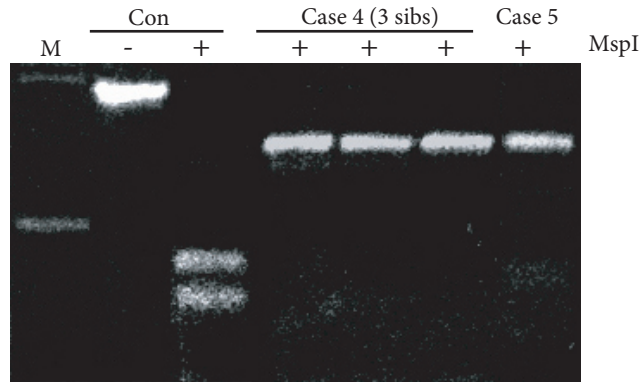
4A



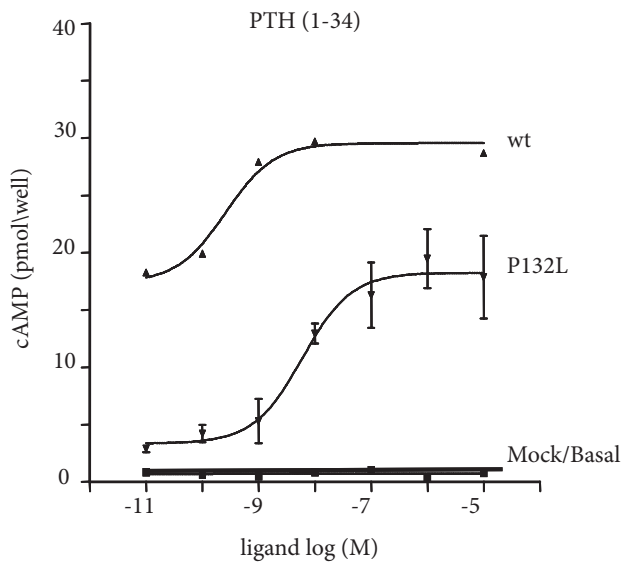
4B



4C



4D



**Figure 4. Case 4 and 5: P132L missense mutation.**

A) Dermal fibroblast of case 4 and control dermal fibroblasts were treated with a high dose (10<sup>-7</sup> M) PTH(1-34) or PTHrP(1-34) and cAMP accumulation was measured. Dermal fibroblasts of the affected foetus showed no cAMP accumulation, whereas in control fibroblasts cAMP accumulation was induced. B) Comparison of the DNA and amino acid sequences of the wild type (wt) and the mutant (mt) receptors. Sequence analysis of PTHR1 gene revealed a homozygous 423C>T substitution (capital and bold), resulting in an amino acid change in the extracellular part of the receptor (P132L). The position of the mutation is shown in a schematic representation of the PTHR1. C) Exon E3 was PCR amplified and subjected to restriction enzyme analysis. The mutation resulted in a loss of an MspI restriction site in case 4 and 5. M is marker. D) COS-7 cells were transiently transfected with an expression vector encoding wild type PTHR1 (wt), mutant P132L PTHR1 or an empty expression vector (mock). Subsequently, cells were treated with a dose range of PTH(1-34) and cAMP accumulation was measured.

### Case 5

Sequence analysis also revealed the homozygous mutation 423C>T in the PTHR1 gene as described for case 4, resulting in a P132L substitution. The presence of the mutation was verified by restriction fragment length analysis (fig 4C).

## Discussion

In the present study we have performed mutation and functional analysis in 5 cases with BOCD. The presence of inactivating mutations in 4 out of 5 cases further confirms the causative role of the PTHR in BOCD. In one patient (case 2 in this study) we did not identify a mutation in the coding exons and flanking exon-intron boundaries of the PTHR1 gene. Possibly, a mutation is present in an intron or in the promoter region, which inhibits the expression of the PTHR1 gene. Such a mutation probably also underlies the absence of expression of the paternal allele in the case described by Jobert et al.<sup>(13)</sup>. Another explanation could be the presence of a mutation in another gene, like PTHrP. Unfortunately, we were unable to test this hypothesis, since only a small amount of DNA of poor quality could be isolated from a limited number of paraffin blocks.

Including this study, 5 different mutations have been described in the PTHR1 causing BOCD<sup>(11-14, this study)</sup>. Each family had its own mutation, with exception of the P132L. This data suggests that this conserved proline residue serves as a hotspot for mutations. Hotspots have already been described in other chondrodysplasias, like the H223R mutation in the PTHR1 causing JMC, and the A380G mutation in the FGFR3 causing achondrodysplasia<sup>(4;30)</sup>. Another explanation for the relatively high frequency of the P132L mutation could be that the patients originate from the same ancestor. Pedigree analysis revealed no indications for this. However, since the 3 families with P132L live in the same region of England and originate from the same ethnic population, such a founder effect cannot be ruled out.

The topic we addressed was the difference between type I and type II BOCD. Oostra et al. postulated that type I BOCD is caused by a completely inactivating mutation in the PTHR1 gene<sup>(16)</sup>, while the less severe abnormalities in type II BOCD are caused by incomplete inactivation of the PTHR1. We present evidence in concordance with this hypothesis. The mutations in the type I BOCD cases described in this study and in previous studies are all completely inactivating mutations<sup>(13;14)</sup>. In the three families classified by Oostra et al. as type II BOCD, the P132L mutation was found<sup>(11;12;16, this study)</sup>. Functional analysis of the P132L mutation, after transient transfections in COS-7 cells, revealed residual activity. Also in the study of Zhang et al. some residual activity was present with respect to activation of the cAMP/PKA pathway, but not to activation of the PLC $\beta$ /PKC pathway<sup>(12)</sup>.

The residual activity of the P132L mutation in COS-7 cells contrasts our observation in dermal fibroblasts of case 4, in which no cAMP accumulation was found after stimulation with PTH or PTHrP (not shown). This discrepancy is most likely explained by a substantial difference in the number of receptors present in dermal fibroblasts and transiently transfected COS-7 cells. Only in the presence of high receptor numbers residual activity might become evident. Since chondrocytes and osteoblasts express higher levels of PTHR1 mRNA and, therefore, are more responsive than dermal fibroblasts, the residual activity of the P132L mutation *in vivo* may only become evident in the developing skeleton, resulting in a milder clinical presentation.

An alternative explanation is that the milder phenotype in the three type II BOCD cases is associated with the same ethnic background. It has been described that the phenotype of PTHR1 knock out mice strongly depends on this genetic composition<sup>(3)</sup>. This latter explanation seems, however, unlikely, since we have identified in this study a new type II BOCD case of distinct ethnic background, caused by a novel mutation (intronM4+27C>T). Like in case 4, dermal fibroblasts of this patient did not reveal any responsiveness to PTH or PTHrP, using intracellular cAMP accumulation as a read out. However, by sensitive RT-PCR we show that the native splice site is also used, although at very low levels. This will result in the presence of low levels of wild type mRNAs. Apparently, in dermal fibroblasts, a cell type with low levels of PTHR1 transcripts, this expression is too low to result in a measurable functional response. In skeletal cells with high levels of mRNA and high responsiveness, low residual activity due to native splicing may become evident, resulting in the milder skeletal manifestations. Taken together, the relatively milder presentation of BOCD type II, particularly in the skeleton, is most likely caused by a partial inactivating mutation of the PTHR1 gene, resulting in low amounts of residual activity during endochondral bone formation.

The heterogeneity in clinical manifestations, depending on the kind of mutation, is not only shown by differences in skeletal abnormalities, but also by differences in extra-skeletal abnormalities. A characteristic of PTHR1 knockout mice is the absence of breast and nipple formation<sup>(18)</sup>. This finding is confirmed in a patient with BOCD<sup>(17)</sup>. Also in case 3 reported in this study, absence of nipple formation was noted during obduction. However, in one case (specimen M684 in the study by Oostra et al.), having the typical skeletal characteristics of BOCD, nipple formation is clearly visible (see figure 4 in<sup>(20)</sup>). We postulate that the presence or absence of nipple formation may also depend on the nature of the mutation in the PTHR1, affecting skeletal development but not nipple formation. Unfortunately, because of long-term preservation (over 100 years in formalin), DNA isolation from this case for sequence analysis was not possible to test this hypothesis.

Heterogeneity in clinical manifestations due to the nature of the mutation in the PTHR1 is not only shown in BOCD families, but also in JMC families. Two missense mutations at the same amino acid position in the PTHR1 (T140P and T140R) have distinct effects on receptor activity. Compared to T140P, the T140R substitution resulted in a less severe form of ligand dependent activation of the AC/PKA pathway<sup>(7)</sup>. This became clinically evident in a less severe presentation of JMC<sup>(8)</sup>. The impact of a mutation on receptor function critically determines the clinical presentation.

The mutations in the PTHR1 gene identified in this study and in previous studies are shown in table 2. It summarizes the type and position of the mutations identified in 4 disorders, caused by defective PTHR1 signalling. They are divided into dominant and recessive disorders. Not only the type of the mutation, but also the position of the mutation influences the kind and the severity of the disease. The mutations are evenly distributed over the open reading frame. The striking differences in clinical presentations caused by distinct mutations further underscores the critical role of the PTHR1 in normal endochondral bone formation. Multiple signalling pathways are activated by the PTHR1. It seems feasible that distinct mutations affect only subsets of these pathways, resulting in differences in signalling properties and hence in clinical manifestations. Detailed functional analysis of mutations in the PTHR1 identified in human patients may, therefore, provide novel insights in the role of each of these signalling pathways

in the biological effects of PTHR1.

## Acknowledgements

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| dominant/<br>recessive | disease           | nucleotide<br>change | amino acid<br>change | Type of<br>mutation | Impact on receptor                       | reference           |
|------------------------|-------------------|----------------------|----------------------|---------------------|--|---------------------|
| dominant               | JMC               | 696A>G               | H223R                | missense            | constitutively activating                | (6)                 |
|                        |                   | 1401T>G              | I458R                | missense            | constitutively activating                | (4)                 |
|                        |                   | 1256A>C              | T410P                | missense            | constitutively activating                | (7)                 |
|                        | JMC (less severe) | 1257C>G              | T410R                | missense            | less severe constitutively activating    | (8)                 |
|                        | Ollier syndrome   | 476C>T               | R150C                | missense            | disturbed regulation of Hedgehog pathway | (5)                 |
| recessive              | Type I<br>BOCD    | 338C>T               | R104X                | nonsense            | Inactivating                             | This report         |
|                        |                   | 1122delG             | V365fsX505           | frameshift          | Inactivating                             | (14)                |
|                        |                   | 1176G>A              |                      | splicing defect     | Inactivating                             | (13)                |
|                        | Type II<br>BOCD   | 423C>T               | P132L                | missense            | partial Inactivating                     | This report (11;12) |
|                        |                   | intronM4+<br>27C>T   |                      | splicing defect     | partial inactivating                     | This report         |
|                        | Eiken syndrome    | 1656C>T              | R485X                | nonsense            | partial inactivating                     | (10)                |

**Table 2: PTHR1 mutations.**



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